Remodeling of the Intestinal Brush Border Underlies Adhesion and Virulence of an Enteric Pathogen

The Harvard community has made this article openly available. Please share how this access benefits you. Your story matters

Citation

Published Version
doi:10.1128/mBio.01639-14

Citable link
http://nrs.harvard.edu/urn-3:HUL.InstRepos:12785933

Terms of Use
This article was downloaded from Harvard University’s DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA
Remodeling of the Intestinal Brush Border Underlies Adhesion and Virulence of an Enteric Pathogen

Xiaohui Zhou, Ramiro H. Massol, Fumihiko Nakamura, Xiang Chen, Benjamin E. Gewurz, Brigid M. Davis, Wayne I. Lencer, Matthew K. Waldor

Division of Infectious Diseases, Brigham and Women’s Hospital, Boston, Massachusetts, USA; Department of Microbiology and Immunobiology, Harvard Medical School, Boston, Massachusetts, USA; Department of Pathobiology and Veterinary Science, University of Connecticut, Storrs, Connecticut, USA; Division of Gastroenterology, Boston Children’s Hospital, Boston, Massachusetts, USA; Department of Pediatrics, Harvard Medical School, Boston, Massachusetts, USA; Harvard Digestive Diseases Center, Boston, Massachusetts, USA; Translational Medicine Division, Department of Medicine, Brigham and Women’s Hospital, Boston, Massachusetts, USA

* Present address: Xiang Chen, Laboratory of Zoonosis, Yangzhou University, Yangzhou, China.

ABSTRACT  Intestinal colonization by *Vibrio parahaemolyticus*—the most common cause of seafood-borne bacterial enteritis worldwide—induces extensive disruption of intestinal microvilli. In orogastrically infected infant rabbits, reorganization of the apical brush border membrane includes effacement of some microvilli and marked elongation of others. All diarrhea, inflammation, and intestinal pathology associated with *V. parahaemolyticus* infection are dependent upon one of its type 3 secretion systems (T3SS2); however, translocated effectors that directly mediate brush border restructuring and bacterial adhesion are not known. Here, we demonstrate that the effector VopV is essential for *V. parahaemolyticus* intestinal colonization and therefore its pathogenicity, that it induces effacement of brush border microvilli, and that this effacement is required for adhesion of *V. parahaemolyticus* to enterocytes. VopV contains multiple functionally independent and mechanistically distinct domains through which it disrupts microvilli. We show that interaction between VopV and filamin, as well as VopV’s previously noted interaction with actin, mediates enterocyte cytoskeletal reorganization. VopV’s multipronged approach to epithelial restructuring, coupled with its impact on colonization, suggests that remodeling of the epithelial brush border is a critical step in pathogenesis.

IMPORTANCE  Colonization of the small bowel by *Vibrio parahaemolyticus*, the most common bacterial agent of seafood-borne enteric disease, induces extensive structural changes in the intestinal epithelium. Here, we show that this diarrheal pathogen’s colonization and virulence depend upon VopV, a bacterial protein that is transferred into host epithelial cells. VopV induces marked rearrangement of the apical epithelial cell membrane, including elimination of microvilli, by two means: through interaction with actin and through a previously unrecognized interaction with the actin-cross-linking protein filamin. VopV-mediated “effacement” of microvilli enables *V. parahaemolyticus* to adhere to host cells, although VopV may not directly mediate adhesion. VopV’s effects on microvillus structure and bacterial adhesion likely account for its essential role in *V. parahaemolyticus* intestinal pathogenesis. Our findings suggest a new role for filamin in brush border maintenance and raise the possibility that microvillus effacement is a common strategy among enteric pathogens for enhancing adhesion to host cells.
inject (translocate) diverse proteins into host cells (4). These “effector” proteins are known to modulate a wide variety of host cell processes, and T3SS are essential for the virulence of numerous bacterial pathogens (5).

