environmental microbiology

Environmental Microbiology (2013) **15**(2), 419–432 doi:10.1111/j.1462-2920.2012.02854.x

MARTX of *Vibrio vulnificus* biotype 2 is a virulence and survival factor

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Summary

Vibrio vulnificus **biotype 2 is a polyphyletic group whose virulence for fish relies on a plasmid. This plasmid contains an *rtxA* gene duplicated in the small chromosome that encodes a MARTX (Multifunctional, Autoprocessing Repeats-in-Toxin) unique within the species in domain structure (MARTX type III). To discover the role of this toxin in the fitness of this biotype in the fish-farming environment, single- and double-knockout mutants were isolated from a zoonotic strain and analysed in a series of *in vivo* and *in vitro* experiments with eel, fish cell lines and amoebae isolated from gills. Mice, murine and human cell lines were also assayed for comparative purposes. The results suggest that MARTX type III is involved in the lysis of a wide range of eukaryotic cells, including the amoebae, erythrocytes, epithelial cells and phagocytes after bacterium–cell contact. In fish, MARTX type III may act as a toxin involved in the onset of septic shock, while in mice it may promote bacterial colonization by preventing phagocytosis of bacterial cells. Moreover, this toxin could protect bacteria from predation by amoebae, which would increase bacterial survival outside the host and would explain the fitness of this biotype in the fish-farming environment.**

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Introduction

Vibrio vulnificus is a bacterial species native to estuarine environments in temperate and tropical areas, where it lives associated to fish and filter-feeding bivalves (mainly oysters) (Jones and Oliver, 2009). The species is subdivided into three biotypes: biotype 1 is related to sporadic human septicemia cases after oyster consumption or wound infections; biotype 2 is linked to vibriosis outbreaks in fish (mainly eel, *Anguilla anguilla*) as well as sporadic cases of secondary septicemia in humans; and biotype 3 is associated with outbreaks of secondary septicemia caused by wounds from tilapia spines (Tison *et al.*, 1982; Amaro and Biosca, 1996; Bisharat *et al.*, 1999; Jones and Oliver, 2009). Recent studies into the phylogeny of *V. vulnificus* demonstrate that biotype 2 is a polyphyletic group (Cohen *et al.*, 2007; Sanjuán *et al.*, 2011). One of the aforementioned studies suggests that biotype 2 is, in fact, a pathovar specifically pathogenic for fish (Sanjuán *et al.*, 2011). This suggestion is strongly supported by three findings: (i) all biotype 2 strains possess a virulence plasmid of 68–70 kb that encodes resistance to eel innate immunity (Lee *et al.*, 2008; Valiente *et al.*, 2008); (ii) the virulence plasmid can be transmitted among strains by conjugation, aided by a conjugative plasmid (Lee *et al.*, 2008); and (iii) some biotype 1 transconjugants are resistant to innate immunity and virulent for eels (Huang *et al.*, 2010).

The biotype 2 virulence plasmid contains only five genes showing significant homology to previously described virulence genes (Lee *et al.*, 2008). These genes constitute an *rtx* gene cluster, organized in two divergent operons; *rtxC-A1* encodes a RTX (repeats-in-toxin) toxin (*rtxA1*) plus an enzyme required for toxin modification, and *rtxB-D-E* encodes a toxin transport system (Lee *et al.*, 2008). RTX have been related to virulence for mammals and/or resistance to amoebal predation (Satchel, 2011). The plasmid-encoded RTX belongs to the MARTX subfamily (multifunctional autoprocessing RTX) (Lee *et al.*, 2008). MARTX share a modular structure formed by two conserved external modules (N- and C-termini) harbouring the repeated motifs, and one variable internal module containing different functional

Table 1. *V. vulnificus* biotype 2 strains used in this study; virulence, toxicity and resistance to the bactericidal effect of fresh eel plasma (EP) or fresh human plasma (HP).

Strain	Description	Reference	Virulence LD_{50}[a]			Toxicity TD_{50}[b]	Serum resistance[c]	
			Mice (i.p.)	Eel (i.p.)	Eel (imm.)	Eel (i.p.)	EP	HP
CECT4999	Wild-type strain	Lee *et al.* (2008)	5.7×10^5	1.5×10^2	1.5×10^6	1.8	++	+
CT218	Plasmid-cured CECT4999	Lee *et al.* (2008)	6×10^5	$> 1 \times 10^7$	$> 1.0 \times 10^8$	1.7	–	+
CT281	CT218 Δc*rtxA1*$_3$	This study	ND	$> 1 \times 10^7$	$> 1.0 \times 10^8$	ND	ND	ND
CT284	CECT4999Δp*rtxA1*$_3$	This study	4.7×10^6	1.5×10^2	1.5×10^6	ND	++	+
CT285	CECT4999Δp*rtxA1*$_3$Δc*rtxA1*$_3$	This study	5.4×10^7	$> 1.7 \times 10^7$	$> 7.0 \times 10^7$	1.8	++	+
CT302	CECT4999Δc*rtxA1*$_3$	This study	1.7×10^6	1.7×10^2	2×10^6	ND	++	+
CT310	Revertant from CT285 (Δp*rtxA1*$_3$ reverted)	This study	ND	3.5×10^2	ND	ND	++	+
CT316	Revertant from CT285 (Δp*rtxA1*$_3$ and Δc*rtxA1*$_3$)	This study	5×10^5	3×10^2	ND	ND	++	+

a. Virulence was calculated as LD_{50} after intraperitoneal injection (i.p.) (cfu per animal) or bath immersion (imm.) (cfu per ml).
b. Toxicity degree is expressed as TD_{50} (μg of ECP per g fish).
c. Bacterial growth after 4 h of incubation in eel plasma (EP) and human plasma (HP) is expressed as the ratio final versus initial counts (–, no growth; +, between 1 and 10; ++, between 10 and 100; +++, between 100 and 1000).
ND, not done.

domains related to the specific toxin activity (Satchel, 2011). A previous *in silico* study on the evolution of the *rtxA1* gene in *V. vulnificus* suggests that it is a mosaic gene that has emerged by recombination and that each domain has a different evolutionary history (Roig *et al.*, 2011). Consequently, the species produces at least four different types of MARTX (types I–IV), three of which are present in the virulent strains (types I–III) (Kwak *et al.*, 2011; Roig *et al.*, 2011). The structure of MARTX types I, II and III is shown in Fig. S1. The plasmid-encoded MARTX corresponds to type III (or $RtxA1_3$), which is structurally and evolutionarily different to MARTX types I and II (Roig *et al.*, 2011). Interestingly, *rtxA1*$_3$ is present in all analysed biotype 2 strains, regardless of their clonal origin; also a second copy of the gene is present in chromosome II (Lee *et al.*, 2008; Roig *et al.*, 2011).

Considering the above, we hypothesized that *rtxA1*$_3$ is an essential gene for the survival of biotype 2 in its main reservoir: the fish farming environment. To test this hypothesis, we obtained single- and double-knockout mutants in the chromosomal and plasmid *rtxA1*$_3$ genes, together with the corresponding revertants from a serovar E strain. All these mutants and revertants, together with the wild-type strain, were used in a series of *in vivo* and *in vitro* experiments with fish, fish cell lines and amoebae freshly isolated from fish gills. Since serovar E is zoonotic, we also included mice, a human cell line and murine macrophages to test the potential role of this toxin in pathogenesis towards humans.

Results

$rtxA1_3$ *is a virulence gene for eels and mice*

The virulence of the wild-type strain and its derivatives were assayed (Table 1) in the eel and mouse. As shown in Table 1, the single mutants showed the same virulence degree for eels as the wild-type strain while the double mutant was completely avirulent by either i.p. injection or immersion. When the strains were tested in mice, the LD_{50} of the double mutant was about two-log units higher than that of the wild-type strain, while the LD_{50} of the single mutants was three- to eightfold higher than that of the wild-type strain (Table 1). As expected, the plasmid-cured strain (CT218) was avirulent for eels [the loss of the plasmid makes the bacterium sensitive to the eel innate immunity (Valiente *et al.*, 2008)] and as virulent for mice as the wild-type strain while the plasmid-cured strain with Δc*rtxA1*$_3$ (CT281) showed the same changes in virulence degree as the double mutant (Table 1). In addition, the single revertant CT310 (Δp*rtxA1*$_3$ reverted) and the double revertant CT316 (Δp*rtxA1*$_3$ and Δc*rtxA1*$_3$ reverted) exhibited the wild-type level of virulence in both eel and mouse (Table 1). This result confirmed that attenuated virulence was not caused by an unexpected mutation that had occurred elsewhere.

