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Identification and Characterization of the *Vibrio vulnificus rtxA* Essential for Cytotoxicity *in vitro* and Virulence in Mice

Jeong Hyun Lee[1], Myung Won Kim[1], Byoung Sik Kim[1], Seung Min Kim[1], Byung Cheol Lee[2], Tae Sung Kim[2], and Sang Ho Choi[1]*

[1]*Department of Agricultural Biotechnology and Center for Agricultural Biomaterials, Seoul National University, Seoul 151-742, Republic of Korea*
[2] *School of Life Sciences and Biotechnology, Korea University, Seoul 136-701, Republic of Korea*

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A mutant exhibiting decreased cytotoxic activity toward INT-407 intestinal epithelial cells and carrying a mutation in the *rtx* gene cluster that consists of *rtxCA* and *rtxBDE* operons was screened from a library of *V. vulnificus* mutants. The functions of the *rtxA* gene, assessed by constructing an isogenic mutant and evaluating its phenotypic changes, demonstrated that RtxA is essential for the virulence of *V. vulnificus* in mice as well as in tissue cultures.

Keywords: *Vibrio vulnificus*, RtxA, cytotoxicity, virulence

The pathogenic marine bacterium *Vibrio vulnificus* is an agent of food-borne diseases such as life-threatening septicemia and possibly gastroenteritis in individuals with predisposed conditions, including liver damage, excess iron levels, and immunocompromised conditions (Linkous and Oliver, 1999; Strom and Paranjpye, 2000). Wound infections can also result from exposure to seawater or the handling of shellfish contaminated with *V. vulnificus*. Mortality from septicemia is very high (>50%), and death can occur within one to two days after the first signs of illness (Linkous and Oliver, 1999; Strom and Paranjpye, 2000).

Understanding the molecular pathogenesis of host-pathogen interaction is critical in elucidating how *V. vulnificus* can circumvent host defenses, multiply in the host, and cause disease. Disease caused by infection with *V. vulnificus* is remarkable for the invasive nature of the infection, ensuing severe tissue damage, and a fulminating progression, indicating that pathogenicity of the bacteria is multifactorial and a complex process that involves the products of many genes. Although several potential virulence factors have been identified in *V. vulnificus*, such as an endotoxin, polysaccharide capsule, iron sequestering systems, cytolytic hemolysin, elastase, phospholipase A2, and other exotoxins, only few including the capsule and iron acquisition systems have been confirmed to be essential for virulence by the use of molecular Koch's postulates (Linkous and Oliver, 1999; Strom and Paranjpye, 2000; Kim *et al*., 2003; Gulig *et al*., 2005; Park *et al*., 2006b). Therefore, the identification and characterization of more virulence factors of *V. vulnificus* are necessary in the development of improved treatment and prevention as well as for discovering novel approaches in the control this pathogen.

The successful transmission of a disease depends primarily on the survival and multiplication of the infecting microorganisms. Hence, it is generally accepted that virulence factors include all those factors contributing to survival and multiplication on or within host as well as to disease (Mekalanos, 1992). Thus far, several experimental approaches have been used to exploit transposon mutagenesis for the extensive screening of bacterial genes required for virulence of bacteria. The technique of signature-tagged transposon mutagenesis (STM) allows for the isolation of genes which are essential for survival within the host (Hensel *et al*., 1995) and in vivo expression technology (IVET) has been successfully used in identifying bacterial genes that are highly expressed in host tissue (Mahan *et al*., 1993). These screens and subsequent characterizations have allowed identification of many bacterial genes encoding potential virulence factors (Lee and Camilli, 2000), yet when compared with the substantial body of literature concerned with the identification of virulence factors in other bacterial pathogens, only a few studies have reported on the extensive screening and identification of the genes involved in the virulence of *V. vulnificus* (Kim *et al*., 2003). Accordingly, the present study screened a mutant exhibiting a decreased cytotoxicity from a library of *V. vulnificus* mutants constructed by random transposon mutagenesis, and a homologue of *V. cholerae rtx* gene cluster was identified by a transposon-tagging method. The functions of the *rtxA* among the genes of *rtx* gene cluster as regards virulence were assessed by the construction of an isogenic mutant of *V. vulnificus*, in which the *rtxA* gene had been inactivated by allelic exchanges, and by evaluating its phenotypic changes *in vitro* and in mice.

