All subtypes of the cytotoxin VacA adsorb to the surface of *Helicobacter pylori* post-secretion

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The major secreted virulence factor of *Helicobacter pylori*, the vacuolating cytotoxin VacA, is known to insert into eukaryotic membranes and has been observed in association with the surface of *H. pylori* cells that are actively producing it. Here, it is demonstrated that VacA is capable of interacting with the surface of *H. pylori* and *Escherichia coli* after secretion. It is shown that this interaction is resistant to disruption of electrostatic and hydrophobic forces, and that it appears to occur despite truncation of LPS and the removal of trypsin-accessible surface proteins. Adsorption to bacterial cell surfaces was independent of the VacA subtype, suggesting that it is not mediated through recognition of a known receptor by the VacA p58 subunit. Similarly, adsorption to bacterial cell surfaces is unlikely to be instigated by the extreme N-terminus of VacA, since a hydrophilic extension at this location that is known to disrupt VacA-induced vacuolation did not interfere with adsorption to *H. pylori* cells.

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INTRODUCTION

Infection by the gastric pathogen *Helicobacter pylori* causes chronic gastritis and is a major risk factor in the development of a number of diseases, including peptic ulcers, gastric lymphoma and gastric adenocarcinoma (Ernst & Gold, 2000). Among the many virulence factors of *H. pylori* (CagA, urease, outer-membrane proteins, adhesins, mucinase), one of the most extensively studied is the vacuolating toxin VacA (Cover, 1996).

VacA possesses the three characteristic functional domains that denote a member of the autotransporter family of proteins: an N-terminal signal peptide, a central passenger domain and a C-terminal domain (Cover, 1996; Telford *et al.*, 1994). The N-terminal targeting domain promotes the protein's delivery to the bacterial inner membrane and is cleaved during translocation of the protein to the periplasm. This translocation may be achieved via the SRP/Sec pathway (Henderson *et al.*, 1998; Sijbrandi *et al.*, 2003). The C-terminal domain is proposed to insert subsequently into the outer membrane, forming a β -barrel (Oomen *et al.*, 2004).

Cleavage of this C-terminal domain, which may be mediated by the passenger protein domain itself or surface proteases (Henderson *et al.*, 1998, 2000), then releases the central domain, which forms the mature protein. The original models of autotransporter secretion suggested that the β -barrel forms a conduit through which the mature protein (or passenger domain) is translocated to the extracellular medium. Recently, it has been proposed that the C-terminal domains of multiple autotransporters may associate to generate the outer-membrane translocation pore (Veiga *et al.*, 2002) and that the outer-membrane protein Omp85 may facilitate the process of outer-membrane translocation (Voulhoux *et al.*, 2003). VacA shows homology to autotransporters both by possession of an extended N-terminal signal peptide and by the presence of a C-terminal domain that shows homology to other autotransporters and is cleaved during secretion (Cover, 1996; Schmitt & Haas, 1994). Once secreted, the ~90 kDa passenger domain of VacA may be processed further, yielding subunits of ~58 kDa and ~37 kDa. The ~90 kDa VacA monomers can combine to form dodecamers or tetradecamers with a flower-like rosette structure (Cover *et al.*, 1997; Lupetti *et al.*, 1996). In acidic conditions, these oligomers dissociate back into monomers, exposing hydrophobic regions and allowing efficient insertion into target membranes (Molinari *et al.*, 1998b). VacA attack on eukaryotic cells can result in

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Abbreviations: FPE, fluorescein phosphatidylethanolamine; RPTP, receptor-like protein tyrosine phosphatases.

mitochondrial damage (Galmiche *et al.*, 2000; Kimura *et al.*, 1999; Menaker *et al.*, 2004; Willhite *et al.*, 2003; Willhite & Blanke, 2004), induction of apoptosis (Galmiche *et al.*, 2000; Kuck *et al.*, 2001), increased permeability of polarized monolayers (Papini *et al.*, 1998; Pelicic *et al.*, 1999), disruption of antigen presentation (Molinari *et al.*, 1998a), inhibition of T-cell proliferation (Sundrud *et al.*, 2004) and interference with vacuolar transport in the late endosomal pathway leading to the formation of characteristic vacuoles (Cover & Blaser, 1992; Li *et al.*, 2004; Molinari *et al.*, 1997). It is presumed that VacA exerts these effects by binding to the plasma membrane of mammalian cells, possibly via interaction with components such as the receptor-like protein tyrosine phosphatases [RPTP β (PTPrz), Fujikawa *et al.*, 2003; Yahiro *et al.*, 1999; RPTP α , Yahiro *et al.*, 2003], the epidermal growth factor receptor (Seto *et al.*, 1998) and lipid raft membrane microdomains (Patel *et al.*, 2002; Schraw *et al.*, 2002). Subsequently, VacA is internalized by cells and localizes to the membranes of vesicles in the endosomal pathway, and can also be found associated with mitochondrial membranes. The former appears to cause clustering and redistribution of late endocytic compartments, which is a critical mechanistic step towards vacuolation (Li *et al.*, 2004). The vacuolation process is proposed to require channel formation by VacA, resulting in an influx of anions into the endosomes, which in turn stimulates increased proton pumping by the vacuolar ATPase. Damage of mitochondrial membranes by VacA leads to apoptosis through induction of cytochrome *c* (see Gebert *et al.*, 2004 for a review of VacA functions). The ability to form channels presumably enables VacA to permeabilize epithelial cells. The mechanisms underlying the suppression of the immune system by VacA (reduction of T-cell proliferation and antigen presentation) are not yet resolved, but have been proposed to be among the features of *H. pylori* pathogenesis that promote its long-term survival in the stomach of its host (Merrell & Falkow, 2004; Sundrud *et al.*, 2004).

Although all *H. pylori* strains analysed to date contain *vacA*, its sequence varies. The nucleotide sequence encoding the N-terminal signal region may be one of two main variants (s1 or s2), while that encoding the mature passenger domain contains a region that may be an m1 or m2 (Atherton *et al.*, 1995; Van Doorn *et al.*, 1998) subtype. *vacA* alleles can comprise signal and mid-regions in all four possible combinations, although the s2/m1 combination is rare (Letley *et al.*, 1999; Atherton *et al.*, 1995). The s1 and m1 types have been associated with increased virulence of *H. pylori*, since strains bearing either s1/m1 or s1/m2 *vacA* are more frequently associated with peptic ulceration than those with s2/m2 (Atherton *et al.*, 1995), and strains harbouring s1/m1 *vacA* are more frequently associated with gastric adenocarcinoma than those containing s1/m2 or s2/m2 *vacA* alleles (Kidd *et al.*, 1999; Miehlke *et al.*, 2000). However, as vacuolating (s1/m1), selectively vacuolating (s1/m2) and non-vacuolating (s2/m2) forms of VacA are maintained in the *H. pylori* gene pool, the existence of a positive selection pressure for

each is implied. Indeed, *H. pylori* producing VacA with reduced toxigenicity (s1/m2) out-competed a mutant devoid of VacA production in a mouse model (Salama *et al.*, 2001).

