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NOTE

Regulatory Characteristics of the *Vibrio vulnificus rtxHCA* Operon Encoding a MARTX Toxin

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***Vibrio vulnificus* MARTX encoded by *rtxA*, an open reading frame of the *rtxHCA* operon, is essential for virulence *in vitro* and in mice. In this study, a primer extension analysis revealed that transcription of the *rtxHCA* operon begins at a single site, and is under the direction of a single promoter, P_{rtxHCA}. P_{rtxHCA} activity appeared at the beginning of growth and reached a maximum in mid-exponential phase. P_{rtxHCA} activity was induced by exposure to INT-407 cells, and the membrane fraction of INT-407 cells was the most effective for the induction.**

Keywords: *V. vulnificus rtxA*, MARTX

The pathogenic marine bacterium *Vibrio vulnificus* is the causative agent of food-borne diseases such as gastroenteritis in healthy persons, and life-threatening septicemia in individuals with underlying predisposing conditions (for a recent review, see Jones and Oliver, 2009). *V. vulnificus* infections are remarkable for their invasiveness, severe tissue damage, and rapidly fulminating course of disease. The characterization of somatic as well as secreted products of *V. vulnificus* has yielded a large list of putative virulence factors, whose known or putative functions are consistent with disease pathology (Jones and Oliver, 2009). Among the putative virulence factors is the multifunctional-autoprocessing RTX toxin (MARTX) encoded by the *rtxA* gene (Lee *et al*., 2007; Kim *et al*., 2008). The MARTX can lyse a variety type of cells including red blood cells (RBC), epithelial cells, and macrophages by forming small pores in the cytoplasmic membrane (Kim *et al*., 2008; Lo *et al*., 2011; Satchell, 2011). This function of MARTX is essential for the virulence of *V. vulnificus* in mice as well as in tissue cultures (Lee *et al*., 2007; Lo *et al*., 2011; Jeong and Satchell, 2012).

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Reports have indicated that the *rtx* gene cluster of *V. vulnificus* consists of the *rtxCA* and *rtxBDE* operons (Lee *et al*., 2007; Liu *et al*., 2007; Kim *et al*., 2008). The *rtxCA* operon is preceded by an open reading frame (ORF), hereafter designated *rtxH* based on its sequence homology (76% identity in nucleotide sequence) to that of *Vibrio anguillarum rtxH* (Li *et al*., 2008). A deletion of *rtxH* and *rtxC*, each encoding a hypothetical protein, leads to a marginal decrease in virulence but with no effect in cytotoxicity of *V. vulnificus* (Liu *et al*., 2007). RT-PCR analysis demonstrated that the *rtxHCA* genes are transcribed as a single transcriptional operon, as are the *rtxBDE* genes (Liu *et al*., 2007; Lee *et al*., 2008). Expression of the *rtxA* gene appeared to be regulated by the HlyU regulator by direct binding to the upstream region of the *rtxHCA* operon acting as an H-NS antirepressor (Liu *et al*., 2007, 2009, 2011). Here we extended our understanding of the regulatory mechanisms of *rtxA* expression and demonstrated that *rtxHCA* genes are transcribed from a single promoter P_{rtxHCA} and that the activity of P_{rtxHCA} is growth-phase specific and dependent on exposure to host cells.

The bacterial strains and plasmids used in this study are listed in Table 1. Unless noted otherwise, the *V. vulnificus* strains were grown in Luria-Bertani (LB) media supplemented with 2.0% (w/v) NaCl (LBS). Total cellular RNA was isolated from *V. vulnificus* MO6-24/O cells grown to mid-exponential phase in different media, such as LBS and MEM (minimum essential medium, Invitrogen-GIBCO™, USA), using an RNeasy® mini kit (QIAGEN, USA). Also, the RNA was prepared from MO6-24/O exposed to INT-407 (ATCC CCL-6) human intestine epithelial cells. For this purpose, MO6-24/O was incubated with INT-407 cells at MOI 10 for 2 h and the bacterial cells were then harvested as previously described (Lee *et al*., 2008). The prepared RNA was used for primer extension analyses. The primer extension products were visualized and quantified using a phosphorimage analyzer (BAS1500, Fuji Photo Film Co. Ltd, Japan) and the Image Gauge (version 3.12) program.