Materials and Methods

Strains, plasmids, and culture media

The bacterial strains and plasmids used in this study are listed in Table 1. The primary DNA cloning and manipulation was conducted on *E. coli* DH5α, plus restriction

* To whom correspondence should be addressed.
(Tel) 82-2-880-4857; (Fax) 82-2-873-5095
(E-mail) choish@snu.ac.kr

mapping was used to confirm that the transformants contained the appropriate plasmids. Unless noted otherwise, the *V. vulnificus* strains were grown in a Luria-Bertani (LB) medium supplemented with 2.0% (w/v) NaCl (LBS).

Cytotoxicity assay

Two different assays were performed for cytotoxicity assays using INT-407 (ATCC CCL-6) human intestinal epithelial cells. The preparation of the INT-407 cells and infection with the bacterial cultures were performed in a 96-well tissue culture plates (Nunc, Denmark) as described previously (Lee *et al*., 2003a; Park *et al*., 2006a, 2006b). The cytotoxicity was then determined by measuring the activity of lactate dehydrogenase (LDH) in the supernatant using a Cytotoxicity Detection Kit (Roche, Germany), and expressed using the total LDH activity of the cells completely lysed by 1% Triton-X 100 as 100%. Morphologic studies were also carried out using INT-407 cells, which were seeded onto glass cover slips placed at the bottom of the tissue culture plate. The INT-407 cells were then infected with the *V. vulnificus* strains as described previously (Ju *et al*., 2005; Park *et al*., 2006b). For data analysis, the averages and standard errors of the mean (SEM) were calculated from at least three independent determinations.

Mouse model of infection

The role of the *V. vulnificus rtxA* gene in virulence was further examined using a mouse model. The 50% lethal doses (LD_{50}s) of the wild type and the *rtxA* mutant were compared using ICR mice (Specific Pathogen-Free; Seoul National University), as described previously (Jeong *et al*., 2000; Lee *et al*., 2003b; Park *et al*., 2006b). To determine the LD_{50}, the bacteria grown overnight in LBS broth at 30°C were harvested and suspended in PBS to appropriate concentrations, ranging from 10^0 to 10^7 CFU in 10 fold increments. Groups of (n=6) 7 week old normal female mice were injected intraperitoneally with 0.1 ml serial dilutions of the bacterial suspensions. The infected mice were then observed for 24 h, and the LD_{50}s were calculated using the method of Reed and Muench (Reed and Muench, 1938). The mice were also injected intraperitoneally with 250 μg of iron dextran for each gram of body weight immediately before being injected with the bacterial cells.

Results and Discussion

Identification of rtx gene cluster

Past studies have provided a library of *V. vulnificus* mutants by random transposon mutagenesis using a mini-Tn5 *lacZ*1 (de Lorenzo *et al*., 1990; Rhee *et al*., 2002). As such, mutants exhibiting decreased cytotoxic activity toward INT-407 intestinal epithelial cells were screened from this mutant library. For primary screening, the neutral red staining assay measures the amount of cell mass remaining attached to 96 wells of culture dishes after infection, as previously described (Valdivieso-Garcia *et al*., 1993). Two hundred six mutants identified as being less cytotoxic, were isolated after measuring the relative concentration of neutral red retained in the wells. These attenuated cytotoxic mutants represented less than 1% of the total mutants screened. Of the 206 mutants, 12 showing reproducibly lower cytotoxic activity, determined by measuring lactate dehydrogenase (LDH) activity released into the culture supernatant by lysed cells as described above, were selected. The mutations in each of the 12 mutants with attenuated cytotoxic activity were identified by a transposon-tagging method as described previously (Kim *et al*., 2002; Rhee *et al*., 2002) (data not shown).

