Type III secretion system 1 of *Vibrio parahaemolyticus* induces oncosis in both epithelial and monocytic cell lines

Xiaohui Zhou, Michael E. Konkel and Douglas R. Call

1Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, WA, USA

2School of Molecular Biosciences, Washington State University, Pullman, WA, USA

The *Vibrio parahaemolyticus* type III secretion system 1 (T3SS1) induces cytotoxicity in mammalian epithelial cells. We characterized the cell death phenotype in both epithelial (HeLa) and monocytic (U937) cell lines following infection with *V. parahaemolyticus*. Using a combination of the wild-type strain and gene knockouts, we confirmed that *V. parahaemolyticus* strain NY-4 was able to induce cell death in both cell lines via a T3SS1-dependent mechanism. Bacterial contact, but not internalization, was required for T3SS1-induced cytotoxicity. The mechanism of cell death involves formation of a pore structure on the surface of infected HeLa and U937 cells, as demonstrated by cellular swelling, uptake of cell membrane-impermeable dye and protection of cytotoxicity by osmoprotectant (PEG3350). Western blot analysis showed that poly ADP ribose polymerase (PARP) was not cleaved and remained in its full-length active form. This result was evident for seven different *V. parahaemolyticus* strains.

*V. parahaemolyticus*-induced cytotoxicity was not inhibited by addition of the pan-caspase inhibitor carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (Z-VAD-FMK) or the caspase-1 inhibitor *N*-acetyl-tyrosyl-valyl-alanyl-aspartyl-aldehyde (Ac-YVAD-CHO); thus, caspases were not involved in T3SS1-induced cytotoxicity. DNA fragmentation was not evident following infection and autophagic vacuoles were not observed after monodansylcadaverine staining. We conclude that T3SS1 of *V. parahaemolyticus* strain NY-4 induces a host cell death primarily via oncosis rather than apoptosis, pyroptosis or autophagy.

INTRODUCTION

*Vibrio parahaemolyticus* is a Gram-negative food-borne pathogen that is commonly associated with consumption of raw or undercooked seafood (Joseph *et al.*, 1982). Symptoms of infection include diarrhoea, nausea, vomiting, headache, fever and chills. In addition to typical gastrointestinal infections, approximately 5% of *V. parahaemolyticus* infections advance to septicemia (Hlady & Klontz, 1996) and these infections may be fatal, especially in immunocompromised patients or those with a preexisting medical condition such as liver disease or diabetes (Yeung & Boor, 2004).

Thermostable direct haemolysin (TDH) is considered the primary virulence factor in *V. parahaemolyticus*. In addition to its ability to form pores in red blood cells, TDH causes increased short circuit current, increased Cl\(^-\) secretion in human colonic epithelial cells, and enhanced Ca\(^{2+}\) entry from the extracellular medium (Takahashi *et al.*, 2001). An early study of a *tdh* deletion mutant demonstrated significantly reduced ability to cause fluid accumulation in ileal loops of a rabbit model (Nishibuchi *et al.*, 1992). In contrast, Park *et al.* (2004) showed that a *tdh* deletion mutant has the ability to cause fluid accumulation in the ligated intestine of rabbits. Furthermore, both TDH-positive and -negative strains of *V. parahaemolyticus* can disrupt the epithelial tight junction, a precursor to dissemination of bacteria into the circulation (Lynch *et al.*, 2005). These studies indicate that there are factors in addition to TDH that contribute to the pathogenesis of *V. parahaemolyticus*.

During the early stage of infection, bacterial-induced epithelial cell death may contribute to the translocation of bacteria from the apical side to the basal side of the intestinal epithelium. Thereafter, some bacteria have adapted a pathogenic mechanism whereby they can...
highjack the resident macrophages in the Peyer’s patches, allowing dissemination of bacteria via the circulatory system. For example, *Listeria monocytogenes* and *Legionella pneumophila* reside intracellularly in the macrophage, thereby avoiding an antimicrobial environment and promoting their dissemination throughout the host without being exposed to antibodies (Duclos & Desjardins, 2000; Pizarro-Cerda & Cossart, 2006; Swanson & Fernandez-Moreira, 2002). In contrast, *Salmonella* and *Shigella* can survive within macrophages as well as kill macrophages, depending on the physiological state of the macrophage (Navarre & Zychlinsky, 2000). Finally, *Yersinia* is also known to induce macrophage death (Hersh et al., 1999; Mills et al., 1997; Palmer et al., 1999).

Apoptosis, pyroptosis, autophagy and oncosis are four distinct pathways used by bacteria to trigger host cell death (Fink & Cookson, 2005). The pathogenic outcomes of these four pathways are distinct, whereby cell death by an apoptotic or autophagy pathway occurs without significant tissue disruption and inflammation, while host cell death by oncosis or pyroptosis leads to secretion of inflammatory cytokines, such as interleukin (IL)-1β and IL-8, and subsequent recruitment of activated neutrophils to the site of infection (Navarre & Zychlinsky, 2000). Interestingly, the same species of bacteria may use more than one mechanism to induce the death of epithelial cells and macrophages. For example, *Salmonella* can induce apoptosis of macrophages in a type III secretion system (T3SS)-dependent manner (Chen et al., 1996). *Salmonella* can also cause pyroptosis of macrophages, which is characterized by the activation of caspase-1 (Brennan & Cookson, 2000). *Salmonella* can also induce autophagy of macrophages that features degradation of cellular components, vacuolization and slight chromatin condensation. Finally, T3SS SPI of *Salmonella* can form pores in the host cell membrane, leading to oncosis of infected host macrophages characterized by cellular swelling, increased membrane permeability, and depletion of cellular energy (Fink & Cookson, 2006).