The secretion pathway that VacA shares with autotransporter proteins predicts that mature VacA would be found in the culture supernatant. However, we (Fitchen *et al.*, 2003, and this study) and others (Ilver *et al.*, 2004; Telford *et al.*, 1994) have observed that up to 50 % associates with the bacterial cells that produce it. This *H. pylori*-associated VacA forms tight foci that can be observed by immunofluorescence microscopy (Fitchen *et al.*, 2003; Ilver *et al.*, 2004) and is likely to be more physiologically relevant than the oligomeric form isolated from culture supernatants, since it does not require acid activation for activity and can be directly delivered to eukaryotic cells upon cell-cell contact (Ilver *et al.*, 2004). We set out to determine whether the interaction between VacA and the *H. pylori* cell surface occurs post- or co-secretion, and whether VacA could also associate with the surface of other Gram-negative bacteria.

METHODS

Bacterial strains and growth conditions. Each *H. pylori* strain was grown on a blood agar plate (5 % horse blood) at 37 °C either in a gas jar with BBL CampyPak Plus (Becton Dickinson) or in a VA cabinet (MACS VA500 microaerophilic workstation; dwScientific) and inoculated into 10 ml brain heart infusion (BHI) broth (Oxoid) supplemented with 0.2 % β -cyclodextrin. The broths were cultured for 48 h, unless otherwise indicated, at 37 °C, either in a gas jar with CampyPak Plus at 200 r.p.m. or in a VA cabinet at 120 r.p.m. A single colony of *Escherichia coli* was inoculated into LB broth, supplemented with appropriate antibiotics where necessary, and incubated overnight at 37 °C at 200 r.p.m. unless otherwise indicated. Antibiotics in bacterial cultures were used at concentrations of 50 $\mu\text{g ml}^{-1}$ for kanamycin and 100 $\mu\text{g ml}^{-1}$ for carbenicillin, streptomycin and tetracycline. All chemicals and reagents were supplied by Sigma unless otherwise indicated. *H. pylori* strains were: 60190 (s1/m1 *vacA*; Leunk *et al.*, 1988), 60190/M2 (s1/m2 *vacA*; Letley *et al.*, 2003), Tx30a (s2/m2 *vacA*; Leunk *et al.*, 1988), 60190/P2S2 (s2/m1 *vacA*; Letley *et al.*, 2003), 60190v1 ($\Delta vacA$; Bebb *et al.*, 2003), *rfbM*⁻ (s1a/m1 VacA; Edwards *et al.*, 2000) and *galE*⁻ (s1a/m1 VacA; Edwards *et al.*, 2000). *E. coli* strains were: D21 (Boman *et al.*, 1968), D21f2 (Boman & Monner, 1975), WA834 (Wood, 1966), WA707 (Wood, 1966) and MC4100 (Casadaban, 1976).

Eukaryotic cell culture. RK13 cells were routinely grown in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL) supplemented with 10 % FCS and 3 mM L-glutamine (Gibco BRL) at 37 °C in 5 % CO₂. When confluent, adherent cells were removed from the flask surface by incubation with 2 ml trypsin/EDTA (Gibco BRL) at 37 °C, 5 % CO₂, for 10 min, resuspended in 10 ml fresh medium and passaged by inoculation (1:25 dilution) into new flasks containing fresh media.

SDS-PAGE and Western blotting. *H. pylori* whole-cell lysates were prepared from 1 ml samples of broth cultures. Cells were pelleted by microcentrifugation and the supernatant was transferred to a fresh tube. The cell pellet was resuspended in 100 μl SDS-PAGE sample buffer and sonicated for 15 s using an MSE Soniprep 150 at an amplitude of 7 μm . The supernatant was concentrated by TCA precipitation, if necessary. Briefly, TCA from a 100 % stock was added to the sample to give a final concentration of 10 % TCA and incubated on ice for at least 30 min. Samples were centrifuged at 13 600 g for 15 min and the supernatant was discarded. Cold acetone (500 μl) was added and the sample was

centrifuged in the same way for 5 min. Supernatant was removed and the pellet was allowed to dry, before resuspension in sample buffer containing 5 mM NaOH. In all cases, samples were stored at -20°C until required. SDS-PAGE and Western blotting were performed as described by Hardie *et al.* (2003). Antisera 929 and 927, raised against the m1 (Garner & Cover, 1996) and m2 regions of VacA, respectively (kindly supplied by T. Cover, Dept of Medicine, Vanderbilt University, Nashville, TN 37232, USA), were used at a dilution of 1 : 20 000, with a secondary antibody of horseradish-peroxidase-conjugated donkey anti-rabbit IgG used at a 1 : 3000 dilution.

Vacuolation assays. Ninety-six-well plates were seeded with 1×10^4 eukaryotic cells (RK13) per well and incubated overnight at 37°C , 5 % CO_2 , to allow adherence. Medium containing 10 mM NH_4Cl was added to each well, prior to addition of an appropriate volume of test supernatant or bacteria. Several dilutions of supernatant or bacteria were used in each assay. Plates were incubated overnight at 37°C , 5 % CO_2 , and the extent of vacuolation was visually assessed by light microscopy.

RESULTS

VacA adsorbs to bacterial cell membranes post-secretion

VacA is produced by *H. pylori* throughout growth and, depending on the strain, up to 50 % of mature 90 kDa VacA can associate with *H. pylori* cells (Fig. 1; Ilver *et al.*, 2004). To determine whether mature VacA was being secreted and then readsorbing to the bacteria, culture supernatants containing VacA were incubated with non-toxin-producing *H. pylori* isogenic mutant cells (strain 60190v1; Bebb *et al.*, 2003). Mature, secreted VacA was clearly able to adsorb to *H. pylori* cells (Fig. 2a). Consistent with the localization of endogenously produced VacA on the surface of *H. pylori* cells seen by immunofluorescence microscopy (Fitchen *et al.*, 2003; Ilver *et al.*, 2004), cell-associated VacA was accessible to trypsin digestion (Fig. 3).

