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Translocation of a *Vibrio cholerae* Type VI Secretion Effector Requires Bacterial Endocytosis by Host Cells

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SUMMARY

The type VI secretion system (T6SS) is a virulence mechanism common to several Gram-negative pathogens. In *Vibrio cholerae,* VgrG-1 is required for T6SS-dependent secretion. VgrG-1 is also secreted by T6SS and displays a C-terminal actin crosslinking domain (ACD). Using a heterologous reporter enzyme in place of the ACD, we show that the effector and secretion functions of VgrG-1 are genetically dissociable with the ACD being dispensable for secretion but required for T6SS-dependent phenotypes. Furthermore, internalization of bacteria is required for ACD translocation into phagocytic target cells. Inhibiting bacterial uptake abolishes actin crosslinking, while improving intracellular survival enhances it. Otherwise resistant nonphagocytic cells become susceptible to T6SS-mediated actin crosslinking when engineered to take up bacteria. Our results support a model for translocation of VgrG C-terminal effector domains into target cell cytosol by a process that requires trafficking of bacterial cells into an endocytic compartment where translocation is triggered by an unknown signal.

INTRODUCTION

Vibrio cholerae is a Gram-negative pathogen that causes the diarrheal disease cholera. It is a diverse species that includes over 200 serogroups, including O1 and O139 strains that cause epidemic and pandemic disease and non-O1/non-O139 strains that more typically cause sporadic outbreaks of gastroenteritis or extraintestinal infections (Rahman et al., 2008). The diversity displayed by *V. cholerae* is reflective of the range of environments it inhabits, including aquatic environments with their associated organisms (Abd et al., 2005; Chiavelli et al., 2001; Rawlings et al., 2007) and also the environment within a human host. *V. cholerae* has acquired a variety of horizontally transferred elements encoding human virulence factors, including the CTX phage encoding cholera toxin (Waldor and Mekalanos, 1996) and a chromosomal island encoding a toxin-coregulated pilus and other intestinal colonization factors (Everiss et al., 1994; Taylor et al., 1987). However, virtually all strains of *V. cholerae*, including both clinical and environmental isolates, encode the genes for well-conserved "accessory virulence factors" whose role in human disease is less clear, but which may play essential roles in the environmental fitness of *V. cholerae* and thus its transmission in natural settings. These include HlyA hemolysin (Manning et al., 1984), HapA hemagglutinin/protease (Wu et al., 1996), and RtxA toxin (Fullner and Mekalanos, 2000). Together, these three virulence factors are thought to contribute to long-term colonization of adult mice (Olivier et al., 2007), but their role in human disease has not been fully evaluated. The type III secretion system islands (Tam et al., 2007) and the recently identified type VI secretion system (T6SS) (Pukatzki et al., 2006) are also virulence factors that are present in clinical and environmental strains of *V. cholerae*. All these products and gene clusters are associated with toxicity for eukaryotic cells and therefore may play a role in human disease or other pathobiological interactions with environmental organisms.

Genes encoding a putative T6SS are present in nearly 25% of all sequenced Gram-negative bacterial species and are mainly restricted to pathogens (Bingle et al., 2008). T6SS has been implicated in virulence in *V. cholerae* (Pukatzki et al., 2006), *Pseudomonas aeruginosa* (Mougous et al., 2006), *Edwardsiella tarda* (Rao et al., 2004; Zheng and Leung, 2007), *Burkholderia* species (Aubert et al., 2008; Pilatz et al., 2006; Schell et al., 2007), and *Aeromonas hydrophila* (Suarez et al., 2008), among others. A T6SS is defined by a canonical group of 15–20 genes, and secretion via this pathway requires orthologs of conserved T6SS components, which include IcmF and DotU (Pukatzki et al., 2006; Zheng and Leung, 2007), outer membrane lipoprotein SciN (Aschtgen et al., 2008), interacting pair VipA/MglA and VipB/MglB (de Bruin et al., 2007), and ClpV (Bonemann et al., 2009). Also genetically or functionally associated with T6SS clusters are *hcp* and *vgrG* genes. In *V. cholerae*, Hcp and VgrG proteins are both required for T6SS-dependent secretion and are themselves secreted in a T6SS-dependent fashion (Pukatzki et al., 2006). Thus, deletion of *hcp-1* and *hcp-2* in *V. cholerae* results in a secretion defect of VgrG proteins (Pukatzki et al., 2006), and deletion of *vgrG-1* or *vgrG-2* results in a secretion defect of Hcp (Pukatzki et al., 2007). This reciprocal requirement for secretion suggests that Hcp and VgrG are secretion substrates that are transported through a putative core T6SS complex and could also comprise components of an

extracellular portion of the T6SS apparatus that can then shear off from bacterial cells.

In vitro secretion of Hcp has been observed in many T6SS-containing bacterial species (Aschtgen et al., 2008; Dudley et al., 2006; Mattinen et al., 2007; Mougous et al., 2006; Pukatzki et al., 2006; Schell et al., 2007; Suarez et al., 2008; Wu et al., 2008; Zheng and Leung, 2007), but secretion of VgrG homologs has been reported for only several of these organisms. EvpI is a VgrG ortholog whose secretion by *Edwardsiella tarda* requires many of the proteins found in its T6SS locus, and secretion of Hcp and VgrG is also mutually dependent in *E. tarda* (Zheng and Leung, 2007). VgrG ortholog ECA3427 is secreted by *Pectobacterium atrosepticum* (Mattinen et al., 2007), and expression of five VgrG orthologs and various other T6SS components is regulated by quorum sensing in planta or exposure to host extracts (Liu et al., 2008; Mattinen et al., 2007; Mattinen et al., 2008).

VgrG-1 is required for T6SS-dependent cytotoxic effects of *V. cholerae* on eukaryotic cells, including *Dictyostelium discoideum* amoebae and J774 macrophages (Pukatzki et al., 2007, 2006). The actin crosslinking domain (ACD) at the C terminus of VgrG-1 is closely homologous to the ACD domain present within a secreted toxin of *V. cholerae* called RtxA (Fullner and Mekalanos, 2000; Sheahan et al., 2004), which inhibits actin polymerization by catalyzing intramolecular isopeptide bond formation between E270 and K50 residues of monomeric actin (Kudryashov et al., 2008). The appearance of covalently crosslinked actin in J774 cells incubated with T6SS+ RtxA− but not with T6SS− RtxA− *V. cholerae* provides strong evidence that the ACD of VgrG-1 can enter target cells by a T6SS-dependent process. Concentrated supernatants containing T6SS-secreted substrates and purified, enzymatically active VgrG-1 do not crosslink actin in host cells, suggesting that contact between *V. cholerae* and the target cell is required for this translocation mechanism (Pukatzki et al., 2007).

