

***Vibrio alginolyticus* VepA Induces Lysosomal Membrane Permeability and Cathepsin-Independent Cell Death**

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The bacterium *Vibrio alginolyticus*, an opportunistic pathogen in humans, has a type III secretion system (T3SS) that is responsible for its cytotoxicity toward eukaryotic cells. The effector of T3SS that is responsible for the cytotoxicity had not been identified. Here we demonstrate that VepA, a homolog of the T3SS effector in *V. parahaemolyticus*, is required for cytotoxicity in *V. alginolyticus*. VepA induces lysosomal membrane permeabilization, and it allows the leakage of only small molecules into the cytosol. Our findings revealed that VepA induces cathepsin-independent cell death in mammalian cells. The ferrous ion, one of the small molecules in the lysosome contents, appears to be involved in the cell death caused by *V. alginolyticus* VepA.

Key words: cell death, lysosomal membrane permeability, VepA, *Vibrio alginolyticus*

Vibrio alginolyticus is a halophilic, Gram-negative rod-shaped bacterium naturally distributed in marine and estuarine waters. This bacterium is known as an opportunistic pathogen for both humans and marine animals [1-3]. In humans, it causes medical problems such as wound infection [4-7], ear infection [8-12], and eye infection [13-15]. The incidence of *V. alginolyticus* infection was reported to be increasing, and this infection may develop into an emerging disease due to climate change issues [16]. A complete understanding of the virulence mechanism of *V. alginolyticus* is necessary to prevent future outbreaks.

The pathogenicity of *V. alginolyticus* has been described both *in vivo* (lethality in a mouse model) [17] and *in vitro* (cytotoxic activity toward several mammalian cell lines) [18]. The killing potency shown in those studies indicated that *V. alginolyticus* possesses virulence factor(s) that allow infection through the ability to

overcome antibacterial immune responses such as phagocytosis, and the ability to obtain nutrition from the dead cells. As such a virulence factor in *V. alginolyticus*, Zhao *et al.* [17] reported that the deletion of type III secretion system (T3SS) apparatus gene reduces the cytotoxicity toward mammalian cell lines. A T3SS is a syringe-like apparatus that introduces specific proteins known as effectors into the cytoplasm of eukaryotic host cells. Injected effectors allow bacteria to manipulate the host cells' functions and cause diseases [19-21]. A variety of effectors are known to show specific cytotoxicity mechanisms in various bacterial species, but the effector of T3SS that is responsible for the cytotoxicity in *V. alginolyticus* has been unclear.

The *V. alginolyticus* T3SS gene cluster is evolutionally close to T3SS1 in *V. parahaemolyticus*, which is dominantly responsible for cytotoxicity [22]. Another T3SS in *V. parahaemolyticus*, T3SS2, which is involved in enterotoxicity, is absent in *V. alginolyticus*. Among the

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effectors of T3SS1 in *V. parahaemolyticus*, VepA (which has also been referred to as VopQ and as VP1680) plays a significant role in its cytotoxicity [23,24]. Since *V. alginolyticus* has a gene encoding a VepA homolog in the T3SS gene cluster, we hypothesized that the VepA homolog also has an important contribution to the cytotoxicity.

In this study, we constructed a *vepA*-deletion mutant in *V. alginolyticus* to analyze its role in the cytotoxicity of *V. alginolyticus*. We observed that *V. alginolyticus* infection led to size-specific lysosome membrane permeabilization (LMP) in a VepA-dependent manner. We also use our results in a discussion of why the small-molecule leakage from lysosomes is related to cell death in *V. alginolyticus* infection.

Materials and Methods

Bacterial strains, plasmids, and culture conditions.

The bacterial strains and plasmids used in this study are listed in Table 1. We used *V. alginolyticus* ATCC 17749 [25] as the parent strain for the construction of gene-deletion mutant. We used the *Escherichia coli* DH5 α [26] for general plasmid manipulation; *E. coli* DH5 α (*λpir*) was used to amplify the R6K-origin-containing plasmids, and *E. coli* RHO3 [27] was used as a conjugation donor for *V. alginolyticus*. *V. alginolyticus* strains were routinely cultured in Luria-Bertani (LB) 3% NaCl medium (1% tryptone, 0.5% yeast extract, 3% NaCl) at 37°C, and the *E. coli* strains were grown in LB medium (Lennox) (1% tryptone, 0.5% yeast extract, 0.5% NaCl) at 37°C. *E. coli* RHO3 was grown in LB medium supplemented with 100 μg/ml 2, 6-diaminopimelic acid. Chloramphenicol (30 μg/ml), ampicillin (50 μg/ml), or kanamycin (100 μg/ml) was added to grow bacteria harboring the plasmid containing the corresponding resistance gene.