Among the mutants, MW23-7A (Table 1) exhibited the least cytotoxicity and appeared to have a transposon insertion in the middle of coding region of *rtxE*, a member of *rtx* gene cluster (data not shown). Reports have indicated that the *rtx* gene cluster consists of *rtxCA* and *rtxBDE* operons in *V. cholerae*, a species closely related to *V. vulnificus*, and is responsible for activation and secretion of RtxA, a member of RTX (repeats in toxin) toxin family (Fig. 1) (Lin *et al*., 1999; Fullner and Mekalanos, 2000). The *V. cholerae* RtxA (Vc-RtxA) is the most potent cytotoxin with actin cross-linking activities and secreted to the cell exterior by the cell by type I secretion systems (TISS) consisting of RtxB, RtxD, and RtxE (Fullner and Mekalanos, 2000; Boardman and Satchell, 2004; Sheahan *et al*., 2004). A database search using the genomic sequence of *V. vulnificus* YJ016 (GenBank accession number NC_005140, www.ncbi. nlm.nih.gov) revealed that the *V. vulnificus rtx* gene cluster (*Vv-rtx*) is organized in the same orientation as in *V. cholerae rtxCA* and *rtxBDE*. As such, a homologue of the *V. cholerae rtxA* which is 15,621 bp in length and physically linked to the *rtx* gene cluster is named the *V. vulnificus rtxA* (Fig. 1). The amino acid sequence deduced from the *V. vulnificus rtxA* coding sequence revealed

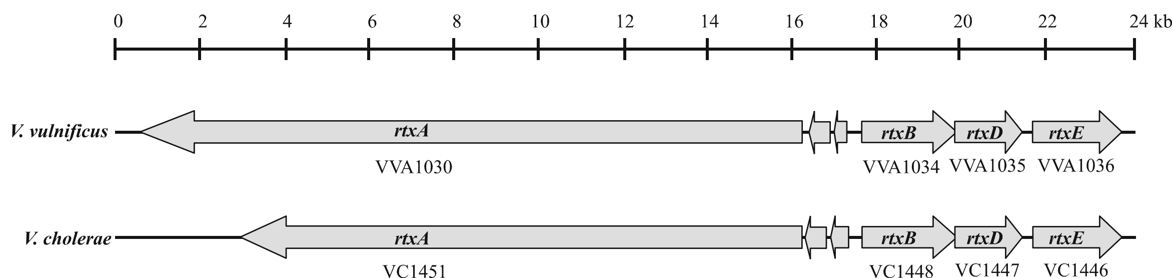

Fig. 1. Schematic representation of the *Vibrio rtx* operons. The arrows represent the transcriptional directions and coding regions of the genes. The figure was derived using the nucleotide sequences of *V. vulnificus* YJ016 (accession no. NC_005140) and *V. cholerae* (accession no. NC_002505) genomes in the GenBank databases (NCBI). The gene ids are shown below each coding region.

Table 1. Plasmids and bacterial strains used in this study

Strain or plasmid	Relevant characteristics[a]	Reference or source
Bacterial strain		
V. vulnificus		
MO6-24/O	Clinical isolate; virulent	Laboratory collection
MW23-7A	MO6-24/O with transposon insertion in *rtxE*	Present study
MW064	MO6-24/O with Δ*rtxA:: nptI*; Km^r	Present study
E. coli		
DH5α	*supE44 D lacU169 (f80 lacz* DM15*) hsdR17 recA1 endA1 gyrA96 thi-1 relAI relA1 relA1*	Laboratory collection
SM10λpir	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu l *pir*; Km^r; host for p-requiring plasmids; conjugal donor	(Miller and Mekalanos, 1988)
Plasmids		
Mini-Tn5 *lacZ*1	R6K*γori*; suicide vector; *oriT* of RP4; Ap^r	(de Lorenzo *et al*., 1990)
pDM4	Suicide vector; *ori*R6K; Cm^r	(Milton *et al*., 1996)
pGEM-T easy	PCR product cloning vector; Ap^r	Promega
pMW0613	pDM4 with *ΔrtxA::nptI*; Cm^r Km^r	Present study

[a] Ap^r, ampicillin resistant; Km^r, kanamycin resistant; Cm^r, chloramphenicol resistant

