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Characterization of *V. cholerae* T3SS-dependent cytotoxicity in cultured intestinal epithelial cells

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Summary

AM-19226 is a pathogenic, non-O1/non-O139 serogroup strain of *Vibrio cholerae* that uses a Type 3 Secretion System (T3SS) mediated mechanism to colonize host tissues and disrupt homeostasis, causing cholera. Co-culturing the Caco2-BBE human intestinal epithelial cell line with AM-19226 in the presence of bile results in rapid mammalian cell death that requires a functional T3SS. We examined the role of bile, sought to identify the mechanism, and evaluated the contributions of T3SS translocated effectors in *in vitro* cell death. Our results suggest that Caco2-BBE cytotoxicity does not proceed by apoptotic or necrotic mechanisms, but rather displays characteristics consistent with osmotic lysis. Cell death was preceded by disassembly of epithelial junctions and reorganization of the cortical membrane skeleton, although neither cell death nor cell-cell disruption required VopM or VopF, two effectors known to alter actin dynamics. Using deletion strains, we identified a subset of AM-19226 Vops that are required for host cell death, which were previously assigned roles in protein translocation and colonization, suggesting that they function other than to promote cytotoxicity. The collective results therefore suggest that cooperative Vop activities are required to achieve cytotoxicity *in vitro*, or alternatively, that translocon pores destabilize the membrane in a bile dependent manner.

Introduction

Although best known as an epidemic disease caused by O1 and O139 serogroup strains of *Vibrio cholerae*, the diarrheal disease cholera can also result from infection by strains of the

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more than 200 other defined serogroups, collectively known as non-O1/non-O139 serogroup strains (Sack *et al.*, 2004, Harris *et al.*, 2012). Clinical reports document similar disease symptoms from infection by all serogroups, although non-O1/non-O139 serogroup strains are associated with sporadic outbreaks that occur both temporally and geographically outside of the patterns associated with epidemic disease. In fact, sporadic cholera has been reported worldwide, and the rising incidence of non-O1/non-O139 serogroup associated disease in endemic areas has prompted investigation of the molecular mechanisms responsible for disease (Morris *et al.*, 1981, Bagchi *et al.*, 1993, Ramamurthy *et al.*, 1993, Dalsgaard *et al.*, 1995, Sharma *et al.*, 1998, Dalsgaard *et al.*, 1999, Sack *et al.*, 2003, Faruque *et al.*, 2004, Lee *et al.*, 2007, Chatterjee *et al.*, 2009, Newton *et al.*, 2012, Dutta *et al.*, 2013, Luo *et al.*, 2013).

Despite the similarity of clinical outcomes, there are numerous genetic differences between epidemic associated O1 and O139 serogroup strains, which are largely clonal and conserved in genetic content, and pathogenic non-O1/non-O139 serogroup strains, which have a more diverse genetic repertoire (Bagchi *et al.*, 1993, Dalsgaard *et al.*, 1995, Dalsgaard *et al.*, 1999, Faruque *et al.*, 2003, Faruque *et al.*, 2004, Feng *et al.*, 2008, Chun *et al.*, 2009). Invariably, O1 and O139 serogroup strains colonize the human intestinal epithelium using the toxin co-regulated pilus (TCP), and cause profuse secretory diarrhea as a result of the enzymatic activity of cholera toxin (CT) (Matson *et al.*, 2007, Harris *et al.*, 2012). In contrast, most clinically isolated non-O1/non-O139 strains do not encode TCP or CT, utilizing less well understood mechanisms to colonize host tissues and alter intestinal cell homeostasis. We now know that a subset of non-O1/non-O139 serogroup strains isolated from cholera patients carry genes for a Type 3 Secretion System (T3SS), and studies have shown that the T3SS is required for disease in murine and rabbit animal models of infection (Dziejman *et al.*, 2005, Tam *et al.*, 2007, Rahman *et al.*, 2008, Chatterjee *et al.*, 2009, Tam *et al.*, 2010, Shin *et al.*, 2011, Dutta *et al.*, 2013, Hasan *et al.*, 2013, Luo *et al.*, 2013, Morita *et al.*, 2013, Octavia *et al.*, 2013).

We previously reported that co-culture of T3SS positive strain AM-19226 with the Caco2-BBE intestinal epithelial cell line resulted in rapid eukaryotic cell death (Miller *et al.*, 2012). The phenotype did not require the HlyA hemolysin or the putative thermostable direct-related hemolysin, TRH, but did require a functional T3SS. In other bacterial pathogens, T3SS-mediated *in vitro* cell death can involve different effector protein activities and pathogen specific mechanisms. For example, *Yersinia* spp. induce apoptosis in naïve macrophages *in vitro*, using YopJ and YopP effector activity to interfere with MAPK and NF-κB signaling (Bergsbaken *et al.*, 2009a, Philip *et al.*, 2012). In the case of *Salmonella* infection, both flagellin and a functional T3SS are required to induce pyroptosis in host cells (Fink *et al.*, 2007). It is also important to note that some pathogens use T3SS translocated proteins to interfere with host pathways in order to prevent cell death and promote colonization and infection. For example, enterohemorrhagic *E. coli* inhibits apoptosis by translocation of two T3SS effector proteins, NleH and NleD (Wong *et al.*, 2011). We therefore further investigated *V. cholerae* T3SS-mediated Caco2-BBE cytotoxicity by interrogating the mechanism and identifying T3SS translocated proteins required for cell death in our co-culture model.

Results and Discussion

Role of bile in promoting cytotoxicity

Previous studies determined that in the presence of 0.2% bile, co-culture of *V. cholerae* strain AM-19226 with the intestinal epithelial cell line Caco2-BBE resulted in ~70–95% cytotoxicity 3h after the start of co-culture, as measured by lactate dehydrogenase (LDH) release (Miller *et al.*, 2012). In contrast, there is no LDH release in the absence of bile, even after six hours of co-culture with *V. cholerae*. We also identified bile as an inducer of T3SS gene expression for bacteria grown *in vitro* overnight in LB (Alam *et al.*, 2010).

We varied the bile concentration during co-culture from 0.025% to 0.2%, while keeping the AM-19226 multiplicity of infection (MOI) constant at ~10. Compared to the results achieved with 0.2% bile, 0.1% bile results in an overall decrease in cytotoxicity, with 30% cytotoxicity measured after 3h, and 50% measured after 5h (Figure 1). When the bile concentration was further decreased to 0.05%, we observed only 25% cytotoxicity after 5h. 0.025% bile does not result in measurable Caco2-BBE cell death after 5h (Figure 1). The results suggest that 0.2% is the optimal concentration of bile for potentiating the effect of *V. cholerae*. Increased bile concentrations have been reported to reduce bacterial growth (Provenzano *et al.*, 2000, Hung *et al.*, 2005). We typically recovered similar bacterial counts from the Caco2-BBE co-culture supernatants in the presence and absence of 0.2% bile (*e.g.* $1.93*10^7$ CFU/mL in the absence of bile vs. $3.18*10^7$ CFU/mL in the presence). We also observed bacterial multiplication in the presence of 0.2% bile during co-culture (15–20 fold increase 3 hpi compared to initial inoculum). We did notice a trend towards ~2-fold increased bacterial adherence in the presence of bile (data not shown), but the relationship between MOI, adherence and cytotoxicity requires further investigation. Having previously demonstrated that cytotoxicity requires both a functional T3SS and bile (Miller *et al.*, 2012) and given the current data, it is possible that a small increase in adherence acts synergistically with T3SS effector protein activity and bile-mediated destabilization of mammalian cell membranes to result in cytotoxicity.