HeLa cells were cultured in Dulbecco's modified

Table 1 Bacterial strains and plasmids

Strain/Plasmid	Relevant Genotype or Properties	Reference or Source
<i>E. coli</i> strains		
DH5 α	F ⁻ Φ 80/ <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>) U169 <i>deoR recA1 endA1 hsdR17</i> (<i>r_K⁻ m_K⁻</i>) <i>phoA supE44</i>	[26]
DH5 α (<i>λpir</i>)	DH5 α <i>λpir</i>	H.P. Schweizer
RHO3	SM10(<i>λpir</i>) Δ <i>asd</i> : <i>FRT</i> Δ <i>aphA</i> : <i>FRT</i>	[27]
<i>V. alginolyticus</i> strains		
ATCC 17749	Wild-type strain	[25]
Δ <i>vepA</i>	ATCC 17749 Δ <i>vepA</i>	This study
Δ <i>vscC</i>	ATCC 17749 Δ <i>vscC</i>	This study
Δ <i>vepA</i> / <i>vepA</i>	Δ <i>vepA</i> harboring pOU550	This study
Δ <i>vepA</i> /vector	Δ <i>vepA</i> harboring pHSG398T	This study
Plasmids		
pGEM-T Easy	AMP ^r ; TA cloning vector	Promega
pSG76-C	CM ^r ; Suicide plasmid vector; I-SceI	[28]
pKU66	CM ^r ; pSG76-C with <i>oriT</i>	This study
pHSG398	CM ^r ; pUC-type cloning vector	Takara Bio.
pHSG398T	CM ^r ; pHSG398 with <i>oriT</i>	This study
pEX18Ap	AMP ^r ; cloning vector; <i>sacB</i> ; <i>oriT</i>	[30]
pEX18Km	KM ^r ; pEX18Ap with AMP ^r replacement to KM ^r	This study
pHSG298	KM ^r ; source of kanamycin resistance gene	Takara Bio.
pUC18T-mini-Tn7T-Gm	AMP ^r , GM ^r ; source of <i>oriT</i>	[29]
pST76A-SceP	AMP ^r ; source of I-SceI	[28]
pOU246	AMP ^r ; pGEM-T Easy with EF-Tu promoter and I-SceI	This study
pOU257	KM ^r ; pEX18Km with EF-Tu promoter and I-SceI	This study
pOU549	CM ^r ; pKU66 with Δ <i>vepA</i> recombinant DNA fragment	This study
pOU550	CM ^r ; pHSG398T with <i>vepA</i>	This study
pOU553	CM ^r ; pKU66 with Δ <i>vscC</i> recombinant DNA fragment	This study

AMP, ampicillin; CM, chloramphenicol; GM, gentamycin; KM, kanamycin.

Eagle's medium (DMEM, Sigma, St. Louis, MO, USA) supplemented with 10% (v/v) inactivated fetal bovine serum (FBS) (Biowest, Nuaille, France) at 37°C under 5% CO₂. DMEM or minimum essential medium/Earle's balanced salts (MEM/EBSS, Gibco, Grand Island, NY, USA) without phenol red supplemented with 10% (v/v) inactivated FBS was used during incubation in the infection experiment.

Construction of a mobilizable vector for gene replacement. We constructed a mobilizable vector for gene replacement carrying the recognition site of meganuclease I-SceI, pKU66, by inserting the *oriT* region into pSG76-C [28] at the *NotI* site. We amplified the *oriT* region from pUC18T-mini-Tn7T-Gm [29] using primers P064 (5'-GCGGCCGCTATCAGAGCTTATCGGCCAG-3'; the underlining indicates a *NotI* site) and P065 (5'-GCGGCCGCGGGGATTCCTTAAGGTATAC-3'; the underlining indicates a *NotI* site).

Construction of mobilizable vectors for gene expression. We constructed pEX18Km by replacing the ampicillin resistance gene on pEX18Ap [30] with the kanamycin resistance gene at the *AatII* and *AhdI* sites. We amplified the kanamycin marker from pHSG298 (Takara Bio, Shiga, Japan) using primers P394 (5'-GACGTCGATCTGATCCTTCAACTCAG-3'; the underlining indicates an *AatII* site) and P395 (5'-GACTCCCCGTCGCTCTGCCAGTGTTACAAC-3'; the underlining indicates an *AhdI* site).

We constructed pHSG398T by inserting *oriT* into the *AflIII* site of pHSG398 (Takara Bio). The *oriT* region was amplified by using primers P127 (5'-ACATGTATCAGAGCTTATCGGCCAG-3'; the underlining indicates an *AflIII* site) and P128 (5'-ACATGTGGGGATTCCCTTAAGGTATAC-3'; the underlining indicates an *AflIII* site).

Construction of an I-SceI expression vector. We amplified the promoter region of the elongation factor thermo unstable (EF-Tu) in *V. alginolyticus* I.029 by using primers P406 (5'-GAATTCAGCGGGTTACCTGTACTAG-3'; the underlining indicates an *EcoRI* site) and P407 (5'-GATCGTGTTCCTTCC TAGTTATG-3').

The I-SceI gene was amplified from pST76-ASceP [28] with the use of the primers P408 (5'-CATAACTAGGAAGGAACACGATCATGCATCAAAAAACCA GGTA-3'; the underlining indicates a sequence overlapping to the 3' region of the EF-Tu promoter) and P409 (5'-CAAAGGGAAAACACTGTCCATAC-3'). The

two DNA fragments were spliced by overlap extension polymerase chain reaction (PCR). The recombinant fragment was ligated into pGEM-T Easy (Promega, Madison, WI) to obtain pOU246. We cloned an *EcoRI*-*SphI* fragment of pOU246 carrying the I-SceI gene with the EF-Tu promoter into the same sites of pEX18Km, resulting in pOU257.