The extracellular products (ECP) from the wild-type, the cured and the double mutant strains were equally toxic for eels, exhibiting similar mean toxic dose (TD_{50}) values (Table 1). This result suggests that MARTX type III, if present, is not active in the ECPs and that other vibrio toxins could contribute to eel virulence.

$rtxA1_3$ *is not required for resistance to mammal and fish serum*

Resistance to the bactericidal effect of serum was tested by growing the bacteria in fresh eel plasma (EP) or human plasma (HP) for 4 h. No differences were found in terms of bacterial growth in plasma among the different strains, with the exception of the cured strain, which was sensitive to fresh EP (Table 1).

rtxA1$_3$ *is not essential for eel colonization and invasion*

To examine whether the *rtxA1$_3$* gene plays a role in fish colonization and invasion, eels were infected by immersion with the wild-type and the double-mutant strains. Contrary to expected, the double-mutant was not visibly deficient in eel colonization and invasion capacity (Fig. 1A). Thus, it was able to adhere to gills, establishing a stable population similar in size to that of the wild-type strain, and cause septicemia but without killing the eels (Fig. 1A). In addition, bacterial population size in the internal organs did not differ significantly to that of the wild-type strain at 9, 24 and 72 h post infection (Fig. 1A). This result strongly suggests that MARTX type III is a lethal factor for eels. Additional co-infection experiments with both the wild-type and double-mutant strains by either injection or immersion revealed that the former was recovered in higher proportions from the blood and head kidney (Fig. 1B and data not shown), which suggests that MARTX type III could also confer some advantages to the bacterium during eel colonization and invasion.

The external and internal organs of the infected eels were examined histologically. In accordance with the low bacterial counts in internal organs, either an absence of bacteria or very few bacteria were observed in infected eel tissues by electron microscopy, and they were mostly close or within the lumen of capillary vessels (data not shown). Haemorrhaging was the only evident alteration observed in tissues of the eel challenged with the wild-type strain. Although haemolysis was not obvious, non-specific changes, such as a slight alteration in the mitochondrial structure in the haematopoietic cells of head kidney or a mild increase in the number of phagocytosed erythrocytes in the spleen at 24 and 48 h after challenge were observed (Fig. 2A). Finally, the granulocytes were the main cell type that showed clear signs of damage (Fig. 2B–D). Granulocyte damage was observed very early in kidneys (at 1 h post challenge) and later (from 9 h post infection) in the head kidney, the main haematopoietic tissue in fish, and was mainly evidenced by release of cytoplasmic content, including granules (Fig. 2B–D).

rtxA1$_3$ *acts as a cytotoxin for mammal and fish cells*

We determined cytotoxicity of the wild-type strain, mutants and revertants to EP-1 (eel epidermis), EPC (fathead minnow epidermis) and HEp-2 (human epidermis) cells as well as to eel and human erythrocytes. The wild-type strain proved toxic towards the three cell lines tested (Fig. 3). Mutants lacking one copy of *rtxA1$_3$* exhibited wild-type cytotoxicity levels, while mutants lacking both copies of *rtxA1$_3$* showed significant reductions in cytotoxicity levels in relation to the wild-type strain (Fig. 3). No significant differences were observed in the cytopathic effects of the revertants compared with the wild-type strain (Fig. 3A and B, and data not shown).

The lytic effect of live bacteria was also tested in eel and human erythrocytes. As shown in Fig. 4A and B, significant differences were found in haemolysis between the double mutant and the wild-type/double revertant strains. Interestingly, the double mutant became immotile and aggregated in presence of wild-eel erythrocytes (Fig. 4C). Bacterial aggregation was not observed when the double mutant was incubated with cultured-eel or human erythrocytes (data not shown).

In all cases, significant differences in lytic activity in all the tested cell types between double mutant and wild-type/revertant strains were only noticeable up until 1.5 h of incubation (Fig. 4 and data not shown) after which all strains underwent complete cell lysis.

Finally, no significant differences were observed in the cytopathic effects produced by the ECP of any of the strains (Fig. 3C and data not shown), which confirmed the results obtained in the eel toxicity assays. In fact, cytopathic effects were observed within 1 h and were manifested by rounding, shrinking, dendritic elongation and, finally, cell detachment, but not by cell lysis (Fig. 3D).

rtxA1$_3$ *partially protects from phagocytosis by professional phagocytes*

To ascertain whether MARTX type III provides protection against phagocytosis, the strains were incubated in presence of eel phagocytes from the peritoneal exudates (PEC) as well as murine macrophages (RAW264.7 cell line). Microscopic observations of PEC preparations revealed that they were enriched in neutrophils (Fig. 5). As shown in Fig. 5A and E, the wild-type strain and the double-revertant resisted phagocytosis by eel PEC; they were not internalized, multiplied extracellularly (bacterial counts between approximately 1×10^6 at time 0 and 1×10^7 cfu per well at 90 min of incubation) and destroyed the monolayer in less than 60 min. The double mutant also multiplied extracellularly to a similar extent (counts between approximately 1×10^6 at time 0 and 1×10^7 cfu per well at 90 min of incubation) but it was poorly phagocytosed (maximal efficiency of 1 per 10^4 bacteria) (Fig. 5A) and did not destroy the monolayer within 60 min of incubation (Fig. 5E). In addition, the internalized double mutant cells did not survive intracellularly, since they were destroyed by eel PEC within 90 min (Fig. 5C). In contrast, the murine macrophages phagocytosed all the analysed strains much more efficiently than eel PEC, specially the double mutant, which was internalized in significantly higher numbers than the other two strains (Fig. 5B). In all cases, the internalized bacteria were also killed by the mouse macrophages (Fig. 5D). These results suggest