Recently, *V. parahaemolyticus* has been shown to induce cell death in HeLa cells by apoptosis, as evidenced by DNA fragmentation, and this apoptosis is dependent on T3SS1 (Ono et al., 2006). Nevertheless, the clinical manifestation of vibriosis is consistent with an inflammatory response, including neutrophil recruitment to the site of intestinal infection (Qadri et al., 2003); this inflammatory response is more consistent with oncosis or pyroptosis than apoptosis or autophagy. Furthermore, Burdette et al. (2008) have recently reported that *V. parahaemolyticus* induces autophagy in cell culture, but blocking this pathway does not block cell death. Thus, it is very likely that alternative pathways are primarily responsible for cell death.

In this report we further characterized *V. parahaemolyticus*-induced cell death in both epithelial (HeLa) and monocytic (U937) cells, and evaluated invasiveness in these cell types. We confirmed that *V. parahaemolyticus* causes cytotoxicity to both HeLa and U937 cells in a T3SS1-dependent manner, but that cell death occurred by oncosis, as demonstrated by morphological and biochemical analysis; this process does not require intracellular *V. parahaemolyticus*, although the process appears to be contact-dependent.

**METHODS**

**Bacterial strains and growth conditions.** Wild-type *V. parahaemolyticus* strains used are listed in Supplementary Table S1. *Escherichia coli* S-17 was used in the gene deletion experiments. *V. parahaemolyticus* was cultured in Luria–Bertani (LB) medium supplemented with 2.5 % sodium chloride. *E. coli* was cultured in LB medium.

**Cell lines.** HeLa cells (CCL-2) and U937 cells (CRL-1593.2) were purchased from the American Type Culture Collection. HeLa cells were maintained as monolayer in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented with 10 % fetal calf serum (FCS) at 37 °C. HeLa cells were passaged every 1–2 days and were washed once before inoculation. U937 cells were maintained with RPMI 1640 (ATCC 30-2001) supplemented with 10 % FCS at 37 °C in a humidified 5 % (v/v) CO₂ incubator and were passaged every 2–3 days. Cells were resuspended in RPMI 1640 culture medium supplemented with phosphol 12-myristate 13-acetate (PMA; Sigma) at a final concentration of 10 ng ml⁻¹ to induce differentiation (Tsuchiya et al., 1982). Cells were dispensed into 12-well tissue-culture plates at a density of approximately 1 × 10⁶ cells per well and incubated for 24 h at a 37 °C humidified 5 % (v/v) CO₂ incubator. After incubation, PMA-containing medium was aspirated and adherent (differentiated) cells were washed once with culture medium before inoculation.

**Secretion of Vp1656 by *V. parahaemolyticus*.** Protein secretion was determined as described previously (Zhao et al., 2008). Briefly, to collect extracellular proteins, an overnight culture was diluted (1:100) in LB medium supplemented with 2.5 % NaCl and grown for 2 h at 37 °C with shaking. Expression of ExsA was induced by the addition of IPTG to a final concentration of 1 mM. Bacterial culture (15 ml) was centrifuged at ~3000 g for 15 min, and the supernatant for each sample was passed through a 0.22 µm pore-size syringe filter to exclude the remaining bacteria within the supernatant. Proteins in the supernatant were precipitated by adding TCA (10 %, v/v). To collect the total proteins for detection of Vp1656, pellets from each sample (15 ml) were resuspended in PBS (pH 7.4, 1 ml) and sonicated for 1 min to lyse the bacteria and shear the chromosomal DNA. Supernatant and pellet samples were loaded onto a 12 % SDS-PAGE gel and Western blot analysis was performed with polyclonal anti-Vp1656 antibody (Zhao et al., 2008).

**Infection and lactate dehydrogenase (LDH) assay.** *V. parahaemolyticus* was harvested from overnight culture and pelleted by centrifugation at 10 000 g at room temperature. The bacterial pellets were resuspended in DMEM containing 1 % (v/v) FCS. Bacterial suspensions (10 µl) were added to each well, containing approximately 10⁵ host cells, to achieve an m.o.i. of 100 c.f.u. per cell. Plates were centrifuged at 1000 g to synchronize bacteria–host cell contact. In experiments using caspase inhibitors, cells were incubated with the pan-caspase inhibitor carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (Z-VAD-FMK; 100 µM; R&D Systems) or the caspase-1 inhibitor N-acetyl-tyrosyl-valyl-alanyl-aspartyl-aldehyde (Ac-YVAD-CHO; 20 µM) (CalBiochem) for 1 h prior to inoculation or treatment with staurosporine (STS; Sigma; 2 µM). To inhibit bacterial internalization, cells were treated with
cytochalasin D (CalBiochem) (1 μg ml$^{-1}$) 1 h prior to inoculation. For the LDH release assay, the medium was free of antibiotics before inoculation. Supernatant was collected at different time points after infection and LDH activity was measured with a Cytotoxicity Detection kit (Promega) according to the manufacturer's instructions. Maximum release was achieved by lysis of cells with the lysis buffer provided in the kit. Spontaneous LDH release in the supernatant of uninfected cells was also measured as described above. Percentage cytotoxicity was calculated using the formula:

\[
\text{Percentage cytotoxicity} = \frac{\text{test LDH release} - \text{spontaneous release}}{\text{maximal release}} \\
\]

Transmission electron microscopy. For transmission electron microscopy, *V. parahaemolyticus* strains and U937 cells were co-incubated at 37 °C for 3 h within 12-well cell-culture plates. Infected U937 cells were scraped from the well, transferred to 1.5 ml microfuge tubes, and fixed overnight at 4 °C in 2 % glutaraldehyde and 2 % paraformaldehyde. Samples were then rinsed in cacodylate buffer, incubated overnight at 4 °C in 1 % osmium tetroxide, and incubated for 1 h in 1 % tannic acid at room temperature. The samples were dehydrated, embedded in Spurr's solution, sectioned to 70–100 nm thin sections, and post-stained using 1 % uranyl acetate and lead citrate. Sections were examined with a transmission electron microscope at the Washington State University Electron Microscopy Center. All the reagents were provided by the Electron Microscopy Center.