Bioinformatic analysis predicts that VgrGs are homologs of the bacteriophage T4 cell-puncturing device called the "tail spike" (Pukatzki et al., 2007). This complex is used by T4 bacteriophage to puncture the bacterial cell envelope during infection and is composed of a $(gp5)_3$-$(gp27)_3$ complex that adopts a needle shape with a central channel (Kanamaru et al., 2002). The N-terminal region of a VgrG ortholog from uropathogenic *E. coli* has since been crystallized and corresponds to gp27 (Leiman et al., 2009). VgrG proteins are fusion proteins between gp27- and gp5-like domains and are predicted to form a similar trimeric structure. Consistent with this model, interactions between VgrG-1, -2, and -3 have been demonstrated in supernatant fluids of *V. cholerae* V52 (Pukatzki et al., 2007). Many VgrG proteins in other T6SS+ bacterial species contain various C-terminal domains that correspond to the position of the ACD of VgrG-1, but translocation of these putative effector domains and their cognate VgrG proteins has not been reported.

Here we report that the effector and secretion functions of VgrG-1 can be genetically dissociated. We find that the ACD is necessary for T6SS-dependent host cell cytotoxicity and impairment of phagocytosis. Furthermore, the ACD is not required for secretion and can be substituted with a heterologous reporter enzyme that is similarly secreted and translocated into target cells. In studying the mechanism of VgrG-1 translocation into target cells, we find that this process requires endocytosis of *V. cholerae* cells, and the same requirements exist for a heterologous reporter enzyme. Our results support a model for the translocation of C-terminal effector domains of VgrG proteins into the cytosol of target cells by a process that requires trafficking of bacterial cells into an endocytic compartment, where translocation is triggered by an unknown signal.

RESULTS

VgrG-1 ACD Is Dispensable for Secretion but Is Required for T6SS-Mediated Host Cell Cytotoxic Effects

VgrG-1 is the first secreted T6SS protein with demonstrated effector function, and *vgrG-1* mutant ATM-4 is phenotypically avirulent toward J774 cells and *Dictyostelium discoideum* (Figures 1D–1F). However, this mutation impairs secretion of the other T6SS secretion substrates (Figure 1A). To dissociate the effector and structural apparatus roles of VgrG-1, we constructed strain ATM-6, a *V. cholerae* V52 strain that lacks the ACD of *vgrG-1*. In this strain, endogenous *vgrG-1* is replaced with a truncated *vgrG-1* gene that encodes for amino acids 1–715 and contains a VSV-G epitope tag in place of the ACD. ATM-6 was able to secrete the truncated VgrG-1 and the other known T6SS substrates, VgrG-2, VgrG-3, and Hcp, at levels comparable to its parental T6SS+ RtxA− strain, ATM-1 (Figure 1A). Although competent for secretion, ATM-6 is phenotypically avirulent to J774 cells and to *D. discoideum*. ATM-6 does not induce actin crosslinking (Figure 1B) and does cause cell rounding of J774 macrophages (data not shown) like ATM-1. It does not alter cellular actin morphology, as seen after exposure of J774 cells to ATM-1 or ATM-2, which is a T6SS− RtxA+ strain that can crosslink actin (Figure 1D). Transient exposure to ATM-1 causes host cell death that occurs over the span of several days, and lysis can be monitored by measuring lactate dehydrogenase (LDH) release 1 day after exposure (Figure 1C). Host cells similarly exposed to ATM-6 or ATM-3—a strain that lacks *rtxA* and *vasK*, an *icmF* homolog required for T6SS function (Figure 1A)—do not cause host cell lysis (Figure 1C), and recover to proliferate normally (data not shown). Additionally, ATM-6 is not virulent toward *D. discoideum*, which is demonstrated by the appearance of *D. discoideum* plaques that form on a lawn of ATM-6 (Figure 1E), and this plaque formation occurs at levels comparable to *Klebsiella aerogenes*, a commonly used feeder strain for *D. discoideum* (Figure 1F). These data indicate that the ACD is likely the critical translocated effector domain for T6SS-mediated phenotypes observed in host cells.

T6SS-Mediated Actin Crosslinking Requires Endocytosis of *V. cholerae* into Host Cells

J774 macrophages and *D. discoideum* amoebae are both phagocytic cell types that are sensitive to *V. cholerae* T6SS-mediated cytotoxicity. To determine whether phagocytic activity contributes to host cell sensitivity, we surveyed a panel of cell lines for their ability to internalize *V. cholerae* and for their sensitivity to RtxA and T6SS-mediated actin crosslinking (Figures 2A and 2B). Internalization was measured by assaying for *V. cholerae* cells protected from gentamicin, an antibiotic impermeable to host cells (Isberg and Falkow, 1985). HEp-2, A549, and Henle-407 cells are epithelial cell lines that did not measurably take up V52 strains. Similarly, 3T3 cells are fibroblast cells that also do

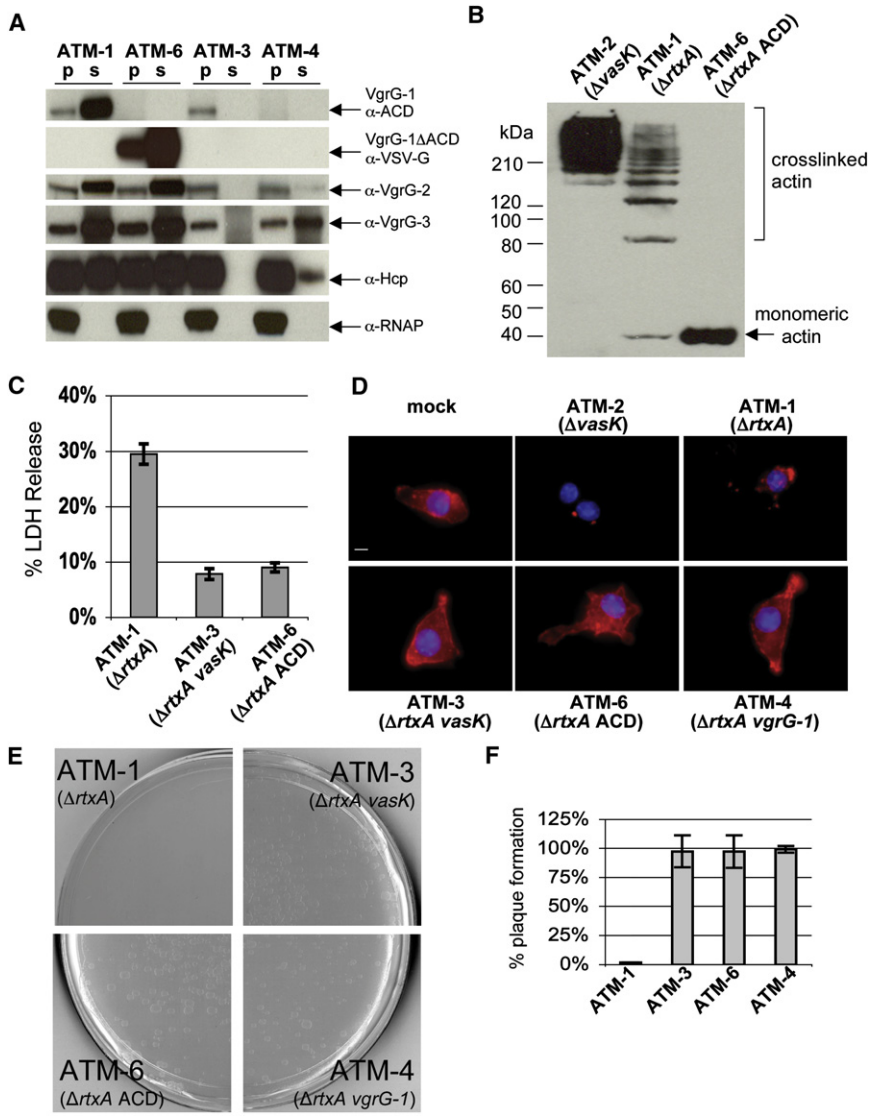

Figure 1. The ACD of VgrG-1 Is Required for Host Cell Phenotypes

(A) Western blots of pellet and supernatant fractions of various V52 strains.