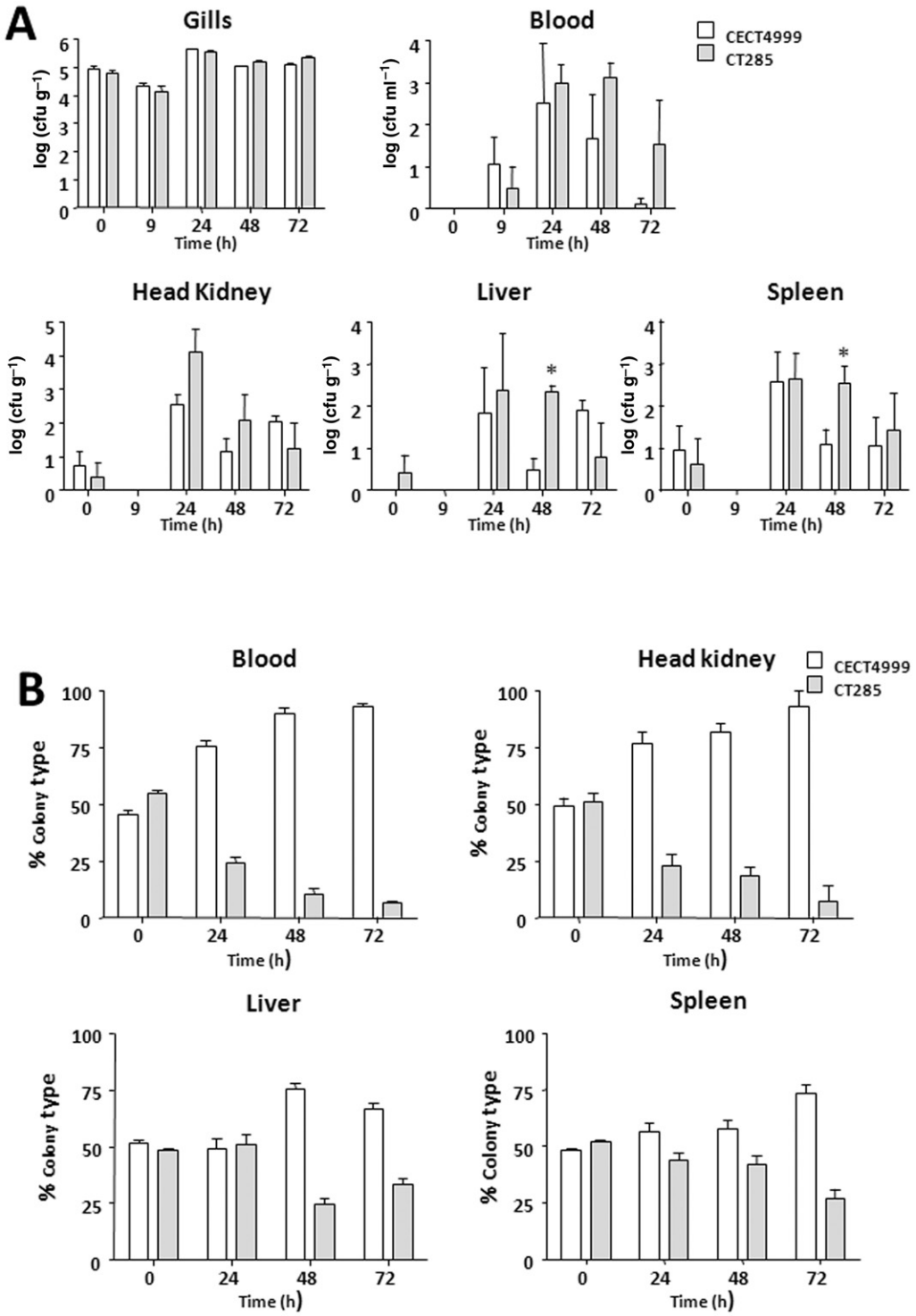

Fig. 1. Eel colonization and invasion assays.
A. Infection experiments: eels were infected by immersion challenge with the wild-type strain (CECT 4999) or with the double mutant in *rtxA1*$_3$ (CT285) and microbial counts on TSA-1 from external and internal organs were performed at different time intervals post challenge. Asterisks indicate the significant differences (Student's *t*-test, $P < 0.05$) when compared with the wild-type strain.
B. Co-infection experiments: eels were co-infected by immersion with strains CT285 and CECT4999 in a ratio 1:1 at a dosis of 1.5×10^6 cfu ml^{-1} and the percentage of each strain recovered on the plates is indicated on the *y*-axis.

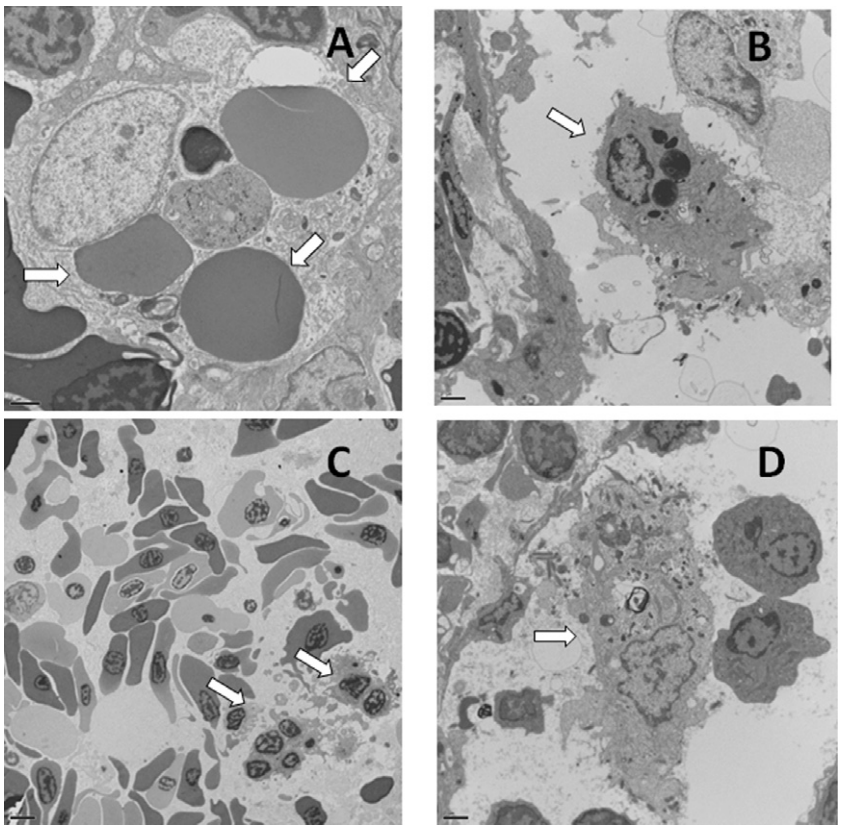

Fig. 2. Histological analysis of the eels infected with the wild-type strain CECT 4999.
A. macrophage with damaged erythrocytes (marked with arrows) engulfed within its cytoplasm. Bar, 1 μm.
B–D. Three images of head kidney showing damaged granulocytes (marked with an arrow): (B) bar, 1 μm; (C) bar, 5 μm, and (D) bar, 2 μm.

that MARTX type III could protect from engulfment by the phagocytes but not from the bacterial destruction mechanisms inside the phagocyte.

rtxA1$_3$ *partially protects from amoeba predation*

To ascertain whether MARTX type III may promote biotype 2 survival in the environment by destroying its putative natural predator, the amoeba, we tried to isolate amoeba from the gills of different cultured fish species. Amoebae were successfully isolated and purified from the turbot (*Scophthalmus maximus*) gills. These amoebae were identified using morphological (Leiro *et al*., 1998) and phylogenetic criteria (Zhang *et al*., 2000) as belonging to the species *Neoparamoeba pemaquidensis*, a gill disease-causing amoebic species. The amoebae were cultured with live bacteria from the wild-type or the double-mutant strain. As shown in Fig. 6A, *N. pemaquidensis* started to grow exponentially from day 14 in the presence of the double mutant; however, the amoebae grew significantly less in the presence of the wild-type strain. In addition, destruction of amoebae, particularly during the first week of incubation, was observed when they were cultured with the wild-type strain but not with the double mutant (Fig. 6B). This destruction seemed to be by cellular apoptosis (Fig. 6B, c)

rtxA1$_3$ *gene is expressed* in vivo *and after cell contact*

To determine the environmental cues involved in *rtxA1$_3$* expression, the transcriptional levels of *rtxA1$_3$* were assayed after growth under a variety of culture conditions mimicking the *in vivo* growth by qRT-PCR. As shown in Fig. 7A, *rtxA1$_3$* expression in cultured bacteria was hardly affected by the presence (by adding ferric chloride, haemoglobin or haemin) or absence (by adding the iron-depleting compound, human apotransferrin) of iron in the culture media. However, *rtxA1$_3$* expression was increased three or fourfold in the presence of either PEC or erythrocytes from eels or, even in the presence of amoeba (Fig. 7B), but only if bacteria came into contact with the eukaryotic cells (Fig. 7C). A significant increase in *rtxA1$_3$* expression was also observed in infected eel blood at 9 h post infection (Fig. 7D). The transcriptional level of *rtxA1$_3$* declined to an undetectable level at 48 h post infection, the time by which 50% of eels had died.

Discussion

The study reported here has tested the hypothesis that MARTX type III is essential for *V. vulnificus* biotype 2 survival in the fish farming environment, in other words both inside and outside its main host, the eel. We selected a strain belonging to the zoonotic serovar with the aim of