Fluorescence microscopy. Pore formation was detected by fluorescence microscopy as described elsewhere (Kirby et al., 1998). Briefly, HeLa and U937 cell monolayers were grown on coverslips before inoculation with strains of *V. parahaemolyticus* and after 3 h of infection coverslips were covered with PBS containing 25 μg ml$^{-1}$ ethidium bromide and 5 μg ml$^{-1}$ acidine orange (AO). Coverslips were observed using FITC (543 nm) and rhodamine (488 nm) filters with a Zeiss confocal microscope. Autophagic vacuoles were detected by monodansylcadaverine (MDC; Sigma) labelling and fluorescence microscopy as described elsewhere (Munafò & Colombo, 2001). Briefly, HeLa cells seeded on the coverslips in a six-well plate overnight to achieve a monolayer were infected with *V. parahaemolyticus* strains for 3 h. The infected HeLa cells were rinsed twice with PBS and incubated with 0.05 mM MDC in PBS at 37 °C for 10 min. After incubation, cells were washed four times with PBS and coverslips were analysed using FITC (543 nm) filters with a Zeiss confocal microscope. For positive and negative controls, HeLa cells were treated with rapamycin (2 μg ml$^{-1}$; Fisher) for 24 h or left untreated, and observed after MDC labelling.

Deletion mutants. Deletion mutants were generated by homologous recombination. A chromosomal deletion in the vcrD (vp1662) gene was constructed by allelic exchange using a suicide vector, pDM4, carrying DNA fragments corresponding to vcrD flanking regions (Milton et al., 1996). PCR-amplified DNA fragments used for constructing an in-frame deletion mutation of vcrD were generated by overlap PCR. Two DNA fragments were amplified by PCR with *V. parahaemolyticus* strain NY-4v1 chromosomal DNA as a template with primer pairs vcrD-1F and vcrD-1R and vcrD-2F and vcrD-2R, respectively (Supplementary Table S2). Primers vcrD-1R and vcrD-2R were complementary to each other. These two fragments were gel-purified and used as templates in a second PCR with primers vcrD-1F and vcrD-2R, resulting in the construction of a fragment with a deletion of 1682 bp in the vcrD gene. The fragment containing the deletion was purified and digested with XhoI and XbaI, and then cloned into a suicide vector, pDM4, that had been pretreated with the same enzymes. This vector was then transferred to NY-4 by conjugation and both ampicillin- and chloramphenicol-resistant transconjugants were selected. Ampicillin (100 μg ml$^{-1}$) was used to select against *E. coli* and chloramphenicol (5 μg ml$^{-1}$) was used to select transconjugants. To complete the allelic exchange, the integrated suicide plasmid was induced to excise from the chromosome by growth on LB medium containing sucrose (5 %). Chloramphenicol-sensitive clones were then obtained and screened by PCR with primers vcrD-up and vcrD-down, which lie adjacent to the flanking regions cloned into the plasmid (Supplementary Table S2). One clone with a vcrD gene deletion hereafter designated NY-4v1 was selected. Construction of an escV deletion mutant was performed in the same manner by using the following primers: escV1F, escV1R, escV2F, escV2R, escV-up and escV-down (Supplementary Table S2). One clone, designated NY-4e1, was selected. VcrD/escV deletion mutants were generated by constructing an escV deletion mutant in strain NY-4v1 to produce strain NY-4v1e1.

Detection of poly ADP ribose polymerase (PARP) cleavage by immunoblotting. Infected, uninfected, STS (2 μM) or t-butylhydroperoxide (TBH; 200 μM)-treated HeLa cells and U937 cells were harvested and lysed in radiolmmunoprecipitation assay (RIPA) buffer, consisting of 25 mM Tris/HCl, pH 7.6, 150 mM NaCl, 1 % Triton X-100, 1 % sodium deoxycholate and 0.1 % SDS. Samples (100 μl) were dissolved in 100 μl SDS sample buffer containing 2.3 % (w/v) SDS, 10 % (w/v) glycerol, 5 % (v/v) 2-mercaptoethanol and 10 μg bromophenol blue ml$^{-1}$ in 125 mM Tris/HCl, pH 6.8, and heated at 100 °C for 5 min and loaded on a 10 % (w/v) polyacrylamide gel. After electrophoresis, proteins were electrophoretically transferred to a nitrocellulose membrane for 12 h. The membrane was blocked with 5 % skimmed milk in Tris-buffered saline (20 mM Tris, 137 mM NaCl, pH 7.6) containing 0.1 % Tween 20 and probed with monoclonal anti-PARP antibody C-20 (Invitrogen) for 1 h at room temperature. The secondary antibody was an anti-mouse immunoglobulin conjugated to horseradish peroxidase. The blots were developed by using the Western Blot kit (Bio-Rad) according to the manufacturer’s instructions.

Osmoprotection assay. HeLa cells and U937 cells were infected with strains of *V. parahaemolyticus* or treated with TBH in the presence of 30 mM of various osmoprotectants. Osmoprotectants included sucrose (diameter~0.9 nm), PEG1450 (diameter~4 nm), and PEG3350 (diameter~3.8 nm). All these chemicals were purchased from Sigma. LDH release was measured after 3 h of infection and experiments were performed at least three times.

Gentamicin protection assay. Invasion of HeLa or U937 cells was assessed by a gentamicin protection assay. Overnight *V. parahaemolyticus* culture (10 μl; ~10⁸ bacterial cells) was resuspended in DMEM and added to each well containing HeLa or U937 monolayers (~10⁸ cells per well). *V. parahaemolyticus*-infected monolayers were incubated at 37 °C for 1 h, after which the DMEM was aspirated and wells were gently washed once with PBS buffer. DMEM containing 200 μg gentamicin ml$^{-1}$ was added to each well for 1 h. Medium was aspirated and cells were washed with PBS two times, and then cell monolayers were lysed by forcibly pulling the culture through a 27 gauge needle 10 times. Intracellular c.f.u. of *V. parahaemolyticus* was determined by serial plating on LB agar with 3 % NaCl and expressed as total c.f.u. minus extracellular c.f.u.