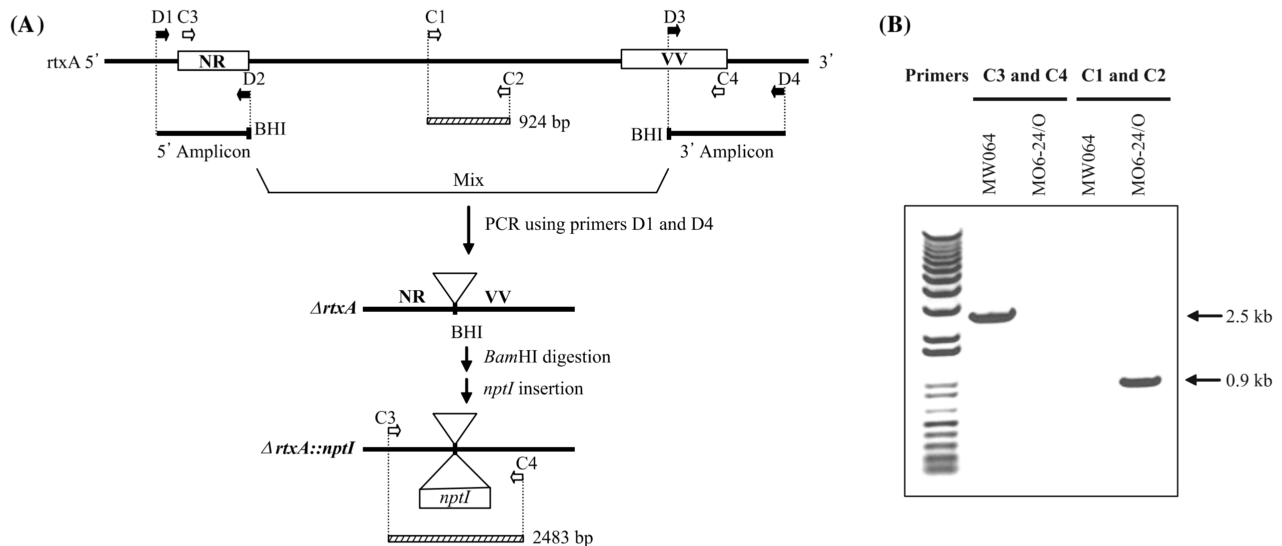

Fig. 2. Generation of the Δ*rtxA*::*nptI* mutant MW064. (A) Diagram for deletion of 10,697 bp of the coding region of *rtxA* by PCR-mediated Linker-Scanning (Murphy *et al*., 2000), and construction of Δ*rtxA*::*nptI* by insertion of the *npt*I cassette conferring kanamycin resistance (Oka *et al*., 1981). Solid lines, chromosomal DNA; shaded lines, PCR products for the deletion of *rtxA* coding region; dashed lines, PCR products for confirmation of the Δ*rtxA*::*nptI* mutant; large triangles, deletion of the coding region of *rtxA*; open boxes, the *nptI* gene; filled arrows (D1, D2, D3, and D4), locations of the oligonucleotide primers used for construction of the Δ*rtxA*::*nptI* mutant; open arrows (C1, C2, C3, and C4), locations of the oligonucleotide primers used for confirmation of the Δ*rtxA*::*nptI* mutant. (B) PCR analysis of M06-24/O and the isogenic mutant MW064 generated by allelic exchanges. Details are in the text. Molecular size markers (1 kb DNA ladder, Invitrogen) and PCR products (in kb) are indicated in figure. Abbreviations for primers listed in Table 2. D1, RTXAN5; D2, RTXAN3; D3, RTXAC5; D4, RTXAC3; C1, RTXA-CF1; C2, RTXA-CF2; C3, RTXA-CF3; C4, RTXA-CF4.

a protein composed of 5,206 amino acids with a theoretical molecular mass of 556,208.62 Da and a PI of 4.98. The alignment of the translated *rtxA* sequences to those of Vc-RtxA revealed about 67% amino acid identity (data not shown, http//www.ebi.ac.uk/clustalw). The similarity in genetic organization and the high level of identity found in the amino acid sequences for RtxA proteins from *V. vulnificus* and *V. cholerae* are an indication that they might perform a similar function in their pathogenesis.