Pathogenic isolates of *V. parahaemolyticus* encode two T3SSs, one on each chromosome (6). T3SS1, which is also present in nonpathogenic isolates of *V. parahaemolyticus*, has been linked to cytotoxicity against a variety of cultured cell lines (reviewed in reference 7); however, analyses of T3SS1-deficient strains in animal models of disease suggest that this apparatus does not play a significant role in induction of intestinal disease (2, 8, 9). In contrast, T3SS2, which is present in the genome of only a subset of (typically virulent) *V. parahaemolyticus* strains, is essential for pathogenesis in several animal models. Inactivation of T3SS2 prevents *V. parahaemolyticus* colonization of the intestines of infant rabbits and all of the associated signs of disease (e.g., diarrhea, inflammation, tissue destruction, and microvillus effacement and elongation), and it similarly reduces enterotoxicity (fluid accumulation) and tissue destruction in surgically ligated ileal loops from adult rabbits (2, 8, 9). However, despite the pivotal role of T3SS2 in disease, it is not known how this pathogen induces such marked alterations in host tissue integrity and structure. Very few of the known T3SS2 effectors make readily detectable contributions to disease, and none have been mechanistically linked to the remodeling of the enterocyte brush border (BB) (10–13). In infant rabbits, only the multifunctional effector VopZ, which inhibits activation of innate immune signaling pathways, is known to be required for colonization (11). In ligated ileal loops, an actin-binding and bundling effector, VopS, is also required for enterotoxicity and is associated with loss of villous architecture (13); however, the role of VopS in an orogastric model of infection and disease and the processes by which this actin-binding effector contributes to pathogenesis have not been defined.

Here, we have further investigated the activity of VopV, which we independently identified via analysis of T3SS2-dependent protein secretion (11), in orogastrically infected infant rabbits, in a cell culture model of the intestinal brush border, and in several assays of protein interactions. We found that VopV is essential for *V. parahaemolyticus* colonization as well as pathogenicity in infant rabbits. Strains lacking VopV fail to induce effacement or elongation of BB microvilli, both in vivo and in tissue culture models of infection. Additionally, our *in vitro* assays indicate that such VopV-mediated remodeling of the apical BB membrane is a necessary precursor to adhesion of *V. parahaemolyticus* to the brush border; however, once effacement occurs, adhesion is VopV independent, suggesting that adhesion is a multistage process. Unexpectedly, VopV can induce effacement by two distinct means: by interacting with actin or through a previously unrecognized interaction with the actin-cross-linking protein filamin. Our findings suggest a new role for filamin in brush border maintenance and raise the possibility that microvillus effacement is a common strategy among enteric pathogens for enhancing adhesion to host cells.

**RESULTS AND DISCUSSION**

The T3SS2 effector protein VopV is a 1,622-amino-acid (aa) protein that consists of an N-terminal secretion signal, a series of interspersed repeat sequences, and a short C-terminal domain (Fig. 1A). A subset of the repeat sequences (“1” in Fig. 1A) and the C terminus were reported to bind actin and to induce fluid accumulation independently in rabbit ligated ileal loops (13), but the significance of VopV in intestinal colonization and pathology in an intact functioning intestine was not assessed. We compared intestinal colonization and associated pathology for infant rabbits orogastrically inoculated with wild-type (wt) *V. parahaemolyticus*, a mutant lacking vopV (ΔvopV mutant), or mutants in which the N-terminal region of VopV (which contains its secretion signal) was either truncated at that point (VopVn mutant), followed by two repeat sequences (VopVnR mutant), or followed by the C-terminal domain (VopVnC mutant) (Fig. 1A). Deletion of vopV was associated with an ~1,000-fold reduction in intestinal colonization relative to the wt strain, minimal intestinal fluid accumulation, and no overt diarrhea (Fig. 1B and C). Scanning electron microscopy (SEM) images of intestinal tissue from rabbits infected with the ΔvopV mutant were indistinguishable from images of uninfected rabbits, in which only the highly uniform tips of microvilli were apparent (Fig. 1D). Additionally, inflammatory cell infiltration and sloughing of epithelial cells that typify *V. parahaemolyticus*-infected tissue (2) were not observed in tissue sections from rabbits infected with the vopV mutant (Fig. 1E and F).

In contrast, rabbits infected with the VopVn or VopVnC strains (but not the VopVn strain) were colonized to the same extent as those infected with wild-type *V. parahaemolyticus* and had equivalent intestinal fluid accumulation, diarrhea, inflammation, and pathological changes in intestinal tissue (Fig. 1B to F). In SEM analyses for the wt, VopVnR, and VopVnC strains, large *V. parahaemolyticus* microcolonies were visible on the intestinal surface, surrounded by and sometimes embedded within highly extended and distorted microvilli. Effacement of the intestinal BB was also apparent in SEM (Fig. 1D), as previously observed using transmission electron microscopy (TEM) (2). These observations indicate that VopV is essential for *V. parahaemolyticus* intestinal colonization and all associated pathology and that VopV contains functionally redundant regions (i.e., R and C) that mediate its effects.