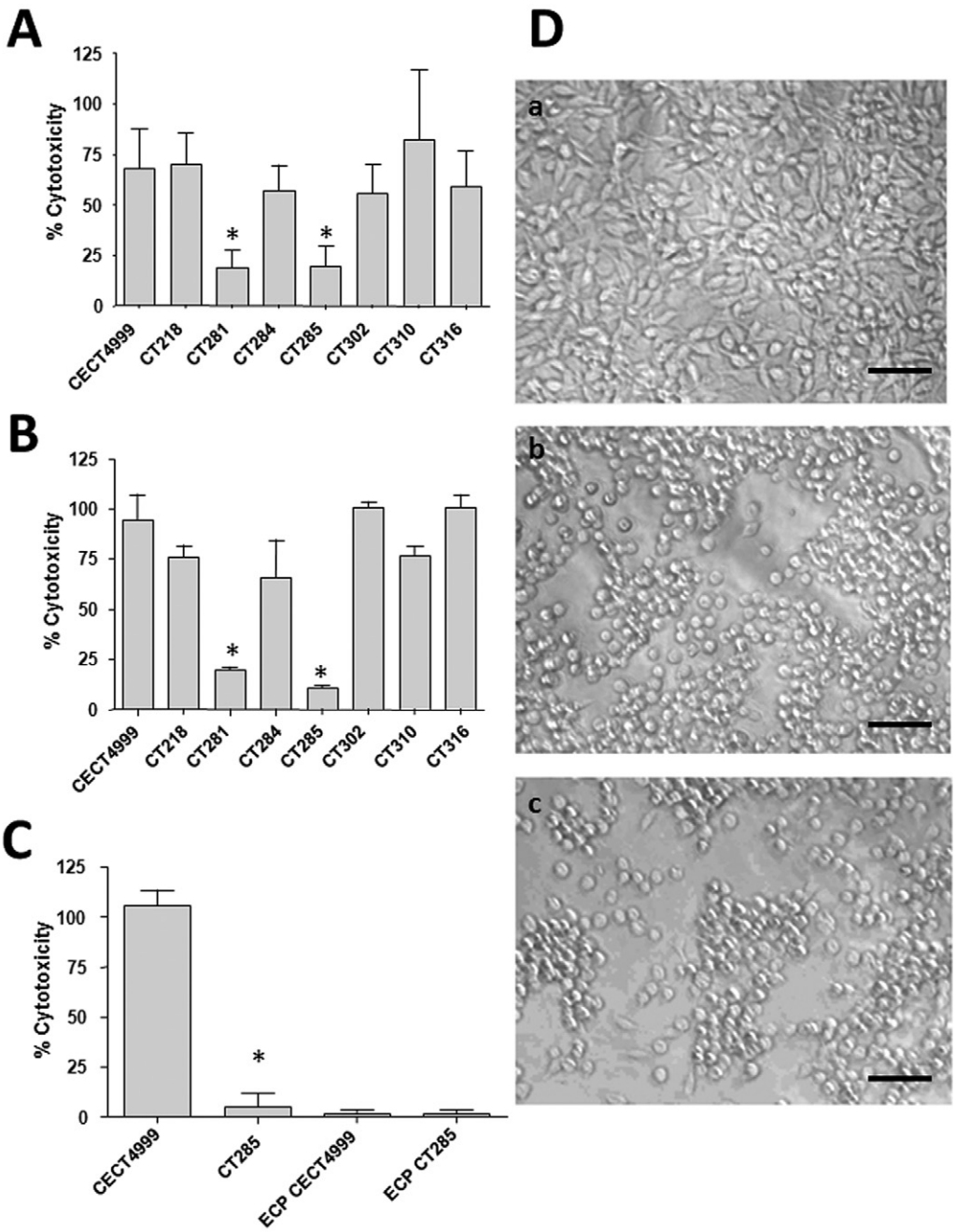

Fig. 3. Cytotoxicity of different *V. vulnificus* biotype 2 strains to EP-1, HEp-2 and EPC cells.
A–C. Cytotoxicity of bacteria or ECPs was determined by the released LDH through measuring the absorbance of the reaction mixture at 490 nm for EP-1 (A), HEp-2 (B) and EPC (C) cell lines. Asterisks indicate the significant differences (Student's *t*-test, $P < 0.05$) when compared with the wild-type strain. The data were from an average of three independent experiments and were taken at time 90 min.
D. Microscopic observation of EPC cells inoculated with *V. vulnificus* ECPs at the minimal protein dose; a, control; b, ECP from CECT 4999; c, ECP from CT285. Bar, 50 μm.

comparing the results obtained in the eel with those obtained in the mouse (the animal model used to predict virulence for humans). The results of virulence and *in vivo* expression assays clearly demonstrate that *rtxA1*$_3$ is a virulence gene, expressed in the internal tissues of eels during the infection process. In addition, *rtxA1*$_3$ also seems to be a virulence determinant for mice. However, the importance of *rtxA1*$_3$ in virulence is not the same in both animal models because inactivation of the two copies implies a complete loss of virulence for eels (increase in LD_{50} of more than 5 log. units), but only attenuated virulence for mice (increase in LD_{50} of two log.

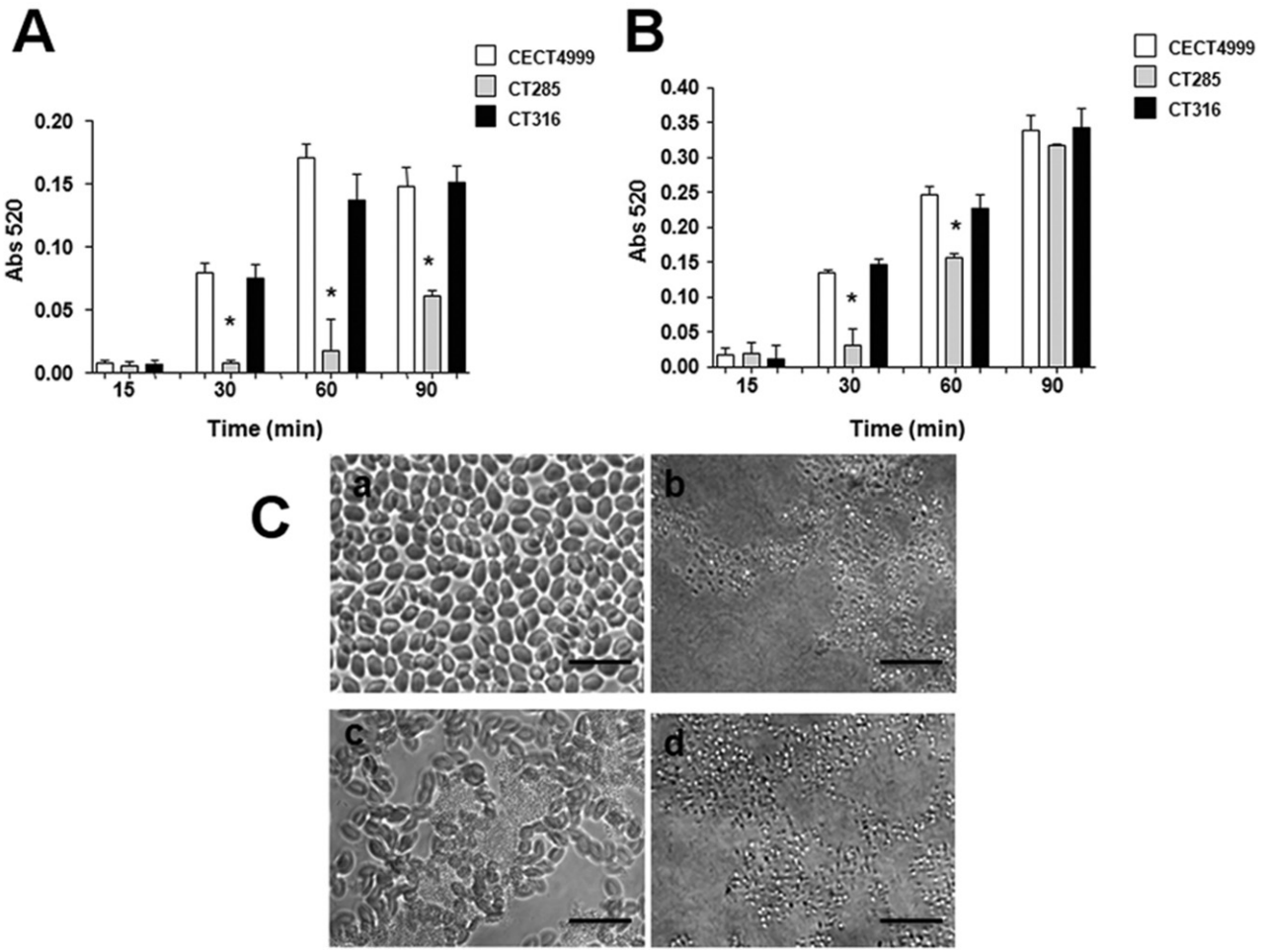

Fig. 4. Cytotoxicity of different *V. vulnificus* biotype 2 to erythrocytes from eels and humans. The wild-type strain and its derivatives were incubated with eel (A) or human (B) erythrocytes in a 96-well plate and haemolysis was determined by measuring the OD_{520} of the supernatant at different time intervals. Asterisks indicate the significant differences (Student's *t*-test, $P < 0.05$) when compared with the wild-type strain. The data were from an average of three independent experiments.
C. Microscopic observation of wild-eel erythrocytes infected with CECT4999 (b), CT285 (c) and CT316 (d) at 60 min post infection. Control (a), non-infected wild-eel erythrocytes. Bar, 100 μm.

units). Another important difference is that only one copy of *rtxA1*$_3$ seems necessary for full virulence in eels while two copies are required for mice. Previous studies have also reported a two-log-unit attenuation in virulence for *rtxA1*$_1$ defective mutants in mice (Lee *et al*., 2007; Liu *et al*., 2007; Kim *et al*., 2008; Lo *et al*., 2011), suggesting that MARTX types I and III, although structurally different, could act similarly in mice.

