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 *H. pylori* 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.

**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 N-terminal 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. 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
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; Pellici et al., 1999), disruption of antigen presentation (Moliniari 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 [RPTPβ (PTPrz), Fujikawa et al., 2003; Yahiro et al., 1999; RPTPα, 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; Mielikke 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 (Iver 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; Iver 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 (Iver 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 µg ml⁻¹ for kanamycin and 100 µg 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 (AvAcA; Bebb et al., 2003), rfhM⁻ (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 passed 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.

VacA adsors 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 reabsorbing 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).

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).
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; RPTPa, 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; Czajkowski 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). Pretreatment 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).

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.
Bacterial-associated VacA

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 (rfbM– 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.)
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 phosphatidyethanolamine (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...
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/C0 mutant (which produces an S-form of LPS lacking only fucose) and also to the *H. pylori* galE/C0 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/C0 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.

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; Szabò 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 \textit{H. pylori} cells (Schraw \textit{et al.}, 1999) and suggested to facilitate the persistence of \textit{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 \textit{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, \textit{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 \textit{rfBM} mutant of \textit{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 \textit{et al.}, 2000), the uptake of membrane blebs with their associated VacA by the gastric epithelium (Fiocca \textit{et al.}, 1999) and previous descriptions of contact-dependent delivery from \textit{H. pylori} (Iivering \textit{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 \beta-barrel translocator domain (Fiocca \textit{et al.}, 1999). Likewise, the study by Iivering \textit{et al.} (2004) utilized \textit{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 \textit{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 Iivering \textit{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 \textit{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 the study of the bacterial surface-adsorbed VacA will reveal an alternative function of nontoxicogenic forms that is advantageous for the survival of \textit{H. pylori} in its gastric niche.

**ACKNOWLEDGEMENTS**

Many thanks are extended to Joanne Rhead for technical assistance. In addition, we thank the \textit{E. coli} genetic stock culture and Dr N. High for strains. For funding we are grateful to the British Society of Antimicrobial Chemotherapy, the University of Nottingham, Engineering and Physical Sciences Research Council and Biotecnology and Biological Sciences Research Council, and the Medical Research Council.

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