Construction of a vepA-deletion mutant and a vscC-deletion mutant. We constructed $\Delta vepA$ as described [28] with several modifications. A DNA fragment with the *vepA*-deletion was constructed by overlap extension PCR as described [31] used the following primers: P787 (5'-GGATCCAACGTGGAGTAAGGATGTGAAAA-3'; underlining indicates a *BamHI* site), P788 (5'-TGAAATTACACCCAGCCTTCTGCGCTGATTTTTTGTGTTGTATTAACCAT-3'; underlining indicates complementary region to P789), P789 (5'-GCAGAAAGGCTGGGTGTAATTTCA-3') and P790 (5'-GAGCTCGAAGTCACTGAAGAGAGATTTTCGA-3'; underlining indicates a *SacI* site).

We cloned this fragment into pKU66 at the *BamHI* and *SacI* sites, resulting in pOU549. *E. coli* RHO3 harboring plasmid pOU549 was conjugated with *V. alginolyticus* ATCC 17749, and the resultant transconjugant was conjugated to *E. coli* RHO3 harboring pOU257. The transconjugants were selected on LB 2% NaCl agar plates containing antibiotic for the responsible plasmid. The *vepA*-deletion was confirmed by colony PCR using primers P787 and P790, and by sequencing of the PCR products. The remaining pOU257 was eliminated by inoculation into LB 3% NaCl medium containing 5% sucrose.

The construction of $\Delta vscC$ was performed using the same method as that used for the $\Delta vepA$ construction. The primers used to amplify the *vscC*-deletion fragment were P793 (5'-GAATTCCGGTTGCGAAAGTATG GCAATG-3'; the underlining indicates an *EcoRI* site), P794 (5'-AGGAACAACACTCACTGCGCATA-3'), P795 (5'-TATGCGCAGTGAGTGTGTTCCTCGCCCTTCAGAGGAGTCTTAACC-3'; the underlining indicates complementary region to P794) and P796 (5'-GGATCCGCAGATCGAGTTTCTGTGTTCGG-3'; the underlining indicates a *BamHI* site). This fragment was cloned into pKU66 at *EcoRI* and *BamHI* sites, resulting in pOU553. The *vscC* deletion was confirmed by colony PCR using primers P793 and P796 and by sequencing of the PCR products.

Construction of a vepA expression vector. We

amplified the DNA fragment containing *vepA* from the gDNA by using primers P791 (5'-GCTGAATTCAATG GTTAATACAACACAAAAAATCAGCCAAAGC-3'; underlining indicates an *EcoRI* site) and P792 (5'-GCTGGATCCTTACACCCAGCCTTCTG CCAAG-3'; underlining indicates a *BamHI* site). The PCR product was cloned into *EcoRI* and *BamHI* sites of pHSG398T, which yielded pOU550.

Cytotoxicity assay. HeLa cells were seeded into a 96-well plate at 3×10^4 cells/well, and then incubated at 37°C under 5% CO₂ for 48 h. The cells were washed with DMEM or MEM/EBSS medium without phenol red, and then infected with *V. alginolyticus* at a multiplicity of infection (MOI) of 100 for 4 h. A ten-times dilution of *V. alginolyticus* at the optical density at 600 nm (OD₆₀₀) of 1.0 is equal to $3\text{--}4 \times 10^8$ CFU/ml on an LB 1% NaCl agar plate. We added 10 µl of these dilution cultures to $3\text{--}4 \times 10^4$ of HeLa cell culture to obtain the MOI of 100.

We measured the release of lactate dehydrogenase (LDH) into the medium by using a Cytotoxicity Detection Kit^{PLUS} (LDH) (Roche, Mannheim, Germany) according to the manufacturer's instructions. The percentage of LDH release was calculated based on the following equation: $[\text{OD}_{490} \text{ of experimental release} - \text{OD}_{490} \text{ of spontaneous release}] / (\text{OD}_{490} \text{ of maximum release} - \text{OD}_{490} \text{ of spontaneous release}) \times 100$. The spontaneous release is the amount of LDH released from the cytoplasm of uninfected cells, and the maximum release is the total amount of LDH released upon the complete lysis of uninfected cells.

AO relocation assay. The acridine orange (AO) relocation assay was performed as described [24]. We used fluorescence microscopy (Biozero BZ-8000, Keyence, Tokyo, Japan) to analyze the AO-stained cells.

Fluorescent-dextran translocation. Fluorescent-dextran translocation was observed as described [32]. Before infection, HeLa cells were incubated in 100 µg/ml fluorescein isothiocyanate (FITC)-dextran for 16 h. We analyzed fluorescent dextran in the HeLa cells by fluorescence microscopy.

Inhibition of cathepsin and reactive oxygen species (ROS), LMP induction, and iron chelation. HeLa cells were pretreated with 100 µM E-64d, a broad-spectrum cathepsin inhibitor, for 16 h before infection. The cells were challenged with 3 mM L-Leucyl-L-Leucine methyl ester (LLOMe) for 4 h to induce LMP. To chelate iron, we used 100 µM 2,2'-bipyridyl (BIP).

To inhibit reactive oxygen species (ROS), we used 5 mM N-acetyl cysteine (NAC) or 250 µM Trolox.

Statistical analysis. The two-tailed Student's *t*-test was used for the statistical analyses.

Results

VepA contributes to HeLa cell cytotoxicity in *V. alginolyticus* infection. We chose *V. alginolyticus* strain ATCC 17749, which was shown to be highly virulent in an *in vivo* infection model [18]. VepA (N646_0746) in this strain is homologous to that in *V. parahaemolyticus*, with 88% sequence similarity (435 aa/493 aa).