MARTX type I is recognized as a colonization and invasion factor for mice (Lo *et al*., 2011). To ascertain whether *rtxA1*$_3$ mutants are avirulent because they are defective in eel colonization and invasion, *in vivo* colonization assays were performed by immersion. Contrary to that reported for Δ*rtxA1* mutants in mice, the double mutant in *rtxA1*$_3$ was not apparently deficient in either colonization or invasion in the eel. This mutant was able to attach to the gills and spread to the blood and to the internal organs, where it survived for at least 72 h in numbers that did not differ significantly from those reached by the wild-type strain. Nevertheless, we cannot discount the possibility that the toxin increases the survival rate in blood and head kidney because the double mutant was recovered in a lower proportion than the wild-type strain in the co-infection experiments. Regarding the clinical signs, the double-mutant infected animals did not show any apparent external or internal sign and survived throughout the experimental period. In contrast, the eels infected with the wild-type strain died in the expected proportion (50%) within 72 h showing external and internal haemorrhaging, which would suggest that MARTX acts as a lethal factor for fish.

To ascertain what underlay the toxic effect caused by MARTX type III, tissues taken from wild-type and double mutant infected animals were microscopically analysed

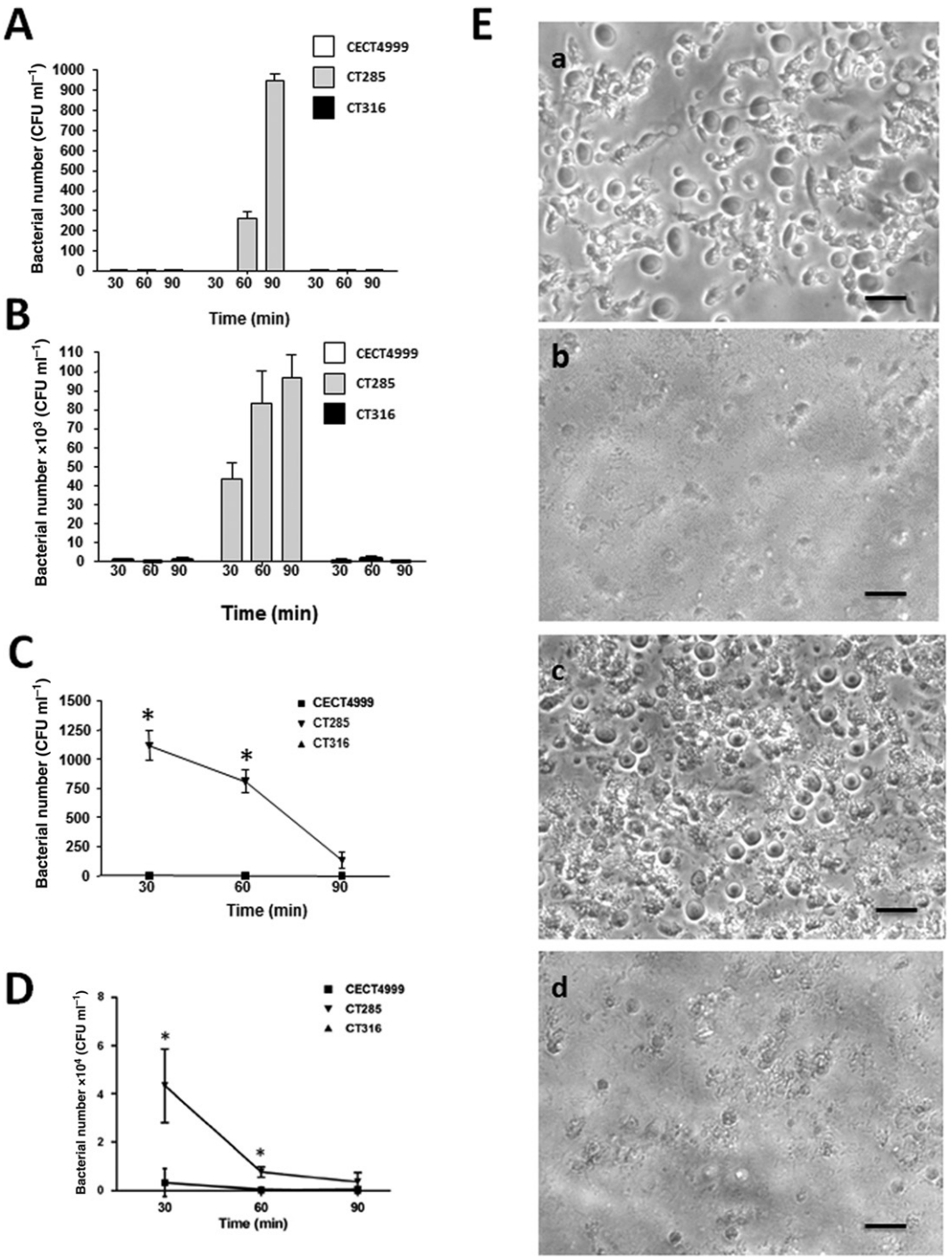

Fig. 5. Interaction of different strains of *V. vulnificus* biotype 2 with phagocytes. Number of intracellular bacteria after 30, 60 and 90 min of incubation of CECT4999, CT285 and CT316 in monolayers of fresh eel PEC (A) or murine macrophages (RAW264.7) at a moi of 10 (B). The data were from an average of three independent experiments. Survival inside eel PEC (C) or murine macrophages (D) after 30, 60 and 90 min was determined as bacterial counts as described in *Experimental procedures*. The data were from an average of three independent experiments. Asterisks indicate the significant differences (Student's *t*-test, $P < 0.05$) when compared with the wild-type strain.
E. Lysis of eel PEC produced by CECT4999 (b) and CT316 (d) but not by CT285 (c) at 60 min. post infection. Control (a), non-inoculated eel PEC.

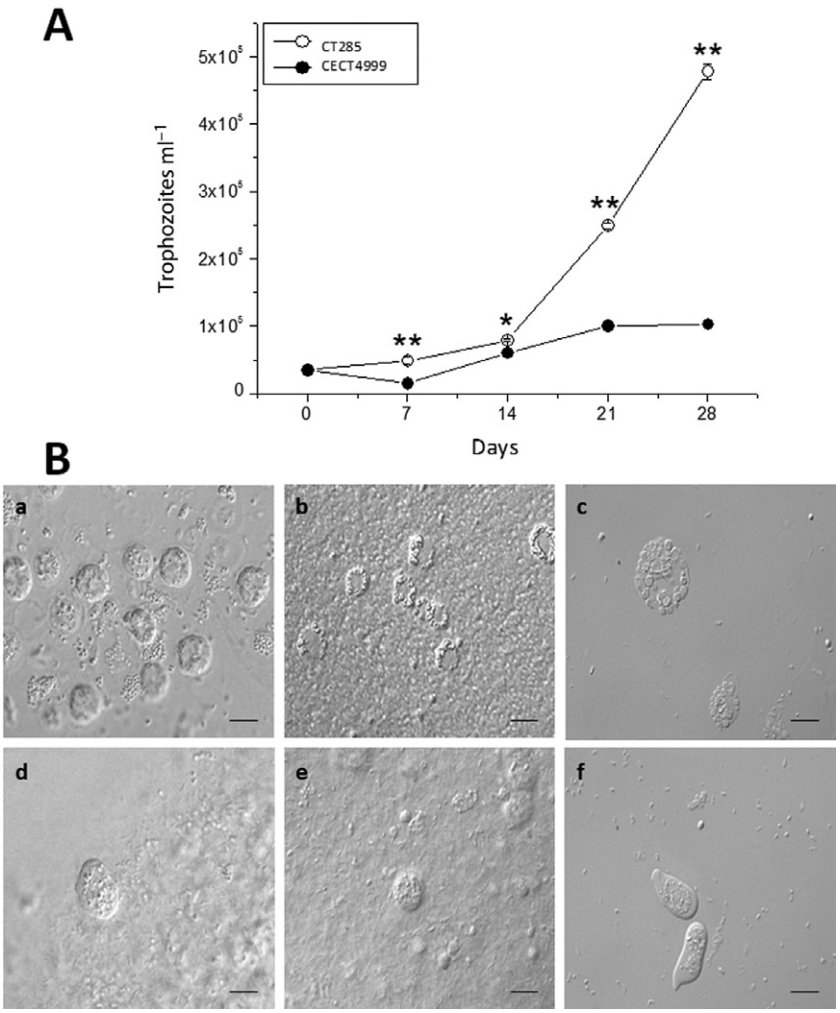

Fig. 6. Interaction of different strains of *V. vulnificus* biotype 2 with amoeba.
A. Growth curve of amoeba (*Neoparamoeba pemaquidensis*) incubated with CECT4999 or CT285. The data were from an average of three independent experiments. Asterisks indicate the significant differences (Student's *t*-test, $*P < 0.05$: $**P < 0.01$).
B. Differential interference contrast of amoeba grown with CECT4999 at time 0 (a) and 3 days (b and c) post incubation (notice that all amoeba are plasmolysed) or with CT285 at time 0 (d) and 3 days (e and f) post incubation. Bar, 5 μm.

and compared. The only cell alteration that could be clearly linked with MARTX type III was cell damage and release of cytoplasmic content, including granules, of granulocytes (a class of cells that includes neutrophils), mainly from the haematopoietic tissues. Indirect evidence of alterations affecting erythrocytes was also observed.

