

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

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4 **Running title:** *Vibrio alginolyticus* VepA and Cell Death

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25

26 **Abstract**

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

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37

38 **Keywords:** cell death, lysosomal membrane permeability, VepA, *Vibrio alginolyticus*

39

## 40 Introduction

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

48 The pathogenicity of *V. alginolyticus* has been described both *in vivo* (lethality in a  
49 mouse model) [17] and *in vitro* (cytotoxic activity toward several mammalian cell lines) [18].  
50 The killing potency shown in those studies indicated that *V. alginolyticus* possesses virulence  
51 factor(s) that allow infection through the ability to overcome antibacterial immune responses  
52 such as phagocytosis, and the ability to obtain nutrition from the dead cells. As such a  
53 virulence factor in *V. alginolyticus*, Zhao *et al.* [17] reported that the deletion of type III  
54 secretion system (T3SS) apparatus gene reduces the cytotoxicity toward mammalian cell  
55 lines. A T3SS is a syringe-like apparatus that introduces specific proteins known as effectors  
56 into the cytoplasm of eukaryotic host cells. Injected effectors allow bacteria to manipulate the  
57 host cells' functions and cause diseases [19–21]. A variety of effectors are known to show  
58 specific cytotoxicity mechanisms in various bacterial species, but the effector of T3SS that is  
59 responsible for the cytotoxicity in *V. alginolyticus* has been unclear.

60 The *V. alginolyticus* T3SS gene cluster is evolutionally close to T3SS1 in  
61 *V. parahaemolyticus*, which is dominantly responsible for cytotoxicity [22]. Another T3SS in  
62 *V. parahaemolyticus*, T3SS2, which is involved in enterotoxicity, is absent in *V. alginolyticus*.  
63 Among the effectors of T3SS1 in *V. parahaemolyticus*, VepA (which has also been referred to  
64 as VopQ and as VP1680) plays a significant role in its cytotoxicity [23, 24]. Since

65 *V. alginolyticus* has a gene encoding a VepA homolog in the T3SS gene cluster, we  
66 hypothesized that the VepA homolog also has an important contribution to the cytotoxicity.

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

72

73

## Materials and Methods

### 74 *Bacterial strains, plasmids, and culture conditions*

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

86 HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma, St.  
87 Louis, MO) supplemented with 10% (v/v) inactivated fetal bovine serum (FBS) (Biowest,  
88 Nuaille, France) at 37°C under 5% CO<sub>2</sub>. DMEM or minimum essential medium/Earle's

89 balanced salts (MEM/EBSS, Gibco, Grand Island, NY) without phenol red supplemented with  
90 10% (v/v) inactivated FBS was used during incubation in the infection experiment.

91

### 92 ***Construction of a mobilizable vector for gene replacement***

93 We constructed a mobilizable vector for gene replacement carrying the recognition site of  
94 meganuclease I-*SceI*, pKU66, by inserting the *oriT* region into pSG76-C [28] at the *NotI* site.

95 We amplified the *oriT* region from pUC18T-mini-Tn7T-Gm [29] using primers P064 (5'-  
96 GCGGCCGCTATCAGAGCTTATCGGCCAG-3'; the underlining indicates a *NotI* site) and  
97 P065 (5'-GCGGCCGCGGGGATTCCTTAAGGTATAC-3'; the underlining indicates a *NotI*  
98 site).

99

### 100 ***Construction of mobilizable vectors for gene expression***

101 We constructed pEX18Km by replacing the ampicillin resistance gene on pEX18Ap [30] with  
102 the kanamycin resistance gene at the *AatII* and *AhdI* sites. We amplified the kanamycin  
103 marker from pHSG298 (Takara Bio, Shiga, Japan) using primers P394 (5'-  
104 GACGTCGATCTGATCCTTCAACTCAG-3'; the underlining indicates an *AatII* site) and  
105 P395 (5'-GACTCCCCGTCTGCTCTGCCAGTGTTACAAC-3'; the underlining indicates an  
106 *AhdI* site).