To examine the contribution of VepA to the cytotoxicity toward human cells, we performed the LDH release cytotoxicity assay after 4 h of bacterial infection against HeLa cells (Fig. 1). The T3SS apparatus gene mutant, $\Delta vscC$, showed significantly less cytotoxicity than the wild-type (WT). $\Delta vepA$ also showed signifi-

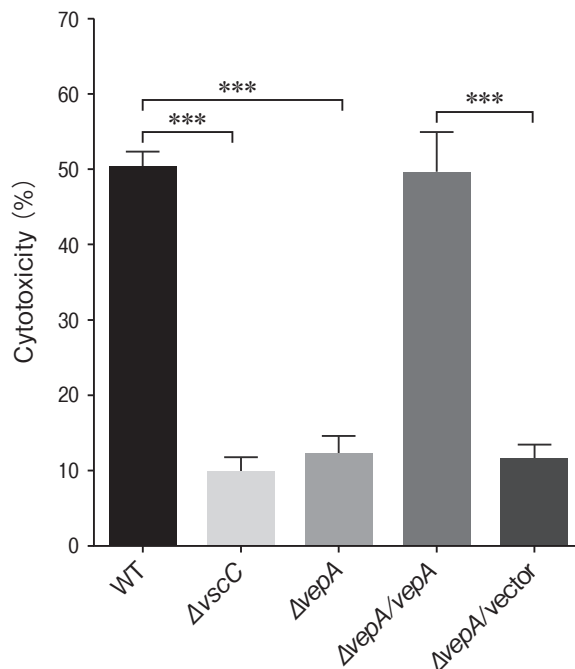


Fig. 1 VepA contribution to cytotoxicity toward HeLa cells. HeLa cells were infected with the wild-type strain (WT), a *vscC*-deletion mutant ($\Delta vscC$), a *vepA*-deletion mutant ($\Delta vepA$), or a *vepA*-deletion mutant carrying a *vepA*-expression plasmid or the empty vector ($\Delta vepA/vepA$ or $\Delta vepA/vector$, respectively). We evaluated cytotoxicity at 4 h after infection by measuring the amount of LDH released into the culture supernatant. Data are mean \pm SD. ****p* < 0.001.

cantly less cytotoxicity. The *vepA* complimentary strain, $\Delta vepA/vepA$ completely restored the cytotoxicity (similar to the WT), whereas $\Delta vepA/vector$ did not. To test the results of the LDH release cytotoxicity assay, we also performed propidium iodine (PI) staining. The results were similar to those of the LDH release cytotoxicity assay (data not shown). These results indicate that mainly VepA is responsible for the cytotoxicity of *V. alginolyticus*.

VepA in *V. alginolyticus* induced lysosomal membrane permeabilization (LMP). It was reported that *V. parahaemolyticus* VepA induces LMP in mammalian cells [24, 33]. Here we therefore examined the integrity of lysosomes in *V. alginolyticus*-infected cells by using acridine orange (AO) (Fig. 2). AO is a metachromatic dye that emits red fluorescence at low pH (e.g., in lysosomes) and emits green fluorescence at neutral pH (e.g., in the cytosol) [34].

In uninfected HeLa cells, we observed small red fluorescent dots, which reflect an accumulation of the dye in lysosomes. In the WT infection, the appearance of red dots was disappeared and the intensity of the green fluorescence in the cytosol was increased. In contrast, in the $\Delta vepA$ infection, the appearance of red dots was comparable to that in the uninfected cells, and the

green fluorescence enhancement was not observed. In the $\Delta vepA/vepA$ infection, the fluorescence pattern reverted to one similar to that obtained with the WT. These results indicate that *V. alginolyticus* infection induces the leakage of the lysosomal contents into the cytosol in a VepA-dependent manner.

We observed that infection with either *V. alginolyticus* strain induced a morphological change of HeLa cells, i.e., cell rounding (Fig. 2). Since the $\Delta vepA$ infection also induced cell rounding, we can safely assume that this change is not due to VepA. On the other hand, the $\Delta vscC$ strain did not affect the cell morphology (data not shown). Therefore, this change might be caused by other T3SS effectors, e.g., N646_0751, the homologue of VopS in *V. parahaemolyticus* [35].

The VepA-induced LMP in *V. alginolyticus* was not due to a lysosomal membrane rupture. The molecular mass of AO is approx. 0.3 kDa. To determine whether VepA induces the release of only small molecules or lysosomal rupture, we observed the LMP in *V. alginolyticus* infection using larger molecules: FITC-dextran, with the molecular mass of 4 or 10 kDa. It is well known that such macromolecules can be loaded into lysosomes to yield green fluorescence punctates while the lysosomes are intact. When the lyso-