To investigate how VopV promotes *V. parahaemolyticus* intestinal colonization and pathology, we infected 14-day terminally differentiated monolayers of Caco-2-BBE cells, which form a uniform, well-structured BB (14) with strains carrying wt *vopV* or the mutant alleles described above. For these experiments, all strains also lacked the key T3SS1 gene *vscN1*, in order to avoid the cytotoxicity associated with this secretion system. Strains encoding full-length VopV, VopVnC, or VopVnR all induced marked disruption of BB microvilli, including microvillus effacement and elongation similar to those observed in infected rabbits (Fig. 2A, left). In contrast, the BB of monolayers infected with *V. parahaemolyticus* strains lacking *vopV* or a functional T3SS2 (ΔvscN2 mutant) did not show formation of extended microvilli; instead, the normal microvillus structure was preserved, although a slight reduction of microvillus density was evident (Fig. 2A). Thus, the infected Caco-2-BBE monolayers generally phenocopy findings from *in vivo* studies of infected infant rabbits, thereby validating the cell line as a model system for study of *V. parahaemolyticus* pathogenicity. Visual analyses and enumeration of adherent CFU revealed that the ΔvopV and ΔvscN2 strains adhered with lower frequency (~80% less) to Caco-2-BBE monolayers than strains producing VopV, VopVnC, or VopVnR (Fig. 2A and B). Together, these observations suggest that VopV promotes *V. parahaemolyticus* intestinal colonization and virulence by enhancing the bacteria’s capacity to adhere to lumenal surfaces of enterocytes, perhaps by inducing rearrangement of the BB membrane.

To test if *V. parahaemolyticus* adhesion requires alteration of...
the microvilli’s actin cores, we pretreated Caco-2_Bbe monolayers with Jasplakinolide, a cell-permeable macrocyclic peptide that inhibits F-actin turnover and thereby stabilizes microvilli (15). Jasplakinolide pretreatment markedly reduced adhesion by all strains that normally adhere robustly to untreated monolayers (those expressing VopV, VopVnR, or VopVnC) (Fig. 2A and B). Furthermore, when bacteria of these 3 strains bound to pretreated cells, no BB effacement and microvillus elongation was evident in the associated Caco-2_Bbe cells, suggesting that *V. parahaemolyticus*-induced BB remodeling requires actin depolymerization and that such remodeling is required for robust adherence (Fig. 2A, right). Notably, Jasplakinolide pretreatment did not affect the minimal adhesion of the ΔvopV or vscN2 (T3SS2-deficient) strains (Fig. 2B); thus, the adherence of these strains is not dependent on actin-based cytoskeletal rearrangements. Interestingly, the bacteria that minimally adhered to Caco-2_Bbe in the absence of microvillus restructuring were present as single cells, rather than the bacterial clusters observed on effaced tissue. It is possible that microvillus effacement also facilitates interbacterial contacts and that such contacts promote *V. parahaemolyticus* adhesion to the intestinal surface.

We also assessed the consequences of pretreating differentiated Caco-2_Bbe monolayers with cytochalasin D, a small molecule that depolymerizes actin filaments and effaces microvilli (16), prior to infection. Cytochalasin D pretreatment slightly decreased the adherence of the VopV*, VopVnR, and VopVnC strains, all of which...
induce effacement of untreated Caco-2BBe cells, but it markedly augmented the adherence of the typically nonadherent and non-effacing ΔvopV mutant (Fig. 2B and C). Furthermore, we found that a VopV+ strain could largely rescue the adherence of the ΔvopV mutant (to >80% of wt levels) when untreated Caco-2BBe monolayers were coinfect ed with the two strains (Fig. 2D). Finally,
we found that deletion of vopV did not impair adhesion of *V. parahaemolyticus* to human microvascular epithelial cells (HMEC-1), which lack microvilli (Fig. 3A and B). These results all suggest that VopV’s key role during colonization is to efface the BB membrane, which is a prerequisite for bacterial adhesion. VopV is not required for adhesion of *V. parahaemolyticus* to host cells, although it may enhance this process, potentially along with other T3SS2 proteins. Our data also suggest that the elongated microvilli that often surround attached *V. parahaemolyticus* are dispensable for bacterial adhesion, since extensive adhesion is evident in the presence of cytochalasin, which largely prevents formation of these actin-rich structures. VopV’s role as a modulator of the brush border architecture highlights the importance of analyzing host-pathogen interactions using physiologically relevant model systems.