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

112

### 113 ***Construction of an I-*SceI* expression vector***

114 We amplified the promoter region of the elongation factor thermo unstable (EF-Tu) in  
 115 *V. alginolyticus* I.029 by using primers P406 (5'- GAATTCAGCGGGTTACCCTGTACTAG-  
 116 3'; the underlining indicates an *EcoRI* site) and P407 (5'-  
 117 GATCGTGTTCCTTCCTAGTTATG-3').

118 The *I-SceI* gene was amplified from pST76-ASceP [28] with the use of the primers  
 119 P408 (5'-CATAACTAGGAAGGAACACGATCATGCATCAAAAAACCAGGTA-3'; the  
 120 underlining indicates a sequence overlapping to the 3' region of the EF-Tu promoter) and  
 121 P409 (5'-CAAAGGGAAAACACTGTCCATAC-3'). The two DNA fragments were spliced by  
 122 overlap extension polymerase chain reaction (PCR). The recombinant fragment was ligated  
 123 into pGEM-T Easy (Promega, Madison, WI) to obtain pOU246. We cloned an *EcoRI-SphI*  
 124 fragment of pOU246 carrying the *I-SceI* gene with the EF-Tu promoter into the same sites of  
 125 pEX18Km, resulting in pOU257.

126

### 127 ***Construction of a vepA-deletion mutant and a vscC-deletion mutant***

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

136 We cloned this fragment into pKU66 at the *BamHI* and *SacI* sites, resulting in pOU549.  
 137 *E. coli* RHO3 harboring plasmid pOU549 was conjugated with *V. alginolyticus* ATCC 17749,  
 138 and the resultant transconjugant was conjugated to *E. coli* RHO3 harboring pOU257. The

139 transconjugants were selected on LB 2% NaCl agar plates containing antibiotic for the  
 140 responsible plasmid. The *vepA*-deletion was confirmed by colony PCR using primers P787  
 141 and P790, and by sequencing of the PCR products. The remaining pOU257 was eliminated by  
 142 inoculation into LB 3% NaCl medium containing 5% sucrose.

143 The construction of  $\Delta vscC$  was performed using the same method as that used for the  
 144  $\Delta vepA$  construction. The primers used to amplify the *vscC*-deletion fragment were P793 (5'-  
 145 GAATTCCGGTTGCGAAAGTATGGCAATG-3'; the underlining indicates an *Eco*RI site),  
 146 P794 (5'-AGGAACAACACTCACTGCGCATA-3'), P795 (5'-  
 147 TATGCGCAGTGAGTGTTGTTTCCTCGCCCTTCAGAGGAGTCTTAACC-3'; the  
 148 underlining indicates complementary region to P794) and P796 (5'-  
 149 GGATCCGCAGATCGAGTTTCTGTGTTTCGG-3'; the underlining indicates a *Bam*HI site).  
 150 This fragment was cloned into pKU66 at *Eco*RI and *Bam*HI sites, resulting in pOU553. The  
 151 *vscC* deletion was confirmed by colony PCR using primers P793 and P796 and by sequencing  
 152 of the PCR products.

153

#### 154 ***Construction of a vepA expression vector***

155 We amplified the DNA fragment containing *vepA* from the gDNA by using primers P791 (5'-  
 156 GCTGAATTCAATGGTTAATACAACACAAAAAATCAGCCAAAGC-3'; underlining  
 157 indicates an *Eco*RI site) and P792 (5'-GCTGGATCCTTACACCCAGCCTTCTGCCAAG-3';  
 158 underlining indicates a *Bam*HI site). The PCR product was cloned into *Eco*RI and *Bam*HI  
 159 sites of pHSG398T, which yielded pOU550.

160

#### 161 ***Cytotoxicity assay***

162 HeLa cells were seeded into a 96-well plate at  $3 \times 10^4$  cells/well, and then incubated at 37°C  
 163 under 5% CO<sub>2</sub> for 48 hr. The cells were washed with DMEM or MEM/EBBS medium

164 without phenol red, and then infected with *V. alginolyticus* at a multiplicity of infection  
165 (MOI) of 100 for 4 hr. A ten-times dilution of *V. alginolyticus* at the optical density at 600 nm  
166 (OD<sub>600</sub>) of 1.0 is equal to 3–4×10<sup>8</sup> CFU/ml on an LB 1% NaCl agar plate. We added 10 μl of  
167 these dilution cultures to 3–4×10<sup>4</sup> of HeLa cell culture to obtain the MOI of 100.