Fig. 1. Mature VacA is associated with the cell surface of *H. pylori*. *H. pylori* strain 60190 producing VacA subtype s1/m1 was grown in BHI containing 0.2 % cyclodextrin for up to 74 h and observed to enter the stationary phase after 50 h growth. At the indicated times, the OD_{600} of the culture was monitored and samples were taken. Samples were centrifuged to yield the culture supernatant (SN) and whole-cell lysate (WC) and corrected for cell number. Aliquots of these fractions harvested from equivalent numbers of cells were separated by 9 % SDS-PAGE and subjected to Western blotting with anti-VacA serum 929. The ~ 90 kDa VacA form is shown.

To assess whether this ability to associate with bacterial membranes was limited to the cell surface of *H. pylori*, VacA was applied to *E. coli* cells and shown to adsorb rapidly (Fig. 2b). The quantity of VacA adsorbed to the *E. coli* cell surface was reduced over time during incubation at 37°C , but was stable during incubation on ice (Fig. 2b), suggesting digestion by a surface protease. The enterobacterial surface protease OmpT appears to have a housekeeping function directed towards the turnover or degradation of proteins that come into contact with the outer membrane, particularly secreted proteins (Kukkonen & Korhonen, 2004); therefore its influence upon adsorbed VacA was investigated. Two independent OmpT mutants (*E. coli* strains WA834 and WA707; Wood, 1966) remained capable of degrading adsorbed VacA, indicating that a different protease was responsible (data not shown).

Fig. 2. Mature VacA adsorbs to the surface of bacteria post-secretion. (a) *H. pylori* strain 60190v1, which does not produce VacA, was grown for 48 h and the cells were collected by centrifugation. These cells were resuspended in BHI/cyclodextrin broth (–) or a filter-sterilized culture supernatant harvested from *H. pylori* strain 60190 grown for 48 h in BHI/cyclodextrin (which therefore contained VacA subtype s1/m1) (+). The resuspended cells were incubated for 10 min with shaking at 37°C in a gas jar containing CampyPak Plus. Whole-cell lysates prepared from these cells were separated through 9 % SDS-PAGE and subjected to Western blotting with the anti-VacA serum 929. (b) *E. coli* strain DH5 α cells were resuspended in spent supernatant from *H. pylori* wild-type strain 60190, containing s1/m1 type VacA, and incubated for the indicated lengths of time (min) at 37°C or on ice. Cells were briefly washed by resuspension in culture medium and harvested into SDS-PAGE sample buffer. Proteins were separated by 9 % SDS-PAGE prior to analysis by Western blotting using the anti-VacA serum 929. The ~ 90 kDa VacA form is shown.

Fig. 3. Cell-associated VacA is accessible to trypsin digestion. *H. pylori* 60190 wild-type cells (s1/m1 VacA) were incubated at 37 °C, 5% CO₂, in the presence of 250 µg trypsin ml⁻¹ for the indicated lengths of time (min) before the addition of soybean trypsin inhibitor (1.25 mg ml⁻¹). Cells were harvested by centrifugation and resuspended in SDS-PAGE sample buffer before proteins were separated by 9% SDS-PAGE and analysed by Western blotting with anti-VacA serum 929. The position of mature ~90 kDa VacA is indicated with an arrow and the digestion products with asterisks. Levels of VacA remained constant in the absence of trypsin.

VacA associates tightly with bacterial membranes

The interaction of secreted VacA with the target eukaryotic cell is dependent on specific protein receptors [the epidermal growth factor receptor, Seto *et al.*, 1998; RPTPβ (PTPrz), Fujikawa *et al.*, 2003; Yahiro *et al.*, 1999; RPTPα, Yahiro *et al.*, 2003]. However, VacA has been shown to insert into artificial lipid bilayers in the absence of such receptors (Molinari *et al.*, 1998a; Moll *et al.*, 1995; Pagliaccia *et al.*, 2000), where it has been visualized as hexameric rosettes by atomic force microscopy and deep-etch electron microscopy (Adrian *et al.*, 2002; Czajkowsky *et al.*, 1999). Evidence exists demonstrating that VacA displays a particular preference for lipid rafts when binding to eukaryotic-type lipid membranes both *in vitro* and *in vivo* (Gauthier *et al.*, 2004; Geisse *et al.*, 2004). Despite this extensive investigation of insertion into membranes consisting of lipids that are characteristic of eukaryotic membranes and the demonstration of VacA delivery to them from the cell surface of bacteria (Ilver *et al.*, 2004), the nature of the interaction of VacA with the bacterial cell surface is unknown. We therefore characterized the strength of the association between VacA and the cell surface of bacteria.

The majority of the VacA adsorbed to *H. pylori* cells could not be removed by simple washing with growth medium, or with agents that interfere with electrostatic (up to 1 M NaCl) or hydrophobic (up to 0.5 M urea) interactions (Fig. 4). Likewise, the *H. pylori*–VacA association was stable to treatments that disrupt the membrane (up to 0.05% Triton X-100). Pre-treatment of bacterial cells with trypsin did not prevent VacA adsorption, indicating that the interaction was unlikely to be protein-mediated (Fig. 5). Indeed, there was a small increase in the amount of VacA adsorbed following trypsin treatment, suggesting that surface-exposed proteins may hinder the interaction. Direct interaction of VacA with LPS is unlikely, since VacA successfully associated with LPS mutants (Fig. 6) of both *H. pylori* (*rfbM*⁻ and *galE*⁻) and *E. coli* (*rfa-1*, *rfa-31*).

Fig. 4. VacA adsorbs tightly to bacterial cells. (a) *H. pylori* strain 60190v1 (lacking VacA) cells were resuspended in spent supernatant from wild-type *H. pylori* strain 60190 (containing VacA subtype s1/m1) and incubated for 45 min with shaking at 37 °C in 5% CO₂ in a VA cabinet. Cells were then washed sequentially four times with BHI plus 0.2% cyclodextrin growth medium before resuspension in SDS-PAGE sample buffer. Proteins in the supernatant following each wash were concentrated 10-fold by TCA precipitation before solubilization in SDS-PAGE sample buffer. Proteins were separated by 9% SDS-PAGE before Western blotting with anti-VacA serum 929. Lanes 1–4, supernatants from sequential washes 1–4, respectively; adsorbed cells prior to (Pre) and following (Post) washing are shown for comparison. (b)–(d) *H. pylori* 60190v1 *vacA*⁻ mutant cells were incubated in spent supernatants from different VacA variant-producing strains (variant shown on left) prior to sequential washing with 0.5 M or 1 M NaCl (b), 0.25 M and 0.5 M urea (c) or 0.025% and 0.05% Triton X-100 (d). Proteins in the wash supernatants were concentrated by TCA precipitation before solubilization in SDS-PAGE sample buffer and cells were harvested directly into SDS-PAGE sample buffer prior to separation of proteins by 9% SDS-PAGE and analysis by Western blotting with anti-VacA serum 929. VacA-adsorbed cells prior to (Pre) and following (Post) washing are shown for comparison. The ~90 kDa VacA form is shown.