Statistical analysis. Student’s t test was used to compare groups in osmoprotectant- and caspase inhibitor-treatment assays, and P<0.05 was considered statistically significant. Pearson’s correlation coefficient (r) was used to examine relationships between intracellular c.f.u. and LDH release.
RESULTS

T3SS1 of *V. parahaemolyticus* induces rapid death of both HeLa and U937 cells

Earlier studies have shown that T3SS1 induces cytotoxicity in epithelial cells after 4 h infection and that this process involves an apoptotic pathway (Ono et al., 2006; Park et al., 2004). In the present study, we examined T3SS1 of *V. parahaemolyticus*-induced cell death for both HeLa and U937 cells. We generated T3SS1 mutant (NY-4v1), T3SS2 mutant (NY-4e1) and T3SS1–T3SS2 double mutant (NY-4ve1) strains and determined the secretion of Vp1656 (a known T3SS1 secretion protein) by the wild-type and each mutant strain. Previous work had shown that T3SS1 genes are expressed when the T3SS1 positive regulator (ExsA) is overexpressed in *V. parahaemolyticus* (Zhou et al., 2008). To confirm that our knockout strains specifically blocked the T3SS1 secretion pathway, we transformed the wild-type and each mutant strain of *V. parahaemolyticus* with an *exsA* plasmid and determined whether they secreted a known T3SS1 secretion protein, Vp1656. Vp1656 was expressed in all strains when they were transformed with an *exsA* plasmid, pexsA (Fig. 1, upper panel). However, Vp1656 was secreted only by wild-type and T3SS2 mutant strains, and was not secreted by the T3SS1 mutant and T3SS1–T3SS2 double mutant (Fig. 1, lower panel). These results confirmed that T3SS1-dependent secretion of Vp1656 was eliminated in the T3SS1 mutant and T3SS1–T3SS2 double mutant strains, while wild-type and T3SS2 mutant strains were competent for the secretion of Vp1656.

We then infected HeLa and U937 cells with wild-type, T3SS1 mutant (NY-4v1), T3SS2 mutant (NY-4e1) and T3SS1–T3SS2 double mutant (NY-4ve1) strains. After 3 h of infection, the wild-type and T3SS2 mutant strains caused ~95 and 58 % of HeLa cell death, respectively, as measured by an LDH-release assay (Fig. 2a, white bars). In contrast, both the T3SS1 mutant and the T3SS1–T3SS2 double mutant strains killed <20 % of HeLa cells (Fig. 2a). HeLa cells infected with wild-type and T3SS2 mutant strains exhibited rounding and appeared to be swelling (Fig. 2b, d). In contrast, HeLa cells infected with the T3SS1 mutant or the T3SS1–T3SS2 double mutant strains maintained a uniform monolayer and normal cell appearance compared with uninfected cells (Fig. 2c, e and f).

**T3SS1-induced cytotoxicity is invasion-independent and cell contact-dependent**

*Salmonella*-induced cytotoxicity in macrophages is dependent on its invasive phenotype (Chen et al., 1996; Hersh et al., 1999); thus, we hypothesized that the cytotoxic differences between wild-type and T3SS1 mutant strains are due to different invasive ability. To test this hypothesis, we performed a gentamicin protection assay to measure the invasion rate of both wild-type and T3SS mutant strains in HeLa and U937 cells. The c.f.u. was similar for intracellular wild-type and T3SS mutant strains recovered from HeLa cells and U937 cells (Fig. 3a). These results indicate that different levels of cytotoxicity caused by wild-type and T3SS mutant strains are not due to a differential invasive phenotype. To explore this further, we also measured the invasiveness and cytotoxicity for an additional 12 strains of *V. parahaemolyticus* and one strain of *Salmonella typhimurium*. *Salmonella* invasion was ~10–100 times greater than that of *V. parahaemolyticus*. Strains NY-2 and B-4 showed the highest level of invasiveness; nevertheless, while strain NY-2 induced 80 % cell death for HeLa cells, strain B-4 induced only 20 % cell death 4 h post-infection (Fig. 3b, c), indicating that the high cell death induction of strain B-4 was not related to its invasiveness. In contrast, strains NY-10, NY-11 and NY-12 induced high levels of cytotoxicity with low levels of invasiveness (Fig. 3b, c), suggesting that the high cytotoxicity of these strains was not due to their invasive phenotypes. Overall, there was no correlation between recovery of intracellular c.f.u. and LDH release (r = −0.005).

To further test if intracellular bacteria are necessary for cytotoxicity, we treated HeLa cells for 1 h with cytochalasin D, which inhibits actin polymerization and therefore bacterial invasion of mammalian cells, and then infected...
the cells with wild-type *V. parahaemolyticus*. As reported elsewhere (Akeda *et al.*, 1997), bacterial c.f.u. recovered from HeLa cells treated with cytochalasin D was ~1–2% of that recovered from HeLa cells that were not treated with cytochalasin D (Fig. 3d), indicating that cytochalasin D significantly inhibited bacterial internalization by HeLa cells. Nevertheless, cell killing was not inhibited by treatment with cytochalasin D (Fig. 3e), confirming that bacterial invasiveness was not responsible for the cell killing by T3SS1. In addition, cytochalasin D treatment without infection did not cause significant LDH release (Fig. 3e), indicating that cytochalasin D did not promote LDH release during these experiments.