168 We measured the release of lactate dehydrogenase (LDH) into the medium by using a  
169 Cytotoxicity Detection Kit<sup>PLUS</sup> (LDH) (Roche, Mannheim, Germany) according to the  
170 manufacturer's instructions. The percentage of LDH release was calculated based on the  
171 following equation: [OD<sub>490</sub> of experimental release – OD<sub>490</sub> of spontaneous release]/(OD<sub>490</sub> of  
172 maximum release – OD<sub>490</sub> of spontaneous release) × 100. The spontaneous release is the  
173 amount of LDH released from the cytoplasm of uninfected cells, and the maximum release is  
174 the total amount of LDH released upon the complete lysis of uninfected cells.

175

#### 176 ***AO relocation assay***

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

180

#### 181 ***Fluorescent-dextran translocation***

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

185

#### 186 ***Inhibition of cathepsin and reactive oxygen species (ROS), LMP induction, and iron*** 187 ***chelation***

188 HeLa cells were pretreated with 100 μM E-64d, a broad-spectrum cathepsin inhibitor, for 16



189 hr before infection. The cells were challenged with 3 mM L-Leucyl-L-Leucine methyl ester  
190 (LLOMe) for 4 hr to induce LMP. To chelate iron, we used 100  $\mu$ M 2,2'-bipyridyl (BIP). To  
191 inhibit reactive oxygen species (ROS), we used 5 mM N-acetyl cysteine (NAC) or 250  $\mu$ M  
192 Trolox.

193

#### 194 ***Statistical analysis***

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

196

197

## Results

### 198 ***VepA contributes to HeLa cell cytotoxicity in V. alginolyticus infection***

199 We chose *V. alginolyticus* strain ATCC17749, which was shown to be highly virulent in an *in*  
200 *vivo* infection model [18]. VepA (N646\_0746) in this strain is homologous to that in  
201 *V. parahaemolyticus*, with 88% sequence similarity (435 aa/493 aa).

202 To examine the contribution of VepA to the cytotoxicity toward human cells, we  
203 performed the LDH release cytotoxicity assay after 4 hr of bacterial infection against HeLa  
204 cells (Fig. 1). The T3SS apparatus gene mutant,  $\Delta$ *vscC*, showed significantly less cytotoxicity  
205 than the wild-type (WT).  $\Delta$ *vepA* also showed significantly less cytotoxicity. The *vepA*  
206 complimentary strain,  $\Delta$ *vepA/vepA* completely restored the cytotoxicity (similar to the WT),  
207 whereas  $\Delta$ *vepA/vector* did not. To test the results of the LDH release cytotoxicity assay, we  
208 also performed propidium iodine (PI) staining. The results were similar to those of the LDH  
209 release cytotoxicity assay (data not shown). These results indicate that mainly VepA is  
210 responsible for the cytotoxicity of *V. alginolyticus*.

211

### 212 ***VepA in V. alginolyticus induced lysosomal membrane permeabilization (LMP)***

213 It was reported that *V. parahaemolyticus* VepA induces LMP in mammalian cells [24, 33].  
214 Here we therefore examined the integrity of lysosomes in *V. alginolyticus*-infected cells by  
215 using acridine orange (AO) (Fig. 2). AO is a metachromatic dye that emits red fluorescence at  
216 low pH (e.g., in lysosomes) and emits green fluorescence at neutral pH (e.g., in the cytosol)  
217 [34].

218 In uninfected HeLa cells, we observed small red fluorescent dots, which reflect an  
219 accumulation of the dye in lysosomes. In the WT infection, the appearance of red dots was  
220 disappeared and the intensity of the green fluorescence in the cytosol was increased. In  
221 contrast, in the  $\Delta vepA$  infection, the appearance of red dots was comparable to that in the  
222 uninfected cells, and the green fluorescence enhancement was not observed. In the  
223  $\Delta vepA/vepA$  infection, the fluorescence pattern reverted to one similar to that obtained with  
224 the WT. These results indicate that *V. alginolyticus* infection induces the leakage of the  
225 lysosomal contents into the cytosol in a VepA-dependent manner.