Together, these results suggest that if VacA adsorbs to bacteria via a specific receptor, the receptor is shared by different bacterial species and is resistant to digestion by trypsin.

Fig. 5. Pre-treatment of *H. pylori* cells with trypsin does not prevent VacA adsorption. *H. pylori* strain 60190v1 cells (lacking VacA) were incubated in the presence (+) or absence (–) of 250 µg trypsin ml^{–1} (as indicated above blot) for 2 h at 37 °C with shaking in a gas jar with CampyPak Plus, before addition of soybean trypsin inhibitor to 1.25 mg ml^{–1}. Cells were then incubated in supernatant containing (+) or lacking (–) VacA subtype s1/m1 (as indicated below blot) for 45 min in the same way. Cells were briefly washed with culture medium following adsorption and harvested into SDS-PAGE sample buffer (WC). Proteins were separated by 9% SDS-PAGE prior to Western blotting with anti-VacA serum 929. The ~90 kDa VacA form is shown.

VacA association with bacterial membranes is independent of VacA subtype

Although prior treatment of bacterial cells with trypsin did not reduce VacA adsorption, suggesting that protein–protein interactions were not involved, the possibility remains that a specific receptor (which is resistant to trypsin cleavage) is involved. Since the mid-region of VacA has been shown to direct binding to specific eukaryotic receptors (Garner & Cover, 1996), a specific isogenic construct that generated s1/m2 VacA rather than s1/m1 VacA was employed (60190/M2) to determine whether this region was also involved in bacterial membrane interactions. Analysis of *H. pylori* strains 60190 and 60190/M2 revealed that the processed form of VacA was present in whole-cell lysates from both strains, showing that the ability of mature VacA to associate with the bacterial cell surface was shared by *H. pylori* strains producing the s1/m2 and s1/m1 subtypes. This finding was confirmed using *H. pylori* strains that naturally produce s1/m2 VacA (data not shown). Both VacA subtypes were capable of adsorbing to the bacteria post-secretion, and the adsorbed toxin was generally resistant to removal by agents that disrupt the membrane or electrostatic and hydrophobic interactions (Fig. 4). In addition, the adsorption to the *E. coli* LPS mutant (*rfa-1*, *rfa-31*) was independent of the mid-region type (Fig. 6). Thus interaction with bacterial membranes does not occur through known VacA receptor-binding mechanisms.

The N-terminus of VacA can be naturally hydrophobic (s1 subtype) or hydrophilic (s2 subtype). To address the possibility that the hydrophobic N-terminus of p37 was involved in bacterial cell adsorption, a form of VacA with a hydrophilic N-terminus was analysed to determine its ability to adsorb to bacterial cell surfaces. This extension has previously been shown to disrupt vacuolation (Letley &

Atherton, 2000; Letley *et al.*, 2003); therefore, if the N-terminus of VacA is involved in binding to bacterial cells, it would be predicted that addition of the hydrophilic extension would at least partially disrupt this interaction. Like strain 60190 (producing s1/m1 VacA), whole cells of the isogenic strain producing s2/m1 VacA (60190/P2S2) bore processed VacA on their surface (data not shown). In addition, the s2/m1 VacA could adsorb tightly to 60190v1 (Fig. 4) and LPS mutants (Fig. 6) of *H. pylori* (*rfaM*[–] and *galE*[–]) and *E. coli* (*rfa-1*, *rfa-31*). These findings were confirmed with *H. pylori* strains producing VacA with a natural hydrophilic N-terminus, and together these data suggested that membrane insertion is unlikely to be mediated through the extreme N-terminus of VacA.

Surface-associated VacA does not cause vacuolation in RK13 cells

VacA affects eukaryotic cells in many ways (Galmiche *et al.*, 2000; Kimura *et al.*, 1999; Kuck *et al.*, 2001; Molinari *et al.*, 1998a; Papini *et al.*, 1998; Pelicic *et al.*, 1999), but perhaps the most striking effect is the induction of large cytoplasmic vacuoles (Cover & Blaser, 1992). Bacterial cell-associated VacA has recently been reported to be capable of inducing vacuolation (Ilver *et al.*, 2004); however, these assays were performed with bacterial cells retaining the ability to secrete VacA. We therefore investigated whether adsorbed VacA (attached to the cell surface of an *H. pylori* strain incapable of *de novo* synthesis and secretion of VacA) was as capable as free VacA to induce vacuolation of eukaryotic cells. To do this, RK13 cells were co-cultured with *H. pylori* strain 60190v1 cells that had undergone prior incubation with culture supernatants containing VacA. The results of assays with this rabbit kidney epithelial cell line are presented in preference to those obtained with other cell lines, even though similar results were obtained, because the gastric cell line AGS requires comparatively higher levels of VacA to induce visible vacuolation and HeLa cells respond selectively to m1 and not m2 subtype (Pagliaccia *et al.*, 1998). Extensive vacuolation of RK13 cells occurred when incubated with either spent culture supernatant isolated from *H. pylori* strain 60190 or 60190 cells themselves (Fig. 7a, d); however, none was evident following incubation with *H. pylori* 60190v1 cells or culture supernatant (Fig. 7b, e). The minimum concentration of free VacA required to induce vacuolation was determined by serial dilution (data not shown). The amount of VacA of subtype s1/m1 adsorbed to *H. pylori* strain 60190v1 shown in Fig. 7(f) was equivalent to the amount of VacA of subtype s1/m1 required to induce vacuolation in 50 % of the RK13 cells. Thus despite provision of concentrations sufficient for soluble VacA to induce vacuolation, VacA adsorbed to *H. pylori* strain 60190v1 did not induce vacuolation of RK13 cells (Fig. 7c). Similar results were obtained with Hep-2 cells and acid-activated VacA (data not shown). (It should be noted that acid activation is not required for vacuolation when VacA is delivered from intact bacterial cells.)

