Repression of VvpM Protease Expression by Quorum Sensing and the cAMP-cAMP Receptor Protein Complex in Vibrio vulnificus

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ABSTRACT Septicemia-causing Vibrio vulnificus produces at least three exoproteases, VvpE, VvpS, and VvpM, all of which participate in interactions with human cells. Expression of VvpE and VvpS is induced in the stationary phase by multiple transcription factors, including sigma factor S, SmcR, and the cAMP-cAMP receptor protein (cAMP-CRP) complex. Distinct roles of VvpM, such as induction of apoptosis, lead us to hypothesize VvpM expression is different from that of the other exoproteases. Its transcription, which was found to be independent of sigma S, is induced at the early exponential phase and then becomes negligible upon entry into the stationary phase. SmcR and CRP were studied regarding the control of vvpM expression. Transcription of vvpM was repressed by SmcR and cAMP-CRP complex individually, which specifically bound to the regions −2 to +20 and +6 to +27, respectively, relative to the vvpM transcription initiation site. Derepression of vvpM gene expression was 10- to 40-fold greater in an smcR crp double mutant than in single-gene mutants. Therefore, these results show that the expression of V. vulnificus exoproteases is differentially regulated, and in this way, distinct proteases can engage in specific interactions with a host.

IMPORTANCE An opportunistic human pathogen, Vibrio vulnificus produces multiple extracellular proteases that are involved in diverse interactions with a host. The total exoproteolytic activity is detected mainly in the supernatants of the high-cell-density cultures. However, some proteolytic activity derived from a metalloprotease, VvpM, was present in the supernatants of the low-cell-density cultures sampled at the early growth period. In this study, we present the regulatory mechanism for VvpM expression via repression by at least two transcription factors. This type of transcriptional regulation is the exact opposite of those for expression of the other V. vulnificus exoproteases. Differential regulation of each exoprotease’s production then facilitates the pathogen’s participation in the distinct interactions with a host.

KEYWORDS metalloprotease VvpM, quorum sensing, cAMP-CRP, Vibrio vulnificus

A number of bacterial secreted proteins and surface factors are involved in the virulence of Vibrio vulnificus, an opportunistic human pathogen causing fatal septicemia through rapid pathogenic progression (1). One of the extracellular enzymes responsible for the pathology caused by V. vulnificus is a zinc metalloprotease, VvpE (2, 3), which enhances vascular permeability, thus causing hemorrhagic damage, degrading type IV collagen in the vascular basement membrane, and leading to membrane destruction and capillary vessel breakdown (4). Another extracellular protease of V. vulnificus is serine protease VvpS, whose N-terminal and C-terminal domains contain a protease catalytic domain and a carbohydrate-binding domain, respectively (5). Due to its peptidoglycan-hydrolyzing activity, VvpS shows the ability to interact with carbo-
hydrates in the cell wall, and the enzymatic activity may be related to the pathogenesis of V. vulnificus in an animal model (5).

Zymogram analysis of the proteases secreted by V. vulnificus has revealed the presence of several exoproteases in addition to VvpE and VvpS (M.-A. Lee, J.-A. Kim, and K.-H. Lee, unpublished data). In an effort to identify another exoprotease playing a role in the interactions with host cells, we recently isolated another zinc metalloprotease (VVMO6_03483) and named it VvpM (6). The recombinant VvpM (rVvpM) has been shown to induce apoptosis in human cells via a signaling pathway consisting of activation of ERK, a release of cytochrome c, and activation of caspases 9 and 3 (6). In addition, rVvpM-exposed cells show apparent changes in their morphology, such as formation of autophagic vesicles, which is mediated by lipidated light chain 3B II (LC3B-II) (7). Furthermore, it also regulates the pathogenic pathway stimulating NF-κB-dependent inter leukin-1β (IL-1β) production (8).