Due to the effect of VopV on bacterial association with host cells, we hypothesized that VopV might facilitate the transfer of other T3SS effectors. We therefore monitored the effect of VopV on translocation of T3SS2 effector-CyaA fusion proteins into differentiated Caco-2BBe monolayers. Surprisingly, we found that deletion of an essential T3SS2 component (VscN2), but not deletion of VopV alone, markedly diminished translocation of the T3SS reporter molecules VopT-CyaA and VopL-CyaA (Fig. 2E). Thus, the reduced BB effacement associated with VopV-deficient strains appears to be a direct consequence of the absence of VopV alone. These data also suggest that relatively loose adhesion of *V. parahaemolyticus* to host cells is sufficient to enable translocation of T3SS2 effectors.

We explored the molecular basis of VopV’s activity by using the full-length protein as bait in a yeast two-hybrid screen. Unex-
expectedly, this screen did not detect an interaction between VopV and actin; however, it yielded >30 distinct hits to filamins A, B, and C, which are members of a family of large actin-binding proteins that promote orthogonal actin branching (17) and were not previously known to bind VopV. The N-terminal region of filamin binds actin, while the remainder of the protein, which consists largely of 24 repeat units, has numerous interaction partners, including factors that modulate cell adhesion, receptors, channels, and intracellular signaling molecules (18). Affinity purification of VopV, followed by mass spectrometry-based identification of interacting proteins in eukaryotic cell lysates, confirmed that all 3 filamin isoforms interact with VopV. Additionally, it revealed that filamins bind the C-terminal 200 aa of VopV (VopVC) but not a subset of VopV’s repeat sequences (repeats 1 and 2, henceforth termed VopVR) (Fig. 4A). In contrast, our copurification studies suggest that actin binds VopVR (as previously observed [13]) but not VopVC. It is not clear why our results regarding actin binding of the C terminus of VopV differ from those of a previous report (13). Finally, pulldown studies using purified His-tagged filamin A (FLNA) repeats 1 to 8, 8 to 15, and 16 to 24 revealed that VopVC directly binds protein fragments containing repeats 8 to 15 and 16 to 24 and that this binding is dependent upon two bioinformatically predicted filamin-binding domains (FBDs) (Fig. 1A and 4B, C, and D). The FBDs are also required for colocalization of filamin and VopVC in transfected HMEC-1 cells, which was investigated using immunofluorescence microscopy (Fig. 4D). Colocalization was explored in HMEC-1 cells because we were unable to transfec
t fully differentiated Caco-2ＢBe cells.

Infection analyses, using both infant rabbits and differentiated Caco-2ＢBe cells, revealed that VopV’s FBDs are important for the effector’s activity against host cells. When the FBDs were absent from VopV’s C terminus (VopVnCΔFBD), *V. parahaemolyticus* could not colonize infant rabbits (Fig. 1B) and did not induce intestinal fluid accumulation or disrupt intestinal microvilli (Fig. 1C and D). A strain producing VopVnCΔFBD also displayed markedly reduced adhesion to differentiated Caco-2ＢBe cells, comparable to that exhibited by the T3SS2- and VopV-deficient strains (Fig. 2B). Furthermore, immunostaining of infected Caco-2ＢBe cells revealed that the FBDs contribute to the cytoskeletal alterations induced by infection with *V. parahaemolyticus*. Differen
tiated monolayers infected with a T3SS2-deficient strain or the VopVnCΔFBD strain or mock infected retained a uniform surface of actin-rich microvilli, and an FLNA-rich zone was often present immediately below. In contrast, actin in monolayers infected with adherent strains (VopV+, VopVnR, and VopVnC) was disorganized, and an underlying filamin layer was rarely evident (Fig. 5). These data provide additional evidence that VopV enables *V. parahaemolyticus* to remodel the host cell cytoskeleton via binding to filamin as well as to actin. Interactions between effec
tors and filamin have been observed for *Salmonella* and atypical *V. cholerae* but have not previously been shown to modulate BB dynamics (19, 20).

