S1-3

Regulation and sensory transduction of the genes involved in pathogenesis of *Vibrio vulnificus*

Sang Ho Choi, Ph. D.

Dept. of Food Sci. & Technol., Institute of Biotechnology, Chonnam National University, Kwang-Ju, 500-757, S. Korea

The pathogenic marine bacterium *Vibrio vulnificus* is the causative agent of food-borne diseases such as life-threatening septicemia and possibly gastroenteritis in individuals with underlying predisposing conditions such as liver damage, excess levels of iron, and immuno-compromised conditions. Wound infections result from exposure to seawater or from the handling of shellfish contaminated with *V. vulnificus*. Mortality from septicemia is very high (>50%), and death may occur within one to two days after the first signs of illness. Several potential virulence factors including endotoxin, polysaccharide capsule, iron sequestering systems, a cytolytic hemolysin, an elastase, a phospholipase A2, and other exotoxins have been identified for *V. vulnificus*.

Transmembrane transcription activator ToxRS is involved in the expression of the *V. vulnificus* virulence genes

In an attempt to dissect the virulence regulatory mechanism in *Vibrio vulnificus*, we tried to identify the transmembrane virulence regulator *Vibrio cholerae toxRS* (Vc-*toxRS*) homologs in *V. vulnificus*. By comparing the sequences of *toxRS* of *V. cholerae* and *Vibrio parahaemolyticus* (Vp), we designed a degenerate primer set targeting well-conserved sequences. Using the PCR product as the authentic probe for Southern blot hybridization, a 1.6-kb *Bgl*II-*Hin*dIII and a 1.2-kb *Hin*dIII fragment containing two complete and one partial open reading frames attributable to Vv-*toxR*, Vv-*toxS*, and Vv-*htpG* were cloned. The Vv-ToxR shared sequence homology of 55.0% and 63.0% with Vc-ToxR and Vp-ToxR, respectively. Vv-ToxS was 71.5% and 65.7% homologous to Vc-ToxS and Vp-ToxS, respectively. The amino acid sequences of Vv-ToxRS showed similar transmembrane and activity domains

observed in Vc-ToxRS and Vp-ToxRS. Western blot analysis proved the expression of Vv-ToxR in *V. vulnificus*. Vv-ToxRS promoted, in an *Escherichia coli* background, the expression of *V. vulnificus* hemolysin gene (*vvhA*) by 5-fold. Vv-ToxRS also activated Vc-ToxR-regulated *ctx* promoter incorporated in an *E. coli* chromosome. Vv-*toxR* null mutation decreased hemolysin production. The defect in the hemolysin production could be complemented by a plasmid harboring the wild type gene. The Vv-*toxR* mutation also showed reversed outer membrane protein expression profile in comparison to the isogenic wild type strain. These results demonstrate Vv-ToxR may regulate the virulence expression of *V. vulnificus*.

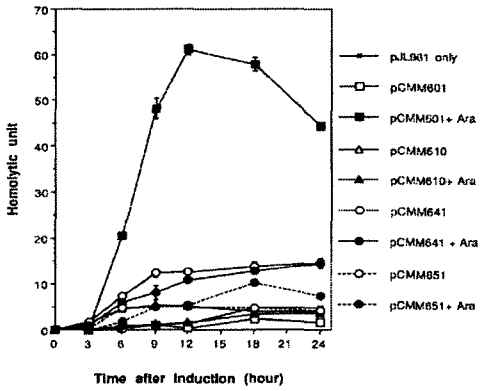

Figure 1. **Stimulation of the *vvh* gene by Vv-ToxRS in an *E. coli* background.** The compatible plasmid carrying the 3.4-kb *vvh* gene fragment (pJL961) was cotransformed with the plasmids carrying Vv-*toxRS* (pCMM601), Vv-*toxR* (pCMM610), Vc-*toxRS* (pCMM641) or Vc-*toxR* (pCMM651) under the control of P_{BAD} promoter. Hemolytic activity in the culture supernatant was assayed at the indicated time intervals. Cultures were induced with 0.2% arabinose. The error bar indicates SEM from triplicate experiments. Symbols: I, pJL961 only; G and B, pCMM601 before and after induction; C and H, pCMM610 before and after induction; E and J with solid line, pCMM641 before and after induction; E and J with broken line, pCMM651 before and after induction, respectively.

