Functional Genomics of *Vibrio vulnificus*: From Survival to Toxigenesis

Sang Ho Choi

Chonnam National University, Kwang-Ju, 500-757, South Korea

Understanding the molecular pathogenesis of the multifaceted host-pathogen interaction is critical in the development of improved treatment and prevention, as well as elucidating how certain bacteria can circumvent host defenses, multiply in the host, and cause such extensive damage. Disease caused by infection with *V. vulnificus* is remarkable for the invasive nature of the infection, ensuing severe tissue damage, and rapidly fulminating course. The characterization of somatic as well as secreted products of *V. vulnificus* has yielded a large list of putative virulence attributes, whose known functions are easily imagined to explain the pathology of disease. These putative virulence factors include a carbohydrate capsule, lipopolysaccharide, a cytolysin/hemolysin, elastolytic metalloprotease, iron sequestering systems, lipase, and pili. However, only few among the putative virulence factors has been confirmed to be essential for virulence by the use of molecular Koch's postulates. This presentation describes molecular biological characterization of the virulence factors contributing to survival as well as to toxigenesis of *V. vulnificus*.

Characterization of the *Vibrio vulnificus putAP* Operon Encoding Proline Dehydrogenase and Proline Permease and Its Differential Expression in Response to Osmotic Stress

The *Vibrio vulnificus putAP* genes encode proline dehydrogenase and proline permease involved in proline catabolism and proline uptake, respectively. The gene product of *putP* also contributes to the osmotic tolerance of *V. vulnificus*. Transcriptional orientations of the *Vibrio vulnificus putAP* genes are in the same direction. Proline dehydrogenase activity and the level of *putA* transcript were determined to reach a maximum in exponential phase and were then repressed when growth slowed down. Northern blot and primer extension analyses revealed that transcription of *putAP* genes results in two different transcripts, transcript A (*putA* transcript) and transcript AP (*putAP* transcript). Expression of *putAP* genes was directed by two promoters, promoter P_{putA} and promoter P_{putAP}. A *crp* null mutation decreased proline dehydrogenase activity and the level of the *put* transcripts, indicating transcription of *putAP* is under the positive control of CRP (cyclic AMP receptor protein). Proline dehydrogenase and the level of both *put* transcripts were increased by proline, but repressed by glutamate. In contrast to this, the level of transcript A, not transcript AP, increased when proline dehydrogenase was induced by NaCl. It is apparent that since P_{putA} activity, not P_{putAP} activity, increased by NaCl, transcript A and transcript AP are transcribed through P_{putA} and P_{putAP}, respectively. Cells challenged with NaCl and various hyperosmotic stresses accumulated higher levels of glutamate, indicating that glutamate is a compatible solute in *V. vulnificus* [Fig. 1].

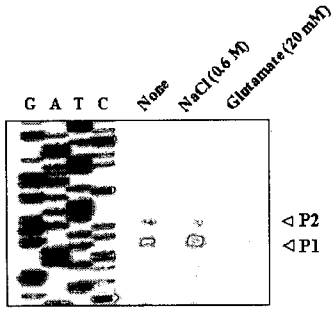

Fig. 1. Effects of NaCl and glutamate on the activities of *put* promoters. Promoter activities were determined by primer extension of the RNA derived from the culture of ATCC29307 grown to log phase in M9-N (None), and in M9-N supplemented with NaCl (0.6 M) or glutamate (20 mM) as indicated. Lanes G, A, T, and C represent the nucleotide sequencing ladders of pHJK002. The sites of the transcription start for P_{putA} (P1) and P_{putAP} (P2) are indicated by arrows.

Modulation of Acid-Induced Expression of the *Vibrio vulnificus cadBA* Genes by CadC

Like many other food-borne pathogenic bacteria, *V. vulnificus* has to cope with ever-changing acidity in their growth environments to ensure developing illness. The *V. vulnifcius cadBA* genes encode a lysine/cadaverine antiporter and a lysine decarboxylase, whose combined activity leads to the synthesis and excretion of cadaverine to counteract external acidification. Lysine decarboxylase activity of *V. vulnificus* was induced at a low pH (pH 5.8), and the induction of lysine decarboxylase was regulated at the level of transcription. A primer extension analysis revealed that *cadBA* genes are organized as a single transcriptional unit, and that the transcription of *cadBA* begins at a specific site, consisting of a putative promoter P_{cad}. An open reading frame, *cadC*, consisting of 526 amino acids, was identified upstream of P_{cad}. The production of lysine decarboxylase and the cellular level of *cadBA* transcript decreased in *cadC* mutant, which was constructed by allelic exchange. This decrease in the level of *cadBA* transcript in the *cadC* mutant appeared to be mediated by the reduced activity of P_{cad}. These results establish that *cadBA* expression is directed by P_{cad} in a pH-dependent manner, and activated by the gene product of *cadC* [Fig. 2]