Finally, we used SEM to assess the consequences of VopV on the eukaryotic cell cytokoskeleton in the absence of other T3SS2 proteins. For these experiments, VopVC, VopVR, and VopVCAFBDB were each transfected into HMEC-1 cells, which lack microvilli; consequently, we could investigate formation of elongated microvilli but not VopV’s sufficiency to induce microvillus effacement. Notably, VopVC and VopVR both induced formation of elongated microvillus-like appendages reminiscent of those seen *in vivo* and on differentiated Caco-2ＢBe cells after *V. parahaemolyticus* infection. In contrast, VopVCAFBDB did not alter HMEC-1 surfaces; VopVCAFBDB-transfected cells, like untransfected cells, had smooth cell membranes (Fig. 3B). Thus, even in the absence of other T3SS2-secreted factors, VopV’s actin and filamin-binding activities are each sufficient to induce actin-based remodeling of the host cell surface, likely by directly
In summary, we demonstrate that VopV is essential for *V. parahaemolyticus* to colonize the intestine and induce diarrhea and histopathological evidence of infection/disease. Differentiated Caco-2BB monolayers infected with *V. parahaemolyticus* likewise demonstrate VopV-dependent microvillus effacement and elongation. *V. parahaemolyticus* lacking VopV does not adhere to differentiated Caco-2BBW cells, which likely underlies the mutant’s marked attenuation in vivo. VopV does not appear to be critical as a direct mediator of adhesion; instead, our data suggest that VopV’s primary activity is to promote BB rearrangement, through its binding to actin and filamin, and that the resulting effacement is a necessary precursor to bacterial adhesion and pathogenesis. Whether effacement merely provides an altered surface for adhesion, or exposes membrane domains and/or a specific host receptor that the pathogen does not have access to in the intact BB, remains to be determined. Additional studies to define *V. parahaemolyticus* factors that directly mediate bacterial adhesion are also warranted.

VopV’s actin-binding repeats and its filamin-binding C terminus are functionally redundant, in that translocation of either by *V. parahaemolyticus* is sufficient for BB remodeling, enterocyte adherence, intestinal colonization, and virulence. By interacting with the highly networked protein filamin as well as actin, VopV likely provides *V. parahaemolyticus* with greater scope to modulate host cell processes linked to the actin cytoskeleton. The T3SS2 effectors VopL and VopC have also been reported to influence (either directly or indirectly) the actin-based cytoskeleton, although unlike VopV, these factors are not required for *V. parahaemolyticus* pathogenicity (10–12, 21). Determining if and how the activities of these 3 effectors are coordinated will be an interesting challenge for future research. Further studies of cytoskeletal rearrangement may also provide more understanding of the role of extended microvilli in *V. parahaemolyticus*-associated disease.

*V. parahaemolyticus*’ remodeling of the enterocyte BB is reminiscent of, but not identical to, the effects of traditional attaching and effacing (A/E) pathogens, e.g., EHEC, EPEC, and *Citrobacter rodentium*, which all induce effacement of microvilli underneath host cell-associated bacteria (1, 22, 23). However, *V. parahaemolyticus*, unlike the A/E pathogens, does not induce formation of actin-rich pedestals in host cells, nor does it induce the extensive bacterium-host cell contact that A/E pathogens trigger via binding of the bacterial protein intimin and its translocated receptor, Tir. Instead, *V. parahaemolyticus* appears to form relatively limited contacts with host cells, and interbacterial contacts are more evident than with A/E pathogens. Our analyses also suggest that VopV plays a more pivotal role in effacement than any of the effacement-linked effectors in A/E pathogens (e.g., Map, EspF, Tir), which are collectively, rather than individually, required (24, 25). Notably, VopV has no homology to any of these other effectors, suggesting that the various species have independently evolved means to alter the host BB. At least in vitro, neither the absence of VopV nor of EPEC effacement-mediating effectors (25) reduces effector translocation overall, despite the marked effects of their absences on bacterial adhesion to host cells; thus, the phenotypes associated with these mutants can be attributed to the absence of specific effectors in host cells rather than a general translocation deficiency. In vivo, the failure of VopV-deficient *V. parahaemolyticus* to colonize the intestine, like that seen for EHEC with impaired effacement (e.g., *tir* mutants) (26), suggests that disruption of the enterocyte BB is a key step underlying attachment and pathogenicity of noninvasive T3SS-bearing enteric pathogens.