Expression of *Vibrio vulnificus* hemolysin gene is dependent on cyclic-AMP receptor protein

Hemolysin is a potent virulence factor of the pathogenic bacterium *V. vulnificus*, and shows cytolytic activity. Physiological studies have shown that the hemolytic activity in *V. vulnificus* decreases in the presence of glucose. To test the effect of

glucose further, the regulatory region of *vvh* operon encoding hemolysin was cloned upstream of a promoterless *luxCDABE*. Measurement of luminescence in *E. coli* CH1105 carrying the resulting *vvh-lux* fusion plasmid, pBY9802, indicated that the glucose effect was due to repression of promoter activity of *vvh*. When transformed with pBY9802, *E.coli cya* or *crp* isogenic mutants showed much lower level of luminescence than that in wild type CH1105. Luminescence from pBY9802 in the *E. coli cya* mutant CH1019 was restored and reached even greater levels than that in CH1105 by addition of increasing amounts of cAMP. The repression of luminescence in *E.coli crp* mutant CH1133 containing *vvh-lux* was complemented by introducing *E. coli crp* gene. To examine if the cAMP and CRP can regulate *vvh* genes as demonstrated in *E. coli*, the effects of cAMP addition and *crp* mutation on the production of *V. vulnificus* were examined. Hemolytic activity increase by adding cAMP in a dose dependent fashion and decrease to undetectable levels in the *crp* mutant. These results support that the synthesis of hemolysin in *V. vulnificus* is dependent on cAMP and *crp* gene function.

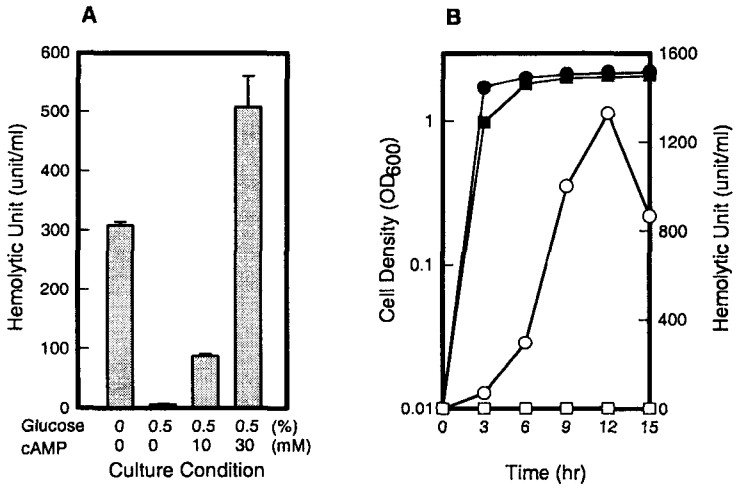

Figure 2. **Dependency of hemolysin production of *V. vulnificus* on cAMP and cAMP receptor protein.** For panel A, cultures of strain MO6-24/O were grown in heart infusion broth with varied supplement(s) as indicated. Samples removed at 12 hr were analyzed for hemolytic activity on each bar. Error bars represent standard errors of the mean. For panel B, growth and hemolytic activity of wild type (●, ○) and *crp* mutant CMM988 (■, □) were compared. Samples removed at indicated times from cultures were analyzed for determination of growth (filled symbols), and hemolytic activity (open symbols). For both panels, sRBC were used for determination of hemolytic activities.