(B) Western blot of host cell actin from J774 cells incubated with various V52 strains at an moi of 10 for 2 hr.

(C) Supernatants of J774 cells were analyzed for LDH release one day after exposure to *V. cholerae*. Values are from triplicate wells and are expressed as percentage of LDH released from lysed mock-treated cells. Error bars indicate ± one SD.

(D) Fluorescence microscopy of cells visualizing actin with rhodamine phalloidin (red) and nuclei with DAPI (blue). Scale bar indicates 10 μm.

(E) *Dictyostelium discoideum* plaque assay with various V52 strains.

(F) Quantification of plaque formation normalized to plaque formation on *K. aerogenes*.

not take up V52 cells (Figure 2A). When exposed to ATM-2, actin was crosslinked efficiently, indicating that these cell lines were fully sensitive to RtxA-mediated actin crosslinking. However, when incubated with ATM-1, host cell actin remained in monomeric form, indicating a resistance to T6SS-mediated actin crosslinking (Figure 2B). In contrast, two different macrophage cells lines, J774 and RAW 264.7, both internalized ATM-1 and ATM-3 (Figure 2A) and all other V52 strains listed in Table 1 to similar levels (data not shown), except for ATM-2, which is discussed below. Both macrophage cell lines are sensitive to T6SS-mediated actin crosslinking (Figure 2B).

To determine whether J774 cells remain sensitive to T6SS actin crosslinking if internalization is inhibited, incubations were performed in the presence of cytochalasin D, which inhibits phagocytosis by inhibiting actin polymerization, resulting in depolymerized actin filaments. Cytochalasin D had little effect on RtxA-mediated actin crosslinking, as has been previously reported (Fullner and Mekalanos, 2000). In contrast, T6SS-mediated actin crosslinking was abolished (Figure 3A). Inhibition of bacterial uptake by cytochalasin D was verified by gentamicin protection assay (Figure 3B). Furthermore, cytochalasin D had no effect on the catalytic activity of VgrG-1 ACD in vitro (Figure 3C). Thus, the ability of cytochalasin D to inhibit VgrG-1-mediated actin crosslinking indicates that bacterial internalization is likely required for delivery of the ACD into the target cell cytosol.

Inhibition of Endosomal Acidification Improves Intracellular Viability of *V. cholerae*, Resulting in Increased T6SS-Mediated Actin Crosslinking

A variety of bacterial toxins "sense" their internalization by target cells by undergoing conformational changes induced by endosomal acidification (Glomski et al., 2002; Qa'Dan et al., 2000). To determine whether endosomal pH triggers T6SS-mediated translocation of the ACD into target cells, we incubated J774 cells with *V. cholerae* in the presence of bafilomycin A, a specific inhibitor of vacuolar ATPases (Yoshimori et al., 1991). To exaggerate the effects of bafilomycin A on intracellular bacteria, a time course of J774 cells exposed to *V. cholerae* at a low multiplicity of infection (moi) was performed. Higher order actin multimers formed more quickly and were more abundant in incubations with bafilomycin A-treated cells compared to untreated cells (Figure 4A). Because this drug does not affect in vitro actin crosslinking by VgrG-1 (Figure 4D) and is not known to affect rates of endocytosis, the effect of bafilomycin A on actin crosslinking is likely due to efficient inhibition of phagosome acidification. This would have a protective effect on *V. cholerae*, since it is an acid-sensitive organism (Merrell and Camilli, 1999), resulting in increased translocation of ACD. Indeed, we observed a significant increase in the number of intracellular bacteria in incubations performed in the presence of bafilomycin A (Figure 4B). This effect is abrogated by

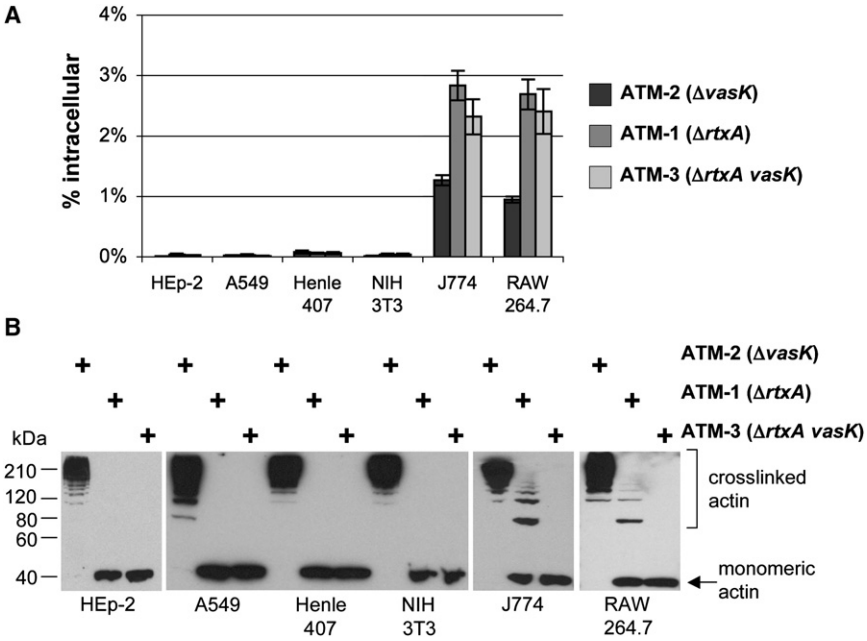

Figure 2. T6SS-Mediated Actin Crosslinking Correlates with Endocytic Uptake into Host Cells

(A) Gentamicin protection assay of various host cell lines exposed to various V52 strains at an moi of 10 for 1 hr, followed by gentamicin treatment and enumeration of intracellular bacteria. Values are of triplicate wells and are expressed as percentage of initial inoculum recovered. Error bars indicate ± one SD.

(B) Actin crosslinking in host cells detected by western blot after exposure to various V52 strains at an moi of 10 for 2 hr.

cytochalasin D treatment, since it blocks endocytosis altogether (Figures 4B and 4C). Furthermore, translocation apparently requires active bacterial protein synthesis after internalization, given that chloramphenicol blocks the appearance of crosslinked actin in J774 cells exposed to ATM-1 (data not shown). Enhancement of actin crosslinking by bafilomycin A is thus consistent with its ability to improve bacterial viability and function by maintaining a neutral vacuolar pH rather than enhancing ACD translocation per se.