226 We observed that infection with either *V. alginolyticus* strain induced a morphological  
227 change of HeLa cells, i.e., cell rounding (Fig. 2). Since the  $\Delta vepA$  infection also induced cell  
228 rounding, we can safely assume that this change is not due to VepA. On the other hand, the  
229  $\Delta vscC$  strain did not affect the cell morphology (data not shown). Therefore, this change  
230 might be caused by other T3SS effectors, e.g., N646\_0751, the homologue of VopS in  
231 *V. parahaemolyticus* [35].

232

### 233 ***The VepA-induced LMP in V. alginolyticus was not due to a lysosomal membrane rupture***

234 The molecular mass of AO is approx. 0.3 kDa. To determine whether VepA induces the  
235 release of only small molecules or lysosomal rupture, we observed the LMP in  
236 *V. alginolyticus* infection using larger molecules: FITC-dextran, with the molecular mass of 4  
237 or 10 kDa. It is well known that such macromolecules can be loaded into lysosomes to yield

238 green fluorescence punctates while the lysosomes are intact. When the lysosomes are  
239 ruptured, the FITC-dextran is released into the cytosol to yield diffuse green fluorescence.

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

248

#### 249 ***VepA-dependent cytotoxicity is not due to lysosomal cathepsin***

250 LMP-associated cell death is usually related to lysosomal cathepsin release [36]. Since the  
251 VepA-induced LMP in *V. alginolyticus* infection allowed the release of only molecules <4  
252 kDa, we hypothesized that lysosomal proteases with the molecular sizes >4 kDa (including  
253 cathepsins, the molecular masses of which is approx. 30 kDa) could not pass through the  
254 lysosomal membrane. To determine whether the VepA-mediated cell death is due to cathepsin  
255 release from lysosomes, we examined the effect of a broad-spectrum cathepsin inhibitor, E-  
256 64d, on cell death. HeLa cells were treated with E-64d for 16 hr before challenge with  
257 LLOMe or a *V. alginolyticus* strain. The lysosomal rupture induced by LLOMe subsequently  
258 resulted in cell death, as reported [36], and cytotoxicity was reduced by E-64d treatment as  
259 expected (Fig. 4A). When the cells were challenged with the WT strain, the cell death rates  
260 were similar regardless of the E-64d treatment (Fig. 4A). These results suggest that cathepsin  
261 is not the major causative factor of *V. alginolyticus* cytotoxicity.

262 Since the lysosome is one of the iron-rich intracellular organelles, VepA might allow  
263 excessive iron release into the cytosol to induce cell death via ROS [37]. To investigate the  
264 roles of iron and ROS in cell death in *V. alginolyticus* infection, we compared their cytotoxic  
265 effects in the presence of an iron chelator, BIP, and an ROS scavenger, NAC or Trolox. In the  
266 presence of BIP, the cytotoxicity was significantly reduced. However, after the ROS  
267 scavenger treatment, the cytotoxicity was slightly reduced or not changed, respectively (Fig.  
268 4B). When we directly examined hydroxyl radicals by using hydroxyphenyl fluorescein, we  
269 observed no fluorescence in *V. alginolyticus*-infected cells (data not shown).

270

## 271 Discussion

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

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

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

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

303 Since it is well known that iron triggers cell death via ROS accumulation [37, 39], we  
304 examined the effects of ROS scavengers. NAC inhibited the cell death only slightly, but  
305 Trolox did not inhibit the cell death at all. Moreover, the growth rate of *V. alginolyticus* was  
306 not affected by the presence of BIP, NAC or Trolox. We therefore propose that iron induces  
307 cell death without ROS accumulation in this infection model. A study on yeast demonstrated  
308 that iron overload stimulates the sphingolipid production that leads to cell death, without ROS  
309 accumulation [37]. Additional studies are required to elucidate the function of iron in VepA-  
310 induced cell death.