Averages and standard errors of the mean (SEM) were calculated from at least three independent experiments. Data were analyzed by Student's *t* test with the SAS program (SAS software; SAS Institute Inc., USA). Significance of differences between experimental groups was accepted at a *P* value of <0.05.

Table 1. Bacterial strain and pasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Reference or source
Strain		
V. vulnificus		
MO6-24/O	Clinical isolate; virulent	Laboratory collection
Plasmid		
pGEM-T Easy	PCR product cloning vector; Ap^r	Promega
pBBR-*lux*	Broad host range vector within promoterless *luxCDABE*; Cm^r	Lenz *et al.* (2004)
pJS0901	pGEM-T Easy with 448-bp fragment of *rtxHCA* upstream region; Ap^r	This study
pJS0904	pBBR-*lux* with 448-bp fragment of *rtxHCA* upstream region; Cm^r	This study

[a] Ap^r, ampicillin resistant; Cm^r, Chloramphenicol resistant

Table 2. Oligonucleotides used in this study

Oligonucleotide	Oligonucleotide sequence, 5′→3′[a]	Description
RtxA-PE1	CCATTCTTGGATCTTCTAGCATGT	Used for primer extension
RtxHCAF	GGATCCTATGGACAACTTGCTTAAGTTC	Used for amplification of *rtxHCA* promoter region
RtxHCAR	GGATCCATTCAAATGAATGATGCA	

[a] Regions of oligonucleotides not complementary to corresponding genes are underlined.

Transcription of the *rtxHCA* operon begins at a single site

For the primer extension, an end-labeled 24-base primer RtxA-PE1 complementary to the coding region of *rtxH* was added to the RNA and then extended with SuperScript II RNase H-Reverse Transcriptase (Invitrogen, USA) as previously described (Kim *et al.*, 2011). The cDNA products were purified and resolved on a sequencing gel alongside sequencing ladders generated from pJS0901 (Table 1) with the same primer used for the primer extension. For construction of pJS0901, the 448-bp DNA fragment containing the *rtxHCA* promoter region and part of *rtxH* ORF (from -221 to +227) was amplified by PCR using primers RtxHCAF and RtxHCAR (Table 2) and subcloned into pGEM-T Easy (Promega, USA) (Table 1).

A single reverse transcript was produced from primer extension of RNA isolated from *V. vulnificus* grown in LBS (Fig. 1A). The 5′ end of the *rtxHCA* transcript is located 166-bp upstream of the RtxH translation initiation codon, and subsequently designed +1 (Fig. 1B). This +1 site is at the one base downstream from the 5′-end of the posttranscriptionally processed transcript of *rtxHCA* that was previously determined in an experiment using an RNA ligase mediated rapid amplification of cDNA ends by Crosa and colleagues (Liu *et al.*, 2009). The putative promoter upstream of the transcription start site was named P_{rtxHCA}. Using several different sets of primers, we were unable to identify any other transcription start sites by primer extension (data not shown). This indicated that a single transcription start site was used for the transcription of *rtxHCA* genes, consistent with the previous report that *rtxHCA* genes are cotranscribed as a single operon (Liu *et al.*, 2007).