Translocation of VgrG-1-Bla Fusion Proteins into J774 Cells

Many VgrG orthologs in other T6SS+ bacteria display C-terminal extension domains, which might correspond to effector domains similarly transported into target cells (Pukatzki et al., 2007). Thus, heterologous protein domains fused to the C terminus of VgrG-1 might also be translocated in the cytosol of target cells. Accordingly, we constructed two strains that encode fusions between endogenous *vgrG-1* or *vgrG-1*ΔACD and the *blaM* gene that encodes β-lactamase enzyme (Bla). β-lactamase activity can be measured using CCF2, a FRET substrate that emits green fluorescence but emits blue fluorescence after cleavage by β-lactamase (Zlokarnik et al., 1998). The Bla domain replaces the C-terminal ACD domain of VgrG-1 in strain ATM-11 and extends from the C terminus of the ACD in strain ATM-7. This *vgrG-1*::*bla* gene was also introduced into T6SS mutant backgrounds, creating strains ATM-8 (Δ*vasK vgrG-1*::*bla*), ATM-9 (Δ*hcp vgrG-1*::*bla*), and ATM-10 (Δ*vgrG-2 vgrG-1*::*bla*). All these strains produced β-lactamase fusion proteins, but only VgrG-1-Bla and VgrG-1-ΔACD-Bla were secreted in vitro by ATM-7 and ATM-11 (Figure 5A). Additionally, secreted VgrG-1-Bla and VgrG-1-ΔACD-Bla were able to cleave a free acid form of CCF (Figure 5B), indicating β-lactamase activity was intact in the fusion proteins. Extracellular transport of VgrG-1-ΔACD-Bla also confirms that the ACD domain of VgrG-1 is not required for secretion function of the T6SS apparatus.

We next tested whether VgrG-1-Bla fusion proteins could be translocated into target cells. To monitor translocation of β-lactamase, we used CCF2/AM, an esterified form of CCF2 that accumulates in host cells and fluoresces green; after cleavage by cytosolic β-lactamase, its product emits blue fluorescence (Charpentier and Oswald, 2004; Marketon et al., 2005). In order to optimize translocation into J774 cells, we took advantage of the increased translocation elicited by treatment of cells with bafilomycin A, observed earlier for the natural ACD reporter of VgrG-1, and also treated host cells with a higher moi. After J774 cells were incubated with various VgrG-1-Bla fusion strains, translocation of β-lactamase into the cytosol was monitored by quantitatively measuring substrate cleavage and by fluorescence microscopy. All nonsecreting T6SS mutants had CCF2/AM cleaved/uncleaved ratios similar to mock-treated cells and incubations with ATM-1, which lacks the β-lactamase fusion, while ATM-7 had a significantly higher cleaved/uncleaved ratio, which was inhibited by cytochalasin D (Figure 5C). Substrate cleavage in incubations with ATM-7 varied with moi

Table 1. Bacterial Strains Constructed for this Study

Strain	Genotype
ATM-1	V52 Δ*rtxA*[a] Δ*hlyA*[a] Δ*hapA*[a]
ATM-2	V52 Δ*hlyA* Δ*hapA* Δ*vasK*[b]
ATM-3	V52 Δ*rtxA* Δ*hlyA* Δ*hapA* Δ*vasK*
ATM-4	V52 Δ*rtxA* Δ*hlyA* Δ*hapA* Δ*vgrG-1*[b]
ATM-5	V52 Δ*rtxA* Δ*hlyA* Δ*hapA* Δ*hcp-1*[b] Δ*hcp-2*[b]
ATM-6	V52 Δ*rtxA* Δ*hlyA* Δ*hapA vgrG-1* ΔACD::VSV-G[c]
ATM-7	V52 Δ*rtxA* Δ*hlyA* Δ*hapA vgrG-1*::*bla*[c]
ATM-8	V52 Δ*rtxA* Δ*hlyA* Δ*hapA* Δ*vasK vgrG-1*::*bla*
ATM-9	V52 Δ*rtxA* Δ*hlyA* Δ*hapA* Δ*hcp-1* Δ*hcp-2 vgrG-1*::*bla*
ATM-10	V52 Δ*rtxA* Δ*hlyA* Δ*hapA* Δ*vgrG-2*[b] *vgrG-1*::*bla*
ATM-11	V52 Δ*rtxA* Δ*hlyA* Δ*hapA vgrG-1*ΔACD::*bla*[c]

[a] Accessory toxin in-frame deletions as constructed in Tam et al., 2007.
[b] T6SS in-frame deletions as constructed in Pukatzki et al., 2006, 2007.
[c] New deletions and fusions were constructed as described in Experimental Procedures.

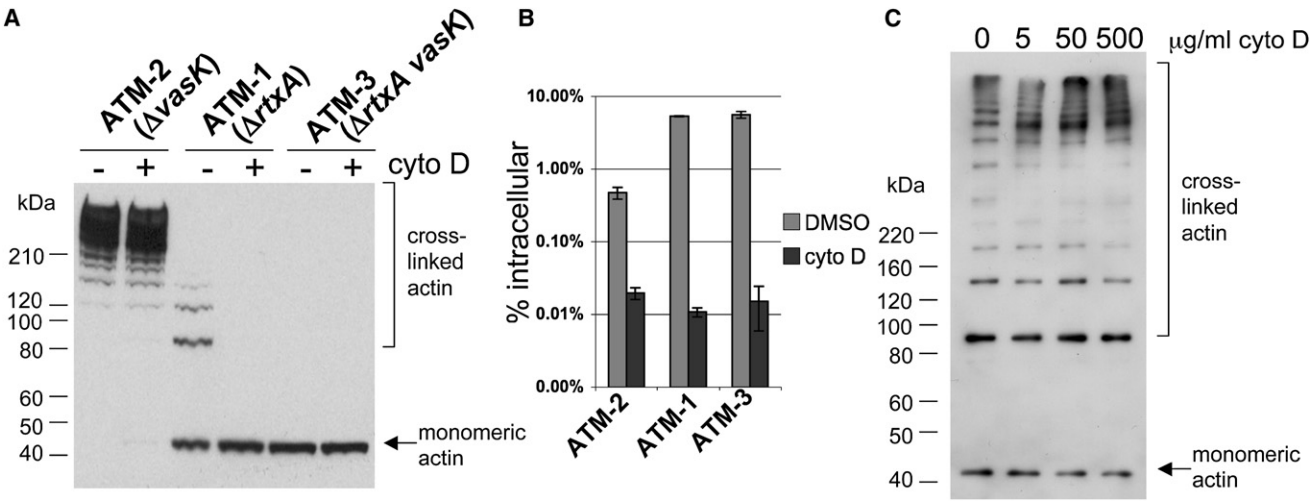

Figure 3. Cytochalasin D Inhibits Uptake into J774 Cells and In Vivo Actin Crosslinking

(A) Western blot against actin from J774 cells in the presence or absence of cytochalasin D. Exposure to *V. cholerae* was at an moi of 10 for 2 hr.

(B) Gentamicin protection assays were performed to verify inhibition of uptake. One hour exposure to *V. cholerae* at an moi of 10 was followed by gentamicin treatment and enumeration of intracellular bacteria. Values are of triplicate wells and are expressed as percentage of initial inoculum recovered. Error bars indicate ± one SD.