Fig. 6. VacA is associated with the surface of LPS mutants of *H. pylori* and adsorbs to the surface of LPS mutants of *E. coli*. (a) Schematic representation of the LPS structure of the *H. pylori* wild-type strain NCTC 11637. The position beyond which sugars are absent in the *H. pylori* *rfbM*⁻ mutant (Edwards *et al.*, 2000) is marked with a solid line and with a dotted line for the *H. pylori* *galE*⁻ mutant (Edwards *et al.*, 2000). Additional sugars present in the *H. pylori* *galE*⁻ mutant are shown in boxes surrounded by broken lines (adapted from Edwards *et al.*, 2000). KDO, 2-keto-3-deoxyoctonic acid; Hep, heptose; P, phosphate; Gal, galactose; Glc, glucose; Fuc, fucose; GlcNac, *N*-acetylglucosamine; LacNac, *N*-acetylglucosamine. (b) *H. pylori* strains *rfbM*⁻ and *galE*⁻ were grown for 48 h in BHI plus 0.2% cyclodextrin media with shaking in a gas jar with CampyPak Plus at 37 °C. Cells were washed briefly in culture medium before being harvested into SDS-PAGE sample buffer (WC). Culture supernatant samples were also taken and the proteins were concentrated by TCA precipitation before solubilization in SDS-PAGE sample buffer (SN). Proteins were separated through 9% SDS-PAGE prior to analysis by Western blotting with anti-VacA serum 929. (c) Schematic representation of the LPS structure of the *E. coli* wild-type strain D21. The position beyond which sugars are absent in the *E. coli* *rfa*⁻ strain D21f2 is marked with the broken line (adapted from Boman & Monner, 1975). KDO, 2-keto-3-deoxyoctonic acid; Hep, heptose; Glc, glucose; Gal, galactose; Rha, rhamnose. (d) Spent supernatants from *H. pylori* strains producing different VacA variants [*H. pylori* strains 60190 (s1/m1), 60190/M2 (s1/m2), Tx30a (s2/m2) and 60190/P2S2 (s2/m1) shown on left] were diluted 1:2. *E. coli* parent strain D21 and LPS mutant strain D21f2 cells were resuspended in the neat and diluted supernatants (as indicated) and incubated for 45 min with shaking at 37 °C. Cells were washed briefly in culture medium and harvested into SDS-PAGE sample buffer. Proteins were separated by 9% SDS-PAGE prior to analysis by Western blotting, using the anti-VacA serum 929. The ~90 kDa VacA form is shown.

DISCUSSION

In the present study, we showed that mature VacA adsorbs to bacterial cell surfaces post-secretion. The interaction of VacA with bacterial membranes appeared to be tight, intimate and not adversely affected by the removal of surface proteins or alterations in the structure of LPS. Furthermore, this interaction was independent of VacA subtype (s1b/m1, s2/m1, s1a/m2, s1b/m2, s2/m2), suggesting that it is not mediated through a specific mid-region sequence binding to a known receptor or via the hydrophobic N-terminus. It seems, therefore, that VacA is most likely to insert directly into the

lipid leaflet of the outer membrane, a conclusion supported by insertion of VacA into artificial lipid bilayers (Adrian *et al.*, 2002; Czajkowsky *et al.*, 1999; Molinari *et al.*, 1998a; Moll *et al.*, 1995; Pagliaccia *et al.*, 2000; Tombola *et al.*, 1999) and modulation of the fluorescence of fluorescein phosphatidylethanolamine (FPE)-labelled bacterial cells by the p37 domain of VacA (Fitchen *et al.*, 2003).

Comparison of the interaction of p37 with FPE-labelled artificial lipid bilayers or FPE-labelled bacteria cells (Fitchen *et al.*, 2003) suggested the existence of a bacterial factor that assisted VacA adsorption to bacterial cell surfaces. As VacA

Fig. 7. *H. pylori* cell-surface adsorbed VacA does not vacuolate RK13 cells. VacA⁻ 60190v1 mutant cells were incubated in the spent supernatant from the wild-type s1/m1-producing strain 60190 to adsorb VacA to the bacterial surface. (a)–(c) Vacuolation assay using RK13 cells. Bacteria were resuspended in DMEM to an OD₆₀₀ of 1 and serially diluted. Each concentration of bacteria was added to RK13 cells at a 1 : 5 dilution and several concentrations of each bacterial strain were used. Supernatants were treated in the same way. RK13 cells co-cultured overnight at 37 °C, 5 % CO₂, with 60190 wild-type bacteria (a), 60190v1 vacA⁻ mutant strain prior to VacA adsorption (b) or 60190v1 cells with adsorbed VacA (c). (d, e) RK13 cells cultured with 60190 supernatant (d) or 60190v1 supernatant (e). (f) Western blot of proteins separated by 9 % SDS-PAGE and probed with the anti-VacA serum 929. Lane 1, cell sonicate of wild-type 60190; lanes 2 and 3, cell sonicates of 60190v1 vacA⁻ mutant strain prior to and following VacA adsorption, respectively. The ~90 kDa VacA form is shown. Examples of vacuoles produced following incubation with 60190 cells or supernatant are marked with arrows.

adsorbed to both *E. coli* and *H. pylori* cells in the study described here, a component common to both is likely to be involved in this binding. However, the bacterial factor implicated in VacA binding to *H. pylori* cells appears to be distinct from trypsin-accessible surface proteins. VacA adsorption to cells with altered LPS structure indicates that if this interaction occurs via LPS, it must be mediated by the lipid A core. Any contribution of the O-polysaccharide is unlikely, as VacA adsorbed to the *H. pylori* *rfbM*⁻ mutant (which produces an S-form of LPS lacking only fucose) and also to the *H. pylori* *galE*⁻ mutant (which produces an R-form of LPS).