311 In conclusion, our experiments demonstrated that VepA induced cathepsin-independent

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

317

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- 428

429 **Table 1.** Bacterial strains and plasmids

Strain/Plasmid	Relevant Genotype or Properties	Reference or Source
<b><i>E. coli</i> strains</b>		
DH5 $\alpha$	F <sup>-</sup> $\Phi$ 80 <i>lacZ</i> $\Delta$ M15 $\Delta$ ( <i>lacZYA-argF</i> )U169 <i>deoR</i> <i>recA1 endA1 hsdR17</i> (r <sub>K</sub> <sup>-</sup> m <sub>K</sub> <sup>-</sup> ) <i>phoA supE44</i>	[26]
DH5 $\alpha$ ( $\lambda$ <i>pir</i> )	DH5 $\alpha$ $\lambda$ <i>pir</i>	H.P. Schweizer
RHO3	SM10( $\lambda$ <i>pir</i> ) $\Delta$ <i>asd:FRT</i> $\Delta$ <i>aphA:FRT</i>	[27]
<b><i>V. alginolyticus</i> strains</b>		
ATCC 17749	Wild-type strain	[25]
$\Delta$ <i>vepA</i>	ATCC 17749 $\Delta$ <i>vepA</i>	This study
$\Delta$ <i>vscC</i>	ATCC 17749 $\Delta$ <i>vscC</i>	This study
$\Delta$ <i>vepA/vepA</i>	$\Delta$ <i>vepA</i> harboring pOU550	This study
$\Delta$ <i>vepA/vector</i>	$\Delta$ <i>vepA</i> harboring pHSG398T	This study
<b>Plasmids</b>		
pGEM-T Easy	AMP <sup>r</sup> ; TA cloning vector	Promega
pSG76-C	CM <sup>r</sup> ; Suicide plasmid vector; I-SceI	[28]
pKU66	CM <sup>r</sup> ; pSG76-C with <i>oriT</i>	This study
pHSG398	CM <sup>r</sup> ; pUC-type cloning vector	Takara Bio.
pHSG398T	CM <sup>r</sup> ; pHSG398 with <i>oriT</i>	This study
pEX18Ap	AMP <sup>r</sup> ; cloning vector; <i>sacB</i> ; <i>oriT</i>	[30]
pEX18Km	KM <sup>r</sup> ; pEX18Ap with AMP <sup>r</sup> replacement to KM <sup>r</sup>	This study
pHSG298	KM <sup>r</sup> ; source of kanamycin resistance gene	Takara Bio.
pUC18T-mini-Tn7T-Gm	AMP <sup>r</sup> , GM <sup>r</sup> ; source of <i>oriT</i>	[29]

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pST76A-SceP	AMP <sup>r</sup> ; source of I-SceI	[28]
pOU246	AMP <sup>r</sup> ; pGEM-T Easy with EF-Tu promoter and I-SceI	This study
pOU257	KM <sup>r</sup> ; pEX18Km with EF-Tu promoter and I-SceI	This study
pOU549	CM <sup>r</sup> ; pKU66 with $\Delta$ <i>vepA</i> recombinant DNA fragment	This study
pOU550	CM <sup>r</sup> ; pHSG398T with <i>vepA</i>	This study
pOU553	CM <sup>r</sup> ; pKU66 with $\Delta$ <i>vscC</i> recombinant DNA fragment	This study