Construction and Phenotypic Evaluation of a *Vibrio vulnificus vvpE* Mutant for Elastolytic Protease

Numerous secreted virulence factors have been proposed to account for the fulminating and destructive nature of *V. vulnificus* infections. Among the putative virulence factors is an elastolytic metalloprotease. We cloned and sequenced the *vvpE* gene encoding an elastase of *Vibrio vulnificus* ATCC29307. Functions of the elastase were assessed by constructing a *vvpE* insertional knockout mutant and evaluating phenotypic changes in vitro and in mice. Although other types of protease activity were still observed in the *vvpE* mutant, the elastase activity was completely absent in the mutant and was restored by reintroducing the recombinant *vvpE* gene. In contrast to previous characteristics of elastase as a potential virulence factor demonstrated by injecting the purified protein into animals, inactivation of the *V. vulnificus vvpE* gene did not affect the ability of the bacteria to infect mice and cause damage, either locally in subcutaneous tissues or systemically in the liver in both iron-treated and normal mice. Furthermore, the *vvpE* mutant was not affected for cytolytic activity towards INT407 epithelial cells or detachment of INT407 cells from culture dishes in vitro. Therefore, it appears that elastase is less important in pathogenesis of *V. vulnificus* than would have been predicted from examining effects of administering purified proteins to animals. However, *V. vulnificus* utilizes a variety of virulence factors, hence the effects of inactivation of elastase could be masked by other compensatory virulence factors.

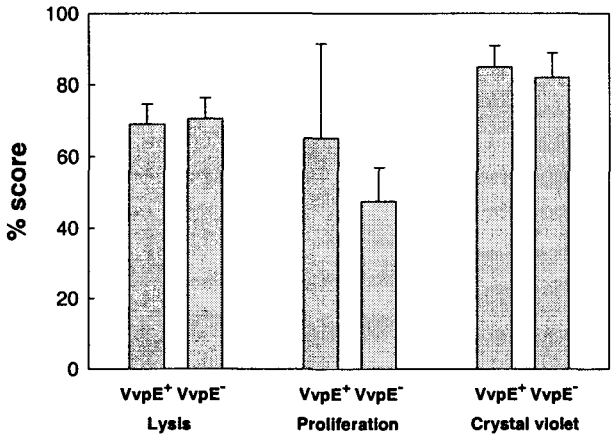

Figure 3. **Lack of effect of the *vvpE* mutation on cytopathology of *V. vulnificus* to INT407 cells.** INT407 intestinal epithelial cells were infected with a multiplicity of infection of 5 for 1 h before addition of gentamicin to 100 g/ml. Twenty-four hours later, lysis

was measured using LDH release into culture supernate; values are percent release from vibrio-infected cells compared with total LDH (Triton-X released) of vibrio-infected cells with the background lysis (15%) of uninfected cells subtracted. The Proliferation assay measures metabolically active cells remaining attached to culture dishes; values are percent loss compared with uninfected wells. The crystal violet assay measures percent loss of crystal violet-staining material for vibrio-infected cell cultures compared with uninfected wells. Triplicates wells of each experiment were run, and these assays were repeated at least once. The differences in values between the VvpE+ and VvpE- strains were not significantly different in any of the three assays ($p>0.2$).

References

1. **Bang, Y. B., S. E. Lee, J. H. Rhee, and S. H. Choi.** 1999. Evidence that expression of the *Vibrio vulnificus* hemolysin gene is dependent on cyclic AMP and cyclic AMP receptor protein. J. Bacteriol. 181:7639-7642.

2. **Jeong, K. C., K. Y. Kim, A. M. Starks, G. M. Escudero, P. A. Gulig, and S. H. Choi**. 2000. Construction and Phenotypic Evaluation of a *Vibrio vulnificus vvpE* Mutant for Elastolytic Protease. Infect. Immun. Submitted for publication.

3. **Lee, S. E. S. H. Shin, S. Y. Kim, Y. R. Kim, D. H. Shin, S. S. Chung, Z. H. Lee, S. H. Choi, and J. H. Rhee.** 2000. *Vibrio vulnificus* has the transmembrane transcription activator ToxRS stimulating the expression of the hemolysin gene *vvh*. J. Bacteriol. In press