Since bile is required for T3SS-dependent cytotoxicity and since the bile concentration can modulate the time course and number of Caco2-BBE cells succumbing to cytotoxicity, we reasoned that bile in the co-culture medium might stimulate signaling pathways in the mammalian host cells leading to cell death. Our previous results suggest that bile does not influence T3SS gene expression under co-culture conditions: when strain AM-19226 is grown in standard tissue culture conditions (described in Experimental Procedures), T3SS gene expression levels remain similar whether or not 0.2% bile and/or Caco2-BBE mammalian cells are present (Miller *et al.*, 2012). Alternatively, however, bile might promote bacterial T3SS activity during co-culture. To differentiate among the possibilities, we conducted experiments where AM-19226 inoculating cultures or Caco2-BBE monolayers were pretreated with bile before co-culture, and varied whether bile was present or not at the time of co-culture (Table 1). Earlier experiments determined that stationary phase bacteria grown in LB + bile display maximal T3SS structural gene expression compared to bacteria in exponential phase (or grown in the presence of sodium deoxycholate alone), thus stationary phase cultures were used (Alam *et al.*, 2010). We found that the

cytotoxicity phenotype requires the presence of bile specifically during the time of mammalian cell co-culture; if bile was left out of the co-culture media, neither pre-treating Caco2-BBE cells with bile before co-culture, nor growing bacteria overnight in LB+0.4% bile, resulted in cell death. One interpretation is that bile acts on epithelial cells by sensitizing them to bacterial protein activities in a reversible manner, such that the phenotype is revealed only during co-culture with bile. Interestingly, *V. parahaemolyticus* grown to exponential phase in the absence of bile can cause Caco-2 T3SS2-mediated death, although when bacteria are grown in the presence of bile prior to co-culture, more rapid cell death is observed (Gotoh *et al.*, 2010, Kodama *et al.*, 2010). It is important to note that different *in vitro* growth conditions promote maximal T3SS gene expression in *V. cholerae* and *V. parahaemolyticus*, and that differences in effector protein repertoires likely contribute to the variations in *in vitro* and *in vivo* phenotypes observed for the two species (Zhang *et al.*, 2013). For example, maximal T3SS gene expression in *V. parahaemolyticus* occurs during exponential growth, compared to stationary phase for *V. cholerae* (Alam *et al.*, 2010, Kodama *et al.*, 2010). Thus, the *V. cholerae* and *V. parahaemolyticus* T3SS gene expression networks and response to environmental signals in the presence of mammalian cells are not identical.

Bile is a complex mixture of different bile salts, cholesterol, pigments, and fatty acids that collectively have detergent-like properties. Importantly, bile salts are commonly used to promote T3SS activity and activate expression of virulence genes in enteric bacteria, including epidemic *V. cholerae* strains (Gupta *et al.*, 1997, Gunn, 2000, Begley *et al.*, 2005a, Begley *et al.*, 2005b, Hung *et al.*, 2005, Olive *et al.*, 2007, Gotoh *et al.*, 2010, Yang *et al.*, 2013). To test whether any single bile component could promote the cell death phenotype, we included individual anionic bile salts (sodium deoxycholate, sodium cholate) or other compounds with detergent properties (anionic (SDS), nonionic (Triton X-100, Tween-80) or zwitterionic (CHAPS) detergents), instead of whole bile during Caco2-BBE co-culture with AM-19226. For each reagent, we first determined the highest concentration that did not result in altered Caco2-BBE cell morphology as visualized by microscopy. We then conducted the co-culture experiment with the highest permissible concentration for each compound as listed in Table 1. We did not observe Caco2-BBE cytotoxicity or changes in cellular morphology during co-culture with AM-19226 in the presence of the detergents and bile salts we tested (Table 1). Because we used each component at the highest concentration possible that alone did not cause cytotoxicity, we propose that other components of bile (*e.g.* cholesterol, fatty acids) or the properties of multiple bile components are required for cytotoxicity.

Investigating apoptosis as the cause of T3SS-dependent cytotoxicity

Results from our earlier studies led to the conclusion that Caco2-BBE cytotoxicity did not require the AM-19226 *trh* gene product, which in *V. parahaemolyticus* functions as a pore forming toxin similar to the thermostable direct hemolysin, TDH (Miller *et al.*, 2012). Nonetheless, the rapid, dramatic disruption of monolayers and presence of debris was easily observed by bright field microscopy and cell death measured by lactate dehydrogenase release (Miller *et al.*, 2012). However, previous experiments did not investigate the

mechanisms of cell death that may involve apoptotic or necrotic programmed cell death pathways or osmotic cell lysis.

Although we did not observe morphological features consistent with apoptosis (*e.g.* bright field microscopy did not reveal cytoplasmic shrinkage or membrane blebbing, data not shown), and the rapid progression to loss of membrane integrity is not characteristic of apoptotic cell death, we sought additional evidence against apoptosis as the mechanism of AM-19226 induced intestinal epithelial cell death. Experiments using pan-caspase inhibitors Z-VAD-fmk and Q-VD-OPh, which block caspase activation, did not inhibit cytotoxicity (Table 2). Since Poly- (ADP Ribose) Polymerase (PARP) cleavage is a hallmark of apoptosis, we examined Caco2-BBE cell lysates using Western blot analysis (Krysko *et al.*, 2008). As expected, we detected PARP cleavage after treatment with the apoptosis inducer, staurosporine (Figure 2, STS). However, Caco2-BBE cells co-cultured with AM-19226 T3SS WT, an AM-19226 T3SS-null strain (Δ*vcsN2*) or mock treated do not exhibit PARP cleavage (Figure 2). Together these results strongly suggest that the observed Caco2-BBE death is not occurring by apoptosis. Interestingly, Tam and coworkers used cell cycle analysis to observe HeLa cells co-cultured with strain AM-19226, and reported a shift of G1 to S phase and an increase in the apoptotic cell population (17%), compared to co-culture with the T3SS-null strain (9%) (Tam *et al.*, 2010). The use of a different cell line, a higher MOI and the absence of bile in their studies may account for the difference in results. However, TUNEL staining identified a much smaller population (<5%) of apoptotic cells in their studies, which is more consistent with our results.

Evaluating necrotic mechanisms of cell death and the role of second messengers

T3SS mediated bacterial infections commonly initiate necrotic cell death mechanisms, including pyroptosis and necroptosis (Fink *et al.*, 2007, Bleriot *et al.*, 2016). We tested chemical inhibitors of different programmed necrotic death mechanisms shown in Table 2. Z-VAD-fmk and Z-VD-OPh are pan-caspase inhibitors that block apoptosis as well as pyroptosis, whereas Z-YVAD-fmk specifically blocks activity of caspases that mediate pyroptosis. Glycine can transiently block pyroptosis by preventing non-specific ion fluxes in mammalian cells (Brennan *et al.*, 2000, Bergsbaken *et al.*, 2009b). Since over activated PARP can lead to cell death (Galluzzi *et al.*, 2012), we blocked PARP activation using the inhibitor, PJ-34. Treatment with PJ-34 decreased cytotoxicity (~30%) only at a 100μM concentration, which is ~100-fold greater than concentrations necessary for selective inhibition, suggesting that PARP activation was not the primary cause of cell death and that off target effects could decrease LDH release (data not shown)(Antolin *et al.*, 2012). We also included necrostatin-1, which inhibits RIP1 kinase activity and therefore necroptosis (Galluzzi *et al.*, 2012). None were able to inhibit cytotoxicity. Neither of two intracellular calcium chelators, BAPTA-AM or EGTA-AM, prevented AM-19226 mediated cell death. Since both compounds function in mammalian cells to suppress transient calcium fluxes that can lead to cell death, the results do not support a mechanism that requires calcium mobilization (Tsien, 1981, Zhivotovsky *et al.*, 2011). We also investigated whether mitochondrial damage was involved in cytotoxicity, since evidence suggests that the VopE effector protein can influence mitochondrial dynamics by interacting with Miro GTPase proteins (Suzuki *et al.*, 2014). However, inhibiting the activity of the mitochondrial

permeability transition pore using cyclosporin A did not affect cytotoxicity. Together these data suggest that AM-19226 does not induce Caco2-BBE cell death by common programmed necrotic mechanisms.

Investigating osmotic cell lysis as a mechanism of T3SS-dependent cytotoxicity

Given that most AM-19226 effector proteins activities are poorly understood, and that bile perturbs mammalian cell membranes and is required for cell death in our model system, we sought to investigate if osmotic lysis contributes to AM-19226 induced intestinal epithelial cell death. Various solutes can be used to attenuate osmotic lysis, and osmoprotectants of known sizes can also decrease water influx into the cell through membrane pores. We therefore added the following molecules to the media during co-culture: sucrose (0.9nm), which also can establish an isotonic environment to prevent water loss across the plasma membrane, raffinose (1.14nm), and PEG3350 (3.80nm), which can also create a hypertonic environment (Clinkenbeard *et al.*, 1991, Fink *et al.*, 2006). Neither sucrose nor raffinose altered cytotoxicity levels, but addition of 30mM PEG3350 decreased cytotoxicity ~2-fold (Figure 3A).