Growth phase-dependent expression of *rtxHCA*

In order to examine whether the expression of *rtxHCA* is influenced by growth phase, the activities of P_{rtxHCA} were monitored during growth. The same amount of total RNA was isolated from MO6-24/O cells at different stages of growth in LBS. P_{rtxHCA} activity appeared at the beginning of growth and reached a maximum in mid-exponential phase. When compared with the exponential cells, gradually decreasing P_{rtxHCA} activities were apparent in the cells of stationary phase (Fig. 2). The results indicated that the *rtxHCA* operon is expressed in a growth-phase dependent manner.

rtxHCA is induced by exposure to host cells

To determine the effect of host cells on the activity of P_{rtxHCA}, total bacterial RNA was isolated from the MO6-24/O cultures exposed to INT-407 cells, and used for primer extension

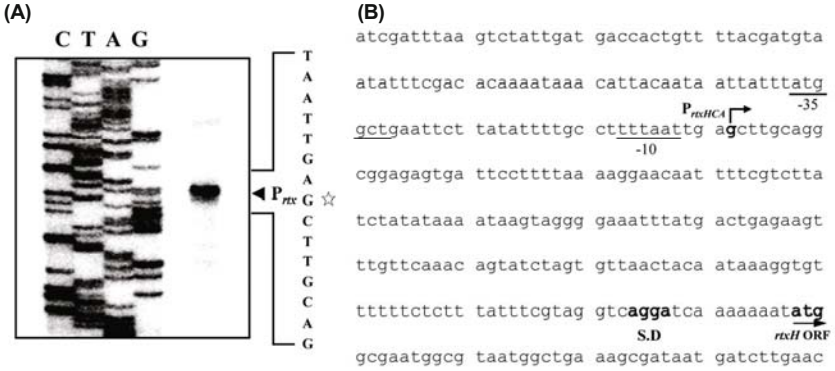

Fig. 1. Primer extension analysis of *rtxHCA* transcript and sequence of *rtxHCA* upstream region. (A) The transcription start site was determined by primer extension of RNA derived from *V. vulnificus* MO6-24/O grown to mid-exponential phase in LBS. Lanes G, A, T, and C represent the nucleotide sequencing ladders of pJS0901. The transcription start site for P_{rtxHCA} is indicated by a white star. (B) Transcription start site is indicated by bent arrow. Possible promoters (-10 and -35) are shown underlined with continuous lines for the *rtxHCA* promoter. The ATG translation initiation codon and putative ribosome binding site (AGGA) are indicated in boldface type. ORF, open reading frame.

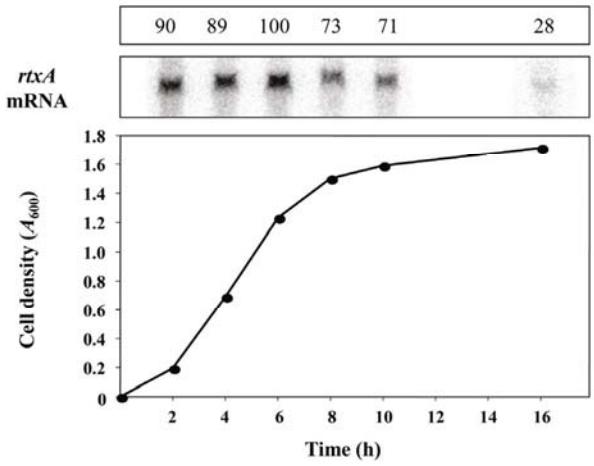

Fig. 2. Growth kinetics and growth phase-dependent expression of *rtxHCA*. Samples of the cultures were removed at the time points as indicated. Samples were analyzed for P_{rtxHCA} activity (top) and cell density (A_{600}) (bottom). The relative levels of the P_{rtxHCA} activity relative to the level of the P_{rtxHCA} activity of cells grown for 6 h are presented. Growth of MO6-24/O is indicated by the filled circles.

analysis. The primer extension analysis performed with RNA isolated from MO6-24/O exposed to INT-407 revealed a band of reverse transcript, and its intensity was almost 2-fold greater than that of the reverse transcript obtained with RNA isolated from MO6-24/O grown in LBS (Fig. 3). Based on the intensity of the bands of the reverse transcripts, it was apparent that the activity of P_{rtxHCA} is induced by exposure of *V. vulnificus* to host cells. It is noteworthy that expression of *rtxHCA* is dependent on a single P_{rtxHCA} with the same transcription start site regardless of exposure of *V. vulnificus* to the host cells (Fig. 3). In order to characterize the effect of host cells on the expression of *rtxHCA* in more detail, primer extension analyses were performed with the RNA isolated from an MO6-24/O culture grown in MEM. When compared to the culture grown in LBS, the intensity of the bands of the reverse transcript was not significantly affected by exposure of the bacterial cells to MEM (Fig. 3).