If VacA recognizes a specific bacterial receptor, this recognition must be mediated by a region of VacA conserved among the different subtypes, since all were similarly able to exhibit this ability. It is unlikely to be mediated through a specific sequence within the mid-region that has previously been implicated in binding to eukaryotic cell receptors (Ji *et al.*, 2000), as this differed among VacA subtypes. Many of the studies characterizing the interaction of VacA with eukaryotic cells have demonstrated a reduced effect with a VacA mutant devoid of amino acids 6–27 of the passenger domain, suggesting a role for this hydrophobic region (Czajkowsky *et*

al., 1999; Galmiche *et al.*, 2000; Li *et al.*, 2004; Willhite *et al.*, 2003; Vinion-Dubiel *et al.*, 1999; McClain *et al.*, 2003; Sundrud *et al.*, 2004; Szabó *et al.*, 1999). Structural modelling has revealed that this domain is strongly reminiscent of the structure of the prokaryotic anion-selective channel MscS (Kim *et al.*, 2004). This link has been suggested to provide an explanation for the ability of VacA to interact with the membranes of mitochondria and induce apoptosis via cytochrome *c* release (Galmiche *et al.*, 2000; Willhite *et al.*, 2003). Since the mitochondrial membrane is more closely related to prokaryotic than eukaryotic membranes, it would therefore be plausible that the extreme N-terminus of VacA may mediate interaction with the bacterial cell surface. However, this appears questionable, as the interaction was not inhibited by addition of a hydrophilic extension that has previously been shown to inhibit the ability of VacA to cause vacuolation in eukaryotic cells (Letley & Atherton, 2000; Letley *et al.*, 2003).

Within eukaryotic and artificial membranes, VacA is capable of forming channels (Gebert *et al.*, 2004) and it is possible that this also occurs when VacA is adsorbed to bacterial cell surfaces. Indeed, this might represent a mechanism to promote autolysis, a phenomenon proposed to result in

the release of proteins such as urease from *H. pylori* cells (Schraw *et al.*, 1999) and suggested to facilitate the persistence of *H. pylori* in the human gastric mucus layer, although such lysis was not observed in these studies.

Whatever the precise mechanism of interaction, VacA bound to *E. coli* is subject to degradation. This degradation was achieved by cell-surface proteases distinct from OmpT. One such protease is DegP, and it is interesting that proteolytic degradation of VacA was not shared by all strains (for example, *E. coli* strain DL21 did not degrade adsorbed VacA). The identity of the protease involved is currently under investigation. The low levels of extracellular VacA present in the *rfbM*⁻ mutant of *H. pylori* may also indicate an increase in VacA degradation in this strain, but the basis of this has not yet been investigated.

Our demonstration that bacterial-adsorbed VacA could not induce vacuolation in eukaryotic cells is at odds with the demonstration of vacuolation using VacA associated with membrane blebs (Keenan *et al.*, 2000), the uptake of membrane blebs with their associated VacA by the gastric epithelium (Fiocca *et al.*, 1999) and previous descriptions of contact-dependent delivery from *H. pylori* (Ilver *et al.*, 2004). Blebs appear to contain VacA within them and not solely on the surface, and it is possible that it is this form that is delivered in an active form to the eukaryotic cell, particularly since bleb-associated VacA maintains its β -barrel translocator domain (Fiocca *et al.*, 1999). Likewise, the latter study by Ilver *et al.* (2004) utilized *H. pylori* cells with an intact *vacA*, which were therefore capable of secreting VacA while attached to the eukaryotic cells. Although they were able to demonstrate that VacA was only delivered to cells in direct contact with *H. pylori*, and used this as evidence to discount the possibility that soluble VacA was being made, the possibility remains that the amount of soluble VacA delivered to neighbouring cells was below the levels detectable by immunofluorescence. The study by Ilver *et al.* (2004) does, however, suggest that cell-surface-attached VacA can be delivered to the eukaryotic cell, since they were able to track its progress over time. In our assay, the use of *H. pylori* strain 60190v1, which is devoid of a functional *vacA* gene, removed the possibility that there was any secreted VacA. However, it is possible that the levels of bacterial-associated VacA required to induce vacuolation are higher than those of soluble VacA or that the VacA adsorbed to the bacterial cells was not efficiently delivered.

There is much discussion regarding the most physiologically relevant effects of VacA (secreted, cell-bound, allelic subtype). Likewise, the identification of the effects most intricately linked with disease progression (vacuolation, induction of apoptosis, depolarization of cellular membrane potential, permeabilization of epithelial monolayers, detachment of epithelial cells from the basement membrane, interference with antigen presentation, inhibition of T-cell proliferation) remains unknown. This uncertainty arises partly because many strains harbour a non-toxicogenic (non-vacuolating) VacA subtype (s2/m2) and yet persist within the

host for many years, merely inflicting mild gastritis. All VacA subtypes retain a similar capacity for bacterial-surface adsorption. Thus perhaps study of the bacterial surface-adsorbed VacA will reveal an alternative function of non-toxicogenic forms that is advantageous for the survival of *H. pylori* in its gastric niche.

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REFERENCES

- Adrian, M., Cover, T. L., Dubochet, J. & Heuser, J. E. (2002). Multiple oligomeric states of the *Helicobacter pylori* vacuolating toxin demonstrated by cryo-electron microscopy. *J Mol Biol* **318**, 121–133.
- 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*. Association of specific *vacA* types with cytotoxin production and peptic ulceration. *J Biol Chem* **270**, 17771–17777.
- 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.
- Boman, H. G. & Monner, D. A. (1975). Characterization of lipopolysaccharides from *Escherichia coli* K-12 mutants. *J Bacteriol* **121**, 455–464.
- Boman, H. G., Eriksson-Grennberg, K. G., Normark, S. & Matsson, E. (1968). Resistance of *Escherichia coli* to penicillins. IV. Genetic study of mutants resistant to D,L-ampicillin concentrations of 100 $\mu\text{g ml}^{-1}$. *Genet Res* **12**, 169–185.
- Casadaban, M. J. (1976). Transposition and fusion of the *lac* genes to selected promoters in *Escherichia coli* using bacteriophage lambda and Mu. *J Mol Biol* **104**, 541–555.
- Cover, T. L. (1996). The vacuolating cytotoxin of *Helicobacter pylori*. *Mol Microbiol* **20**, 241–246.
- Cover, T. L. & Blaser, M. J. (1992). Purification and characterization of the vacuolating toxin from *Helicobacter pylori*. *J Biol Chem* **267**, 10570–10575.
- 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. *J Cell Biol* **138**, 759–769.
- 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.
- Edwards, N. J., Monteiro, M. A., Faller, G., Walsh, E. J., Moran, A. P., Roberts, I. S. & High, N. J. (2000). Lewis X structures in the O antigen side-chain promote adhesion of *Helicobacter pylori* to the gastric epithelium. *Mol Microbiol* **35**, 1530–1539.
- 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**, 615–640.
- 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. *J Pathol* **188**, 220–226.