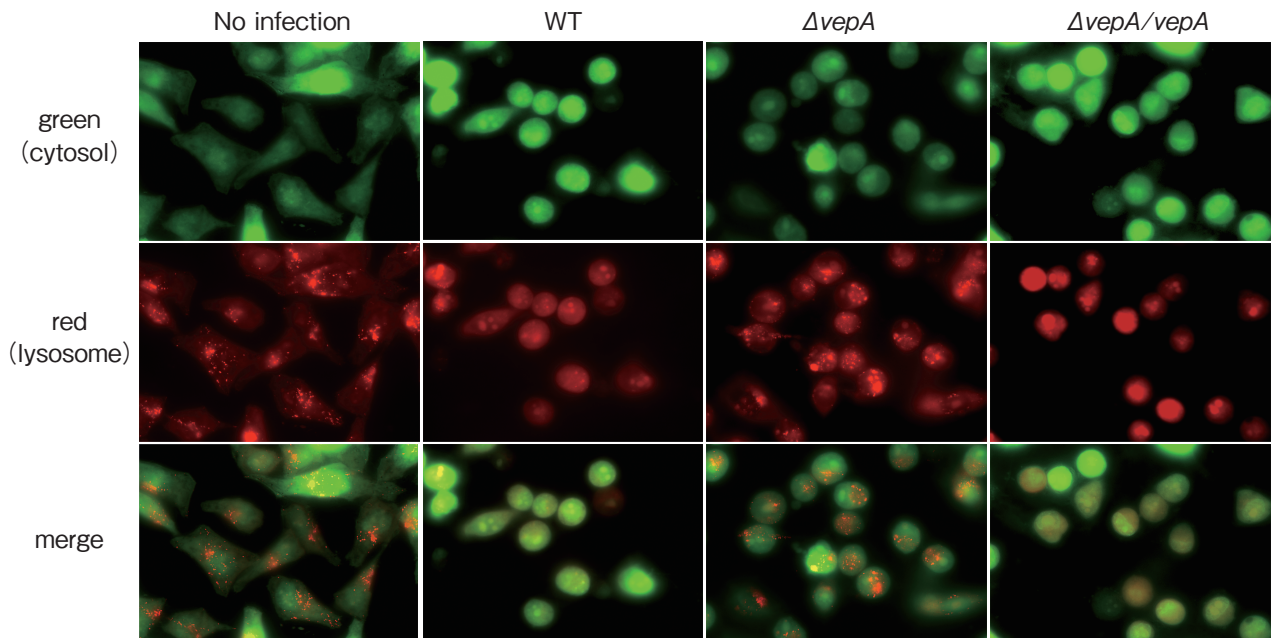


Fig. 2 AO relocation assays results. HeLa cells were incubated with AO and then infected with the WT strain, a *vepA*-deletion mutant ($\Delta vepA$), or a *vepA*-deletion mutant carrying a *vepA*-expression plasmid ($\Delta vepA/vepA$) for 4h. AO emits red fluorescence in the lysosomes and green fluorescence in the cytosol.

somes are ruptured, the FITC-dextran is released into the cytosol to yield diffuse green fluorescence.

HeLa cells were treated with FITC-dextran for 16 h to allow lysosomal uptake before infection or challenge with an LMP inducer, LLOMe. When FITC-dextran with the molecular mass of 10 kDa was preloaded (Fig. 3A), LLOMe-treated cells showed diffuse fluorescence enhancement in the cytosol. In contrast, the cells infected with the WT or $\Delta vepA$ did not exhibit the fluorescence diffusion into the cytosol, but the cells did show fluorescent punctates. Our experiments using FITC-dextran with the molecular mass of 4 kDa gave the same pattern (Fig. 3B). These results indicate that the VepA-mediated LMP allows the translocation of molecules smaller than 4 kDa.

VepA-dependent cytotoxicity is not due to lysosomal cathepsin. LMP-associated cell death is usually related to lysosomal cathepsin release [36]. Since the VepA-induced LMP in *V. alginolyticus* infection allowed the release of only molecules <4 kDa, we hypothesized that lysosomal proteases with the molecular sizes >4 kDa (including cathepsins, the molecular masses of which is approx. 30 kDa) could not pass through the lysosomal membrane. To determine whether the VepA-mediated cell death is due to cathepsin release from lysosomes, we examined the effect of a broad-spectrum cathepsin inhibitor, E-64d, on cell death. HeLa cells were treated with E-64d for 16 h before challenge with LLOMe or a *V. alginolyticus* strain. The lysosomal rupture induced by LLOMe subsequently resulted in cell death, as reported [36], and cytotoxicity was reduced by E-64d treatment as expected

(Fig. 4A). When the cells were challenged with the WT strain, the cell death rates were similar regardless of the E-64d treatment (Fig. 4A). These results suggest that cathepsin is not the major causative factor of *V. alginolyticus* cytotoxicity.

Since the lysosome is one of the iron-rich intracellular organelles, VepA might allow excessive iron release into the cytosol to induce cell death via ROS [37]. To investigate the roles of iron and ROS in cell death in *V. alginolyticus* infection, we compared their cytotoxic effects in the presence of an iron chelator, BIP, and an ROS scavenger, NAC or Trolox. In the presence of BIP, the cytotoxicity was significantly reduced. However, after the ROS scavenger treatment, the cytotoxicity was slightly reduced or not changed, respectively (Fig. 4B). When we directly examined hydroxyl radicals by using hydroxyphenyl fluorescein, we observed no fluorescence in *V. alginolyticus*-infected cells (data not shown).

Discussion

The opportunistic pathogen *V. alginolyticus* has an active T3SS that is involved in its cytotoxicity [3, 17]. In *V. parahaemolyticus*, a T3SS effector, VepA, makes the major contribution to the cytotoxicity. Our present findings demonstrate that the VepA in *V. alginolyticus* plays a significant role in its cytotoxicity (Fig. 1). Since the *vepA* gene is conserved in several *Vibrio* species [38], it is possible that VepA-induced cell death is a common pathogenic mechanism among the species.