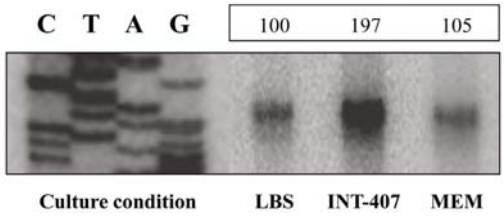

Fig. 3. Activities of P_{rtxHCA} in *V. vulnificus* grown under different conditions. RNA derived from MO6-24/O exposed to INT-407 cells, or grown in different media such as LBS, MEM, as indicated. The activities of P_{rtxHCA} were determined separately by primer extension of the RNA. Lanes G, A, T, and C represent the nucleotide sequencing ladders of pJS0901. The levels of the P_{rtxHCA} activity are presented relative to the level of the P_{rtxHCA} activity of *V. vulnficus* grown in LBS.

The membrane fraction of host cells induces *rtxHCA*

The induction of *rtxHCA* expression on the exposure to INT-407 cells was reconfirmed using a transcriptional fusion reporter (Fig. 4). For this purpose, a plasmid pJS0904 was created by subcloning the 448-bp DNA fragment, used to construct pJS0901 as mentioned above, into pBBR-*lux* (Table 1 and Fig. 4A). The latter plasmid carries the promoterless *luxCDABE* luciferase genes (Lenz *et al.*, 2004). Plasmid pJS0904 was mobilized into MO6-24/O by conjugation to result in MO6-24/O (pJS0904) (Lee and Choi, 2006).

To prepare cellular fractions, harvested cells of INT-407 were resuspended in a lysis buffer (20 mM Na-HEPES, 2 mM $MgCl_2$, 1 mM EDTA, 2 mM Na_3VO_4, 4 μg/ml leupeptin, 30 μg/ml PMSF), disrupted for 2 min, and fractionated into cytoplasm and membrane fractions as previously described (Thodeti *et al.*, 2001). An equal number of MO6-24/O (pJS0904) cells was mixed with 500 μl of the fractions and incubated for 30 min at 30°C. Cellular luminescence of the mixtures was measured with a luminometer (Lumat model 9501, Berthold, Germany) and expressed in arbitrary relative light units (RLUs) (Lee and Choi, 2006).

For MO6-24/O containing pJS0904 incubated in LBS as a negative control, luminescence activity was about 1.0×10^1 RLU/CFU (Fig. 4B). The light produced from the cells incubated with the membrane fraction of INT-407 increased significantly, indicating that the activity of P_{rtxHCA} is dependent on preexposure to the host cell membrane. However, the levels of luminescence from cells incubated in MEM, in