Generation of ΔrtxA::nptI mutant

The role of the gene product of *rtxA* in virulence was examined by constructing a *rtxA* mutant of *V. vulnificus*. PCR-mediated linker-scanning mutagenesis was applied for

Table 2. Oligonucleotides used in this study[a]

Oligonucleotide	Oligonucleotide sequence, 5'→3'[a]	Location[b]	Use
RTXAN5	TACGGCAACATTTCCGTGCACAAAG	Chromosomal DNA	Deletion of *rtxA*
RTXAN3	AACGGGATCCCGCGAATATTCCTAG	Chromosomal DNA	Deletion of *rtxA*
RTXAC5	CGGGATCCCGTTTAACTTTGCTGTG	Chromosomal DNA	Deletion of *rtxA*
RTXAC3	ACCGTTGAATTGACTGAACTTAGAG	Chromosomal DNA	Deletion of *rtxA*
RTXA-CF1	CTAACGGGGTATTACCATCA	*rtxA*	*rtxA* mutant confirmation
RTXA-CF2	ATCCACCAGTTGCTTGATAC	*rtxA*	*rtxA* mutant confirmation
RTXA-CF3	GTGGTTACAACACCCATACC	*rtxA*	*rtxA* mutant confirmation
RTXA-CF4	CAATGACCTCCAACATCTCT	*rtxA*	*rtxA* mutant confirmation

[a] Regions of oligonucleotides not complementary to corresponding gene; *Bam*HI sites are underlined.
[b] Site of nucleotides hybridization.

the construction of an *rtxA* deletion mutant MW064 (Table 1) (Murphy *et al.*, 2000). The primers used to create the linker-scanning mutants are listed in Table 2. The 5' amplicon, approx. 1,377 bp DNA fragment corresponding to the NR (N-terminal novel repeats, Fig. 2A) (Sheahan *et al.*, 2004) of RtxA, was generated using the two primers designated as RTXAN5 and RTXAN3. RTXAN3 is a 25 base antisence primer, containing a *Bam*HI restriction site followed by bases corresponding to the *rtxA* coding region. Similarly, a 978 bp DNA fragment corresponding to *V. vulnificus* specific region (VV in Fig. 2A) (Sheahan *et al.*, 2004) of RtxA was amplified using primers RTXAC5 and RTXAC3 and generated the 3' amplicon. RTXAC5 contains a sequence identical to *rtxA* coding region and a 5'-end BamHI restriction site. The 10,697 bp deleted *rtxA* was amplified by PCR using the mixture of both amplicons as the template and RTXAN5 and RTXAC3 as primers. The 2.3 kb PCR product was cloned into pGEM-T eazy vector and 1.2 kb *nptI* DNA conferring resistance to kanamycin (Oka *et al.*, 1981) was inserted into a unique *Bam*HI site present within the Δ*rtxA* to result in Δ*rtxA*::*nptI* (Fig. 2A).

The 3.5 kb Δ*rtxA*::*nptI* cartridge was then liberated from the resulting construct and ligated with *Sph*I-*Spe*I digested pDM4 (Milton *et al.*, 1996) to form pMW0613 (Table 1). To generate the Δ*rtxA*::*nptI* mutant, MW064, by homologous recombination, *E. coli* SM10 λ*pir*, *tra* (containing pMW0613) (Miller and Mekalanos, 1988) was used as a conjugal donor to *V. vulnificus* M06-24/O. The conjugation and isolation of the transconjugants were achieved using the methods, in previous studies (Jeong *et al.*, 2000; Rhee *et al.*, 2004; Lee *et al.*, 2006; Lee and Choi, 2006) and a double crossover, in which wild-type *rtxA* gene being replaced with the Δ*rtxA*::*nptI* allele, was confirmed by PCR as shown in Fig. 2B. PCR analysis of genomic DNA from the wild type with primers RTXA-CF1 and RTXA-CF2 (Table 2) produced a 924 bp fragment (Fig. 2B). However, genomic DNA from the Δ*rtxA*::*nptI* mutant MW064 was not able to result in amplified DNA, supporting that the *rtxA* region, which are supposed to be hybridized with the primers, are deleted. Similarly, PCR analysis of genomic DNA from the Δ*rtxA*::*nptI* mutant with primers RTXA-CF3 and RTXA-CF4 (Table 2) resulted in an amplified DNA fragment approximately 2.5 kb in length (Fig. 2B). The 2.5 kb fragment is in agreement with the projected size of the DNA fragment containing Δ*rtxA* (1,333 bp) and the *nptI* gene (1.2 kb). PCR amplification of genomic DNA from parental strain MO6-24 with primers RTXA-CF3 and RTXA-CF4, supposed to produce a 12 kb DNA fragment, consisting of 1,333 bp and the deleted 10,697 bp, was not successful (Fig. 2B). These results confirmed the generation of the Δ*rtxA*::*nptI* mutant MW064, in which almost two-thirds of the *rtxA* coding region were deleted.