To determine whether cell contact is required for the T3SS1-induced cytotoxicity, we collected culture media from HeLa cells 4 h post-infection (m.o.i. 100:1) and centrifuged it to remove cells. The supernatant was filtered-sterilized (0.22 μm pore-size) and gentamicin (10 μg ml⁻¹) was added before adding the supernatant back (neat) to fresh HeLa cell monolayers. Supernatant from HeLa cells infected with wild-type, T3SS1 mutant, T3SS2 mutant and T3SS1–T3SS2 double mutant strains caused 5.6 ± 0.7, 4.8 ± 1.5, 5.5 ± 1.5 and 6.5 ± 1.5% cell death, respectively. We concluded from this experiment that cell contact is required for *V. parahaemolyticus* to induce host cell death.

**Pore forming activity of T3SS1**

*Salmonella* uses a T3SS-dependent mechanism to form pores in the host cell membrane, thereby leading to osmotic lysis of host cells (Fink & Cookson, 2006). Based on the morphology (cell rounding and swelling) of infected HeLa cells as observed by light microscopy (Fig. 2b, d), we hypothesized that T3SS1 of *V. parahaemolyticus* can form a pore structure on the host membrane, resulting in osmotic lysis. Infected cells were stained with a membrane-impermeable dye (ethidium bromide; EtBr) and a

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**Fig. 2.** *V. parahaemolyticus* induces rapid death of both HeLa and U937 cells in a T3SS1-dependent manner. (a) HeLa cells (white bars) and U937 cells (grey bars) were killed by the wild-type (NY-4) and T3SS2 mutant (NY-4e1) strains, but much lower cytotoxicity was observed for cells infected with the T3SS1 mutant (NY-4v1) and T3SS1–T3SS2 double mutant (NY-4ve1) strains (3 h infection). The results represent the mean ± SD of three biological replicates. Morphological observations under a light microscope for (b) HeLa cells infected with the wild-type, (c) T3SS1 mutant, (d) T3SS2 mutant and (e) T3SS1–T3SS2 double mutant strains, and (f) uninfected HeLa cells. Bar, 100 μm; ×100 magnification.
To estimate the size of the pores produced by T3SS1 of V. parahaemolyticus, we infected HeLa and U937 cells with the NY-4 strain in the presence of osmoprotectants of different molecular mass. The premise of this assay is to introduce increasingly larger osmoprotectants until one is encountered that provides protection by blocking membrane pores. LDH release was significantly reduced when HeLa and U937 cells were infected with NY-4 in the presence of PEG3350, while PEG1450 and sucrose had no protective effect (Fig. 5). PEG1450 and PEG3350 have diameters of 2.4 and 3.8 nm, respectively, indicating that the pore-size produced by T3SS1 of V. parahaemolyticus is between 2.4 nm and 3.8 nm. LDH release of control U937 cells induced by TBH, an organic hydroperoxide that induces oxidative stress, resulting in ATP depletion and cell death (Nieminen et al., 1995), was not prevented by any of the osmoprotectants (Fig. 5). Collectively, these data indicate that V. parahaemolyticus is causing host cell death by producing pores in HeLa and U937 cells via the T3SS1 pathway.

Morphological and biochemical characterization of T3SS1-induced cell death

After 3 h of infection, intracellular bacteria were evident within the U937 cells infected with wild-type and T3SS mutant strains (white arrows in Fig. 6f–i), while uninfected U937 cells harboured no intracellular bacteria (Fig. 6j). Intracellular bacteria were not distinguishable in the dying HeLa cells (Fig. 6a–e), probably due to the lower invasion rate for HeLa cells compared with U937 cells (Fig. 3a). One interesting finding was that U937 and HeLa cells infected with wild-type and T3SS2 mutant strains displayed severe cytoplasmic vacuolation (black arrows in Fig. 6a, c, f and h) while cells infected with the T3SS1 or T3SS1–T3SS2 double mutant strain have no obvious changes (Fig. 6b, d, g and i) compared with uninfected cells (Fig. 6e, j). The membrane integrity of the cells infected with wild-type and T3SS2 mutant strains was disrupted, while there was no evidence of similar changes in the cells infected with T3SS1 and T3SS1–T3SS2 double mutant strains. Changes typical of apoptosis, such as chromatin condensation, membrane blebbing and apoptotic bodies, were not observed in U937 and HeLa cells infected with either wild-type or mutant strains of V. parahaemolyticus.

PARP is a 116 kDa enzyme that is cleaved into an 89 kDa inactive form to conserve the energy required for apoptosis (Affàr et al., 2001); thus, detection of the cleaved form of PARP is a hallmark of apoptotic cell death. In contrast, for the other three mechanisms of cell death (autophagy, oncosis and pyroptosis), PARP is maintained as a full-length protein and the active form depletes its substrate NAD and ATP, leading to cell death. As shown in Fig. 7(a, b) (lanes 1–4), PARP remained in its active uncleaved state in both wild-type and mutant infected HeLa cells (Fig. 7a) and U937 cells (Fig. 7b). In control cells treated with STS, a protein kinase inhibitor that induces apoptosis, PARP was clearly cleaved into an 89 kDa inactive form (Fig. 7a, b, lanes 5). These results indicated that the apoptotic pathway leading to PARP cleavage does not function during strain NY-4-induced cell death for either U937 or HeLa cells, and therefore apoptosis does not occur when either HeLa or U937 cells are infected with strain NY-4. To determine whether this conclusion is applicable to other strains of V. parahaemolyticus, we infected HeLa cells with six different strains that have been shown to be cytotoxic to HeLa cells (Fig. 3c) and then measured the cleavage of PARP. PARP remained in its active, uncleaved state regardless of the infecting strain (Fig. 7c), indicating that the apoptotic pathway is not induced by any of the seven strains tested in this experiment.