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

431

432 **FIGURE LEGENDS**

433

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

440

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

445

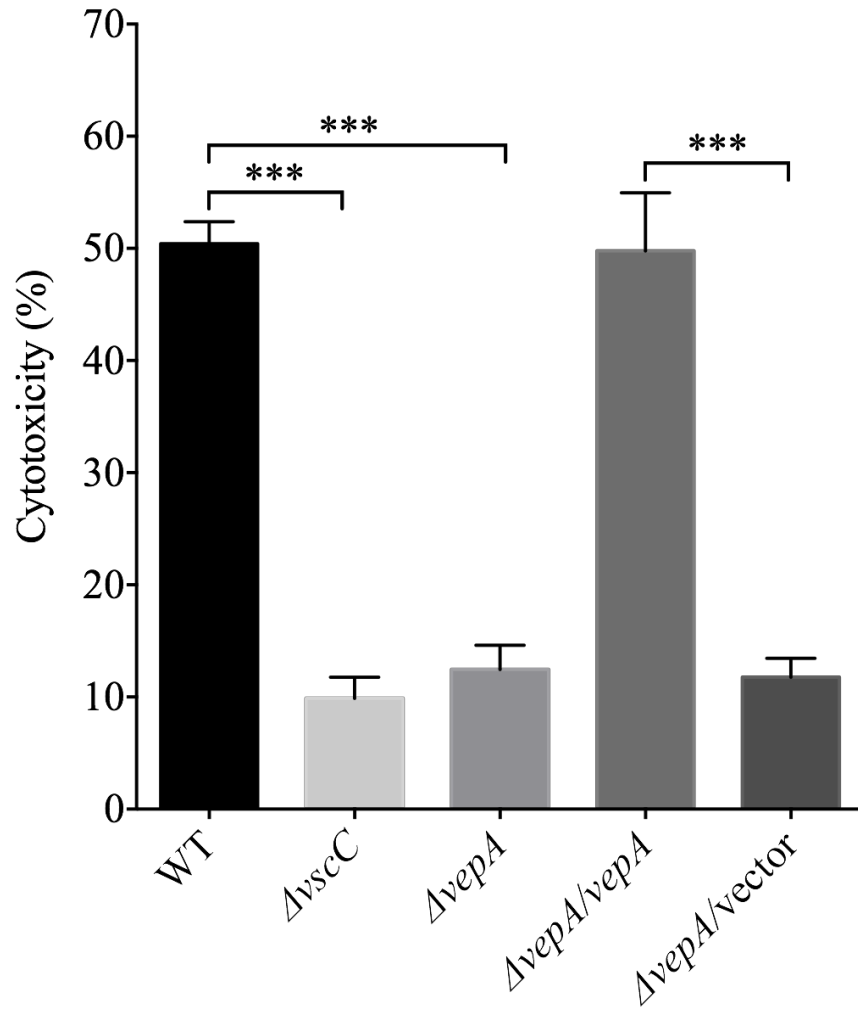
446 **Fig. 3.** LMP visualized by fluorescent dextran release. HeLa cells were incubated with 10-  
447 kDa (**A**) or 4-kDa (**B**) FITC-dextran for 16 hr and then challenged with LLOMe or infected  
448 with the WT strain, *vepA*-deletion mutant ( $\Delta vepA$ ), or *vepA*-deletion mutant carrying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

450

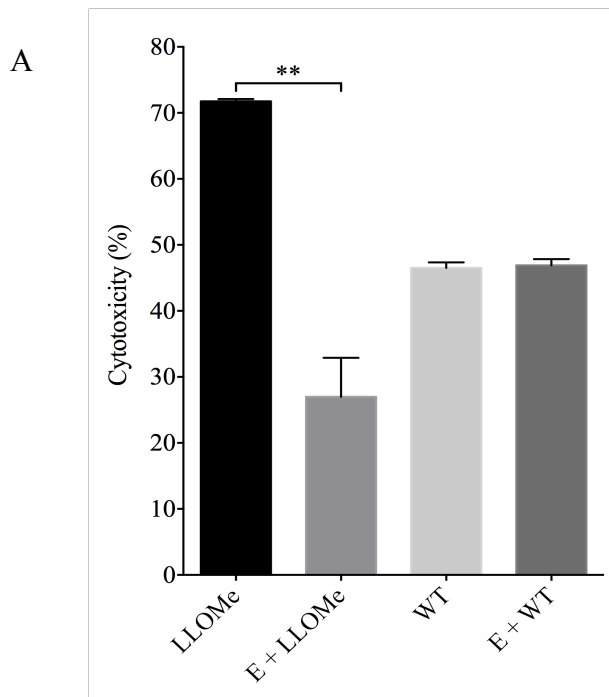
451 **Fig. 4.** Cytotoxicity of *V. alginolyticus* infection in cathepsin inhibition, iron chelation, and  
452 ROS inhibition. **A**, HeLa cells were pretreated with E-64d (E) and then challenged with 3 mM  
453 LLOMe or infected with the WT strain for 4 hr. **B**, HeLa cells were pretreated with an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

456

Fig. 1.



**Fig. 4.**



**B**