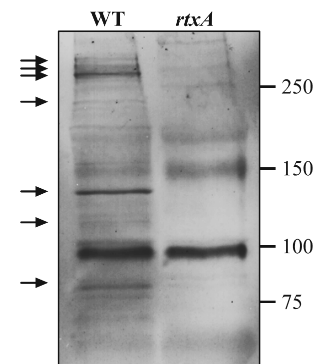

Fig. 3. Western blot analysis of RtxA in the culture supernatants. The culture supernatant of the wild-type and *rtxA* mutant of *V. vulnificus* strains incubated with INT-407 (MOI of 100) were harvested and concentrated as described in text. For each lane, 30 mg of the total protein was loaded, and separated on a SDS-PAGE. RtxA was detected by a Western blot analysis using rabbit anti-*V. vulnificus* RtxA antibody. The protein concentrations were determined by the method of Bradford (Bradford, 1976), using a bovine serum albumin standard. The fragmented RtxA are indicated by the arrows. WT, wild type; *rtxA*, *rtxA* mutant.

Western blot analysis of RtxA

Since the production of *V. vulnificus* RtxA is induced by prior exposure to host cells (SHC, unpublished observation), the INT-407 cells were infected by the wild-type and its isogenic mutant cultures at a multiplicity of infection (MOI) of 100 and incubated for 2 h. For the Western blot analyses, secreted proteins in the culture broth of *V. vulnificus* and INT-407 cells were precipitated by using ammonium sulfate by the methods of Park (Park *et al.*, 2002) with slight mod-

ifications (Kachlany *et al*., 2002). The proteins were spun down, resuspended in a dialysis buffer (40 mM Tris-HCl; pH 7.0, 1 mM EDTA, 1 mM PMSF), dialyzed (Slide-A-Lyzer Dialysis Cassette, Pierce, USA), and finally kept frozen at -80°C until use. The proteins of concentrated supernatants from the wild-type and its isogenic mutant cultures were resolved by SDS-PAGE (Laemmli, 1970). The truncated-RtxA protein which carries C-terminal 640 amino acids was purified as described elsewhere (Lee *et al*., submitted for publication), and used to raise primary antibodies to the RtxA of *V. vulnificus*. Western immunoblotting was performed according to the procedure previously described by Jeong *et al*. (2003). The predicted full length size of RtxA toxin, about 556 kDa, was not detected in supernatants of cultures in either the wild type or the *rtxA* mutant by Western blotting. However numerous bands were detected in concentrated supernatant prepared from the culture with the wild type by the anti-RtxA antiserum, and the size of the majority bands ranged from 125 kDa to 300 kDa (Fig. 3). This fragmentation of RtxA in supernatant is not limited to *V. vulnificus* RtxA, and the *V. cholerae* RtxA were also detected as breakdown products in supernatant fluids (Fullner and Mekalanos, 2000; Boardman and Satchell, 2004). The RtxA breakdown products in the supernatant of cultures with the *rtxA* mutant MW064 were however not detected by Western blot analysis, indicating that deletion of almost two-thirds of the *rtxA* coding region led to the loss of the RtxA in supernatant fluids of MW064 (Fig. 3).

RtxA is required for cytotoxicity to epithelial cells in vitro
LDH activities from monolayers of INT-407 cells which were infected with 20 μl of a suspension of M06-24/O and MW064 strains at different MOI and incubated for 3 h were determined (Fig. 4A). The *rtxA* mutant MW064 exhibited much less LDH activity when the MOI was below 50. At an MOI of 10, the level of LDH activity from the INT-407 cells infected with MW064 was almost 3 fold less than from the cells infected with the wild type. Similarly, LDH activity from INT-407 cells was compared at an MOI of 10 with different incubation times as indicated in Fig. 4A. The cells infected with MW064 exhibited lower levels of LDH activity than cells infected with the wild type, when the cells were incubated with the bacterial suspension as long as 4 h. Therefore, an optimal period of 3 h incubation with a constant MOI of 10 was chosen for further experiments.