We recovered similar bacterial cell numbers from the co-culture medias at 2 h (7.2×10^6 CFU/mL for mock vs. 6.6×10^6 for PEG3350 treated wells), suggesting that PEG3350 does not affect bacterial replication. We next determined whether PEG3350 influences bacterial adherence. We found that the effect of PEG3350 was variable, and the averaged results of 3 experiments suggest that PEG3350 decreased adherence ~2-fold (Figure 3B). While we currently do not understand the precise relationship between adherence, MOI, and cytotoxicity, we do know that ~20–30% of the bacterial population is adherent at 2hrs using an MOI of ~10, and that cytotoxicity remains at ~80% at 3hrs if the MOI drops to ~5. The MOI must be decreased ten-fold to ~1 in order to observe a decrease in cytotoxicity, measured as 2-fold (to ~40% at 3 hrs) (Miller *et al.*, 2016). If the proportion of adherent bacteria at 2hrs, when little replication has occurred, remains similar regardless of MOI, then a 10-fold decrease in adherence is required to elicit the 2-fold decrease in cytotoxicity observed by decreasing the MOI 10-fold to ~1. We therefore propose that in our experiment, a 2-fold decrease in adherence alone is insufficient to affect cytoxicity 2-fold, and suggest that PEG attenuates cytotoxicity due to combined effects on adherence and in a more traditional role as an osmoprotectant.

The phenotype of rapid Caco2-BBE LDH release is consistent with cytotoxicity mediated by pore formation or other mechanisms leading to membrane disruption and osmotic lysis. Currently, none of the identified effector proteins share sequence similarity to pore forming toxins, yet cytotoxicity is T3SS dependent. Examples of T3SS translocon insertion resulting in cell death due to colloid osmotic lysis have been reported for other pathogens, and some translocon components structurally resemble pore-forming toxins (Dacheux *et al.*, 2001, Miki *et al.*, 2010, Barta *et al.*, 2012). However, the precise mechanism of translocon pore-related lysis is difficult to resolve, especially since the internal diameter of the T3SS translocon reportedly ranges from ~1.2–3.5nm, depending on the bacterial species (Galan *et al.*, 2006, Mattei *et al.*, 2011). Effectors that regulate pore formation have been identified in *Yersinia* and *E. coli*, and the process is now recognized as complex, involving repair of

translocon “pores”, and is likely to be highly regulated (Guignot *et al.*, 2015, Guignot *et al.*, 2016). Our collective results are therefore consistent with a hypothesis that AM-19226 induced cell death likely proceeds by currently undefined molecular mechanisms that shares characteristics with osmotic lysis.

Identifying host cell signaling pathways that respond to bile and T3SS-positive *V. cholerae*

We found several reports in the literature documenting the effects of individual purified bile components in stimulating the MEK/ERK mammalian cell signaling pathway (Qiao *et al.*, 2001, Akare *et al.*, 2005). To our knowledge, however, studies have not examined the impact of whole, crude bile on epithelial cell ERK1/2 signaling. We therefore exposed Caco2-BBE monolayers to 0.2% bile for up to 2h (in the absence of bacteria), harvested the monolayers, and then examined lysates for ERK1/2 phosphorylation (Figure 4A). The untreated sample shows basal levels of ERK1/2 phosphorylation after monolayer growth overnight. Addition of fresh culture medium without bile at 0h results in transient ERK1/2 activation, but addition of medium with bile results in slightly elevated ERK1/2 phosphorylation at 0h that further increases at later time points as compared to untreated cells (Figure 4A). Densitometry calculation quantify a ~15-fold increase in ERK1/2 phosphorylation after a 2h bile treatment, compared to the same time point in the absence of bile. We therefore concluded that crude bile induces ERK1/2 activation in Caco2-BBE cells.

Studies in *Saccharomyces cerevisiae* show that the AM-19226 effector protein, VopX, may target proteins in the yeast cell wall integrity pathway, which includes protein orthologs to mammalian MEK/ERK family proteins (Qi *et al.*, 2005, Alam *et al.*, 2011, Seward *et al.*, 2015). We reasoned that both bile and bacterial effector proteins could target the MEK/ERK pathway, thus potentiating ERK1/2 over-activation and detrimental consequences. We therefore examined ERK1/2 phosphorylation in the presence of bile, varying whether bacteria were or were not added to the Caco2-BBE cells. Although we observed ERK1/2 phosphorylation at 0.5h and 2h in the presence of bacteria (Figure 4B, lanes 3 and 7), ERK1/2 phosphorylation also occurred in response to bile when no bacteria were present (Figure 4B, compare lane 1 to 3 and lane 5 to 7). Since both bile and bacteria are required for cell death, we could not definitively determine whether *V. cholerae* alone induced ERK activation that then resulted in cytotoxicity.

However, we recognized the importance of examining whether ERK1/2 phosphorylation (regardless of the stimuli) is important in AM-19226 T3SS-mediated cell death. We therefore blocked the activity of the MEK1/2 kinases that lie upstream of ERK by adding U0126 to the co-culture media, thereby increasing the pool of unphosphorylated, inactive ERK1/2 (Favata *et al.*, 1998, Johnson *et al.*, 2002). We first demonstrated that ERK1/2 phosphorylation was not observed in the presence of U0126 by performing Western blot analysis for ERK1/2 using Caco2-BBE cell lysates from standard co-culture conditions (Figure 4B). To determine if ERK1/2 activation is required for Caco2-BBE cell death, we performed the co-culture assay with bile and AM-19226 in the presence or absence of U0126 and measured LDH release. As shown in Figure 4C, addition of U0126 does not abrogate mammalian cell cytotoxicity, suggesting that ERK1/2 activation does not lead to cell death. Furthermore, we did not observe p38 MAPK or JNK MAPK phosphorylation

during Caco2-BBE-*V. cholerae* co-culture in the presence of bile, suggesting that neither signaling pathway is altered during the time preceding cell death (data not shown). It is therefore plausible that crude bile is acting on the Caco2-BBE membrane in a manner similar to that reported for deoxycholic acid alone, rendering ERK1/2 activation as a marker of cell membrane perturbations rather than a cause of cell death (Akare *et al.*, 2005). Although co-culture conditions differ, in comparison, blocking the activity of upstream kinases MEK1/2 does decrease *V. parahaemolyticus* T3SS1-dependent cell death (Matlawska-Wasowska *et al.*, 2010, Yang *et al.*, 2011). Our results thus far suggest that although MEK/ERK pathway perturbation occurs during AM-19226/Caco2-BBE co-culture in the presence of bile, blocking MEK/ERK pathway signaling does not prevent the cytotoxic effect of AM-19226.

AM-19226 disrupts Caco2-BBE cell-cell junctions and membrane skeleton organization

We next examined the response of host cells by determining whether markers for adherens junctions, which are a well-known target of enteric pathogens, were disrupted in the presence of T3SS-positive bacteria (Viswanathan *et al.*, 2009). After 2h of Caco2-BBE/AM-19226 co-culture in the presence of bile, we fixed and stained cells with antibodies against the adherens junction proteins, E-cadherin and β-catenin, and α-adducin, a membrane skeleton protein that is associated with the basolateral plasma membrane of differentiated epithelial cells (Naydenov *et al.*, 2010). Mock treated and AM-19226 Δ*vcsN2* (T3SS-null) co-cultured Caco2-BBE cells displayed intact cell-cell junctions as evidenced by continuous staining of E-cadherin and β-catenin at the cell periphery (Figure 5A). In contrast, co-culture with the AM-19226 strain carrying wild-type genes encoding a functional T3SS (T3SS WT) results in disruption of the adherens junctions. Furthermore, T3SS activity induces changes in the distribution of α-adducin, including formation of vesicles indicating increased dynamics of the lateral plasma membrane, as compared to both the mock infected and AM-19226 Δ*vcsN2* infected cells.