(C) In vitro actin-crosslinking assay with purified VgrG-1 and actin with various concentrations of cytochalasin D or 0.1% DMSO. After incubation at 37°C for 1 hr, reactions were ran out on a 4%–15% gradient SDS-PAGE gel and monitored by western blot.

and bafilomycin A pretreatment time (Figures S1A and S1B). Substrate cleavage was also monitored by fluorescence microscopy (Figure 5E).

ATM-11, which expresses and secretes VgrG-1-ΔACD-Bla, is also able to translocate the β-lactamase fusion protein into host cells and had a significantly higher cleaved/uncleaved ratio than strain ATM-7. This could be due to an antiphagocytic effect caused by the ACD of VgrG-1-Bla, which crosslinks actin in host cells and thus inhibits subsequent uptake of bacterial cells (Figure 5D). This antiphagocytic effect was observed after shorter incubations with strain ATM-2, which produces RtxA (Figure 2A), during which time robust actin crosslinking occurs. However, with T6SS-mediated actin crosslinking, this effect becomes apparent only after longer exposure to *V. cholerae*. By eliminating the actin-crosslinking activity in strain ATM-11, more bacteria are taken up into J774 cells over the course of the incubation, and thus more intracellular bacteria are able to translocate VgrG-1-ΔACD-Bla.

Opsonophagocytosis by CHO-FcγRII Cells

To determine whether induction of bacterial uptake into a nonphagocytic cell line could stimulate translocation of VgrG-1 into an otherwise resistant cell line, we used Chinese hamster ovary (CHO) cells that express Fcγ receptors (CHO-FcγRII), a cell line which is engineered to induce opsonophagocytosis in an IgG-dependent manner (Joiner et al., 1990). Gentamicin protection assays show that uptake of ATM-1 and ATM-3 is significantly higher than in incubations lacking opsonizing antibodies (Figure 6A). Actin crosslinking occurs after exposure to ATM-1 in the presence of anti-*Vibrio* antibody (Figure 6B), but not after exposure to *vasK* mutant ATM-3. Similar to results seen with J774 cells, cytochalasin D inhibited uptake of ATM-1 and ATM-3 and actin crosslinking induced by ATM-1 (Figures 6A and 6B).

Translocation into CHO-FcγRII cells was also monitored using β-lactamase fusions to VgrG-1 and CCF2/AM substrate. Incubations with T6SS mutants expressing the VgrG-1-Bla fusion protein had cleaved/uncleaved ratios similar to mock-treated cells and incubation with ATM-1, a T6SS+ strain that lacks the β-lactamase fusion. However, incubation with ATM-7 in the presence of opsonizing antibodies had significantly higher cleaved/uncleaved ratios, while incubation in the absence of opsonizing antibody had cleaved/uncleaved ratios similar to mock-treated cells (Figure 6C). Cells were imaged, and CHO-FcγRII cells emitted blue fluorescence when exposed to ATM-7 in the presence of anti-*Vibrio* antibodies (Figure 6D), indicating cytosolic translocation of the C-terminal β-lactamase domain on VgrG-1-Bla had occurred.

DISCUSSION

Bacterial pathogens utilize a variety of mechanisms to manipulate host cell function, and the actin cytoskeleton is a common target (Barbieri et al., 2002). The ACD of RtxA toxin and VgrG-1 of *V. cholerae* represent another biochemical paradigm for microbial manipulation of actin function (Fullner and Mekalanos, 2000; Kudryashov et al., 2008; Pukatzki et al., 2007). Detection of actin crosslinking was a particularly useful tool to monitor T6SS function and allowed us to determine that bacterial endocytosis is required for translocation into host cells. Even nonphagocytic cell lines can be rendered sensitive to T6SS-mediated effects by opsonophagocytosis of *V. cholerae* via expression of the FcγRII receptor. This induced sensitivity is blocked by treatment with cytochalasin D, suggesting that adherence of V52 to CHO-FcγRII cells via IgG-FcγRII interaction is not sufficient to induce translocation. Even without this artificial interaction, V52 and derivative strains adhere to both phagocytic and nonphagocytic

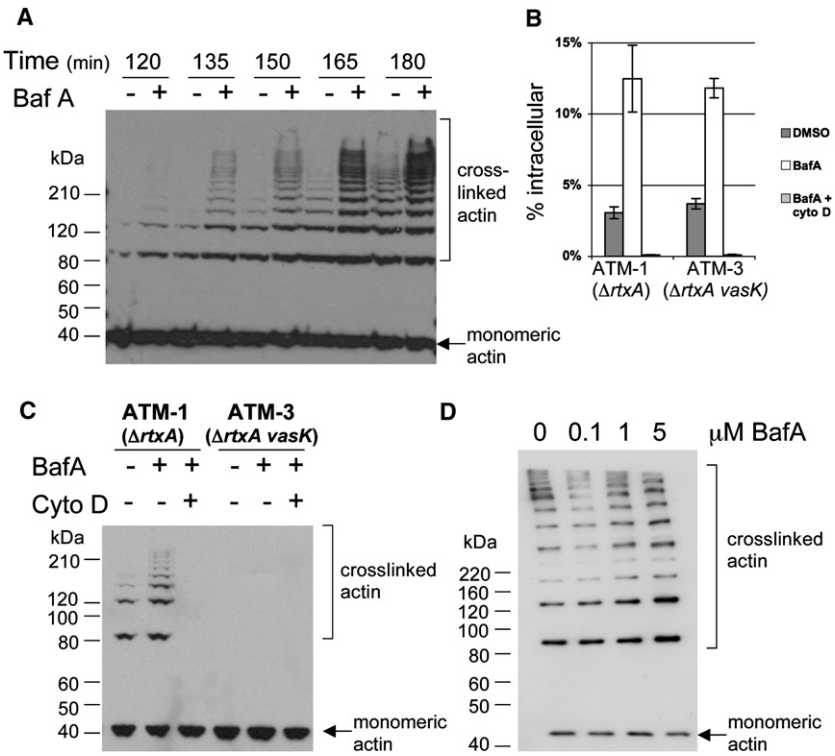

Figure 4. Bafilomycin A Increases ATM-1-Induced Actin Crosslinking in J774 Cells

(A) Time course of J774 cells exposed to ATM-1 at an moi of 1 in the presence or absence of bafilomycin A. Actin was visualized by western blot.

(B) Gentamicin protection assay of J774 in the presence or absence of bafilomycin A and with the addition of cytochalasin D. One hour long incubations at an moi of 10 were followed by gentamicin treatment and enumeration of intracellular bacteria. Values are of triplicate wells and are expressed as percentage of initial inoculum recovered. Error bars indicate ± one SD. Values between different treatment conditions are statistically significant by Student's t test ($p < 0.05$).

(C) Actin crosslinking in host cells in the presence of 0.1% DMSO, bafilomycin A alone, or bafilomycin A with cytochalasin D. Exposures were at moi of 10 and were 2 hr long. Actin was visualized by western blot.