To further investigate the cytotoxicity of the *rtxA* mutant MW064 compared to its parental wild type, morphological studies were also carried out using INT-407 cells infected

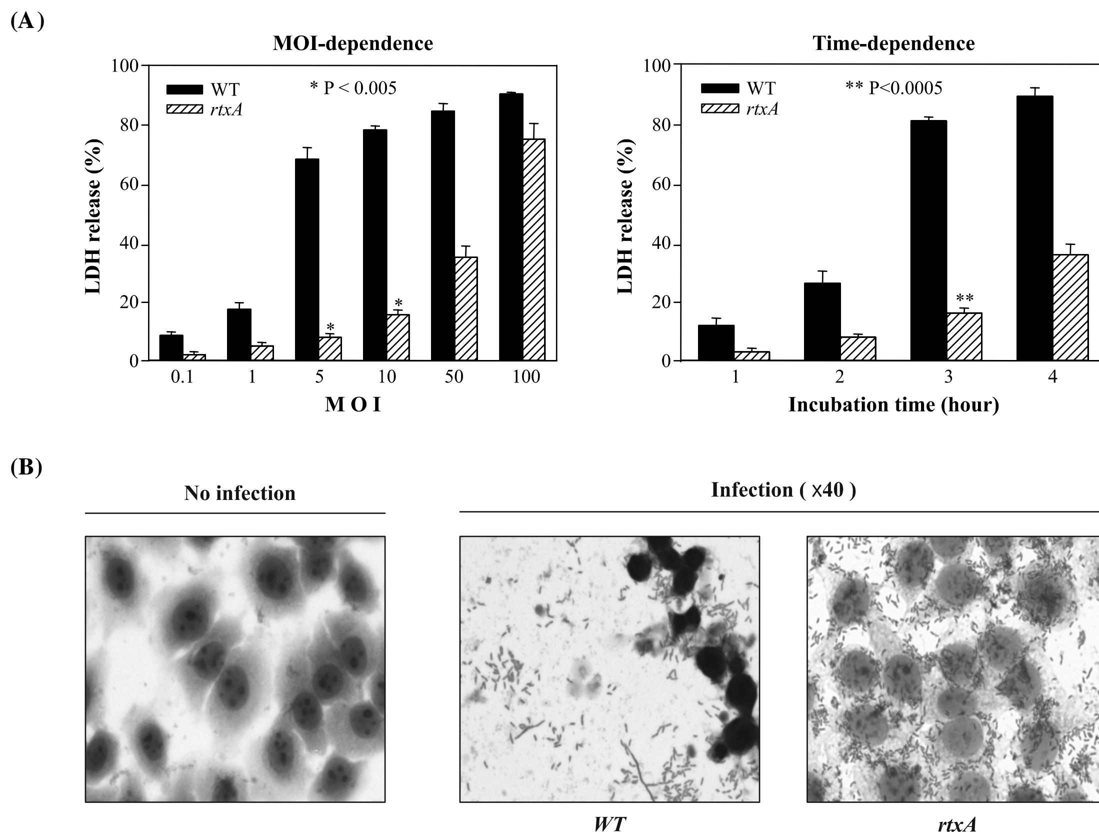

Fig. 4. Effect of *rtxA* mutation on the virulence of *V. vulnificus* towards INT-407 cells. (A) INT-407 cells were infected with the wild type or the *rtxA* mutant of *V. vulnificus* at various MOIs for 3 h (left), or at an MOI of 10 for various incubation times (right). The cell cytotoxicity was determined by an LDH release assay. The data represent the Mean±SEM from three independent experiments. $^{*}P<0.005$, $^{**}P<0.0005$, relative to groups infected with the wild type of *V. vulnificus* at each MOI or incubation time. (B) Microscopic observation of INT-407 infected with the *V. vulnificus* strains at an MOI of 10 for 3 h. From the left, uninfected (control) and infected with wild type (WT) or MW064 (*rtxA*).