**FIG 5** Infected Caco-2BBW cells were stained with phalloidin (to detect actin; red), anti-filamin A (green), and DAPI (to detect Caco-2 and bacterial DNA; blue) and visualized using confocal microscopy. Views show the *xz* (left) and *xy* projection views (rest). For selected regions of the *xy* projection (stippled-line squares), actin and DNA (first cropped image) and filamin alone (last cropped image) are shown. Scale bar, 10 μm. Mutant alleles of vopV are described in the legend to Fig. 1. Strains also contained mutations in vscN1 to avoid the cytotoxic effects of T3SS1.

(VopVR) or indirectly (via filamin; VopVC) altering actin dynamics.

In summary, we demonstrate that VopV is essential for *V. parahaemolyticus* to colonize the intestine and induce diarrhea and histopathological evidence of infection/disease. Differentiated Caco-2BB monolayers infected with *V. parahaemolyticus*
MATERIALS AND METHODS

Bacterial strains and plasmids. All strains are derived from RIMD2210633 (6). The vscN1 and vscN2 strains have been described previously (9). Additional mutants, including vopV, vopVnR, vopVnC, and vopVnCDΔFBDtox were created, as described in reference 27. Plasmid pVopL-CyaA was created by cloning the first 133 codons of VopL in frame with cyaA in pMMB207 (28), and pVopT-CyaA, a similar pMMB207 derivative, was described in reference 29. Expression constructs for glutathione S-transferase (GST)-tagged VopVR or VopVC were generated from pGEX4T1 (GE Healthcare). The expression constructs for His-tagged FLNA fragments are described in reference 30.

Rabbit infections. Infant rabbits were infected as previously described (2, 11). Briefly, rabbit-adapted strains were oroginoculately inoculated with 10⁸ CFU of V. parahaemolyticus, and bacterial colonization (CFU/g intestinal tissue), fluid accumulation, and histological changes were measured 38 h postinfection. Histological scoring was performed by a pathologist as described in reference 2. Samples for SEM of intestinal tissues were fixed with 2% glutaraldehyde. SEM images were captured as previously described (2).

Eukaryotic cell culture and infection. Caco-2 cells were routinely maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 15% fetal bovine serum (FBS). Differentiated Caco-2 cells, cell monolayers were prepared by plating trypsinized cells into 24-well transwells and growing for ~14 days (14). V. parahaemolyticus cells used to infect Caco-2 cells were grown in LB with 0.04% sodium cholate for 2 h prior to infection, in order to induce the expression of T3SS2. All bacterial strains used for infection of Caco-2 cells lacked functional T3SS1 (due to vscN1), in order to avoid the cytotoxicity associated with T3SS1 in vitro. Differentiated Caco-2 cells, monolayers were infected with 10 μl of bacterial culture (~10⁶ CFU) for 1.5 h in the presence of 0.04% sodium cholate. Measurement of bacterial adhesion to Caco-2 cells, monolayers and confocal microscopy analyses are described below. Additionally, infected cells were fixed and processed for SEM. Briefly, after infection, the polycarbonate membrane on which the differentiated cells were grown was excised from the transwell and immediately fixed with fixative containing picric acid and glutaraldehyde. SEM images were captured as previously described (2).

HMEC-1 cells were routinely maintained as previously described (32) and infected using the conditions described above for Caco-2 cells. HMEC-1 cells were transfected with pCMV plasmids encoding VopVC, VopVR, or VopVCAFβD for 24 h. Transfected cells were fixed and processed for SEM analysis.