Expression of both vvpE and vvpS is reported to be induced by the quorum-sensing (QS) regulatory mechanism (9, 10). QS functions in a cell density-dependent manner via chemical communication using signaling molecules called autoinducers (AI). In V. vulnificus, AI-2 is one of the signaling molecules mediating QS (11) and is sensed by a membrane-bound receptor, leading to induction of the HapR-homologous transcription factor SmcR via a series of signal transductions (12, 13). SmcR is the QS master regulator of V. vulnificus, and the set of genes in the SmcR regulon has been identified (14). In the case of vvpE and vvpS, their expression is activated by direct binding of SmcR to the regulatory regions of the corresponding genes, resulting in production of these exoproteases at high cell density (12, 15). A global regulator, cAMP receptor protein (CRP), is also involved in transcription activation of vvpE and vvpS via formation of a complex with 3’,5’-AMP (termed the cAMP-CRP complex) (9, 15). The vvpE and vvpS genes have served as experimental models in studies on the molecular mechanisms of transcription regulation coordinately controlled by SmcR with other transcription factors, such as the cAMP-CRP complex, sigma factor S, IHF, Fur, and LeuO (9, 16, 17).

Because VvpM differs from VvpE in host interactions, it has been speculated that regulation of vvpM expression should be different from the regulation of other cases of exoprotease expression. Thus, in this study, transcriptional characteristics of vvpM were investigated and compared to those of the vvpE and vvpS genes.

RESULTS

Determination of transcription characteristics of the vvpM gene. A primer extension analysis was performed to determine the transcription initiation site (TIS) of the vvpM gene. A single transcript was detected, which started at nucleotide position ~76 relative to the initiation codon (IC) of vvpM (Fig. 1A). Upstream of the identified TIS, the tentative promoter region having ~10 (TACACT) and ~35 (TTGATG) sequences was discernible. To examine the expression characteristics of vvpM in V. vulnificus, a luxAB transcription fusion, pML_VvpM416, containing a DNA fragment ranging from position ~416 to +195 relative to the vvpM TIS, was constructed. During the whole growth period of wild-type V. vulnificus harboring pML_VvpM416 in AB-succinate medium (300 mM NaCl, 50 mM MgSO4, 0.2% [wt/vol] vitamin-free Casamino Acids, 10 mM potassium phosphate, 1 mM L-arginine, 1% [vol/vol] succinate, pH 7.5) supplemented with 3 μg/ml tetracycline, biomass and light production were measured. The expression was maximal in the early exponential phase, where the optical density at 595 nm (OD595) was ~0.1. vvpM expression then gradually decreased and showed a negligible level in the stationary phase (Fig. 1B). Its expression pattern was confirmed by monitoring the cellular contents of the vvpM transcripts. Relative abundance of the vvpM transcripts, which had been estimated by normalizing to the transcripts of the glyceraldehyde-3-phosphate dehydrogenase gene (gap) in each sample, showed the maximal expression of vvpM occurred at the early exponential phase (Fig. 1C). Compared to the vvpM transcript levels in the exponential-phase cells, the cellular levels of the vvpM transcript were highly decreased in the stationary-phase cells. Based upon the observation of the low expression of vvpM in the stationary phase, its expression was predicted to be...
independent of $\sigma^5$. The estimated maximal expression level of the $vvpM$-lux$AB$ transcriptional fusion in the $\Delta rpoS$ mutant was almost the same as that in the wild type (Fig. 1D).

**Effects of mutations of QS components on $vvpM$ transcription.** The overall expression pattern of $vvpM$ was different from those of genes $vvpE$ and $vvpS$. Maximal expression of $vvpM$ occurred in the early exponential phase, whereas the expression levels of $vvpE$ and $vvpS$ were basal during the exponential phase and increased in the stationary phase (15) via modulation by SmcR, which is the QS master regulator in *V. vulnificus* (10, 15). To test whether QS also controls $vvpM$ expression, the $vvpM$-lux$AB$ transcriptional fusion was transferred to various *V. vulnificus* mutant strains defective in one of the components of the QS regulatory cascade, such as lux$S$, lux$O$, hfg, or smcR. All of the regulatory components of QS influenced $vvpM$ expression. LuxO and Hfq are
involved in repressing smcR during the exponential growth period (12). Mutations in these genes decreased the expression of vvpM in the exponential phase (Fig. 2Aa). Deletion of luxS or smcR had a strong effect on vvpM expression, which increased ~8-fold compared to that of wild-type cells in the exponential phase. In contrast, vvpM expression was not significantly influenced by the mutations in luxO and hfq when the cells entered the stationary phase (Fig. 2Ab). The smcR mutant continued to show the greatest effect on vvpM expression, which increased ~100-fold compared to vvpM expression in the wild-type cells in the stationary phase. Primer extension analysis also revealed that the amount of the vvpM transcript highly increased in the smcR mutant (Fig. 2B), as shown in the experiment on the vvpM-luxAB transcriptional fusion.