(D) In vitro actin-crosslinking assay with purified VgrG-1 and actin with various concentrations of bafilomycin A or 0.1% DMSO. After incubation at 37°C for 1 hr, reactions were ran out on a 4%–15% gradient SDS-PAGE gel and monitored by western blot.

host cells (data not shown), suggesting that the correlation between endocytic uptake and translocation is not due to impaired interaction with the host cell surface. In contrast, *Legionella pneumophila* can translocate type IV secretion effectors into CHO-FcγRII cells at wild-type levels even in the presence of cytochalasin D, suggesting that membrane proximity and/or adherence is sufficient to induce translocation (Cambronne and Roy, 2007). This distinction suggests that the one or more putative signals could trigger translocation within the endosome and may represent a strategy to ensure that translocation occurs only in that specific microenvironment. It has yet to be determined whether *V. cholerae* intracellular survival is affected by the action of the ACD, or whether eventual escape from host cells can occur after their actin cytoskeleton has been disrupted. To some extent, this may depend on how fast the phagocytic cell acidifies its endosome, or whether *V. cholerae* is phagocytosed in a physiological state of comparable acid tolerance (Merrell and Camilli, 1999). Since T6SS-dependent translocation of the ACD requires close contact between bacterium and target cell, it seems quite clear that this has not evolved as a mechanism to disrupt the function of host cells at a distance, but rather plays its predominant role in close cell-to-cell interactions. The endocytosis requirement for T6SS suggests that phagocytes are the natural target for *V. cholerae* T6SS, which could include predatory phagocytes encountered in the environment or phagocytic cells of the mammalian host immune system. T6SS-mediated virulence mechanisms could function in both of these environments, similar to *Legionella pneumophila* virulence factors facilitating replication within both amoebae and human macrophages (Swanson and Hammer, 2000). After uptake of *V. cholerae* by J774 cells, T6SS mediates crosslinking of actin, which then results in impaired phagocytic function and eventual host cell death. These effects could provide protection to bystander *V. cholerae* within an infection of a mammalian host or within an environmental reservoir. Indeed, interaction between *V. cholerae* and free living aquatic amoebae *Acanthamoeba castellani* has been reported (Abd et al., 2005). However, determining the biological role of the T6SS of *V. cholerae* will require other experimental approaches and appropriate animal models that are currently under development.

T6SS-mediated virulence targeted at phagocytes seems to be a common theme for T6SS+ bacterial organisms, since many of these phenotypes involve intracellular behavior or interaction with phagocytes. Mutations in *evpC*, an *hcp* homolog, resulted in reduced replication rates of *Edwardsiella tarda* in gourami fish phagocytes (Rao et al., 2004). A homolog of IcmF, SciS, from *Salmonella typhimurium*, is required for limiting intracellular growth in macrophages (Parsons and Heffron, 2005). Components of T6SS were reported to be upregulated in *Burkholderia pseudomallei* after invasion of macrophages (Shalom et al., 2007). These phenotypes and modes of regulation indicate that signals in the endocytic compartment of host cells could trigger the expression and function of T6SS systems in organisms other than *V. cholerae*. The concept that T6SS-dependent virulence factors are specialized for use in the endosomal microenvironment is somewhat reminiscent of specialized T3SS loci that function only after entry of bacteria into endosomes, as has been observed in *Salmonella* species (Holden, 2002).

The results reported in this study indicate that the ACD of VgrG-1 is not required for secretion in vitro or for translocation into host cells, but is required for all known T6SS phenotypes observed in *V. cholerae* incubation with eukaryotic cells. This indicates that VgrG-1 is a bona fide effector of T6SS-dependent cellular pathobiology rather than solely a component of the T6SS

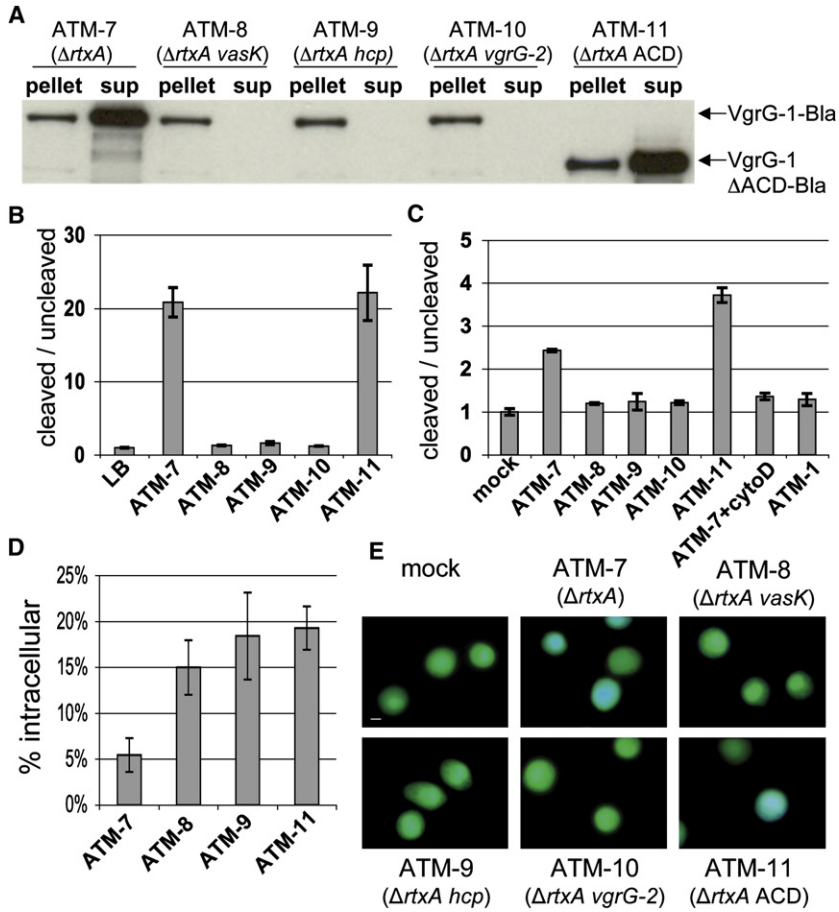

Figure 5. Translocation of VgrG-1-Bla or VgrG-1ΔACD-Bla into J774 Cells

(A) Western blot against β-lactamase of pellet and supernatant fractions of various V52 strains producing VgrG-1-Bla or VgrG-1ΔACD-Bla.

(B) In vitro CCF2-FA cleavage by supernatants of various V52 strains grown in triplicate. Fluorescence was measured with an excitation wavelength of 405 nm and emission wavelengths of 460 nm (cleaved blue channel) and 530 nm (uncleaved green channel). Values are expressed as ratios of cleaved signal to uncleaved signal. Error bars indicate ± one SD.

(C) Translocation of VgrG-1-Bla or VgrG-1ΔACD-Bla into J774 cells incubated with various V52 strains. Cells were pretreated with bafilomycin A, incubated with V52 strains for 2 hr at an moi of 50, and then loaded with CCF2/AM. Cells were harvested and analyzed on a fluorescence plate reader as for in vitro CCF2-FA assay. Ratios were normalized to mock, and error bars indicate ± one SD.

(D) Gentamicin protection assay on host cells incubated with *V. cholerae* as for translocation assay and subsequently treated with gentamicin and enumeration of intracellular bacteria. Values are of triplicate wells and are expressed as percentage of initial inoculum recovered. Error bars indicate ± one SD.