Adhesion assays. Differentiated Caco-2 cells were left untreated, pretreated with lasaplatinolide (0.5 μM; Santa Cruz Biotech) for 2 h, or pretreated with cytochalasin D (0.5 μg/ml; Sigma) for 10 h and then infected with V. parahaemolyticus for 1.5 h as described above. Infected Caco-2 cells were washed with phosphate-buffered saline (PBS) 5 times to remove nonadherent bacteria, and subsequently the number of adherent bacterial CFU was determined via plating. For each experiment, the frequency of adhesion by the ΔvscN1 strain was set to 1, and all other frequencies were calculated relative to this control strain. For coinfected, Caco-2 cells were infected with 10 μl of a ΔvscN1 ΔvopV strain harboring chloramphenicol resistance and 10 μl of the ΔvscN1 mutant and compared to cells infected with each strain singly. The frequency of adherent chloramphenicol-resistant CFU was enumerated as described above for single infections and coinfections and compared to the frequency of adherent ΔvscN1 bacteria.

Adhesion to HMEC-1 cells was performed similarly, except that cells were infected for 1 h.

Immunofluorescence labeling. Infected cells were extensively washed with PBS + + ( supplemented with 1 mM CaCl₂ and 0.1 mM MgCl₂) and fixed with 4% paraformaldehyde (PFA) for 20 to 30 min at room temperature. Samples were permeabilized, quenched, and blocked with PBS + + + 50 mM NH₄Cl-5% bovine serum albumin (BSA)-0.1% Saponin for 10 min at room temperature. Filamin was detected with a rabbit polyclonal antibody raised against recombinant human FLNA repeat 1 (Pacific Immunology Corp.) and affinity purified using the repeat 1 protein immobilized on N-hydroxysuccinimide ( NHS)-Sepharose beads. The purified antibodies, which were confirmed to specifically recognize FLNA, were then visualized with Alexa 488-labeled goat anti-rabbit secondary antibodies (Invitrogen). Actin was visualized using Texas Red phalloidin (Invitrogen). Samples were mounted using Mowiol.

Confocal image acquisition, processing, and analysis. Confocal images were acquired using a spinning disc confocal head (CSU-X1; PerkinElmer Co., Boston, MA) coupled to a fully motorized inverted Zeiss Axiosvert 200-M microscope equipped with a ×63 magnification lens (Plan Apochromat; 1.4 numerical aperture [NA]) and with a 175-W xenon lamp Lambda DG-4 (Sutter Instruments, Novato, CA) for wide-field illumination. Solid-state lasers (473 nm, 568 nm, and 660 nm; Crystal Laser, Reno, NV) coupled to the spinning head through a fiber optic were used as a light source. An acoustic-optical tunable filter (AOTF) was used to switch between different wavelengths. The imaging system operates under control of SlideBook 5 (Intelligent Imaging Innovations Inc., Denver, CO) and includes a computer-controlled spherical aberration correction device (SAC; Intelligent Imaging Innovations, Inc., Denver, CO) installed between the objective lens and the charge-coupled-device (CCD) camera (QuantEM:512SC; Photometrics). Acquisition of sequential optical sections spaced 0.15 μm apart was achieved with the aid of a Piezo electric Z motorized stage (ASI). Representative confocal images were selected and processed to make figures using Adobe Photoshop software.

Effector-CyaA fusion protein-based analysis of effector translocation. Caco-2 cells, cell monolayers were cultured for 14 days to achieve differentiation and then infected for 1.5 h in the presence of 0.04% sodium cholate with vscN1, vscN1 vopV, and vscN1 vscN2 strains containing pVopT-CyaA or pVopL-CyaA. Subsequently, cyclic AMP (cAMP) levels were determined by enzyme-linked immunosorbent assay (ELISA) as described (29).

Yeast two-hybrid and pulldown assay. Yeast two-hybrid analysis was performed by Hybrigenics (France). Briefly, full-length vopV was used to screen a placental cDNA library. Clones expressing proteins with positive interactions with VopV were isolated and sequenced, and all clones with high confidence interactions were found to express isoforms of filamin. The interaction between VopV and filamin was confirmed via copurification using GST-tagged VopV fragments and Caco-2 lysates. Briefly, whole-cell lysates of E. coli BL21 overexpressing GST-tagged VopVC, GST-tagged VopVR, or GST alone were loaded onto spin columns containing 100 μl glutathione agarose and incubated for 1 to 2 h. The agarose was thoroughly washed with PBS 5 to 7 times before being mixed with whole-cell lysates of Caco-2 cells. After 1 h of incubation, the agarose was further washed 5 to 7 times with PBS and eluted with glutathione (10 mM). Eluted fractions were separated by SDS-polyacrylamide gel electrophoresis. A protein band detected only in the eluate containing VopVC-GST was analyzed by mass spectrometry (Taplin Mass Spectrometry Facility, Harvard Medical School) and found to contain filamins A, B, and C. Column eluates were also analyzed by Western blotting using an antiactin antibody. Additional copurification analyses were performed using GST-tagged VopVR and VopVC and derivatives lacking one (VopVCΔFB112) or both (VopVCΔFB112ΔFB112) FB domains, which were incubated with His-tagged filament fragments (30) for 1 h. Eluates from glutathione agarose columns were analyzed using Western blotting with anti-His and anti-GST antibodies.