V. parahaemolyticus-induced host cell death is caspase-independent

Because PARP is cleaved by activated caspase-3 and caspase-7 (Affàr et al., 2001; Germain et al., 1999; Lazebnik et al., 1994; Tewari et al., 1995) and our results showed that PARP is not cleaved during cell death induced by V. parahaemolyticus (Fig. 7), this indicated that caspase-3 and caspase-7 are not activated during the process of cell death. To further confirm that caspase is not involved in the V. parahaemolyticus-induced cell death, we treated HeLa cells with a pan-caspase inhibitor Z-VAD-FMK for
Fig. 4. Pore formation on the membranes of HeLa and U937 cells by T3SS1 of *V. parahaemolyticus*. HeLa cells (a–e) and U937 macrophages (f–j) adherent to glass coverslips were infected with wild-type strain (a, f), T3SS1 mutant strain (b, g), T3SS2 mutant strain (c, h) and T3SS1-T3SS2 double mutant strain (d, i) at an m.o.i. of 100:1 for 3 h. (e) and (j) show uninfected HeLa and U937 cells, respectively. Cells were stained with the membrane-permeable dye AO (green) and the membrane-non-permeable dye ethidium bromide (red). Adherent cells were visualized by confocal fluorescence microscopy. Bars, 50 μm.
1 h prior to infection and measured LDH release; others have shown that at a concentration of 100 μM, Z-VAD-FMK completely inhibits Fas-mediated apoptosis (Kato et al., 2000). Results showed that the pan-caspase inhibitor significantly protected STS-treated cells from death (Fig. 8a), indicating that Z-VAD-FMK inhibited activation of caspasess in STS-induced apoptotic cells. The pan-caspase inhibitor did not prevent TBH-treated cells from dying (Fig. 8a), indicating that, as expected, caspasess are not involved in TBH-induced cell death. The pan-caspase inhibitor did not prevent cell death of HeLa cells infected with wild-type and escV mutant strains. Thus, caspasess are not activated and are not involved in V. parahaemolyticus-induced cytotoxicity. DNA fragmentation analysis showed that neither wild-type nor mutant strains of V. parahaemolyticus induced DNA fragmentation in HeLa cells, while the apoptosis inducer STS induced significant DNA fragmentation (Fig. 8b). These results confirmed that apoptosis is not involved in the host cell death caused by T3SS1 of V. parahaemolyticus.

To verify that pyroptosis does not play a role in V. parahaemolyticus-induced cell death, we treated U937 cells with caspase-1 inhibitor 1 h prior to infection and measured LDH release. Salmonella-induced cell death was significantly inhibited by treatment with caspase-1 inhibitor (Fig. 9), consistent with an earlier report that Salmonella induces caspase-1-dependent pyroptosis (Fink & Cookson, 2006). Cell death induced by wild-type and T3SS2 mutant strains of V. parahaemolyticus was not prevented (Fig. 9), indicating that caspase-1 is not involved in V. parahaemolyticus-induced cytotoxicity.

V. parahaemolyticus does not induce autophagy in HeLa cells

Wild-type and T3SS2 mutant strains induced vacuoles in both HeLa and U937 cells (Fig. 7). To determine whether these vacuoles were autophagic vacuoles, infected cells were labelled with an autofluorescent drug, MDC, a selective marker for autophagic vacuoles (Biederbick et al., 1995). MDC-labelled vesicles were not evident in HeLa cells infected with wild-type (Fig. 10a), T3SS1 mutant (Fig. 10b), T3SS2 mutant (Fig. 10c) and T3SS1–T3SS2 double mutant (Fig. 10d) strains. As a negative control, uninfected HeLa cells did not show any MDC-labelled vesicles (Fig. 10e). As a positive control, HeLa cells were treated with rapamycin, a known autophagy inducer (Iwai-Kanai et al., 2008), for 24 h and then were stained with MDC. As shown in Fig. 10(f), the distinct dot-structures with punctuate MDC fluorescence appeared in the cytoplasm or in the perinuclear regions (white arrows). These results indicated that V. parahaemolyticus does not induce autophagy in HeLa cells.

DISCUSSION

The genome of V. parahaemolyticus harbours two distinct T3SSs of which T3SS1 is similar to the Ysc secretion system in Yersinia, while T3SS2 is similar to Inv–Mxi–Spa secretion system in Salmonella and Shigella (Denecker et al., 2002). The Ysc secretion system is typically associated with cytotoxicity, while the Inv–Mxi–Spa secretion system usually contributes to host cell invasion (Denecker et al., 2002). Earlier studies have shown that one fully sequenced clinical strain of V. parahaemolyticus (RIMD2210633) can infect HeLa cells, which in turn display apoptotic changes including DNA fragmentation and the presence of phosphatidylserine in the outer membrane leaflet of the plasma membrane. These apoptotic changes are dependent on T3SS1 (Ono et al., 2006; Park et al., 2004).

We describe herein another cytopathic effect that differs from apoptosis and that is induced by T3SS1 of strain NY-4 of V. parahaemolyticus in both HeLa and U937 cell lines. The cytopathic effect is manifested by LDH release, cellular swelling, pore formation on the cell membrane, and cytoplasmic vacuolation. In addition, infected cells retained PARP in its active, uncleaved state and DNA fragmentation...
did not occur. These results indicated that the NY-4 strain of *V. parahaemolyticus* does not induce epithelial and monocytic cell death via a mechanism of apoptosis. In addition, a panel of six other strains that are cytotoxic to both HeLa cells and U937 cells did not cause PARP cleavage (Fig. 7c), indicating that these strains also did not cause apoptosis. It is unclear why our results contradicted earlier results, although this may reflect different phenotypes associated with different strains, thereby illustrating phenotypic and genotypic diversity attributable to *V. parahaemolyticus*. For example, it is possible that the wild-type strain (NY-4) and the other six strains used in the present study caused rapid cell death (within 4 h) by unrecognized effector protein(s) of T3SS1 that are absent in the strain used in previous studies (RIMD2210633; unavailable for the present study) and that this rapid cell death prevented detection of apoptosis in our study. Burdette et al. (2008) also reported that host cell death induced by *V. parahaemolyticus* is independent of apoptosis.