Our present results obtained using an *in vitro* infection model also showed that *V. alginolyticus* induces the

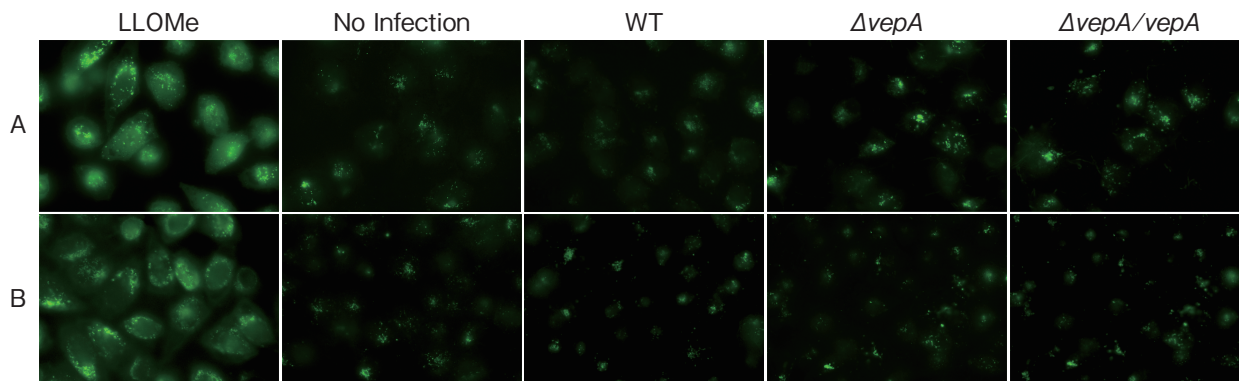


Fig. 3 LMP visualized by fluorescent dextran release. HeLa cells were incubated with 10-kDa (A) or 4-kDa (B) FITC-dextran for 16 h and then challenged with LLOMe or infected with the WT strain, *vepA*-deletion mutant ($\Delta vepA$), or *vepA*-deletion mutant carrying a *vepA*-expression plasmid ($\Delta vepA/vepA$) for 4 h.

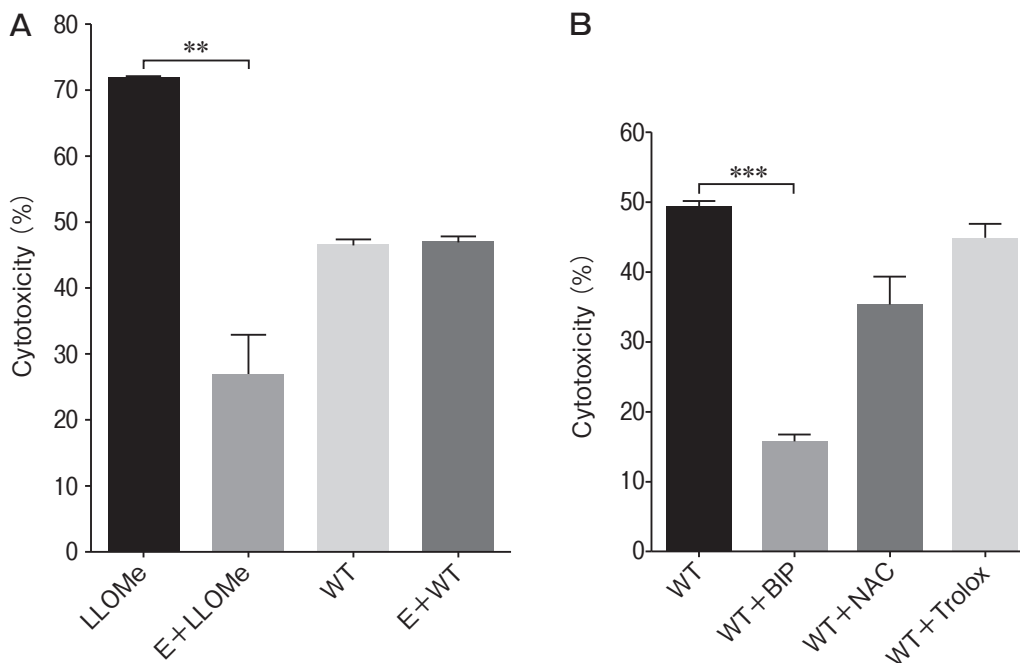


Fig. 4 Cytotoxicity of *V. alginolyticus* infection in cathepsin inhibition, iron chelation, and ROS inhibition. **A**, HeLa cells were pretreated with E-64d (E) and then challenged with 3 mM LLOMe or infected with the WT strain for 4 h; **B**, HeLa cells were pretreated with an iron chelator (BIP) or an ROS scavenger (NAC or Trolox) and then infected with WT strain for 4 h. Data are mean \pm SD. ** $p < 0.01$, *** $p < 0.001$.

leakage of the 0.3-kDa lysosomotropic dye AO into the cytosol in a VepA-dependent manner (Fig. 2). Molecules larger than 4 kDa were retained in the lysosomes (Fig. 3). We thus speculate that *V. alginolyticus* VepA induces size-specific LMP. These results are consistent with those obtained by Sreelatha *et al.* [33], *i.e.*, *V. parahaemolyticus* VepA induces LMP, which only allows the release of molecules < 4 kDa. Sreelatha *et al.* also proposed that VepA forms pores with an estimated diameter of 18 Å in liposomes. Further studies are necessary to determine whether *V. alginolyticus* VepA forms such pores in the lysosomal membrane.