Because tight junctions are stabilized by the actin cytoskeleton, we investigated whether VopM and VopF, two AM-19226 T3SS effector proteins that modulate host actin, are responsible for the phenotype (Tam *et al.*, 2007, Hiyoshi *et al.*, 2011, Suzuki, 2013). Tam *et al.* reported a ~50% decrease in transepithelial resistance (TER) and disruption of the tight junction protein, ZO-1 in a polarized T84 intestinal epithelial cell co-culture model (Tam *et al.*, 2010). However, as shown in Figure 5B, co-culture of Caco2-BBE with VopM or VopF deletion strains triggers similar disruption of adherens junctions and vesiculation of the membrane skeleton as compared to cells co-cultured with the isogenic parental strain (T3SS WT). Interestingly, co-culture with AM-19226 Δ*vopF* resulted in a modest decrease in TER (~30%), suggesting that while VopF is involved in tight junction disruption, other proteins likely contribute to the process or encode redundant functions (Tam *et al.*, 2010). Together, the data suggest that other AM-19226 effector proteins function to remodel host cell adherens and tight junctions via actin reorganization and membranotropic effects. Nonetheless, such changes to the host cell physiology can not only contribute to diarrhea, but also perhaps reveal a niche for increased bacterial colonization (Muza-Moons *et al.*, 2003).

Role of individual effector proteins in cytotoxicity

Reasoning that mammalian cell cytotoxicity is due to the action of specific T3SS effector proteins, we constructed individual, in-frame deletions in each 11 known effector proteins in *V. cholerae* strain AM-19226. Each strain was then assayed for the ability to cause cytotoxicity in our *in vitro* co-culture assay. As shown in Figure 6A, strains deleted for Vops H/A/I/or W caused less than 5% Caco2-BBE cytotoxicity, similar to a T3SS-null strain (Δ*vcsN2*), whereas co-culture with strains that did not encode Vops E/M/F/G/K/Y/or Z still caused wild-type levels of cell death (70%-80% cytotoxicity, Figure 6A). Previous studies determined that a VopX deletion strain had altered T3SS gene expression due to regulatory sequences that overlap the *vopX* coding region, so we constructed and assayed separately an AM-19226 strain carrying a copy of the *vopX* gene that produces a truncated protein (*vopX'*) (Miller *et al.*, 2016). Co-culture of Caco2-BBE cells with a strain unable to produce VopX resulted in cytotoxicity similar to the parental strain (Figure 6B), indicating that the VopX effector alone is not responsible for cell death. To complement the null phenotype in the Vops H/A/I/ and W deletion strains, we transformed each effector null strain with a plasmid expressing the appropriate *vop* gene from an arabinose inducible promoter, and performed the co-culture assay in the presence of 0.2% bile and different concentrations of arabinose (Figure 6C). While we were able to complement the *vopH/A/W* deletions for cytotoxicity with the respective plasmids (Figure 6C), we were unable to complement the *vopI* deletion strain (Figure 6C). VopI is encoded within an operon where downstream gene products include structural apparatus components and the VopM and VopW effector proteins (Alam *et al.*, 2010, Chaand *et al.*, 2013). In the current VopI deletion strain, mRNA stability or translational efficiency could be altered, therefore resulting in unanticipated polar effects if structural protein or effector protein levels are critical for function. Based on our results, we conclude that Vops H/A/W are required for Caco2-BBE cytotoxicity. Interestingly, however, Vops H/A/W are also required for translocation of other Vops, suggesting that Vops H/A/W function as part of the T3SS structural or translocation apparatus rather than exerting activities within the host cell to cause cytotoxicity (Chaand *et al.*, 2015). Determination of the role of VopI awaits construction of a truncation mutant, similar to that used to assess VopX function.

Conclusions

We investigated the conditions that resulted in rapid Caco2-BBE cytotoxicity. Although bile could be acting on the *V. cholerae* cells to increase expression of T3SS genes, expression of the T3SS structural gene operon, *vcsRTCNS2*, during co-culture with 0.2% bile is similar to that in the absence of bile (Miller *et al.*, 2012). Alternatively, bile could increase the secretory activity of the T3SS, as has been demonstrated for *Shigella*. Picking and coworkers presented evidence that sodium deoxycholate increases secretion through the apparatus by recruiting the translocon components, IpaB and IpaD, to the needle tip (Olive *et al.*, 2007, Dickenson *et al.*, 2013). An analogous situation could exist for *V. cholerae*, where in addition, bile could be acting on the Caco2-BBE cells as a sensitizer or stimulator of signaling pathways targeted by effector protein activity. Bile also likely influences the membrane composition and dynamics of the bacterial or eukaryotic cells, altering fatty acid, lipid and/or cholesterol content, perhaps altering membrane sensitivity or response to T3SS

translocon pore formation (Giles *et al.*, 2011). Numerous reports document a role for bile components (specifically deoxycholate) in altering the permeability and structure of epithelial cell junctional barriers, which is consistent with our results demonstrating the bile requirement for cytotoxicity and the disruption and reorganization of junctional proteins during *V. cholerae* co-culture (Raimondi *et al.*, 2008, Deli, 2009). Interestingly, PEG solutions of various molecular weights have been reported to exert protective effects on intestinal cells damaged by environmental and chemical insults (Bharadwaj *et al.*, 2011, Edelstein *et al.*, 2011).

Based on our current results, we were able to provide convincing data for the role of the T3SS in modulating cytotoxicity although we were unable to identify the mechanism of how T3SS activity or specific effectors lead to cell death. While the targets and functions of most AM-19226 T3SS effector proteins are unknown, both *V. cholerae* VopF and *V. parahaemolyticus* VopM homolog, VopV, can reorganize host actin, although it is currently not known whether the proteins encode redundant functions or play separate roles in the host (Tam *et al.*, 2007, Hiyoshi *et al.*, 2011). While our studies were underway, a T3SS2 effector protein was identified in *V. parahaemolyticus* and named VopZ, distinct from the VopZ effector protein identified earlier in strain AM-19226 (Alam *et al.*, 2011, Zhou *et al.*, 2013). In *V. parahaemolyticus*, VopZ is critical for host colonization and diarrheal disease in the infant rabbit model, but is not required for adherence (Zhou *et al.*, 2013). Our initial studies indicate that the *V. cholerae* VopZ homolog, sharing ~68% amino acid similarity with the *V. parahaemolyticus* protein, may encode different functions, and additional experiments are therefore required to determine what role the protein plays in AM-19226 pathogenicity and cytotoxicity.

Because functional redundancy is a common theme among bacterial effector proteins encoded by a single pathogen, an alternative interpretation allows for multiple AM-19226 T3SS effector proteins to act coordinately to cause mammalian cell death (Galan, 2009). Reports of microvillus blunting and disruption of the intestinal epithelial cell surface *in vivo* in the infant rabbit model are consistent with our *in vitro* observations (Shin *et al.*, 2011), and non-apoptotic cell death (e.g. necrosis) is often associated with cell swelling and rupture of the cell membrane leading to release of intracellular contents that is considered pro-inflammatory. Such observations are consistent with reports of inflammation associated with clinical outcomes of non-O1/non-O139 associated disease. We therefore expect the cytotoxicity phenotype will serve as a useful tool to dissect the roles and activities of T3SS-related proteins, and lead to a more complete understanding of how non-epidemic strains can cause gastroenteritis by disrupting host cell homeostasis using novel, cholera-toxin independent mechanisms.

Experimental Procedures

Bacterial strains, growth conditions, and in silico analysis

Strains were maintained and grown under standard conditions and as described. Ampicillin (Amp) and streptomycin (Str) were used at 100μg/ml for *Escherichia coli* and *Vibrio cholerae*. Bile (bile bovine B3883; Sigma) was prepared as previously described (Alam *et*

al., 2010). Clone Manager Professional Suite, version 9 (Sci-Ed Software) was used for sequence analysis.

Strain and plasmid construction

Nucleic acid manipulations were performed using standard molecular biology techniques (Sambrook *et al.*, 1989). Non-polar, in-frame deletions of *vopE, vopH, vopA, vopM, vopI, vopW, vopF, vopG, vopK, vopY*, and *vopZ*, were constructed in *V. cholerae* strain AM-19226 Δ*hap*Δ*hlyA*Δ*rtxA* (Δ3) as previously described (Horton *et al.*, 1989, Donnenberg *et al.*, 1991, Alam *et al.*, 2010, Alam *et al.*, 2011, Chaand *et al.*, 2015). Deletions were confirmed by PCR and Southern blot analyses. pBAD18-*vopA* was cloned using restriction sites so that the *vopA* open reading frame is downstream of the arabinose inducible promoter. pBAD18 was converted to a Gateway compatible vector using the Gateway vector conversion system (Invitrogen) to result in pBAD18rfaKM, which was then used in standard Gateway protocols to produce pBAD18-*vopH*, pBAD18-*vopI*, and pBAD18-*vopW*. The *vopX*' truncation was constructed by introducing a single nucleotide substitution (A157T) resulting in amino acid change K54X, which introduced a unique BglII site as described (Miller *et al.*, 2016).