- Fitch, N., O'Shea, P., Williams, P. & Hardie, K. R. (2003). Electrostatic sensor for identifying interactions between peptides and bacterial membranes. *Mol Immunol* **40**, 407–411.
- Fujikawa, A., Shirasaka, D., Yamamoto, S. & 9 other authors (2003). Mice deficient in protein tyrosine phosphatase receptor type Z are resistant to gastric ulcer induction by VacA of *Helicobacter pylori*. *Nat Genet* **33**, 375–381.
- Galmiche, A., Rassow, J., Doye, A. & 9 other authors (2000). The N-terminal 34 kDa fragment of *Helicobacter pylori* vacuolating cytotoxin targets mitochondria and induces cytochrome *c* release. *EMBO J* **19**, 6361–6370.
- Garner, J. A. & Cover, T. L. (1996). Binding and internalization of the *Helicobacter pylori* vacuolating cytotoxin by epithelial cells. *Infect Immun* **64**, 4197–4203.
- Gauthier, N. C., Ricci, V., Gounon, P., Doye, A., Tauc, M., Poujeol, P. & Boquet, P. (2004). Glycosylphosphatidylinositol-anchored proteins and actin cytoskeleton modulate chloride transport by channels formed by the *Helicobacter pylori* vacuolating cytotoxin VacA in HeLa cells. *J Biol Chem* **279**, 9481–9489.
- Gebert, B., Fischer, W. & Haas, R. (2004). The *Helicobacter pylori* vacuolating cytotoxin: from cellular vacuolation to immunosuppressive activities. *Rev Physiol Biochem Pharmacol* **152**, 205–220.
- Geisse, N. A., Cover, T. L., Henderson, R. M. & Edwardson, J. M. (2004). Targeting of *Helicobacter pylori* vacuolating toxin to lipid raft membrane domains analysed by atomic force microscopy. *Biochem J* **381**, 911–917.
- Hardie, K. R., Cooksley, C., Green, A. D. & Winzer, K. (2003). Autoinducer 2 activity in *Escherichia coli* culture supernatants can be actively reduced despite maintenance of an active synthase, LuxS. *Microbiology* **149**, 715–728.
- Henderson, I. R., Navarro-Garcia, F. & Nataro, J. P. (1998). The great escape: structure and function of the autotransporter proteins. *Trends Microbiol* **6**, 370–378.
- Henderson, I. R., Cappello, R. & Nataro, J. P. (2000). Autotransporter proteins, evolution and redefining protein secretion. *Trends Microbiol* **8**, 529–532.
- 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**, 167–174.
- Ji, X., Fernandez, T., Burroni, D., Pagliaccia, C., Atherton, J. C., Reyat, J. M., Rappuoli, R. & Telford, J. L. (2000). Cell specificity of *Helicobacter pylori* cytotoxin is determined by a short region in the polymorphic midregion. *Infect Immun* **68**, 3754–3757.
- 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**, 259–264.
- 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.
- Kim, S., Chamberlain, A. K. & Bowie, J. U. (2004). Membrane channel structure of *Helicobacter pylori* vacuolating toxin: role of multiple GXXXG motifs in cylindrical channels. *Proc Natl Acad Sci U S A* **101**, 5988–5991.
- Kimura, M., Goto, S., Wada, A., Yahiro, K., Niidome, T., Hatakeyama, T., Aoyagi, H., Hirayama, T. & Kondo, T. (1999). Vacuolating cytotoxin purified from *Helicobacter pylori* causes mitochondrial damage in human gastric cells. *Microb Pathog* **26**, 45–52.
- Kuck, D., Kolmerer, B., Iking-Konert, C., Krammer, P. H., Stremmel, W. & Rudi, J. (2001). Vacuolating cytotoxin of *Helicobacter pylori* induces apoptosis in the human gastric epithelial cell line AGS. *Infect Immun* **69**, 5080–5087.
- Kukkonen, M. & Korhonen, T. K. (2004). The omptin family of enterobacterial surface proteases/adhesins: from housekeeping in *Escherichia coli* to systemic spread of *Yersinia pestis*. *Int J Med Microbiol* **294**, 7–14.
- Letley, D. P. & Atherton, J. C. (2000). Natural diversity in the N terminus of the mature vacuolating cytotoxin of *Helicobacter pylori* determines cytotoxin activity. *J Bacteriol* **182**, 3278–3280.
- 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. *J Clin Microbiol* **37**, 1203–1205.
- 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.
- 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**, 93–99.
- Li, Y., Wandering-Ness, A., Goldenring, J. R. & Cover, T. L. (2004). Clustering and redistribution of late endocytic compartments in response to *Helicobacter pylori* vacuolating toxin. *Mol Biol Cell* **15**, 1946–1959.
- 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. *J Cell Biol* **133**, 801–807.
- McClain, M. S., Iwamoto, H., Cao, P., Vinion-Dubiel, A. D., Li, Y., Szabo, G., Shao, Z. & Cover, T. L. (2003). Essential role of a GXXXG motif for membrane channel formation by *Helicobacter pylori* vacuolating toxin. *J Biol Chem* **278**, 12101–12108.
- 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**, 2889–2898.
- Merrell, D. S. & Falkow, S. (2004). Frontal and stealth attack strategies in microbial pathogenesis. *Nature* **430**, 250–256.
- Miehlke, S., Kirsch, C., Agha-Amiri, K., Gunther, T., Lehn, N., Malfertheiner, P., Stolte, M., Ehninger, G. & Bayerdorffer, E. (2000). The *Helicobacter pylori* *vacA* s1, m1 genotype and *cagA* is associated with gastric carcinoma in Germany. *Int J Cancer* **87**, 322–327.
- Molinari, M., Galli, C., Norais, N., Telford, J., 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**, 25339–25344.
- Molinari, M., Salio, M., Galli, C., Norais, N., Rappuoli, R., Lanzavecchia, A. & Montecucco, C. (1998a). Selective inhibition of Ii-dependent antigen presentation by *Helicobacter pylori* toxin VacA. *J Exp Med* **187**, 135–140.
- Molinari, M., Galli, C., de Bernard, M., Norais, N., Ruyschaert, J.-M., Rappuoli, R. & Montecucco, C. (1998b). The acid activation of *Helicobacter pylori* toxin VacA: structural and membrane binding studies. *Biochem Biophys Res Comm* **248**, 334–340.
- Moll, G., Papini, E., Colonna, R., Burroni, D., Telford, J., Rappuoli, R. & Montecucco, C. (1995). Lipid interaction of the 37-kDa and 58-kDa fragments of the *Helicobacter pylori* cytotoxin. *Eur J Biochem* **234**, 947–952.
- Oomen, C. J., Van Ulsen, P., Van Gelder, P., Feijen, M., Tommassen, J. & Gros, P. (2004). Structure of the translocator domain of a bacterial autotransporter. *EMBO J* **23**, 1257–1266.
- Pagliaccia, C., de Bernard, M., Lupetti, P. & 7 other authors (1998). The m2 form of the *Helicobacter pylori* cytotoxin has cell type-specific vacuolating activity. *Proc Natl Acad Sci U S A* **95**, 10212–10217.