LMP results in cell death, which is usually related to lysosomal cathepsin release [36]. Since *V. alginolyticus* VepA induced size-specific LMP, *i.e.*, < 4 kDa, we can safely assume that this is not the case, since lysosomal proteases (e.g., cathepsins) have much larger molecular masses at approx. 30 kDa. To test this, we used the broad-spectrum cathepsin inhibitor E-64d to determine whether it inhibits the cell death. Since *V. alginolyticus* VepA induced cell death regardless of the presence of this inhibitor (Fig. 4A), the cell death is caused by a factor(s) other than cathepsins. We thus suspect that

the involvement of lysosomal small molecules potentially harmful to the cells is responsible for the VepA-induced cell death.

The lysosome is a pool of redox-active iron capable of generating free radicals via the Fenton reaction [37]. We used the iron chelator BIP to examine whether iron mediates the VepA-induced cell death. Since iron chelation significantly reduced the cell death to a level that was almost the same as that observed with *vepA*-deleted *V. alginolyticus* infection, it appears that iron is an important mediator of VepA-dependent cell death (Fig. 4B). Our results support the observation by Matsuda *et al.* [24] that an iron chelator partially reduced the cytotoxicity in *V. parahaemolyticus* infection.

Since it is well known that iron triggers cell death via ROS accumulation [37, 39], we examined the effects of ROS scavengers. NAC inhibited the cell death only slightly, but Trolox did not inhibit the cell death at all. Moreover, the growth rate of *V. alginolyticus* was not affected by the presence of BIP, NAC or Trolox. We therefore propose that iron induces cell death without ROS accumulation in this infection model. A study on

yeast demonstrated that iron overload stimulates the sphingolipid production that leads to cell death, without ROS accumulation [37]. Additional studies are required to elucidate the function of iron in VepA-induced cell death.

In conclusion, our experiments demonstrated that VepA induced cathepsin-independent cell death. We also observed that VepA induced size-specific LMP that allows only small molecules to be released into the cytosol. We propose that iron leakage from lysosomes plays an important role in this cell death mechanism. Further research is required to elucidate the underlying molecular mechanism, which might provide insights into the development of novel therapeutics that target the VepA-related cell death mechanism.