(E) Fluorescence microscopy of J774 cells loaded with CCF2/AM. Scale bar indicates 10 μm. ATM-7, ATM-8, ATM-9, and ATM-10 all contain the *vgrG-1-bla* fusion gene, and ATM-11 contains the *vgrG-1*-ΔACD-*bla* gene.

apparatus. Using β-lactamase fusions to VgrG-1, we demonstrated T6SS-mediated intracellular delivery of an ectopic reporter domain using the conserved "core" structure of VgrG-1. Our data support a more general model for T6SS-mediated delivery of effector domains located at the C termini of VgrG orthologs. Over 500 VgrG orthologs are present in bacterial genome sequence databases, and many of these VgrG orthologs carry C-terminal extensions, some of which are predicted to have interesting biochemical properties based on their structural similarity to other eukaryotic and prokaryotic proteins (Pukatzki et al., 2007). Thus, these C-terminal domains could be translocated into target cells only after phagocytosis of bacteria capable of expressing a functional T6SS. However, not all T6SS+ organisms contain VgrGs with putative C-terminal effector domains, suggesting that there may be T6SS effectors that are not VgrG orthologs (Pukatzki et al., 2009). It remains to be determined whether VgrG proteins possess innate translocation activity or can serve also as a channel for translocation of other effectors. Attempts to show that secreted VgrG-1 and Hcp proteins display cytotoxicity for phagocytic cells have so far failed (Pukatzki et al., 2006), although it is possible that such secreted complexes are capable of mediating translocation of the ACD only in the microenvironment of the endosomal lumen.

Because the well-conserved core of VgrG-related proteins is predicted to form a trimeric needle-like complex that resembles bacteriophage T4 tail spike, a membrane penetrating device, it is possible that VgrG proteins might play an active role in breaching the target cell membrane. In the model of T4 bacteriophage infection of *E. coli*, the force generated from conformational changes in the base plate and contraction of the tail sheath drives the tail tube and tail spike through host cell membrane (Kostyuchenko et al., 2005). In addition to VgrG, there are other T6SS proteins that are structurally similar to bacteriophage tail components. The structure of Hcp resembles bacteriophage tail tube proteins of bacteriophage T4 and lambda (Leiman et al., 2009; Pell et al., 2009). Another T6SS protein resembles gp25, a bacteriophage T4 tail protein that is present at the interface of the tail tube and tail spike (Leiman et al., 2009). Given the mechanism by which T4 bacteriophage infects host cells and the structural similarities between components of T6SS and T4 bacteriophage tail, we favor a model in which T6SS-dependent phenotypes truly reflect the activity of a T6SS membrane-penetrating, translocation machine rather than simply extracellular secretion within the endosomal compartment. Mechanistic studies of the structure and function of the bacterial associated T6SS core complex and mechanism of translocation are needed to test this model.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Cell Culture

Vibrio cholerae strains were grown in Luria-Bertani (LB) broth supplemented with 100 μg/ml streptomycin. In-frame deletions and β-lactamase fusions

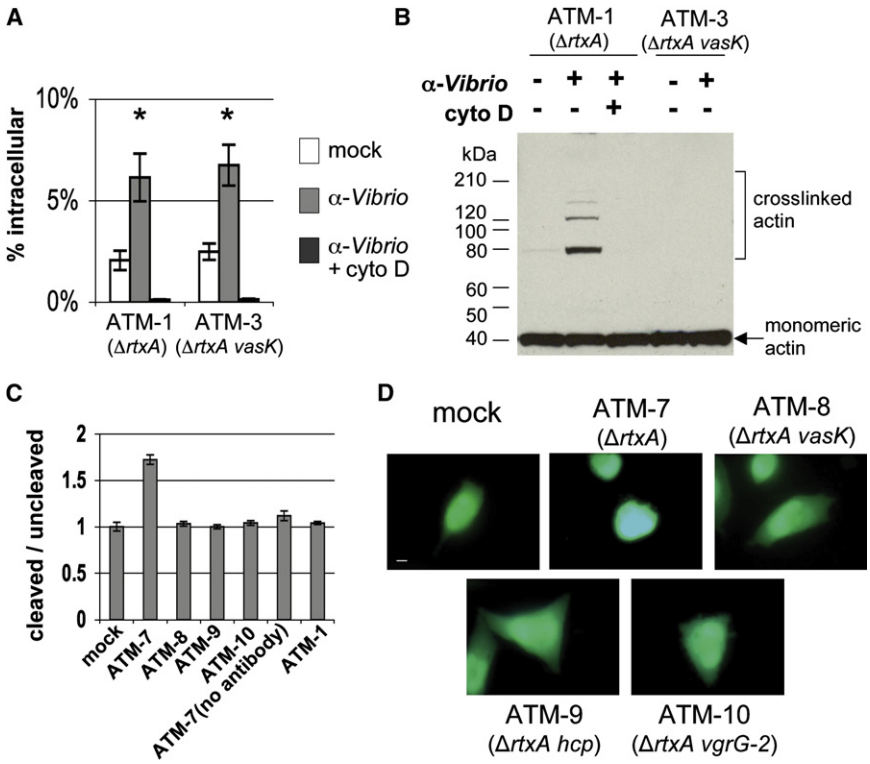

Figure 6. Opsonophagocytosis by CHO-FcRγII Cells Induces Translocation of VgrG-1

(A) Gentamicin protection assay of CHO-FcγRII cells incubated with various V52 strains in the presence or absence of α-*Vibrio* antibody and with cytochalasin D. Values are expressed as percentage of initial inoculum recovered, and error bars indicate ± one SD. Groups marked with asterisks are significantly higher than remaining groups (Student's t test, $p < 0.05$).

(B) Western blot against actin from CHO-FcγRII cells incubated with various V52 strains at an moi of 50 for 30 min under various conditions, then treated with gentamicin for an additional 1.5 hr. Actin was visualized by western blot.

(C) Translocation of VgrG-1-Bla into CHO-FcγRII cells by various V52 strains. Cells were pretreated with α-*Vibrio* antibody, incubated with *V. cholerae* at an moi of 50 for 2 hr, loaded with CCF2/AM, then harvested for quantification. Fluorescence was measured with an excitation wavelength of 405 nm and emission wavelengths of 460 nm (cleaved blue channel) and 530 nm (uncleaved green channel). Values are expressed as ratios of cleaved signal to uncleaved signal and are normalized to mock. Error bars indicate ± one SD.