with *V. vulnificus* strains (Fig. 4B). Microscopic observation of the INT-407 cells infected with M06-24/O at an MOI of 10 for 3 h was carried out as previously described (Park *et al*., 2006b). The stained cells were subsequently assessed for size, regularity of the cell margin, and the morphological characteristics of the nuclei. As shown in Fig. 4B, many Giemsa-stained INT-407 cells exhibited marked cellular damage after infection with the wild type. Cytoplasmic loss and nuclear material condensation which are typical phenotypes of cell death, was observed in the intestinal cells infected with the wild type, and many *V. vulnificus* cells were observed in the disrupted cytoplasmic region of the infected cells. In contrast, fewer dead cells were observed after incubation with MW064. The cells infected with MW064 exhibited less damaged surfaces and cytoplasmic loss, suggesting RtxA is important to the ability of *V. vulnificus* to infect and injure host cells.

Virulence in mice is dependent on rtxA

The LD_{50}s in the iron-overloaded mice after intraperitoneal infection with *V. vulnificus* strains are shown in Table 3. The LD_{50} for MW064 was greater than 10^3 CFU, compared with an LD_{50} lower than 10 CFU for the wild type. Consequently, the *rtxA* mutant in the mouse model of intraperitoneal infection, exhibited more than a 2-log increase in the LD_{50} over the wild type hence the *rtxA* mutant appeared to be less virulent than its parental wild type. This result suggests that the RtxA is an important factor in the pathogenesis of *V. vulnificus*. It is reasonable to conclude that the *rtxA* is essential for the virulence of *V. vulnificus* in mice as well as in tissue cultures.

The RTX (repeats in toxin) family of toxins includes a group of related protein toxins produced by a wide range of Gram-negative pathogens with hemolytic, leucotoxic, and actin cross-linking activities (Lally *et al*., 1999; Fullner and Mekalanos, 2000). These toxins all share distinctive features: post-translational activation becoming biologically active, tandemly repeated acidic glycine-rich nanopeptides in a C-terminal calcium binding domain, and export out of the cell by type I secretion systems (TISS) (Boardman and Satchell, 2004). Despite the existence of some variation, the synthesis, activation, and secretion of RtxA are governed by the *rtx* gene cluster consisting of *rtxCA* and *rtxBDE* operons (Fig. 1) (Lally *et al*., 1999). The pro-RtxA protoxin is matured in the cytosol to the active form by RtxC-directed fatty acid acylation (Lally *et al*., 1999). RtxB and E proteins, members of the ATP binding cassette superfamily of transport proteins (traffic ATPase), and RtxD protein, although its function is less clear, belongs to the membrane fusion protein family, composed of the RtxA secretory apparatus (Lally *et al*., 1999).

Table 3. Effects of the *rtxA* mutation on the lethality of *V. vulnificus* to mice

Strain	Intraperitoneal LD_{50} (CFU)
M06-24/O (n=6)	5.87×10^0
MW064 (n=6)	2.68×10^3

[a] n, number of iron-treated mice for each inoculation group, ranging from 10^0-10^7 CFU in 10 fold increments.

The identity of genetic organization observed in Vv- and Vc-*rtx* operons, and high levels of amino acid sequence homology found between Vv-RtxA and Vc-RtxA indicate that the function of Vv-RtxA would be similar to that of Vc-RtxA. However, Vc-RtxA does not disrupt membrane integrity and thus is not likely to be a typical member of pore-forming toxins (Lin *et al*., 1999; Fullner and Mekalanos, 2000). The cytotoxic activity of Vc-RtxA is associated with its ability to directly alter actin monomers. Specifically Vc -RtxA causes mammalian epithelial HEp-2 cells to die and round-up by cross-linking and depolymerization of cellular actin (Lin *et al*., 1999; Fullner and Mekalanos, 2000). However, a null mutation of *rtxA* decreased the release of LDH by *V. vulnificus*, indicating that cell membrane integrity is compromised by Vv-RtxA (Fig. 4). This result indicates that the Vv-RtxA, unlike Vc-RtxA, is able to disrupt membranes in the system we used, as predicted by its close relatedness to the RTX family of pore-forming toxins. This difference may be a result of the observed variation in virulence of the pathogens, such that *V. vulnificus* is exceedingly more destructive and cytolytic compared to *V. cholerae* (SHC, unpublished observation).

Acknowledgements

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