T3SSs are needle-like structures expressed on the surface of Gram-negative bacteria and upon contact with eukaryotic cells (Cornelis & Van Gijsegem, 2000; Cornelis, 2002, 2006; Hueck, 1998; Mota & Cornelis, 2005). These structures become inserted into the cell membrane to create pores in the membrane surface (Neyt & Cornelis, 1999); these pores assist the translocation of effector proteins from bacteria into host cells (Neyt & Cornelis, 1999). In some cases, pore formation results in the entry of small ions and water from the extracellular milieu into the cytosol of host cells leading to cellular oncotic lysis (Dacheux et al., 2001; Fink & Cookson, 2006; Sun et al., 2005). For example, T3SSs of *Pseudomonas, Salmonella* and *Burkholderia* induce oncosis of macrophages that can be prevented by addition of osmoprotectants such as PEG with a molecular diameter larger than that of the pores. Our results showed that wild-type *V. parahaemolyticus* and T3SS2 mutant strains, not T3SS1 and T3SS1–T3SS2 double mutant strains, induced host cell LDH release, cell rounding (Fig. 2a, b, d), uptake of membrane-impermeable dye (Fig. 4a, c, f, h) and disruption of the integrity of cell membrane (Fig. 6a, c, f, h). These findings are consistent with cell death induced by oncosis; this phenotype was abrogated by addition of PEG3350, but not by osmoprotectants with a molecular diameter less than that of PEG3350, suggesting that the pore sizes on the host membrane are between 2.4 and 3.8 nm. This is larger than pores that are created by *Pseudomonas* (1.3–2.8 nm), *Salmonella* (1.1–2.4 nm) and *Burkholderia* (1.4–2.4 nm) (Dacheux et al., 2001; Fink & Cookson, 2006; Sun et al., 2005).

We can exclude the possibility that *V. parahaemolyticus* induces an apoptotic cell death pathway, because PARP is maintained as a full-length protein (Fig. 7), caspase is not involved in the process of cell death (Fig. 8a) and chromosomal DNA fragmentation does not occur (Fig. 8b). The hallmark of pyroptosis is the activation of caspase-1, and inhibition of caspase-1 reduces the host cell death (Fink & Cookson, 2005). Our results showed that inhibition of caspase-1 did not prevent host cell death induced by T3SS1 of *V. parahaemolyticus* (Fig. 9), thus excluding the possibility of pyroptotic cell death.

One interesting finding is that U937 and HeLa cells infected with wild-type and T3SS2 mutant strains displayed...
severe cytoplasmic vacuolation. The intracellular vacuolation is assumed to represent an early pathophysiological event leading to eventual cell death. This activity has been associated with a few toxins; the most extensively studied cytotoxin that induces vacuolation is VacA in Helicobacter. Cellular vacuolation induced by VacA is the result of

![Fig. 7. PARP was not cleaved in V. parahaemolyticus-infected HeLa and U937 cells.](http://mic.sgmjournals.org)

(a) HeLa and (b) U937 cells were infected with wild-type (NY-4; lane 1), T3SS1 mutant (lane 2), T3SS2 mutant (lane 3) and T3SS1–T3SS2 double mutant (lane 4) strains of V. parahaemolyticus. HeLa and U937 cells were treated with STS (lane 5), TBH (lane 6) or remained untreated (lane 7). (c) HeLa cells were infected with strain NY-1 (lane 3), NY-2 (lane 4), NY-10 (lane 5), NY-11 (lane 6), NY-12 (lane 7) or B-3 (lane 8). Lanes 1 and 2 are samples from HeLa cells treated with STS and untreated, respectively. Cells were lysed and separated by SDS-PAGE and immunoblotted with monoclonal anti-PARP antibody specific for both 116 kDa PARP and 89 kDa cleaved PARP.

![Fig. 8. (a) Effect of the pan-caspase inhibitor Z-VAD-FMK on wild-type and T3SS2 mutant strain-induced cytotoxicity.](http://mic.sgmjournals.org)

(a) Effect of the pan-caspase inhibitor Z-VAD-FMK on wild-type and T3SS2 mutant strain-induced cytotoxicity. HeLa cells were preincubated for 1 h with 100 μM Z-VAD-FMK and then infected with the wild-type and T3SS2 mutant strains prior to the measurement of LDH release. STS- and TBH-treated cells were included as positive and negative controls, respectively. The results represent the mean ± SD of three biological replicates. (b) DNA fragmentation analysis showing that V. parahaemolyticus does not induce apoptosis. HeLa cells were infected with the wild-type and each mutant strain or treated with STS for 3 h, and genomic DNA was isolated before electrophoresis on 1.5% agarose gels.
alteration of intracellular vesicle trafficking leading to the merger of the late endosome and lysosome (Reyrat et al., 1999; Wada et al., 2004). ShlA, a secreted haemolysin of Serratia marcescens, causes vacuoles in several epithelial cell lines that eventually lead to cell lysis (Hertle et al., 1999; Hertle, 2000). ShlA-induced vacuoles, however, do not appear to be derived from late endosomes. Alpha toxin of Xenorhabdus triggers endoplasmic reticulum vacuolation by increasing monovalent cation permeability of the membrane, resulting in cell death by colloid-osmotic lysis (Ribeiro et al., 2003). An early in vivo study showed that V. parahaemolyticus-infected mice displayed congestion, oedema and vacuolation in mucosal and submucosal tissues (Hoashi et al., 1990). Our in vitro observations in cell culture systems corroborate this earlier finding and, in addition, we found that T3SS1 is involved in vacuole formation.