- Pagliaccia, C., Wang, X.-M., Tardy, F., Telford, J. L., Ruyschaert, J.-M. & Cabiliaux, V. (2000). Structure and interaction of VacA of *Helicobacter pylori* with a lipid membrane. *Eur J Biochem* **267**, 104–109.
- Papini, E., Satin, B., Norais, N., de Bernard, M., Telford, J. L., Rappuoli, R. & Montecucco, C. (1998). Selective increase of the permeability of polarized epithelial cell monolayers by *Helicobacter pylori* vacuolating toxin. *J Clin Invest* **102**, 813–820.
- Patel, H. K., Willhite, D. C., Patel, R. M., Ye, D., Williams, C. L., Torres, E. M., Marty, K. B., MacDonald, R. A. & Blanke, S. R. (2002). Plasma membrane cholesterol modulates cellular vacuolation induced by the *Helicobacter pylori* vacuolating cytotoxin. *Infect Immun* **70**, 4112–4123.
- Pellicic, V., Reytrat, J.-M., Sartori, L., Pagliaccia, C., Rappuoli, R., Telford, J. L., Montecucco, C. & Papini, E. (1999). *Helicobacter pylori* VacA cytotoxin associated with the bacteria increases epithelial permeability independently of its vacuolating activity. *Microbiology* **145**, 2043–2050.
- 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. *Infect Immun* **69**, 730–736.
- Schmitt, W. & Haas, R. (1994). Genetic analysis of the *Helicobacter pylori* vacuolating cytotoxin: structural similarities with the IgA protease type of exported protein. *Mol Microbiol* **12**, 307–319.
- Schraw, W., McClain, M. S. & Cover, T. L. (1999). Kinetics and mechanisms of extracellular protein release by *Helicobacter pylori*. *Infect Immun* **67**, 5247–5252.
- Schraw, W., Li, Y., McClain, M. S., van der Goot, F. G. & Cover, T. L. (2002). Association of *Helicobacter pylori* vacuolating toxin (VacA) with lipid rafts. *J Biol Chem* **277**, 34642–34650.
- Seto, K., Hayashi-Kuwabara, Y., Yoneta, T., Suda, H. & Tamaki, H. (1998). Vacuolation induced by cytotoxin from *Helicobacter pylori* is mediated by the EGF receptor in HeLa cells. *FEBS Lett* **431**, 347–350.
- Sijbrandi, R., Urbanus, M. L., ten Hagen-Jongman, C. M., Bernstein, H. D., Ougega, B., Otto, B. R. & Luirink, J. (2003). Signal recognition particle (SRP)-mediated targeting and Sec-dependent translocation of an extracellular *Escherichia coli* protein. *J Biol Chem* **278**, 4654–4659.
- Sundrud, M. S., Torres, V. J., Unutmaz, D. & Cover, T. L. (2004). Inhibition of primary human T cell proliferation by *Helicobacter pylori* vacuolating toxin (VacA) is independent of VacA effects on IL-2 secretion. *Proc Natl Acad Sci U S A* **101**, 7727–7732.
- Szabò, I., Brutsche, S., Tombola, F. & 7 other authors (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**, 5517–5527.
- Telford, J. L., Ghiara, P., Dell'Orco, M. & 7 other authors (1994). Gene structure of the *Helicobacter pylori* cytotoxin and evidence of its key role in gastric disease. *J Exp Med* **179**, 1653–1658.
- Tombola, F., Carlesso, C., Szabò, I. & 7 other authors (1999). *Helicobacter pylori* vacuolating cytotoxin forms anion-selective channels in planar lipid bilayers: possible implications for the mechanism of cellular vacuolation. *Biophys J* **76**, 1401–1409.
- Van Doorn, L.-J., Figueiredo, C., Sanna, R., Pena, S., Midolo, P., Ng, E. K. W., Atherton, J. C., Blaser, M. J. & Quint, W. G. V. (1998). Expanding allelic diversity of *Helicobacter pylori* vacA. *J Clin Microbiol* **36**, 2597–2603.
- Veiga, E., Sugawara, E., Nikaido, H., de Lorenzo, V. & Fernandez, L. A. (2002). Export of autotransported proteins proceeds through an oligomeric ring shaped by C-terminal domains. *EMBO J* **21**, 2122–2131.
- Vinion-Dubiel, A. D., McClain, M. S., Czajkowsky, D. M. & 8 other authors (1999). A dominant negative mutant of *Helicobacter pylori* vacuolating toxin (VacA) inhibits VacA-induced cell vacuolation. *J Biol Chem* **274**, 37736–37742.
- Voulhoux, R., Bos, M. P., Geurtsen, J., Mols, M. & Tommassen, J. (2003). Role of a highly conserved bacterial protein in outer membrane protein assembly. *Science* **299**, 262–265.
- 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. *Cell Microbiol* **6**, 143–154.
- Willhite, D. C., Cover, T. L. & Blanke, S. R. (2003). Cellular vacuolation and mitochondrial cytochrome c release are independent outcomes of *Helicobacter pylori* vacuolating cytotoxin activity that are each dependent on membrane channel formation. *J Biol Chem* **278**, 48204–48209.
- Wood, W. B. (1966). Host specificity of DNA produced by *Escherichia coli*: bacterial mutation affecting the restriction and modification of DNA. *J Mol Biol* **16**, 118–133.
- Yahiro, K., Niidome, T., Kimura, M. & 7 other authors (1999). Activation of *Helicobacter pylori* VacA toxin by alkaline or acid conditions increases its binding to a 250-kDa receptor protein-tyrosine phosphatase β . *J Biol Chem* **274**, 36693–36699.
- Yahiro, K., Wada, A., Nakayama, M. & 8 other authors (2003). Protein-tyrosine phosphatase α , RPTP α , is a *Helicobacter pylori* VacA receptor. *J Biol Chem* **278**, 19183–19189.