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References

- Tantillo GM, Fontanarosa M, Di Pinto A and Musti M: Updated perspectives on emerging vibrios associated with human infections. *Lett Appl Microbiol* (2004) 39: 117–126.
- Austin B: Vibrios as causal agents of zoonoses. *Vet Microbiol* (2010) 140: 310–317.
- Zhao Z, Chen C, Hu CQ, Ren CH, Zhao JJ, Zhang LP, Jiang X, Luo P and Wang QB: The type III secretion system of *Vibrio alginolyticus* induces rapid apoptosis, cell rounding and osmotic lysis of fish cells. *Microbiology* (2010) 156: 2864–2872.
- Rubin SJ and Tilton RC: Isolation of *Vibrio alginolyticus* from wound infections. *J Clin Microbiol* (1975) 2: 556–558.
- Pezzlo M and Burns MJ: Wound infection associated with *Vibrio alginolyticus*. *Am J Clin Pathol* (1979) 71: 476–478.
- Spark RP, Fried ML, Perry C and Watkins C: *Vibrio alginolyticus* wound infection: case report and review. *Ann Clin Lab Sci* (1979) 9: 133–138.
- Matsiota-Bernard P and Nauciel C: *Vibrio alginolyticus* wound infection after exposure to sea water in an air crash. *Eur J Clin Microbiol Infect Dis* (1993) 12: 474–475.
- Ciufecu C, Nănescu N and Florescu D: Middle ear infection due to *Vibrio alginolyticus*. Bacteriological characterization. *Acta Microbiol Acad Sci Hung* (1979) 26: 95–98.
- Drona F, Canton R, Selma JL, Garcia-Ramos F and Martinez-Ferrer M: *Vibrio alginolyticus* and swimmer's otitis externa. 2 cases and review of the literature. *Enferm Infecc Microbiol Clin* (1991) 9: 630–633.
- Tsakris A, Psifidis A and Douboyas J: Complicated suppurative otitis media in a Greek diver due to a marine halophilic *Vibrio* sp. *J Laryngol Otol* (1995) 109: 1082–1084.
- Mukherji A, Schroeder S, Deyling C and Procop GW: An unusual source of *Vibrio alginolyticus*-associated otitis: prolonged colonization or freshwater exposure? *Arch Otolaryngol Head Neck Surg* (2000) 126: 790–791.
- Feingold MH and Kumar ML: Otitis media associated with *Vibrio alginolyticus* in a child with pressure-equalizing tubes. *Pediatr Infect Dis J* (2004) 23: 475–476.
- Schmidt U, Chmel H and Cobbs C: *Vibrio alginolyticus* Infections in Humans. *J Clin Microbiol* (1979) 10: 666–668.
- Penland RL, Boniuk M and Wilhelmus KR: *Vibrio* ocular infections on the U.S. Gulf Coast. *Cornea* (2000) 19: 26–29.
- Li XC, Xiang ZY, Xu XM, Yan WH and Ma JM: Endophthalmitis caused by *Vibrio alginolyticus*. *J Clin Microbiol* (2009) 47: 3379–3381.
- Sganga G, Cozza V, Spanu T, Spada PL and Fadda G: Global climate change and wound care: case study of an off-season *Vibrio alginolyticus* infection in a healthy man. *Ostomy Wound Manage* (2009) 55: 60–62.
- Zhao Z, Zhang L, Ren C, Zhao J, Chen C, Jiang X, Luo P and Hu C: Autophagy is induced by the type III secretion system of *Vibrio alginolyticus* in several mammalian cell lines *Arch Microbiol* (2010) 193: 53–61.
- Liu X-F, Zhang H, Liu X, Gong Y, Chen Y, Cao Y and Hu C: Pathogenic analysis of *Vibrio alginolyticus* infection in a mouse model. *Folia Microbiol (Praha)* (2014) 59: 167–171.
- Muller S, Feldman MF and Cornelis GR: The Type III secretion system of Gram-negative bacteria: a potential therapeutic target? *Expert Opin Ther Targets* (2001) 5: 327–339.
- Galán JE and Wolf-Watz H: Protein delivery into eukaryotic cells by type III secretion machines. *Nature* (2006) 444: 567–573.
- Coburn B, Sekirov I and Finlay BB: Type III Secretion systems and disease. *Clin Microbiol Rev* (2007) 20: 535–549.
- Hiyoshi H, Kodama T, Iida T and Honda T: Contribution of *Vibrio parahaemolyticus* virulence factors to cytotoxicity, enterotoxicity, and lethality in mice. *Infect Immun* (2010) 78: 1772–1780.
- Ono T, Park K-S, Ueta M, Iida T and Honda T: Identification of proteins secreted via *Vibrio parahaemolyticus* type III secretion system 1. *Infect Immun* (2006) 74: 1032–1042.
- Matsuda S, Okada N, Kodama T, Honda T and Iida T: A Cytotoxic type III secretion effector of *Vibrio parahaemolyticus* targets vacuolar H⁺-ATPase subunit c and ruptures host cell lysosomes. *PLoS Pathog* (2012) 8: e1002803.
- Miyamoto Y, Nakamura K and Takizawa K: Pathogenic halophiles. Proposals of a new genus “*Oceanomonas*” and of the amended species names. *Microbiol Immunol* (1961) 5: 477–486.
- Liss L: New M13 host: DH5 α F⁺ competent cells. *Focus* (1987) 9: 13.
- López CM, Rhol DA, Trunck LA and Schweizer HP: Versatile dual-technology system for markerless allele replacement in *Burkholderia pseudomallei*. *Appl Environ Microbiol* (2009) 75: 6496–6503.
- Posfai G, Kolisnychenko V, Bereczki Z and Blattner FR: Markerless gene replacement in *Escherichia coli* stimulated by a double-strand break in the chromosome. *Nucleic Acids Res* (1999) 27: 4409–4415.
- Choi KH, Gaynor JB, White KG, Lopez C, Bosio CM, Karkhoff-Schweizer RR and Schweizer HP: A Tn7-based broad-range bacterial cloning and expression system. *Nat Meth* (2005) 2: 443–448.
- Hoang TT, Karkhoff-Schweizer RR, Kutchma AJ and Schweizer HP: A broad-host-range Flp-FRT recombination system for site-spe-

- cific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. *Gene* (1998) 212: 77–86.
31. Heckman KL and Pease LR: Gene splicing and mutagenesis by PCR-driven overlap extension. *Nat Protoc* (2007) 2: 924–932.
 32. Ellegaard A-M, Jäättelä M and Nylandsted J: Visualizing lysosomal membrane permeabilization by fluorescent dextran release. *Cold Spring Harb Protoc* (2015) 2015: 900–903.
 33. Sreelatha A, Bennett TL, Zheng H, Jiang QX, Orth K and Starai VJ: *Vibrio* effector protein, VopQ, forms a lysosomal gated channel that disrupts host ion homeostasis and autophagic flux. *Proc Natl Acad Sci USA* (2013) 110: 11559–11564.
 34. Pierzynska-Mach A, Janowski PA and Dobrucki JW: Evaluation of acridine orange, LysoTracker Red, and quinacrine as fluorescent probes for long-term tracking of acidic vesicles. *Cytometry A* (2014) 85: 729–737.
 35. Yarbrough ML, Li Y, Kinch LN, Grishin NV, Ball HL and Orth K: AMPylation of Rho GTPases by *Vibrio* VopS disrupts effector binding and downstream signaling. *Science* (2009) 323: 269–272.
 36. Droga-Mazovec G, Bojic L, Petelin A, Ivanova S, Romih R, Repnik U, Salvesen GS, Stoka V, Turk V and Turk B: Cysteine cathepsins trigger caspase-dependent cell death through cleavage of bid and antiapoptotic Bcl-2 homologues. *J Biol Chem* (2008) 283: 19140–19150.
 37. Dixon SJ and Stockwell BR: The role of iron and reactive oxygen species in cell death. *Nat Chem Biol* (2013) 10: 9–17.
 38. Park KS, Ono T, Rokuda M, Jang MH, Okada K, Iida T and Honda T: Functional characterization of two type III secretion systems of *Vibrio parahaemolyticus*. *Infect Immun* (2004) 72: 6659–6665.
 39. Kurz T, Eaton JW and Brunk UT: The role of lysosomes in iron metabolism and recycling. *Int J Biochem Cell Biol* (2011) 43: 1686–1697.