**Effect of a crp mutation on vvpM transcription.** Expression of genes vvpE and vvpS is coactivated by the cAMP-CRP complex via synergistic interactions with SmcR (9, 15). To determine whether CRP also participates in vvpM expression, luciferase activity of the vvpM-luxAB transcriptional fusion was monitored in a Δcrp mutant strain. A V. vulnificus Δcrp mutant carrying pML_VvpM416 was grown in LBS medium (LB medium

![Diagram](http://jb.asm.org/)
containing NaCl) supplemented with 3 μg/ml tetracycline because of the absence of growth of the Δcrp mutant in AB-based media. VvpM expression in this mutant showed a slight increase, up to 4-fold higher than that in wild-type cells grown in the same medium (Fig. 3A). This result indicated that VvpM expression is repressed by CRP, but its effect was not as pronounced as the repression by SmcR. VvpM expression in a smcRΔcrp double mutant was found to be the highest, more than 10-fold higher than VvpM expression in the smcR mutant.

Effects of mutations of smcR and crp on VvpM activity. The production of extracellular VvpM by V. vulnificus strains was examined. The supernatants prepared from LBS medium grown by wild-type, smcR, Δcrp, and smcR Δcrp mutant strains were subjected to a proteolysis-zymographic assay (Fig. 3B). The resultant zymogram showed that VvpM activity was higher in the mutants than the wild type, and the derepression degree of VvpM production in the smcR Δcrp double mutant was far higher than the sum of VvpM production from the two single-gene mutants (Fig. 3C). Relative intensities of the VvpM bands corresponding to smcR, Δcrp, and smcR Δcrp mutants were approximately 45-, 4-, and 90-fold greater, respectively, than the intensity of the VvpM band corresponding to the wild type. Thus, the expression and regulatory mechanism of VvpM production are the exact opposite of those for VvpE and VvpS, suggesting that VvpM exoproteolytic activity plays roles in V. vulnificus pathogenicity under different conditions.

Specific binding of SmcR to the regulatory region of the vvpM gene. To examine whether SmcR and/or CRP directly interact with the regulatory regions of vvpM, gel shift assays were performed using a DNA fragment covering nucleotide positions from −132 to +195 relative to the TIS of vvpM. A 32P-labeled DNA probe was incubated with
various concentrations of recombinant SmcR (rSmcR). The probe DNA appeared as a slowly migrating band with rSmcR concentration-dependent intensity (Fig. 4A). Specificity of the binding was confirmed in a competition experiment using an excess of unlabeled probe DNA. Addition of this DNA to the binding reaction decreased the interaction between rSmcR and the 32P-labeled probe DNA, resulting in the disappearance of the slowly migrating band (Fig. 4A, lanes 6 and 7). In contrast, formation of a complex between rSmcR and the labeled probe DNA persisted when an excess of the noncompetitive gap promoter DNA was added to the reaction (Fig. 4A, lane 8).

To locate the specific SmcR-binding site in the probe DNA, a DNase I footprinting assay was carried out. A 32P-labeled DNA fragment incubated with rSmcR was treated with DNase I, and the resultant DNase I digestion patterns were examined. As a control, labeled probe DNA alone was also treated with DNase I (Fig. 4B, lane 1). When the rSmcR protein was added to the reaction mix, a portion of the sequence located
between positions −2 and +20 (5′-TTAGTGACAAACTCATAAATAG-3′) with respect to the vvpM TIS was protected from DNase I digestion (Fig. 4B). This site is highly homologous to the SmcR-binding consensus sequence (5′-TTATTGATWWRWTWNTNAATAA-3′, where W is A or T, R is G or A, and N is any nucleotide [14]) and to the LuxR-binding consensus sequence found in V. harveyi (5′-TATTGATATAAATTTATCAATA-3′ [18]) (Fig. 4C). To verify the interaction of SmcR with the DNA region identified by the DNase I protection assay, the DNA probe containing the altered sequences in the protected region was used as a probe in a gel shift assay. For a mutated probe (mutant probe I), the nucleotides matching the SmcR-binding consensus sequence in the protected region were changed, as shown in Fig. 4C. While the shifted band of the original probe was apparent due to the binding of rSmcR, no binding of rSmcR with mutant probe I was observed (Fig. 4D).