To test the hypothesis that the target for MARTX type III *in vivo* might be the granulocytes and, secondarily, the erythrocytes, cytotoxicity experiments were performed with freshly isolated eel erythrocytes and PEC. In contrast to that measured in the bacteria grown in different iron-depleted culture media and plasma, transcription of *rtxA1$_3$* was upregulated when the bacteria were co-cultured with both cell types. This result suggests that *V. vulnificus* may need this toxin to survive and/or multiply in the presence of both cell types. In both cases, the wild-type and the double revertant strains lysed a significant proportion of eel PEC and erythrocytes within 90 min while the double mutant was unable to do so. Consistent with this result, none of the wild-type bacteria was phagocytosed while the double mutant was phagocytosed, albeit poorly, by eel PEC, a finding that is compatible with this strain's ability to colonize and invade the eel. Our results also suggest that MARTX type III could lyse the epithelial cells from fish and mammals, as observed with MARTX type I (Liu *et al.*, 2007; Kim *et al.*, 2008; Lo *et al.*, 2011).

It has been reported that MARTX type I exerts its activity only upon bacteria-eukaryotic cell contact (Kim *et al.*, 2008). To test whether cell contact is also required for MARTX type III cytotoxicity, we evaluated *rtxA1$_3$* expression in presence of eel erythrocytes by separating them, or not, with a 0.22-μm-pore filter. The results indicate that expression of MARTX type III, like MARTX type I, requires bacterium-eukaryotic cell contact.

Interestingly, the double mutant agglutinated in the presence of eel erythrocytes from wild eels. This result suggests that eel erythrocytes secrete some anti-bacterial component (possibly an agglutinin) that may be involved in the defence against vibriosis. Recently, Morera and colleagues (2011) described an active role of salmonid erythrocytes against pathogens. According to the results of this work, an active role of erythrocytes against patho-

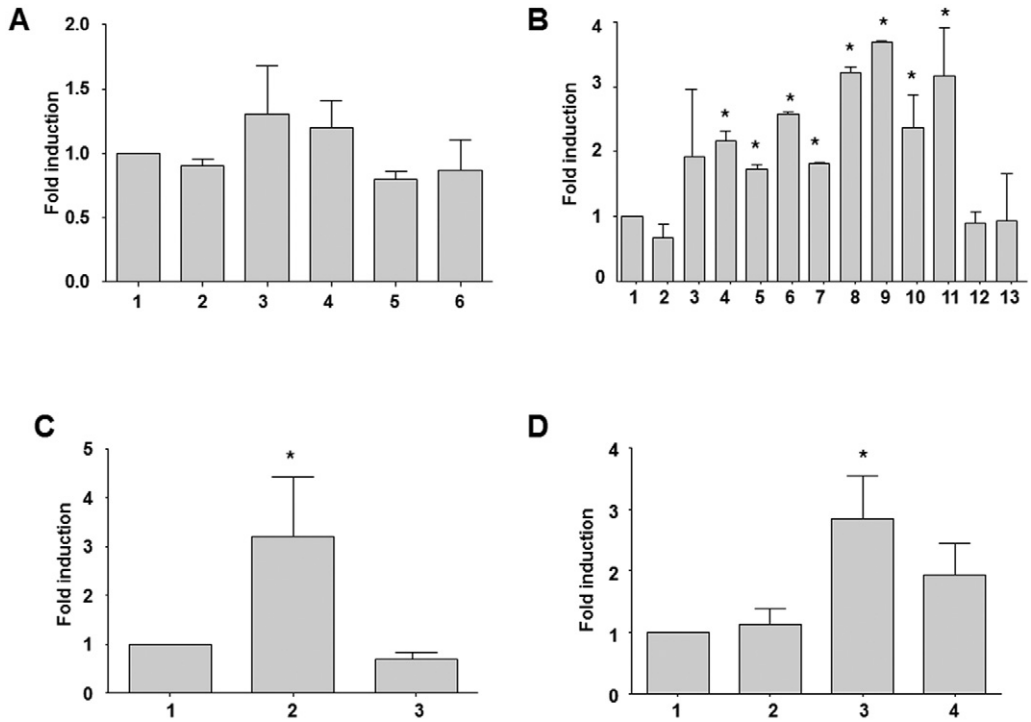

Fig. 7. *rtxA1₃* expression *in vitro* and *in vivo* and contact experiments. Fold induction of *rtxA1₃* in strain CECT4999 growing *in vitro* and *in vivo*.
A. *In culture media*: bacteria were grown in CM9 under a variety of culture conditions mimicking the *in vivo* growth. RNA was extracted from 1 ml of culture at mid-log phase point, which is indicated for each condition in parenthesis. 1, CM9 (6 h); 2, CM9 plus 100 μM $FeCl_3$ (5 h); 3, CM9 plus 40 μM iron-free human apotransferrin (Sigma) (9 h); 4, CM9 plus fresh eel plasma (v/v) (8 h); 5, CM9 plus fresh eel plasma (v/v) and 200 μM $FeCl_3$ (7 h); 6, CM9 plus 10 μM bovine haemoglobin (Sigma) (6 h).
B. *In presence of eukaryotic cells*: Bacteria were cultured in presence of eukaryotic cells maintained in L-15 (fish cells) or PBS (amoeba) at a moi of 10 and RNA was extracted from 1 ml of culture at different times intervals. Control L15 or PBS (1); L15 with eel phagocytes (EP) at 30 min (2), 60 min (3), 90 min (4) and 120 min (5) post incubation; L15 with eel erythrocytes (EE) at 30 min (6), 60 min (7), 90 min (8) and 120 min (9); PBS with amoebae at 2 h (10), 6 h (11), 9 h (12) and 24 h post incubation (13).
C. *Contact experiments*. Bacteria were incubated with or without contact with EE at a moi of 100 and samples were taken after 3 h of incubation. 1, L-15; 2, with contact; 3, without contact.
D. *In vivo experiments*. RNA samples were extracted from blood of immersion-infected eels after 0 h (2), 9 h (3) and 24 h (4) post challenge. Control (1): RNA from 1 ml of culture in CM9 at mid-log phase point. Asterisks indicate the significant differences (Student's *t*-test, $P < 0.05$) when compared with bacteria grown in the respective control culture medium.

gens could be extended to eel erythrocytes. In contrast, no bacterial aggregation was observed with erythrocytes from cultured eels, which correlates with the general immunodepressed state that eels manifest under captivity (R. Barrera, pers. comm.). As expected, no bacterial aggregation was visualized in the presence of human erythrocytes, which are non-nucleated cells.

Contrarily to that observed in the eel, murine macrophages were able to phagocytose the wild-type bacteria, although less efficiently than the double mutant, and all internalized bacteria had been killed by 90 min. Similar results were obtained by Lo and colleagues (2011) and suggest that MARTX types I and III, although structurally different, could act in the same way in mice by protecting the bacteria from phagocytosis.

Rapid eel death without gross clinical signs after being infected with the wild-type strain is congruent with previous studies, suggesting that the eels died from peracute septic shock. Biosca and Amaro (1996) clearly demonstrated that LPS of *V. vulnificus* is not one of the toxic factors involved in septic shock in eels. In fact, most fish species lack orthologues for Toll-like receptor 4, the specific receptor for LPS in mammals (Iliev *et al*., 2005). The results obtained in this work suggest that MARTX type III could be the main toxic factor triggering this septic shock in fish infected with biotype 2. The transcriptome of immunostimulated eels has recently been sequenced (Callol *et al*., 2011) and the genome of CECT4999 is being annotated (T. Prakash, K. M. Wu, T. L. Liao, V. K. Sharma, C. Amaro, L. I. Hor, T. D. Taylor and S. F. Tsai, unpubl. results). Further studies into the host–pathogen interactions at the transcriptomic level are underway to validate this hypothesis.