Mammalian cell lines and culture conditions

Caco2-BBE cells were maintained in Dulbecco's modified Eagle medium (4.5 mg/ml glucose, sodium pyruvate, and L-glutamine; Invitrogen) supplemented with 10% FBS (Gemini Bio-Products) at 37°C with 5% CO_2. Co-culture of Caco2-BBE and *V. cholerae* strain AM-19226 was performed in the presence of DMEM supplemented with 5% FBS as previously described, using DMEM containing 1mg/ml glucose (low glucose medium, Invitrogen) for arabinose induction experiments (Miller *et al.*, 2012). Staurosporine was added to Caco2-BBE cells at 2μM for 14h to induce apoptosis.

Lactate dehydrogenase release assay

Percent cytotoxicity was determined as previously described, using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega) as instructed by the manufacturer (Miller *et al.*, 2012). Briefly, Caco2-BBE cells were seeded in 96 well plates, grown ~24h to ~80% confluency, and infected at an MOI ~10 using strains grown overnight at 37°C in LB medium (or LB containing 0.4% bile). To determine bacterial replication following co-culture, co-culture supernatant was serially diluted and plated on LB agar. Bacterial colony forming units were enumerated and compared to the initial bacterial inoculum. Bile and/or arabinose were added to the culture medium as indicated. Detergents and bile salts were added to the co-culture medium at the following concentrations: 0.05% CHAPS, 0.01% Tween-80, 0.001% Triton X-100, 0.005% SDS, 0.01% sodium deoxycholate, 0.04% sodium cholate. For studies using pharmacological inhibitors, Caco2-BBE cells were pretreated for ~1h in DMEM medium lacking bile and the inhibitors were included in the subsequent co-culture media at the following concentrations: 10μM U0126 (VWR Scientific), 100μM necrostatin-1, 50 and 100μM PJ-34 (Santa Cruz Biotechnology), 50μM Z-VAD-fmk, 30μM Z-VD-OPh (MP Biomedicals), 50μM Z-YVAD-fmk (Santa Cruz Biotechnology), 1, 5, and 10μM cyclosporinA (Sigma), 10μM BAPTA-AM (Santa Cruz Biotechnology), 10μM EGTA-AM (gift from Dr. David Yule). Sucrose, raffinose, or polyethylene glycol 3350

(PEG3350) were included in the co-culture medium at 30mM. Glycine was included in the co-culture medium at 5mM and 100mM.

Adherence Assay

Bacterial adherence to Caco2-BBE cells was performed as previously described (Chaand *et al.*, 2015). Briefly, Caco2-BBE cells were seeded on collagen coated coverslips (Neuvitro) in DMEM + 5% FBS for 48h or in 12-well plates. Confluent cell monolayers were washed with PBS and co-cultured with bacteria at an MOI of ~10 in DMEM + 5% FBS + 0.2% bile for 2h. Additionally, bacterial cells were added to wells containing culture medium alone to calculate bacterial growth (output). Following co-culture, wells were washed 3x with PBS. If coverslips were used they were transferred to fresh wells, and washed again in PBS. Caco2-BBE cells were lysed with 0.1% Triton X-100 in PBS for 1h. Serial dilutions were plated to determine the number of adherent bacteria. Percent adherent bacteria = (coverslip-associated bacteria at 2h/output at 2h) × 100.

Western blot analysis of mammalian proteins

3×10^6 Caco2-BBE cells were seeded into 10cm dishes in DMEM containing 10% FBS, washed the following day (~24h) with PBS and supplied with DMEM containing 5% FBS. Cells were ~80% confluent by the next day (~48h), and were washed with PBS and supplied fresh DMEM containing 5% FBS with or without 0.2% bile. AM-19226 co-culture and a mock co-culture control were performed as described above. At indicated time points mammalian cells were harvested with a cell scraper, washed in PBS, and flash frozen. Mammalian cells were then lysed on ice in RIPA buffer (Tris, EDTA, EGTA, Triton X-100, sodium deoxycholate, SDS, and NaCl) containing mammalian protease and phosphatase inhibitors (Sigma), sonicated for 15s, and cellular debris was pelleted at 13,000 rpm for 10 minutes. Resulting supernatants were electrophoresed on 10% SDS-PAGE gels, transferred to nitrocellulose membranes, and probed with the following antibodies: anti-phosphorylated ERK1/2 (Cell Signaling), anti-total ERK1/2 (Cell Signaling), anti-PARP (Cell Signaling) and anti-GAPDH (Sigma).

Immunofluorescence staining of Caco2-BBE/AM-19226 co-culture

Caco2-BBE cells were seeded on collagen IV coated coverslips (Neuvitro) in 24 well plates. After ~24h, Caco2-BBE cells were infected with AM-19226 strains at an MOI of 10 in the presence of 0.2% bile. Following 2h of co-culture, monolayers were washed with PBS, fixed with 4% paraformaldehyde, and permeabilized with 0.1% TritonX-100. Caco2-BBE cells were probed with antibodies against E-cadherin (Invitrogen), β-catenin (Invitrogen), and α-adducin (Santa Cruz Biotechnology) followed by Alexafluor conjugated secondary antibodies. Proteins were visualized on an Olympus confocal microscope.

Statistical analyses

Statistical analyses were conducted using GraphPad Prism with one-way ANOVA followed by Dunnett post hoc test or two-tailed *t*-test as appropriate.

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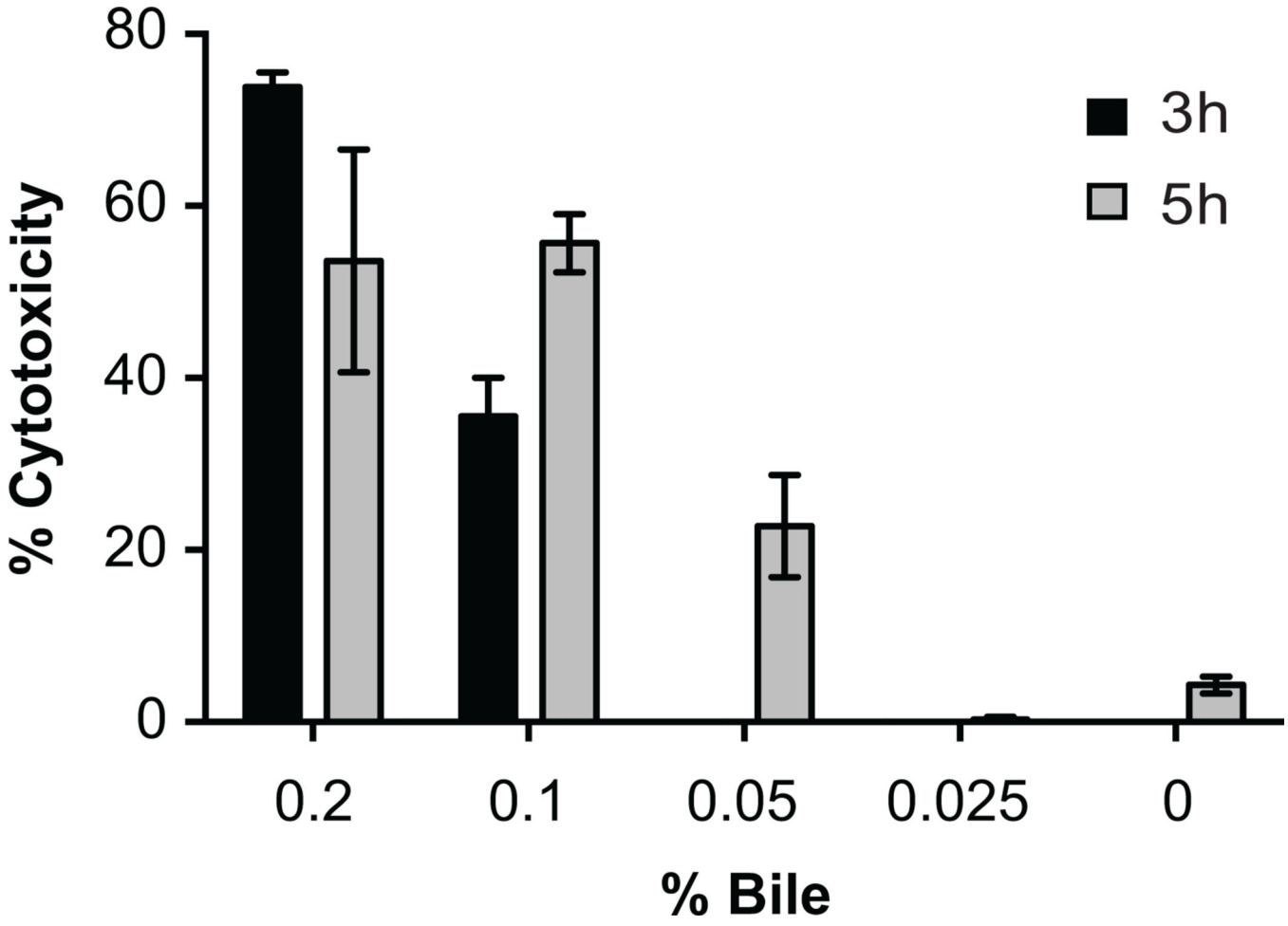