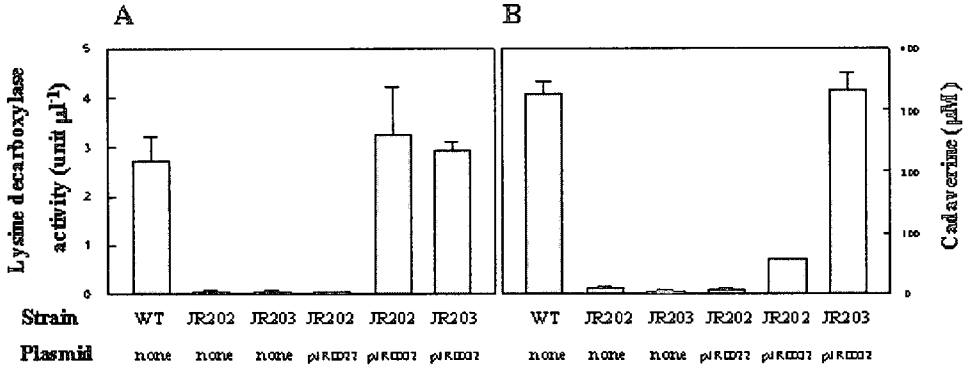

Fig. 2. Effects of mutations in *cad* genes on lysine decarboxylase activity and cadaverine excretion. For both panels, cultures of ATCC 29307 and each isogenic mutant were grown in LBS adjusted to a pH of 5.8. After 4 hr incubation, samples were removed and analyzed for lysine decarboxylase activity (A) and for cadaverine excretion (B). Complementations of the mutants with a functional *cadB* (pJR0022) or *cadA* (pJR0032) are also presented as indicated. Relative activities of lysine decarboxylase, and levels of cadaverine excretion were calculated as described in the text. Error bars represent the SEM.

Promoter Analysis and Regulatory Characteristics of *vvhBA* Encoding Cytolytic Hemolysin of *Vibrio vulnificus*

Hemolysin, a gene product of *vvhA*, is a potent virulence factor of the pathogenic bacterium *V. vulnificus*, and shows cytolytic activity. Hemolysin activity and the level of *vvh* transcript reached a maximum in the late exponential phase and then decreased when cells entered the stationary phase. Northern blot and primer extension analysis revealed that *vvhB* and *vvhA* are organized as one transcriptional unit, and that the transcription of the *vvhBA* operon begins at one site, consisting of a promoter P_{vvh}. A *crp* null mutation decreased hemolysin production and the cellular level of *vvhBA* transcript by reducing the activity of P_{vvh}, indicating that the P_{vvh} activity is under the positive control of CRP. A direct interaction between CRP and the regulatory region of the *vvhBA* operon was demonstrated by a gel-mobility shift assay. The CRP binding site mapped by deletion analysis of the *vvhBA* promoter region and confirmed by DNase I protection assay was centered at the 59.5 base pairs upstream of the transcription start site. These results demonstrate that the *vvhBA* expression is activated by CRP in a growth-dependent manner, and CRP exerts its effects by directly binding to P_{vvh} [Fig. 3].

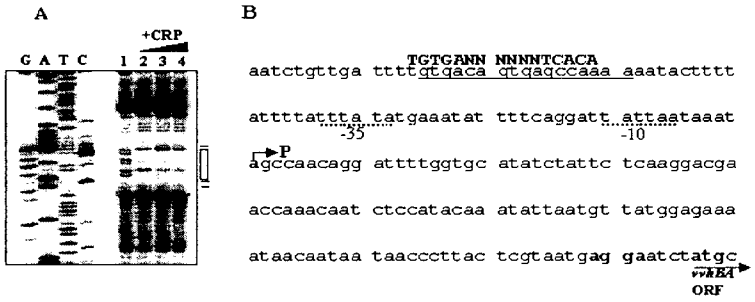

Fig. 3. CRP binding site for the *vvhBA* promoter. (A) DNase I protection analysis of CRP binding to the wild-type *vvh* regulatory region. Lane 1, no CRP added; 2 to 4, CRP at 100 nM, 200 nM, and 300 nM, respectively. Lanes G, A, T, and C represent nucleotide sequencing ladders of pHK0202. Nucleotides showing enhanced cleavage in the presence of CRP are indicated by the thick lines, and the region protected by CRP is indicated by the open box (not all hypersensitive and protected bands are indicated). (B) Sequence analysis of the *vvhBA* upstream region. The transcription start site is indicated by the bent arrow (P). The region protected from DNase I by CRP, and the putative promoter region (-10 and -35) are underlined with continuous and broken lines, respectively. Conserved nucleotide sequences for CRP binding (24) are indicated above the *V. vulnificus* DNA sequence by capital letters. The ATG translation initiation codon and putative ribosome-binding site (AGGA) are indicated in boldface.