The presence of *rtxA1₃* gene in duplicate was confirmed in all the analysed strains of our *V. vulnificus* biotype 2 collection, regardless of clonal origin, serology or virulence degree for eels (F. Roig, F. González-Candelas and C. Amaro, in preparation). It is not clear why this gene

varies in structure and is duplicated in *V. vulnificus* biotype 2 strains. In fact, possession of this gene does not provide a clear evolutionary advantage to the bacterium since this work shows MARTX type III triggers overly rapid animal death, without giving the bacterium time to multiply or reach similar population sizes to other fish pathogenic vibrios (Lamas *et al*., 1994). To test whether MARTX type III could confer survival advantages to the bacterium outside the host, we isolated a fish amoeba from turbot gills and cultured it in the presence of the wild-type strain or the double mutant. We observed destruction of amoebae by the wild-type strain, but not by the double mutant, and detected upregulation of the *rtxA1$_3$* gene in presence of fish amoeba. This indicates that MARTX type III could be involved in bacterial resistance to amoebal predation. Interestingly, the amoebal destruction microscopically resembled to that derived from cellular apoptosis, finding that has to be confirmed in further studies. *Vibrio vulnificus* biotype 2 survives after antibiotic treatment in farms by forming biofilms on the fish surface, mainly on the gills (Marco-Noales *et al*., 2001). The results of the present study suggest that MARTX type III could be used by the bacterium in the biofilms to increase its survival rate in the fish farming environment.

In conclusion, MARTX type III of *V. vulnificus* biotype 2 seems to be involved in the interaction of this organism with a wide range of eukaryotic cells, ranging from amoebae to professional phagocytes. In any event, after bacterium–cell contact this toxin seems to cause cell lysis by an unknown mechanism. While in the mouse MARTX type III seems to act as a colonization factor preventing the bacterial cells from phagocytosis, it may function as a toxin involved in the onset of septic shock in the eel. Furthermore, this toxin may promote *V. vulnificus* biotype 2 survival in the environment by killing the amoeba, putative predator of this organism, which is a plausible explanation for the wide distribution of the *rtx* gene cluster among different clones of this polyphyletic group.

Experimental procedures

Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 1. The bacteria were routinely grown in LB-1/LBA-1 (Luria–Bertani broth/agar, 1% NaCl) or TSB-1/TSA-1 (tryptic soy broth/agar, 1% NaCl). Culture purity and the homogeneity of colony morphology were routinely tested on TSA-1 plates (Biosca *et al*., 1993). In some experiments the bacteria were grown in CM9/CM9A [M9 minimal medium broth/agar supplemented with 0.2% casamino acids (Difco) and 0.3% yeast extract] (Sambrook and Russell, 2001), CM9-Fe (CM9 plus 100 μM $FeCl_3$), CM9-Hb [CM9 plus 10 μM bovine haemoglobin (Sigma)], CM9-Tf [CM9 plus 40 μM iron-free human apotransferrin (Sigma)] (Biosca *et al*., 1996), CM9-HP [CM9 supplemented with human plasma (v/v), see below], CM9-EP [CM9 supplemented with eel plasma (v/v), see below] or CM9-EP-Fe (CM9-EP plus 200 μM $FeCl_3$). In all cases, cultures were inoculated with an overnight starter culture in CM9 at a ratio of 1:100 (v/v) in a final volume of 5 ml and the growth curves were constructed from 0 to 24 h post inoculation. *V. vulnificus* strains were incubated at 28°C and *Escherichia coli* strains at 37°C for 18–24 h. The strains were stored in TSB-1 or LB-1 plus glycerol (17–20%) at −80°C.

PCR, quantitative RT-PCR (qRT-PCR) and Southern hybridization

PCR was performed as described previously (Shao and Hor, 2000). Total RNA was extracted from 1 ml of a mid-log phase culture with TRI reagent (Sigma) and subjected to a DNase treatment with the TURBO™ DNase (Ambion). RNA was cleaned with the RNeasy® MinEute® Cleanup Kit (Qiagen). cDNA was obtained from total RNA (1 μg per reaction mixture) with the M-MLV Reverse Transcriptase (Invitrogen). qRT-PCR of was performed in Power SYBR® Green PCR Master Mix (Applied Biosystems) with StepOne Plus RT-PCR System (Applied Biosystems). The threshold cycle (C_T) values were determined with StepOne Software V2.0 (Applied Biosystems) to establish the relative RNA levels of the tested genes. Primers specific to *recA* (recA-F/recA-R: 5′-CGCCAAAGGCAGAAATCG-3′/5′-ACGAGCTTGAAGAC CCATGTG-3′) and *rtxA1$_3$* (ACD-F/ACD-R: 5′-GAGTGATG ATGGGCGCTTTAC-3′/5′-CAGCCGCGATGAGATG CT-3′) were used to amplify DNA fragments of about 60 bp. DNA polymerization was conducted from 60°C to 95°C to obtain the melting curve for determining the PCR amplification specificity. Southern hybridization was performed as described previously (Shao and Hor, 2000) except that chemiluminescence, instead of ^{32}P, was used for labelling the probe.

Isolation of rtxA1$_3$ *mutants and revertants*

The Δ*rtxA1$_3$* mutants were isolated by *in vivo* allelic exchange as described (Shao and Hor, 2000) (Fig. S2). Briefly, a DNA fragment amplified from CECT4999 with primers RTX7 (5′-CGGTAACGGCACAACCTTAG-3′) and RTX10 (5′-CGCT TTCGCATCCACCAC-3′) was cloned into pGEMT®-easy vector (Promega). The region between two HindIII sites in this amplified DNA fragment was then removed by enzyme digestion and ligation to achieve excision of part of the Actin Cross Linking domain (ACD) and introduction of an early stop codon (Figs S1 and S2). This recombinant DNA fragment was then cloned into pCVD442, a suicide vector, between the SphI and SacI sites. This recombinant suicide plasmid was used to isolate the Δ*rtxA1$_3$* mutants by allelic exchange (Donnenberg and Kaper, 1991). The isolated mutants were checked by southern hybridization for their *rtxA1$_3$* genotype, either wild-type or with the deletion, in the chromosome and plasmid (Fig. S2). The single mutants, Δp*rtxA1$_3$* (deletion in the plasmid) and Δc*rtxA1$_3$* (deletion in the chromosome), and the double mutant, Δp*rtxA1$_3$*Δc*rtxA1$_3$*, were thus identified. To restore the wild-type allele, an alternative strategy to complementation was used. This consisted of replacing the deleted allele in mutant CT285 with the wild-type allele through another allelic exchange to generate ‘the revertant’. In this case, a DNA fragment containing the sequence that

was deleted in the mutants and its flanking regions amplified from strain CECT4999 by PCR with primers RTX13 (5′-GCGAGCTCGGTAACGGCACAACCTTAG-3′) and RTX18 (5′-GCGAGCTCATCTCTGAGTGGAAG-3′) was used instead. The growth of all the mutants and revertants in LB-1 was comparable to that of the wild-type strain (data not shown).

Resistance to serum

Bacterial resistance to serum was assessed by mixing 100 μl of a bacterial suspension in PBS (phosphate buffered saline, pH 7.0) containing 10^3 cfu ml^{-1} with 100 μl of fresh plasma (obtained as described by Amaro *et al*., 1994 and Esteve-Gasent and Amaro, 2004 from humans and eels, respectively) and the mixtures were incubated at 28°C (for EP) or 37°C (for HP) for 4 h. Samples were taken at 0 and 4 h post incubation and the viable bacteria were enumerated by drop plate method.