Figure 1. Cytotoxicity occurs in a bile concentration dependent manner

AM-19226 T3SS WT was grown overnight in LB and used to infect Caco2-BBE cells at an MOI of ~10 in the presence of varying bile concentrations as indicated. After 3h or 5h of co-culture, lactate dehydrogenase (LDH) was measured in the co-culture supernatant as an indicator of membrane permeability. Percent cytotoxicity was calculated relative to a total lysis control sample. Data represent the mean ± standard deviation from one experiment using three individual bacterial colonies. The experiment was repeated twice with similar results.

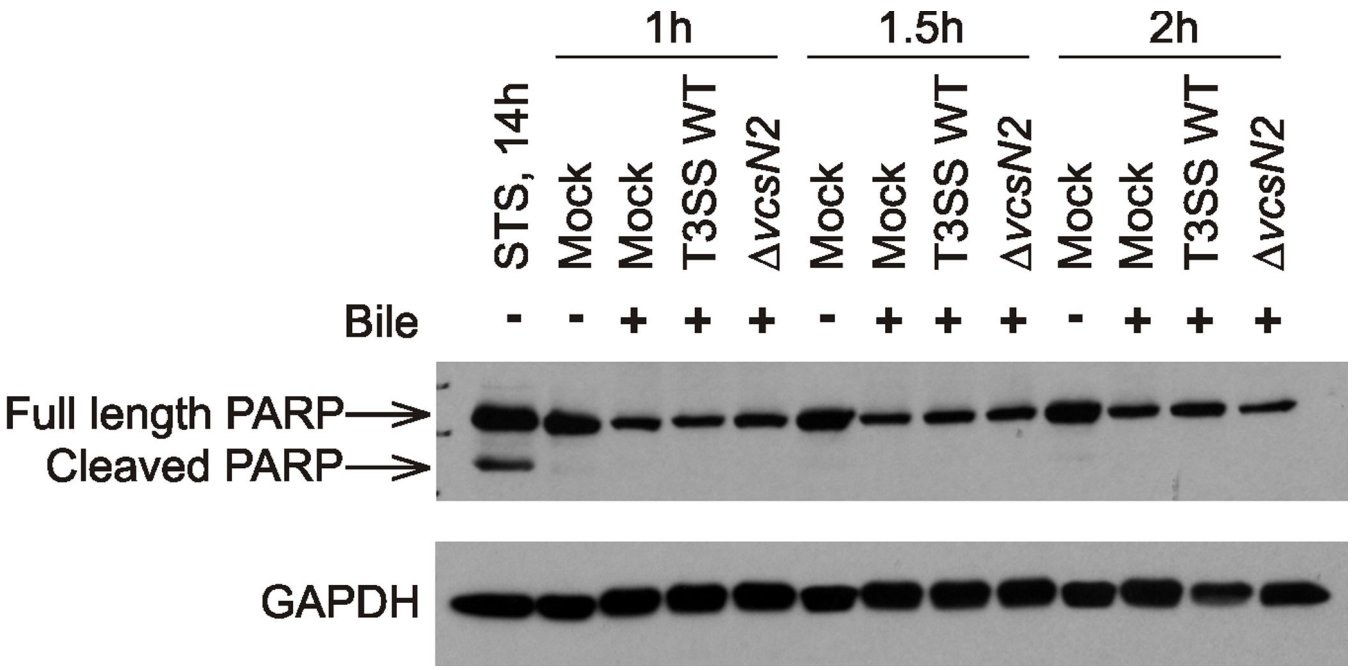

Figure 2. Caco2-BBE PARP is not cleaved during AM-19226 co-culture

AM-19226 T3SS WT and Δ*vcsN2* strains were grown overnight in LB medium and used to infect Caco2-BBE cells at an MOI of ~10 in medium containing 0.2% bile. After 1h, 1.5h, or 2h of co-culture, Caco2-BBE protein lysates were harvested and immunoblotted with anti-poly(ADP-ribose) polymerase (PARP) and anti-GAPDH antibodies. Protein lysates were also harvested from mammalian cells treated with 2μM staurosporine (STS) for 14 hours to induce apoptosis. Experiments were repeated twice and yielded similar results.

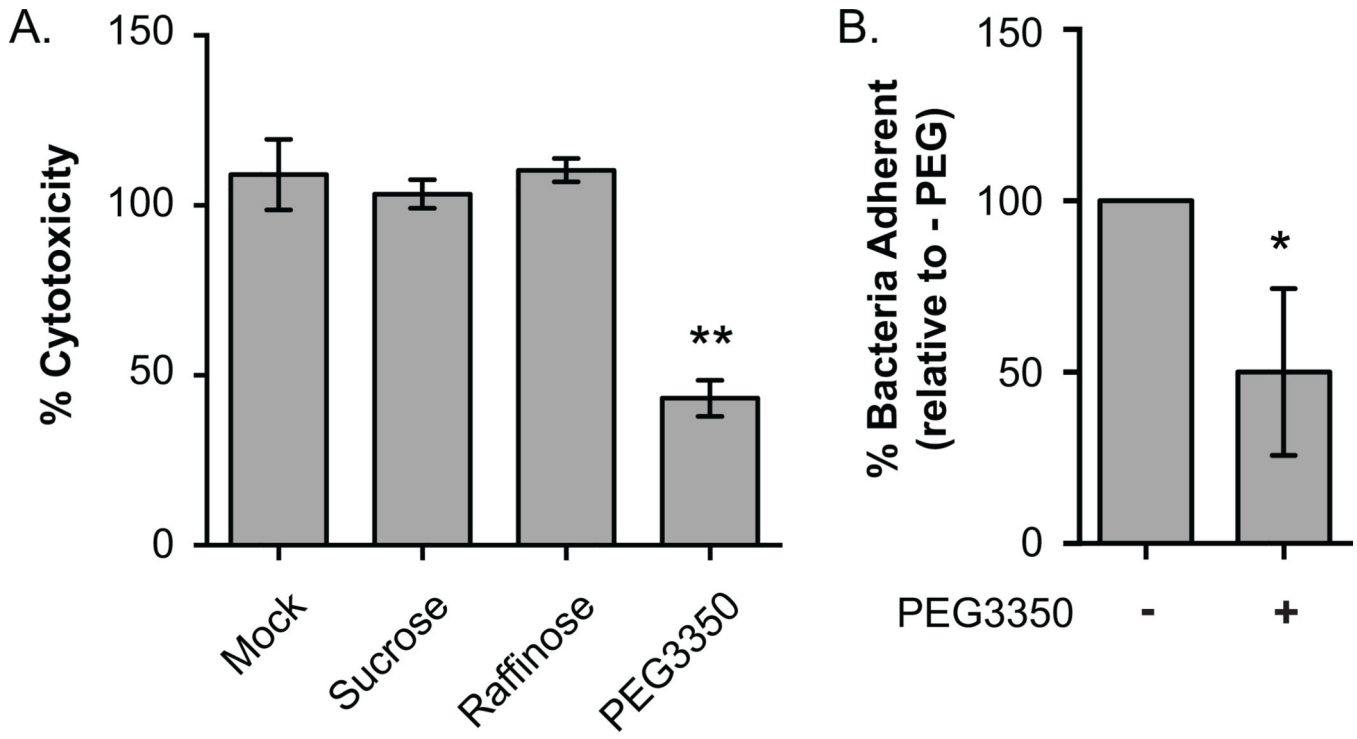

Figure 3.