Specific binding of CRP to the regulatory region of the vvpM gene. Similarly, the original probe used for Fig. 4A was mixed with recombinant CRP (rCRP) in the presence of cAMP. The addition of rCRP shifted the probe DNA to bands with slower mobility (Fig. 5A). Formation of the slowly migrating band was abrogated by addition of an excess of unlabeled probe DNA to the reaction mixture (Fig. 5A, lanes 6 and 7). Binding of the cAMP-CRP complex to the probe DNA was specific, because the interaction between rCRP and the labeled probe DNA persisted when an excess of the noncompetitive gap promoter DNA was added to the reaction (Fig. 5A, lane 8). DNase I footprinting assays revealed two regions, CRP-1 and CRP-2, which were located in regions 6 to 27 relative to the vvpM TIS (Fig. 5B). CRP-1 included the protected site (region +6 to +27 relative to the vvpM TIS) and hypersensitive nucleotides (+11, +24, and +35 relative to the vvpM TIS) in the
The protected region within the CRP-1 site (positions 11001-1127) is 5'-AAATAATAGCAA GTTTCAAATAG-3' (the region overlapping the SmcR-binding site is underlined and hypersensitive nucleotides are italicized), the complementary sequence of which is homologous to the CRP-binding consensus sequence (5'-AAATGTGATCTAGATCACATTT-3' [19]) (Fig. 5C). Fifteen nucleotides of the CRP-1 site overlap the region of the SmcR-binding site, ranging from 11001-1120, as shown in Fig. 4B. DNase I digestion pattern in the region from 11001-1144 also showed the protected region (positions 11001-1134 relative to the vvpM TIS) and the hypersensitive nucleotide (11001 relative to the vvpM TIS) upon CRP binding. The protected region within the CRP-2 site (positions 11001-1134) is 5'-AAATAATAGCAAAGTTTCAAATAG-3' (the IC of vvpM is underlined), which is partially homologous to the known CRP-binding consensus sequence (Fig. 5C).

To verify the interaction of CRP with CRP-1 and CRP-2, oligonucleotides containing CRP-1 or CRP-2 sequences were synthesized and used as probes in gel shift assays using various concentrations of rCRP, ranging from 100 to 900 nM (Fig. 6). The probe containing the CRP-1 site began to bind rCRP in the presence of 200 nM rCRP (Fig. 6A, lane 3). In contrast, the probe containing the CRP-2 site did not show an apparently shifted band until 800 nM rCRP was provided in the reaction mixture (Fig. 6B, lane 9). Thus, both sites were bound by rCRP, but rCRP showed different affinities to each site. The apparent affinities of rCRP to each probe were compared by extrapolating the rCRP concentrations required for 50% binding of the labeled DNA (Fig. 6C).
effective concentrations of rCRP causing half-maximal binding were approximately 340 nM and more than 2.2 µM for CRP-1 and CRP-2, respectively.