Cytotoxicity assay

The cytotoxicity of bacteria or extracellular products (ECP) obtained from 24 h cultures on TSA-1 by the cellophane plate technique (Biosca and Amaro, 1996) was estimated by measuring the absorbance at 490 nm of released lactate dehydrogenase (LDH) from the dead cells. EP-1 (eel mucus-producing epithelial cells) (Kou *et al*., 1995) and EPC (Epithelioma papulosum cyprinid: this cell line was originally deposited as derived from carp, *Cyprinus carpio*, but finally identified by the ATCC (American Type Culture Collection) as derived from fathead minnow, *Pimephales promelas*) were cultured in L-15 (Sigma) without CO_2 at 28°C, and HEp-2 (human laryngeal carcinoma) cells in DMEM (Gibco) with 5% CO_2 at 37°C. The assays were performed in 96-well plates containing $1 \times 10^{4–5}$ cells per well. Fresh eel and human erythrocytes were collected from blood by centrifugation (3000 r.p.m., 15 min, 4°C), washed three times with PBS and resuspended in L-15 at a proportion of 1% (v/v). The monolayers and the suspension of erythrocytes were infected with L-15- or DMEM-washed bacteria (harvested from a 4 h culture in L-15 or DMEM) at a moi of 10. The supernatant was collected from each well at 0, 1, 1.5 and 4 h post infection and the LDH assay was performed with the CytoTox 96 Non-Radioactive Cytotoxicity assay kit (Promega). Haemolysis was estimated by measuring the absorbance at 540 nm as described (Shinoda *et al*., 1985).

Cytotoxicity to amoeba was assayed with the amoebae isolated from the gill homogenates of moribund farmed fish (*Psetta maxima*) and cultured as described (Paniagua *et al*., 2001). A total of 3×10^4 viable trophozoites in 1 ml of marine amoeba medium (0.01% malt and yeast extract, 1% Difco Bactoagar in sterile filtered sea water) in the well of a microplate were co-incubated with PBS-washed bacteria from an overnight culture in LB-1 at a moi of 1000. The viable amoebae were enumerated by haemocytometry.

Phagocytosis assays

Monolayers of RAW264.7 (murine macrophage-like cells), phagocytic cells from the peritoneal exudates (PEC) (Miyazaki and Kurata, 1987), were inoculated with PBS-washed bacteria from a 4 h culture in L-15 at a moi of 10. After 0, 30, 60 and 90 min of co-incubation, two types of bacterial counts were performed: (i) total bacteria that survived to the phagocytosis (externally and internally) and (ii) the bacteria that were phagocyted. In the first case, the phagocytes were lysed with 0.1% Triton X-100, and the bacterial number was determined by drop plate method. In the second case, the cells were treated with gentamicin (100 μg ml^{-1}, Invitrogen) for 30 min, washed with SS-1 and lysed, and the released intracellular bacteria were enumerated by drop plate method. Finally, the intracellular survival rate after 90 min of interaction bacteria/phagocytes was determined by incubating with gentamicin, lysing the phagocytes after 30, 60 and 90 min of additional incubation and performing the corresponding bacterial counts.

Contact of V. vulnificus *with eukaryotic cells*

To test whether contact with eukaryotic cells is essential for the expression of *rtxA1*$_3$, we used Transwell® culture plates with and without polycarbonate filters of 0.2 μm of pore diameter in the wells (Kim *et al*., 2008). The lower chambers of the wells with filter were filled with 0.1 ml of a suspension of 10^6 eel erythrocytes in L-15 and the upper chambers with a suspension of 10^8 log phase cells from CECT 4999 from a 4 h culture in L-15 (moi = 100). In parallel, the wells without filter were filled with eel erythrocytes and bacterial suspensions in L-15 in the same proportions. The plates were incubated at 28°C for 3 h and samples were taken for quantification of *rtxA1*$_3$ expression at 3 h post infection.

Virulence and toxicity assay

The bacterial virulence and the toxicity of the ECPs for the eel, expressed as the LD_{50} (lethal dose to 50% of animal) or TD_{50} (toxic lethal dose to 50% of animal) value, was determined in European elvers of 8–10 g (Amaro *et al*., 1995; Amaro and Biosca, 1996). The bacterial virulence for the mouse was determined in 6- to 8-week-old C3H/HeN mice from the Laboratory Animal Center of National Cheng Kung University. The eels (by peritoneal injection or immersion) and the mice (by peritoneal injection) were infected with 10-fold serially diluted bacterial suspension, and the mortality of infected animal was recorded 72 h post infection. Mortalities were recorded only if the inoculated bacterium was recovered in pure culture from kidney or liver of moribund animals. The LD_{50} was calculated as described (Reed and Münch, 1938). All the protocols of animal experiments were reviewed and approved by the Animal Ethics Committee of NCKU and UV.

Colonization and co-infection assays in the eel

The eels were bath infected with the wild-type strain or with the double mutant strain at a dose equivalent to the LD_{50} of the wild-type strain. In the co-infection experiment, the eels were either injected with or immersed in a bacterial suspension containing equal numbers of the wild-type and the double mutant strains at a dose equivalent to the LD_{50} of the

wild-type strain in each infection model. Then, the gills, blood, head kidney, spleen and liver were taken from live or moribund animals at 0, 9, 24, 48 and 72 h post infection (Valiente and Amaro, 2006). The bacterial number per ml (blood) or g (gills, liver, kidney and spleen) of sample was estimated by the drop plate method. The bacteria recovered from the internal organs were checked by colony hybridization (Roig and Amaro, 2009) with two DNA probes, one for *vvhA* and the other for *rtxA1*$_3$, to determine their identity. The probe for *vvhA* was amplified with vvhA-F (5′-CGCCACCCACTTTCGGGCC-3′) and vvhA-R (5′-CC GCGGTACAG GTTGGCGC-3′); that for *rtxA1*$_3$ was amplified with rtxA1$_3$p-F (5′-GCTCGATGGCGTTCAACG-3′) and rtxA1$_3$p-R (5′-GCATCACGATCACCACGCGA-3′).

Eel histopathology

The eel tissues to be examined by transmission electron microscopy (TEM) were fixed in cold 1% formaldehyde plus 2% glutaraldehyde in phosphate buffer 0.1 M, pH 7.4, for at least 6 h, and postfixed in 2% OsO_4 in the same buffer. After dehydration through a series of alcohol solutions (50–100%), the tissues were embedded in araldite (Durcupan-Fluka). Semithin sections of 1 μm thick were stained with toloudine blue and observed under a light microscope to select the area of interest. Ultrathin sections of 0.120 μm thick obtained with an ultramicrotome (Leica) were stained with uranyl acetate and lead citrate, and examined by TEM (Jeol-1010).

Statistical analysis

All experiments were repeated at least three times and the means were compared by unpaired Student's *t*-tests. All tests were performed with SPSS Statistics 17.0, and statistical significance was defined as $P < 0.05$.

Acknowledgements

This work has been financed by grants AGL2008-03977/ACU, AGL2011-29639 (co-funded with FEDER funds) and Programa Consolider-Ingenio 2010 CSD2009-00006 from MICINN (Spain) and with grant NSC 97-2320-B-006-009-MY3 from National Science Council (Taiwan). The authors also thank Conselleria de Educación Formación y Ocupación de Valencia por la ayuda Gerónimo Forteza FPA/2011/023. The authors also thank the SCSIE of the University of Valencia for technical support in determining the sequences.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. The protein structure of the MARTX types I (approximately 5200 aa), II (approximately 4700 aa) and III (approximately 4600 aa). The external regions, the repeats (vertical lines) and the internal domains for each toxin are colour coded as indicated at the bottom. The putative domains are: RID, Rho-GTPase inactivation; HCR, highly conserved regions; CPD, autocatalytic cysteine protease; DUF, domain with an unknown function; ACD, actin cross-linking; *á*/*β*, *á*/*β* hydrolase; *rtx* PA, *rtxA* of *Photorhabdus asymbiotica*; Efa1/LifA, lymphostatin. Diagrams are drawn to scale. Figure adapted from Roig and colleagues (2011).
Fig. S2. Confirmation of various *rtxA1*$_3$ mutants.
A. The gene structure of *rtxA1*$_3$. The coding region is indicated by an arrow. A 1816 bp DNA fragment between the two HindIII sites that contains part of the putative ACD domain (5886–7269 bp) was deleted to generate the *rtxA1*$_3$ mutants. The probe used in southern hybridization is indicated below.
B. Southern hybridization analysis of the mutants. The plasmid DNA (P) or total DNA (G) was digested with BglII, separated in a 0.8% agarose gel, and probed with a DNA fragment amplified from *rtxA1*$_3$ with primers RTX5 (5′-GAAACACGCAAAGCCGATGC-3′) and RTX16 (5′-CTCAT CTCTGAGTGGAAGCC-3′). CECT4999: wild-type; CT302: Δc*rtxA1*$_3$; CT284: Δp*rtxA1*$_3$; CT285: Δc*rtxA1*$_3$Δp*rtxA1*$_3$. The bands derived from *rtxA1*$_3$ with and without deletions (2.6 and 4.4 kb, respectively) are indicated. M: 1 kb plus DNA markers.