(A) Pore formation influences cytotoxicity. Three independent colonies of the AM-19226 T3SS WT strain were grown overnight in LB and used to infect Caco2-BBE cells at an MOI of ~10 in the presence of 0.2% bile with or without 30mM sucrose, 30mM raffinose, or 30mM PEG3350. Percent cytotoxicity was calculated at 3h after co-culture began by measuring LDH released into the supernatant. Data represent the mean ± standard deviation from one representative experiment. The experiment was repeated once with similar results. **, P<0.0001 by one-way ANOVA with Dunnett post hoc test. **(B) Bacterial adherence in the presence of PEG.** AM-19226 T3SS WT was grown overnight in LB and co-cultured with Caco2-BBE and an MOI of ~10 in the presence of 0.2% bile with or without 30mM PEG3350. After 2h, percent of bacteria adherent to Caco2-BBE cells was determined. Data represent the mean ± standard deviation from three independent experiments using four individual bacterial colonies per experiment. * P<0.05 by t-test.

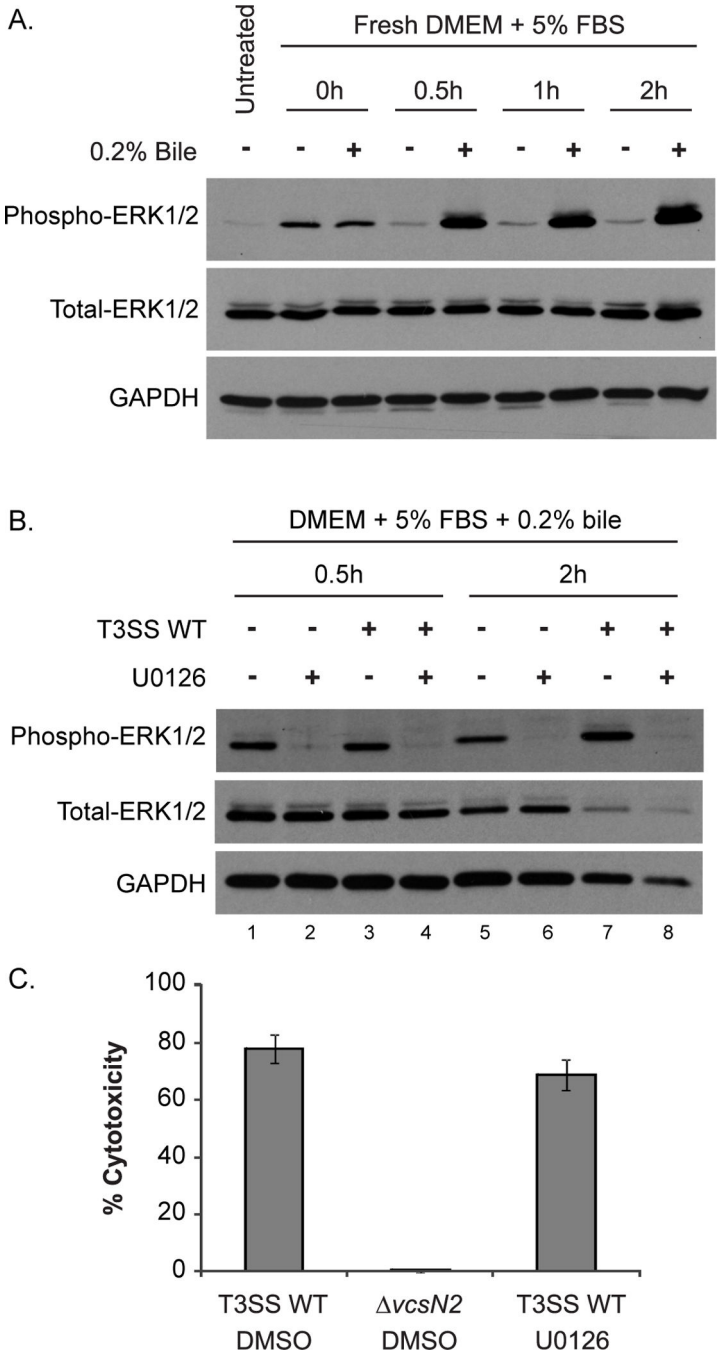

Figure 4.

(A) Bile induces Caco2-BBE ERK1/2 phosphorylation. Caco2-BBE cells were grown for 48h to 80% confluence and protein lysates were harvested from one sample (untreated). Remaining samples were washed with PBS and supplied with fresh DMEM containing 5% FBS with or without 0.2% bile. Protein lysates were harvested 0.5h, 1h, or 2h after media change and probed with antibodies against phosphorylated ERK1/2, total ERK1/2, and GAPDH. The experiment was repeated twice with similar results. **(B) U0126 inhibits bile dependent ERK phosphorylation.** Caco2-BBE cells were grown as in (A) and were then

treated with 10μM U0126 or DMSO in DMEM containing 5% FBS for 1h. Media was removed and replaced with identical medium containing 0.2% bile. Cells were co-cultured with AM-19226 T3SS WT (MOI ~10) grown overnight in LB or were mock infected. Protein lysates were harvested 0.5h or 2h after media change and probed with antibodies against the indicated proteins. The experiment was repeated once with similar results. **(C) Blocking ERK1/2 phosphorylation does not inhibit AM-19226 mediated Caco2-BBE cytotoxicity.** Caco2-BBE cells were treated with 10μM U0126 or DMSO in DMEM containing 5% FBS for 1h. Media was removed and replaced with identical medium containing 0.2% bile. Caco2-BBE cells were infected with AM-19226 T3SS WT or Δ*vcsN2* strains grown overnight in LB medium at an MOI of ~10. After 3h of co-culture, percent cytotoxicity was determined by measuring LDH release in the supernatant. Data shown represents one experiment using five independent colonies per strain. The experiment was repeated once and yielded similar results.