(D) Fluorescence microscopy of CHO-FcγRII cells incubated with various V52 strains and then loaded with CCF2/AM. Scale bar indicates 10 μm. ATM-7, ATM-8, ATM-9, and ATM-10 all encode *vgrG-1-bla* fusion gene.

were introduced into V52 using previously described methods (Skorupski and Taylor, 1996). In-frame deletions of accessory toxins *rtxA*, *hlyA*, and *hapA* were constructed as in Tam et al., 2007. In-frame deletions of T6SS components *vasK*, *hcp-1*, *hcp-2*, *vgrG-1*, and *vgrG-2* were constructed as in Pukatzki et al., 2006 and 2007. Strain ATM-6 was constructed by allelic exchange of *vgrG-1*ΔACD-VSV-G, which encodes amino acids 1–715 of VgrG-1 fused to VSV-G epitope tag. The *blaM* gene encodes a TEM-1-type β-lactamase and was cloned from pVTM30 (Tam et al., 2007) to produce *vgrG-1*::*bla* fusion gene, which was introduced into ATM-7, ATM-8, ATM-9, and ATM-10 by allelic exchange. Strain ATM-11 was constructed similarly with *vgrG-1*ΔACD::*bla*. All resulting strains are listed in Table 1. J774, RAW264.7, NIH 3T3, and CHO-FcγRII cells were maintained in DMEM. HEp-2 and A549 cells were maintained in RPMI 1640. Henle-407 cells were maintained in EMEM. All media were supplemented with 2 mM L-glutamine, penicillin at 100 U/ml, 100 μg/ml streptomycin, and 10% heat-inactivated FBS. Cells were maintained at 37°C and 5% CO_2.

In Vitro Secretion Assay

Various bacterial strains were back-diluted 1:50 into LB broth from overnight cultures. Cultures were grown for approximately 2 hr on a roller drum at 37°C. Pellet fractions were collected and concentrated 2-fold in LDS loading buffer (Invitrogen; Carlsbad, CA). Supernatants were filtered, precipitated with trichloroacetic acid, and resuspended in LDS loading buffer, resulting in a 100-fold concentration, except for α-Hcp western blots, which were not concentrated. VSV-G antibody was obtained from Sigma-Aldrich (St. Louis). RNAP α-subunit antibody was obtained from Neoclone (Madison, WI). β-lactamase antibody was obtained from Abcam (Cambridge, MA). VgrG-1, VgrG-2, VgrG-3, and Hcp polyclonal antibodies were generated by New England Peptide (Gardner, MA). Synthetic peptides encoding unique amino acid sequences of Hcp-1, VgrG-1, VgrG-2, and VgrG-3 were prepared and conjugated to keyhole limpet hemocyanin. The conjugated peptides were injected into New Zealand white rabbits, boosted twice, and then tested on crude cell extracts and culture supernatants from *Vibrio cholerae* V52 and corresponding mutants by western blot to ensure specificity.

In Vitro β-Lactamase Activity Assay

Bacteria were grown in triplicate cultures as for in vitro secretion assays. CCF2-FA (Invitrogen) was added to cell-free supernatant fractions, and the mixture was incubated in the dark for 30 min at room temperature. Fluorescence was quantified on a SpectraMax Gemini XS fluorescent plate reader with an excitation wavelength of 405 nm and emission wavelengths of 460 nm (cleaved blue channel) and 530 nm (uncleaved green channel). Values were corrected for LB blank readings and expressed as a ratio of cleaved to uncleaved signal.

Dictyostelium discoideum Plaque Assay

One hundred microliters of overnight bacterial cultures were plated with dilutions of *Dictyostelium discoideum* on SM/5 plates as previously described (Pukatzki et al., 2007). Plates were incubated at 22°C for 3 days before plaque formation was assessed. For quantification, bacterial strains were plated in triplicate, and the number of plaques was normalized to plaques formed on *K. aerogenes* lawns.

Incubation with Host Cells

Host cells were seeded in 12-well plates at a density of 1–2 × 10^5 per well in drug-free media. The next day, cells were pretreated with 0.1% DMSO, 5 μg/ml cytochalasin D, or 100 nM bafilomycin A for 1 hr unless otherwise indicated. Triplicate incubations were performed at an moi of 10 unless otherwise indicated. For gentamicin protection assays, incubations were allowed to proceed for 1 hr, followed by 1 hr treatment with 100 μg/ml gentamicin. Host cells were washed with PBS, lysed with 1% saponin, and plated for colony-forming units on LB agar plates. For detection of in vivo actin crosslinking, incubations were allowed to proceed for 2 hr, unless otherwise indicated. Host cells were harvested, pooled, and resuspended in LDS loading buffer (Invitrogen) for analysis by western blot using α-actin antibody (Sigma-Aldrich).

CHO-FcγRII Cells

CHO-FcγRII cells were incubated with 1:100 anti-*Vibrio* antibody (Tam et al., 2007) for at least 1 hr before incubation with *V. cholerae*. Bacteria were added at an moi of 10 for gentamicin protection assays or an moi of 50 for

actin-crosslinking assays, and plates were centrifuged at 1000 rpm for 5 min. Cells were incubated with *V. cholerae* for 30 min at 37°C, then treated with 100 μg/ml gentamicin for 1.5 hr. Cells were washed with PBS, lysed with 1% saponin, and plated at LB agar plates to enumerate colony-forming units. Alternatively, cells were harvested for western blot analysis of actin.

Translocation Assay

Host cells were seeded as described above. For fluorescence microscopy experiments, cells were seeded into glass-bottom 12-well plates (MatTek; Ashland, MA). J774 cells were pretreated with 100 nM bafilomycin A (Sigma-Aldrich) for 4 hr, then incubated with various V52 strains at an moi of 50 for 2 hr. CHO-FcγRII cells were incubated as described above for actin-crosslinking assays, except incubations were allowed to proceed for 2 hr. Host cells were then washed with PBS and loaded with CCF2-AM for 1 hr. For fluorescence microscopy, images were acquired with a Nikon inverted epifluorescence microscope. For quantification by fluorescence plate reader, cells were washed after CCF2-AM loading, then allowed to incubate for an additional 30 min. Cells were then harvested in 100 μl HBSS and transferred to an opaque 96-well plate, and fluorescence was quantified as described for in vitro β-lactamase activity assay.

LDH Release Assay

J774 cells were incubated with various bacterial strains in triplicate at an moi of 10 for 2 hr, at which time wells were washed with PBS and treated with 100 μg/ml gentamicin overnight. The next day, supernatant samples or lysed mock-treated cells were collected and analyzed with cytotoxicity detection kit (Roche Applied Science; Indianapolis, IN). Values are expressed as percentage of LDH released from lysed mock-treated wells.

Visualization of Actin Cytoskeleton by Fluorescence Microscopy

J774 cells were seeded onto glass-bottom 12-well plates (MatTek). The next day, J774 cells were incubated with various bacterial strains at an moi of 10 for 2 hr, at which time wells were washed with PBS and fixed with 3.7% paraformaldehyde. Cells were permeabilized with 0.1% Triton X, stained with rhodamine phalloidin (Invitrogen) and DAPI (Sigma-Aldrich), and mounted. Images were acquired on a Nikon inverted epifluorescence microscope.

In Vitro Actin-Crosslinking Assay

This assay was performed as previously described (Cordero et al., 2006; Pukatzki et al., 2007), except for the addition of DMSO, cytochalasin D, or bafilomycin A at the concentrations indicated. Actin derived from rabbit skeletal muscle (Cytoskeleton; Denver) was used as substrate for in vitro crosslinking reactions that were incubated at 37°C for 1 hr.

SUPPLEMENTAL DATA

Supplemental Data include one figure and can be found online at http://www.cell.com/cellhostandmicrobe/supplemental/S1931-3128(09)00068-7.

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