ACKNOWLEDGMENTS

X.Z. was supported by an NERCE Fellowship (U54 AI 057159). F.N. was supported by NIH grant number HL19749. B.E.G. was supported by NIH grant K08 CA140780 and a Burroughs Wellcome Medical Scientist Career Award. W.I.L. was supported by NIH grants DK48106 and DK84424 and...
Hiyoshi H, Kodama T, Saito K, Gotoh K, Matsuda S, Akeda Y, Honda
Liverman AD, Cheng HC, Trosky JE, Leung DW, Yarbrough ML,

We are grateful to Rod Bronson for assistance with analysis of tissue
sections and Marian Neutra and John Mekalanos for insightful comments
on the manuscript.

REFERENCES

1. Wong AR, Pearson JS, Bright MD, Munera D, Robinson KS, Lee SF,

2. Ritchie JM, Rui H, Zhou X, Iida T, Kodoma T, Ito S, Davis BM,

3. Quan P, Alam MS, Ishibuchi M, Rahman T, Alam NH, Chisti J,

4. Galán JE.

5. Dean P. 

6. Makino K, Oshima K, Kurokawa K, Yokoyama K, Uda T, Tagomori K,

7. We are grateful to Rod Bronson for assistance with analysis of tissue

8. Lipid rafting and the assembly of an enteropathogenic bacterial


10. Inflammation and disintegration of

11. The filamins: organizers of

12. Hiyoshi H, Miyoshi Y, Kondo S, Sugiyama J, Bhuiyan NA, Mathan MM,

13. Makino K, Oshima K, Kurokawa K, Yokoyama K, Uda T, Tagomori K,


15. Functional analysis of VopF activity

16. Zhou X, Shah DH, Konkel ME, Call DR.


18. Nakamura F, Stossel TP.

19. Shapiro SS. 

20. Lai Y, Rosenshine I, Leong JM, Frankel G.

21. Morales VM, Bäckman A, Bagdasarian M.

22. The hydrophilic translocator for

23. Morales VM, Bäckman A, Bagdasarian M.


25. The filamins: organizers of

26. Morales VM, Bäckman A, Bagdasarian M.

27. Morales VM, Bäckman A, Bagdasarian M.

28. Morales VM, Bäckman A, Bagdasarian M.

29. Morales VM, Bäckman A, Bagdasarian M.

30. Morales VM, Bäckman A, Bagdasarian M.

31. Morales VM, Bäckman A, Bagdasarian M.

32. Morales VM, Bäckman A, Bagdasarian M.

33. Morales VM, Bäckman A, Bagdasarian M.

34. Morales VM, Bäckman A, Bagdasarian M.

35. Morales VM, Bäckman A, Bagdasarian M.

36. Morales VM, Bäckman A, Bagdasarian M.

37. Morales VM, Bäckman A, Bagdasarian M.

38. Morales VM, Bäckman A, Bagdasarian M.

39. Morales VM, Bäckman A, Bagdasarian M.

40. Morales VM, Bäckman A, Bagdasarian M.

41. Morales VM, Bäckman A, Bagdasarian M.

42. Morales VM, Bäckman A, Bagdasarian M.

43. Morales VM, Bäckman A, Bagdasarian M.

44. Morales VM, Bäckman A, Bagdasarian M.

45. Morales VM, Bäckman A, Bagdasarian M.

46. Morales VM, Bäckman A, Bagdasarian M.

47. Morales VM, Bäckman A, Bagdasarian M.

48. Morales VM, Bäckman A, Bagdasarian M.

49. Morales VM, Bäckman A, Bagdasarian M.

50. Morales VM, Bäckman A, Bagdasarian M.