It is unclear whether the vacuoles observed in the present study were induced by a specific mechanism or were a general consequence of host cell death in response to the V. parahaemolyticus infection. Autophagy is associated with vacuolization, although these vacuoles are usually double-membrane vesicles with sequestrated cytoplasmic materials (Fink & Cookson, 2005). We have no evidence to show that vacuoles induced by T3SS1 of V. parahaemolyticus have double membranes or contain any cytoplasmic materials (Fig. 6). Furthermore, there were apparently no MDC-labelled autophagic vacuoles within the cytoplasm of HeLa cells infected with the wild-type and each mutant strain of V. parahaemolyticus compared with HeLa cells treated with rapamycin, a known autophagy inducer (Fig. 10). Thus, we conclude that if autophagy is occurring, it is not likely to play a major role in host cell death.

In contrast to our findings, Burdette et al. (2008) showed that V. parahaemolyticus is able to induce autophagy in a T3SS1-dependent manner. The authors observed (by electron microscopy) autophagic vesicles within the HeLa cells that were infected with the POR3 strain of V. parahaemolyticus (Burdette et al., 2008). Importantly, however, inhibition of autophagy by addition of wortmannin to the cells made no significant impact on host cell death. Thus, while autophagy may occur, the primary cause of host cell death is independent of this pathway.

![Fig. 9. Effect of caspase-1 inhibitor Ac-YVAD-CHO on cytotoxicity induced by wild-type and T3SS2 mutant strains of V. parahaemolyticus and Salmonella. U937 cells were preincubated for 1 h with 20 μM Ac-YVAD-CHO and then infected with the wild-type or T3SS2 mutant strain, or S. typhimurium prior to the measurement of LDH release. LDH release was determined 8 or 4 h after U937 cells were infected with Salmonella or V. parahaemolyticus strains, respectively. The results represent the mean±SD of three biological replicates.](image)

![Fig. 10. Autophagy is not evident in HeLa cells infected with the wild-type or each mutant strain of V. parahaemolyticus. HeLa cells were infected with (a) wild-type, (b) T3SS1 mutant, (c) T3SS2 mutant and (d) T3SS1–T3SS2 double mutant strains of V. parahaemolyticus for 3 h, and then incubated with MDC for 10 min at 37 °C. (e) Untreated HeLa cells or (f) HeLa cells treated with rapamycin (2 μg ml⁻¹) for 24 h were included as negative and positive controls, respectively. White arrows indicate MDC-labelled vacuoles in rapamycin-treated HeLa cells.](image)
support of this conclusion, Burdette et al. (2008) hypothesized that other signalling pathways are activated by T3SS effectors that in turn result in cell rounding and lysis. Collectively, our results indicate that oncosis is this mechanism, at least with respect to interactions with both U937 and HeLa cells.

Oncosis caused by V. parahaemolyticus may lead to the release of pro-inflammatory cytokines that attract activated neutrophils, thereby leading to inflammatory diarrhea. This is similar to the mechanism by which Salmonella and Shigella cause enteritis by inducing oncosis of macrophages, thereby leading to the release of pro-inflammatory cytokines such as IL-1β (Brennan & Cookson, 2000; Fernandez-Prada et al., 1997; Nonaka et al., 2003). Interestingly, both Salmonella and Shigella must be located intracellularly to induce the death of host cells, which is evident because cytochalasin D inhibits the internalization of bacteria and efficiently prevents cytotoxicity. Our results indicated that intracellular bacteria are not required for V. parahaemolyticus-induced cytotoxicity because cytochalasin D treatment does not prevent cell death, even though invasiveness is significantly reduced (Fig. 3d, e). In addition, there was no significant difference in the invasiveness of wild-type and mutant strains, indicating that invasiveness is not responsible for the differing cytotoxicity of wild-type and mutant strains of V. parahaemolyticus. In this context, V. parahaemolyticus-induced cytotoxicity more closely resembles Yersinia-induced cytotoxicity, which occurs from an extracellular location (Mills et al., 1997). Our results also indicate that cell contact is necessary for V. parahaemolyticus to induce oncosis. It is likely that T3SS1 is activated in a cell-contact-dependent manner and that an effector protein(s) responsible for initiating oncosis is translocated directly into host cells without being released to the extracellular medium. Further work is needed to identify the effector protein(s) involved in this process and to confirm that such proteins are translocated into the host cell.

It is unexpected that neither T3SS1 nor T3SS2 played a role in V. parahaemolyticus invasiveness (Fig. 3a), because T3SS2 belongs to the Inv–Mxi–Spa secretion system that is responsible for efficient invasion; our results showed that T3SS2 belongs to the Inv–Mxi–Spa secretion system that is responsible for efficient invasion; our results showed that T3SS1 and T3SS2 were not responsible for the differing invasiveness (Fig. 3a), because T3SS1 and T3SS2 were not responsible for the differing invasiveness of NY-4 is less than that of other strains of V. parahaemolyticus (e.g. NY-2 and B-4; Fig. 3b) and considerably less than that of S. typhimurium.

Oncosis-induced inflammatory responses result in the breach of tight junctions (Lynch et al., 2005) and lead to the transport of V. parahaemolyticus from the lumen side to the basolateral side of the intestine. To survive within Peyer’s patches and disseminate to internal organs, V. parahaemolyticus must be able to survive phagocytosis by resident macrophages. Our results suggest that the number of intracellular V. parahaemolyticus within U937 cells (a macrophage-like cell line) decreased significantly from 1 to 24 h (data not shown), indicating that macrophages are able to kill intracellular V. parahaemolyticus. These results indicate that V. parahaemolyticus probably survives within the Peyer’s patches by inducing the death of resident macrophages before ingestion and that this precedes systemic infection (Daniels et al., 2000).

In summary, we showed that infection of either epithelial or macrophage-like cell lines with strain NY-4 of V. parahaemolyticus results in death that is due to the rapid induction of oncosis. The mechanism of oncosis involves contact-dependent formation of pores in the host cell membrane and induction of cytoplasmic vacuoles, which are dependent on T3SS1, but not T3SS2. Apoptosis, pyroptosis and autophagy do not appear to play a significant role in this process.

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