**DISCUSSION**

Among various extracellular enzymes released by *V. vulnificus*, such as chondroitinase, DNase, hyaluronidase, lipase, mucinase, protease, and sulfatase (20), exoproteases have potential roles in pathogenic interactions with hosts. Among exoproteases, metalloprotease VvpE can degrade the human vascular basement membrane and capillary vessels (4), whereas serine protease VvpS functions as an autolysin-degrading bacterial peptidoglycan (5). Another metalloprotease, VvpM, is known to evoke marked changes indicative of apoptosis and autophagy in human cell lines (6, 7, 8). In addition, a ΔvvpM mutant exhibited less competitiveness in blood samples when coinfecting mice with wild-type *V. vulnificus*, even though this mutant showed the same colonization and/or survival as the wild type in other mouse tissues, e.g., in the spleen and liver (M.-A. Lee, S.-J. Park, and K.-H. Lee, unpublished data). A lowered competitiveness index in blood samples suggests that VvpM participates in the pathogenesis at specific locations or organs in a host, which remains to be investigated in a future study. Although knowledge about the physiological parameters in each host organ is a prerequisite to understanding the lowered numbers of the ΔvvpM cells in the blood of infected mice, VvpM may be necessary for avoidance of host defense systems mainly operating in blood. For example, aureolysin, a metalloprotease from *Staphylococcus aureus*, is known as a possible inhibitor of complement because of its cleaving of the central complement component C3b (21). Alternatively, lowered numbers of the ΔvvpM cells in the blood may result from the specific nutritional conditions in blood, which are different from those in the spleen and liver, for example, the available concentrations of the iron ion. *Candida albicans* has been found to differentially coordinate its degree of *in vivo* virulence depending upon the site within a host, being pathogenic in the bloodstream but commensalic in the gastrointestinal tract (22).

In pathogenic bacteria, expression levels of their virulence factors are finely and temporally regulated, and many virulence factors have been shown to be controlled by QS regulatory circuits (23, 24). Regulatory cascades for QS have been extensively studied in *V. vulnificus*. Under low-cell-density conditions, the active form of LuxO and sigma factor N (σN) activate luxT transcription. LuxT represses smcR transcription via direct binding to the upstream region of the smcR gene (12). In addition to regulation at the transcription level, smcR expression is repressed posttranscriptionally by the RNA chaperone Hfq and by the small RNA (sRNA) Qrr, the expression of which is induced by LuxO (13, 25). At high-cell density, LuxO becomes inactivated via a dephosphorylation process, resulting in derepression of SmcR. SmcR then induces transcription of many genes, including vvpE and vvpS. Our study indicates that vvpM transcription is strongly repressed by SmcR (Fig. 2). The gel shift assay and DNase I protection assay revealed that this repression is mediated by direct binding of SmcR to the region −2 to +20 relative to the vvpM TIS; this region is highly homologous to the LuxR-binding consensus sequence and SmcR-binding consensus sequence (14, 18) (Fig. 4). The list of genes whose expression is repressed by QS includes the open reading frames encoding the flagellar biosynthesis protein FlhF, methyl-accepting chemotaxis protein I, oligopeptide transport permease OppB, and a Zn-dependent intracellular protease (14). The physiological or pathogenic relatedness of VvpM to these gene products needs to be examined in the near future.

Moreover, vvpM expression was found to be repressed by CRP as well (Fig. 3). cAMP, a major cellular signaling metabolite (26), forms a complex with CRP. The intracellular concentrations of cAMP are determined by its synthesis catalyzed by Cya, its extracellular excretion, and its cleavage into 5’-AMP catalyzed by CpdA (27, 28, 29). The cAMP-CRP complex regulates the expression of numerous virulence factors in pathogenic bacteria. In pathogenic *Vibrio* species, representative genes that are positively regulated by the cAMP-CRP complex include ctxAB (encoding cholera toxin) and tcpPH (encoding the toxin-coregulated pilus) of *V. cholerae* (30) and vvpE, vvpS, and vvhA.
(encoding cytolytic hemolysin) of *V. vulnificus* (31, 32). In addition, the cAMP-CRP complex participates in global regulatory networks by controlling the expression of other global transcription factors, e.g., RpoS or ferric uptake regulator (Fur) (33, 34). Thus, it is likely that cAMP is one of the key molecules for timely expression of virulence factors, including VvpM in pathogenic bacteria (26). It has been shown in *V. vulnificus* that the cellular amount of SmcR increases when cells enter the stationary phase, whereas that of CRP is relatively constant (9). Therefore, the cellular concentration of cAMP is crucial for production of the transcriptionally active form of CRP via formation of the complex with cAMP, whose cellular amounts are known to be dependent upon the growth phase as well as the growth medium (29, 33). Alternatively, other factors, including the posttranslational modification of CRP, could play a role in determining the CRP activity, as shown in *V. fischeri* (35).