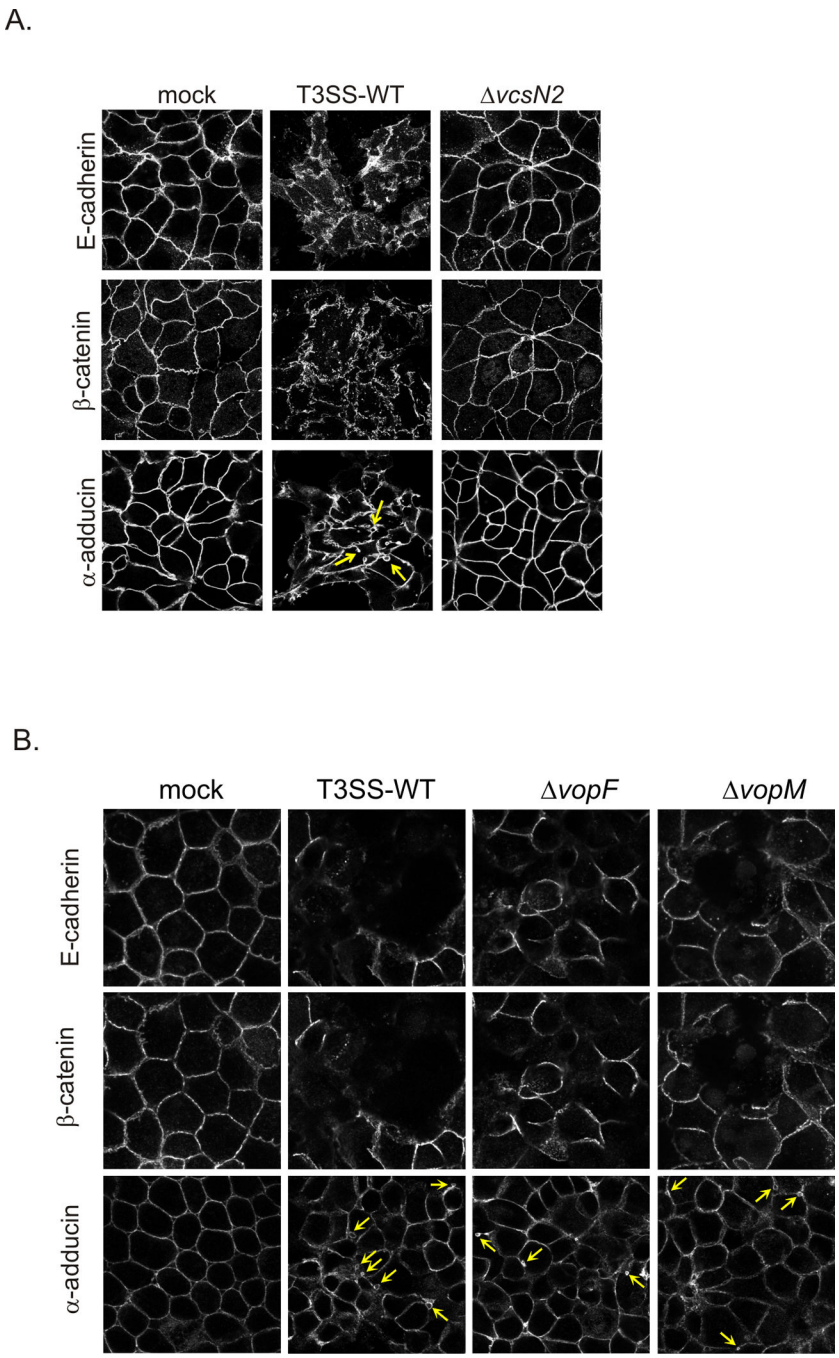

Figure 5. T3SS activity disrupts Caco2-BBE cell-cell junctions

(A) AM-19226 T3SS WT or Δ*vcsN2* strains were grown overnight in LB medium and were used to infect Caco2-BBE cells at an MOI of ~10 in the presence of 0.2% bile. Two independent colonies and wells were infected for each experiment. After 2h of co-culture, the cells were washed once with PBS, fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100. Monolayers were stained with antibodies against E-cadherin, β-catenin, and α-adducin followed by Alexafluor conjugated secondary antibodies. Arrows indicate membrane vesicles. One representative image is shown, and the experiment was

repeated three times with similar results. **(B) VopM and VopF effectors that alter cytoskeletal dynamics do not contribute to junctional protein rearrangement.** The experiment was conducted as described in (A), using AM-19226 T3SS WT, Δ*vcsN2* (T3SS-negative), ΔVopM and ΔVopF strains. Images from one representative experiment of four independent experiments are shown.

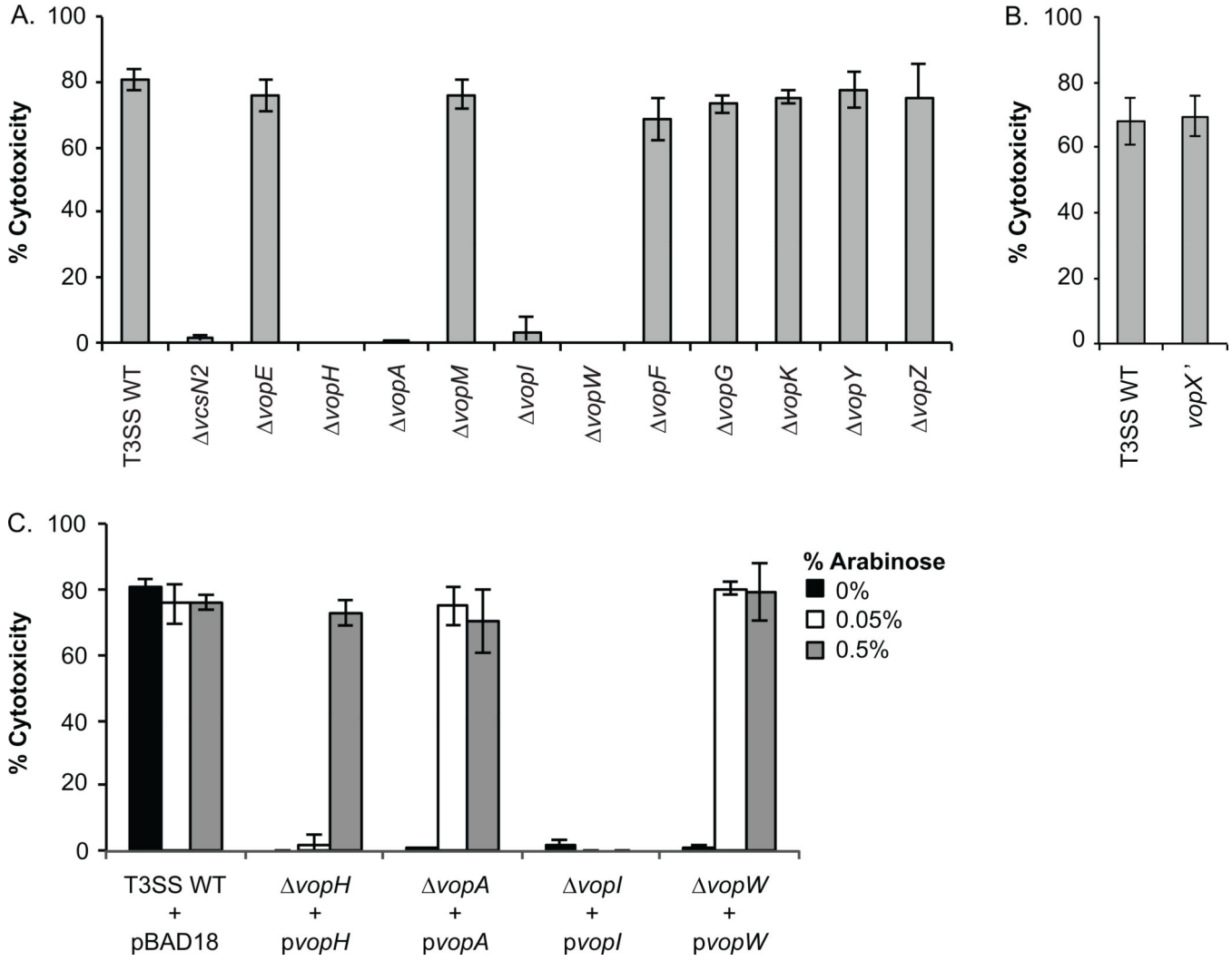

Figure 6. Genetic analysis of Vops required for Caco2-BBE cytotoxicity

AM-19226 strains were grown overnight in LB medium and were used to infect Caco2-BBE cells in the presence 0.2% bile at an MOI of ~10. After 3h of co-culture, percent cytotoxicity was determined by measuring LDH in co-culture supernatants. **(A)** Co-culture was performed with the parental AM-19226 strain, a strain deleted for *vcsN2*, strains deleted for individual effector protein genes (Δ*vop*). **(B)** Co-culture was performed with the parental AM-19226 strain or a *vopX* truncation mutant strain (*vopX'*) **(C)** Co-culture was performed with the parental strain, Δ*vopH*, Δ*vopA*, Δ*vopI*, or Δ*vopW* carrying pBAD18, pBAD18-*vopH*, pBAD18-*vopA*, pBAD18-*vopI*, or pBAD18-*vopW*. Arabinose was added to the co-culture medium at the indicated concentrations to induce *vop* expression from the pBAD18 plasmids. For each panel, data are shown from one experiment using three colonies per strain. Each experiment was repeated twice and produced similar results.

Table 1

Survey of conditions required for Caco2-BBE cytotoxicity.

AM-19226 Overnight	Caco2-BBE Pretreatment	Caco2-BBE/AM-19226 Co-culture	Caco2-BBE Cytotoxicity?
LB	None	0.2% bile	Yes
LB	None	0.05% CHAPS	No
LB	None	0.01% Tween-80	No
LB	None	0.001% Triton X-100	No
LB	None	0.005% SDS	No
LB	None	0.01% Sodium deoxycholate	No
LB	None	0.04% Sodium cholate	No
LB + 0.4% bile	None	No additives	No
LB	0.2% bile 2h	No additives	No

Table 2

Chemicals and inhibitors that do not block cell death.

Chemical / Inhibitor	Function
Z-VAD-fmk	Pan-caspase inhibitor
Z-VD-OPh	Pan-caspase inhibitor
Z-YVAD-fmk	Caspase-1 inhibitor
Necrostatin-1	RIP1 kinase inhibitor
PJ-34	PARP inhibitor
BAPTA-AM	Intracellular calcium chelator
EGTA-AM	Intracellular calcium chelator
Glycine	Prevents non-specific ion flux
Cyclosporin A	Mitochondrial permeability transition pore inhibitor