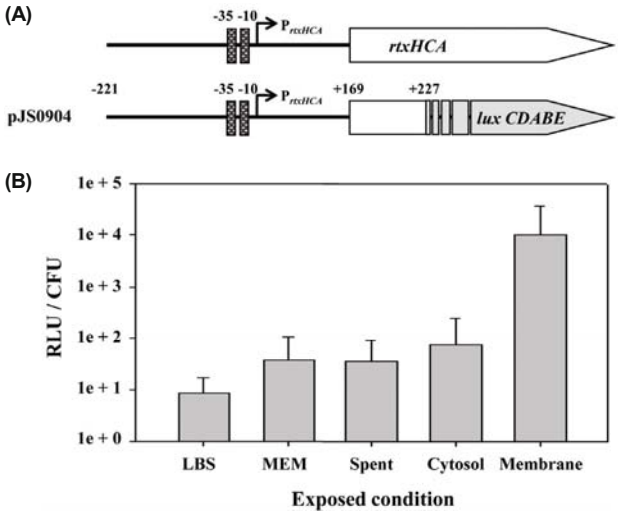

Fig. 4. Activities of P_{rtxHCA} in *V. vulnificus* exposed to different cellular fractions. (A) Construction of *rtxHCA-lux* fusion pJS0904. A PCR fragment carrying the regulatory region of *rtxHCA* and part of the *rtxH* ORF was subcloned into pBBR-*lux* (Lenz *et al.*, 2004). Open blocks, the *rtxH* coding region; filled block, the *luxCDABE* DNA; solid lines, the upstream region of *rtxHCA*. The *rtxHCA* upstream regulatory region and *rtxHCA* ORF are shown on top with the proposed -10 region, -35 region. The -10 and the -35 regions were proposed on the basis of the transcription start site of P_{rtxHCA}. (B) Cellular luminescence values were measured from MO6-24/O grown to mid-exponential phase in LBS and then exposed to different fractions of INT-407 cells as indicated. LBS, MEM, INT-407 spent (Spent), cytoplasmic fraction (Cytosol), membrane fraction (Membrane). Error bars represent the SEM.

the INT-407 spent, and in the cytoplasmic fraction did not significantly differ (Fig. 4B). The supernatant of the INT-407 cell line grown to monolayer in MEM was filtered with Steritop (Millipore, USA) and used as the INT-407 spent (Lee *et al.*, 2008). These data indicated that a component(s), yet unidentified, necessary for activation of P_{rtxHCA} is present in the membrane of the host cells.

In the present study, increased expression of *rtxHCA* was observed only in *V. vulnificus* cells exposed to the membrane fraction of INT-407 cells, and not in *V. vulnificus* cells exposed to MEM, the INT-407 spent or a cytoplasmic fraction of the host cells (Figs. 3 and 4). This observation is consistent with the previous report that the expression of RtxA1 toxin increased after host cell contact in a time dependent manner (Kim *et al.*, 2008). Recently, it has been reported that *rtxHCA* expression in *V. vulnificus*, grown in trypticase soy broth and not exposed to host cells, was dependent on HlyU acting as a derepressor of the H-NS that binds to a region extending upstream and downstream of the *rtxHCA* promoter (Liu *et al.*, 2007, 2009, 2011). These observations suggest the possibility that an unidentified component(s) in the membrane of INT-407 cells may modulate the level (or activity) of HlyU or H-NS. However, additional works are needed to clarify what component(s) is indeed involved in the regulation of the P_{rtxHCA} activity, and how the signals imposed by the host are delivered to the regulator proteins.

It has been generally accepted that bacterial genes that are preferentially expressed within the environment of the host are likely important to pathogenesis (Lee and Camilli, 2000). In addition, the present study demonstrated that expression of *rtxHCA* of *V. vulnificus* is dependent on growth phase, being elevated during exponential phase. It is still difficult to define the implications of the increased expression of *rtxHCA* during exponential phase in the pathogenesis of *V. vulnificus*. However, we speculate that this exponential phase-specific expression of *rtxHCA* is correlated with the functions of MARTX during the initial stage of infection of the pathogen. In agreement with this, it has been reported that MARTX is important for the early growth and survival of *V. vulnificus* at the infection site, and subsequently for dissemination of the pathogen into bloodstream and to other organs (Lo *et al.*, 2011; Jeong and Satchell, 2012). In this context, we postulate that the temporal (eg, exponential phase-specific) expression of *rtxHCA* could ensure the overall success of *V. vulnificus* during pathogenesis.

Thus, when taken together, these results make it reasonable to conclude that the activity of *V. vulnificus* P_{rtxHCA} is expressed in a growth phase-dependent manner, and the expression requires direct contact of the bacteria to the host cell membrane.

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