Molecular Analysis of the Regulatory Effect of SmcR and CRP on the Expression of Elastase in *Vibrio vulnificus*

Among the putative virulence factors of *Vibrio vulnificus* is an elastase, the gene product of *vvpE*. It has been demonstrated that *vvpE* expression is differentially directed by two different types of promoters, PL and PS, and elevated by RpoS, CRP and a trans-acting regulatory protein SmcR. In this study, a primer extension analysis revealed that the activity of log-phase promoter, PL, was unaffected by a null mutation of *smcR*. The mutation of *smcR* reduced the activity of stationary-phase promoter, PS, indicating that the effect of SmcR on the expression of elastase is occurred through PS requiring RpoS. The mutation of *crp* also reduced, even less extent, the PS activity; however, the additional inactivation of CRP did not influence the PS activity in the *smcR* mutant. This indicated that the effect of CRP on the PS activity is mediated by SmcR. GST-pull down and gel-mobility shift analyses revealed that CRP interacted directly with SmcR, and facilitated binding of

SmcR to the promoter. The binding sites for CRP and SmcR were mapped by deletion analysis of the *vvpE* promoter region and confirmed by an *in vitro* DNase I protection assay. Each binding site for CRP and SmcR was juxtapositioned and centered at the 214 and 184 base pairs upstream of the transcription start site of PS, respectively. The combined results demonstrate that the activation of *vvpE* expression by by CRP and SmcR is occurred in a growth-dependent manner through PS, and the regulatory proteins exerts their effects by directly binding to the promoter in the stationary Phase [Fig. 4, 5, and 6].

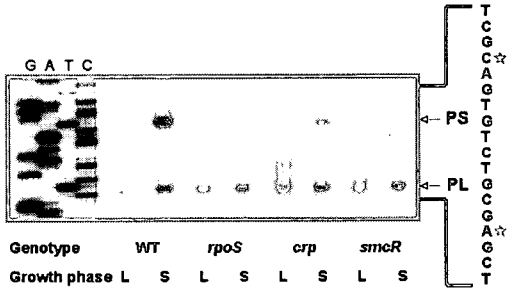

Fig. 4. Activities of the *vvpE* promoters PL and PS in *V. vulnificus* with different genetic background. The PL and PS activities were determined separately by the primer extension of the RNA derived from ATCC29307 and the isogenic mutants as indicated. Total RNAs were prepared in the log phase (L, OD_{600} 0.6) and stationary phase (S, OD_{600} 2.0) of each culture. Lanes G, A, T, and C represent the nucleotide sequencing ladders of pKC980. Asterisks indicate the sites of the transcription start for P1 (A) and P2 (C), respectively.

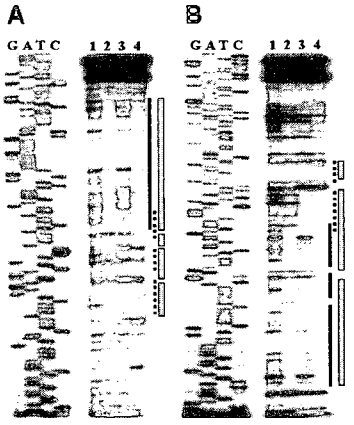

Fig. 5. Interaction between CRP and SmcR as probed with DNase I footprinting. Labeling of the *vvpE* regulatory region was performed on both strands, the coding strand (A) and the noncoding strand (B), independently. The ^{32}-P-labeled 290-bp fragments were incubated with different proteins and then digested with DNase I. Lane 1, no protein added; lane 2, 500nM SmcR; lane 3, 500nM CRP; and lane 4, mixture of 500nM SmcR and 500nM CRP, respectively. Lanes G, A, T, and C represent the nucleotide sequencing ladders of pKC980. The regions protected by independent binding of SmcR or CRP are shown in solid line and dashed line, respectively. Protection by the mixture of SmcR and CRP is indicated by the shaded boxes.

Fig. 6. Sequence of *vvpE* upstream region. Transcription start sites of the log phase (P1) and stationary phase (P2) are indicated by bent arrows. The positions of the putative –10 and –35 regions are underlined with continuous lines for the promoter PS. The sequences proposed for the binding sites of SmcR and CRP, termed SB and CB, respectively, are represented by blue and red box. The ATG translation initiation codon and putative ribosome binding site (AGGA) are indicated in boldface.

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