Regulation of *vvpM* transcription by the cAMP-CRP complex was found to be weak compared to the regulation by SmcR, since the maximal expression of *vvpM* in the Δcrp strain was only 4-fold higher than that in the wild type, whereas its maximal expression in the *smcR* mutant strain was 14 times that in the wild type (Fig. 3). In contrast, the maximal expression of the *vvpM-luxAB* transcription fusion was >100-fold higher in the *smcR Δcrp* double mutant than in the wild type. Accordingly, it is likely that the cAMP-CRP complex is required for SmcR to effectively and maximally repress *vvpM*. For the cooperative repression of *vvpM* expression by SmcR and the cAMP-CRP complex, it seems that CRP would have to directly interact with SmcR bound to the consensus sequence to strengthen the binding of SmcR. Two protected regions were detected in an autoradiogram in which DNase I-digested DNA probes bound to rCRP were separated by gel electrophoresis (Fig. 5B). One of the protected sites is located in the region +78 to +94 (CRP-2 site) relative to the *vvpM* TIS, which includes the IC of *vvpM*. Although repression of gene expression via binding of transcription factors to the regions downstream of the TIS has been observed (17, 36, 37), this mechanism has not yet been reported for the regulation by CRP. Consequently, to verify this CRP-binding site, an oligonucleotide probe containing CRP-2 sequences was applied to a gel shift assay (Fig. 6B). It revealed that the CRP-2 site was bound by cAMP-CRP complex, but its binding affinity with rCRP was much less than the binding affinity of the CRP-1 site (Fig. 6C).

Given that the CRP-1 site overlaps the SmcR-binding site (−2 to +20) (Fig. 5B), we hypothesize that the SmcR dimer binds one side of the DNA double helix and the CRP dimer binds the other side. Some nucleotides of CRP consensus sequence (underlined) are known to be essential for binding to CRP: sense strand, 5′-TGTGN₆TCACA-3′; antisense strand, 5′-ACACTN₆AGTGT-3′ (38). According to *in silico* analysis of structures of the dimeric forms of SmcR and CRP, the positions of those nucleotides required for the CRP interaction are unlikely to be masked by the binding of SmcR, and they appear to be accessible to dimeric CRP recognizing CRP consensus sequences in the complementary strand (K.-J. Lee and K.-H. Lee, unpublished data). Thus, two transcription factors were found to bind to the overlapping sites, which could facilitate the interaction between SmcR and CRP and consequently stabilize the binding of SmcR. In addition, it is further speculated that the bending of DNA strands containing the CRP-1 site, which has been induced by binding of cAMP-CRP complex, could increase the affinity of SmcR to its binding site on the other side of the CRP-1 site. Coactivation by SmcR and CRP was observed during induction of *vvpS* and *vvpE*, where the two binding sites are located at a distance from each other (Fig. 7): for *vvpE*, the CRP and SmcR sites are centered at positions −220 and 196.5, respectively, and for *vvpS*, the SmcR and CRP sites are centered at positions −166.5 and 137.5, respectively. The regulatory mechanism(s) underlying this phenomenon of simultaneous binding of two transcription factors to the same site should be resolved in a future study.

Characteristic expression of *vvpM*, which is tightly repressed by SmcR and CRP, suggests that a pathogenic activity, for example, an apoptotic activity induced by VvpM, operates before cell density becomes high enough to turn on QS regulation. Although most fatal toxins are expressed only after the pathogen reaches high cell
density at a colonizing site (24), some bacterial factors need to be expressed before significant damage to host cells or tissues occurs (39). These proteins include the factors required for the initial pathogenic interactions, such as the colonization and adaptation to a specific environment within a host (39). An apoptotic event in epithelial cells or preferential survival in blood mediated by VvpM may be important at the initial stage of host interactions of V. vulnificus.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The strains and plasmids used in this study are listed in Table 1. Escherichia coli strains were grown in Luria-Bertani (LB) (40) medium supplemented with appropriate antibiotics at 37°C. The medium for V. vulnificus growth was prepared by addition of NaCl to LB at a final concentration of 2.5% (wt/vol) (LBS medium) (2). V. vulnificus strains carrying a vvpM-luxAB transcription reporter fusion, pML_VvpM416 (17), were incubated in LBS or AB-succinate medium (300 mM NaCl, 50 mM MgSO4, 0.2% [wt/vol] vitamin-free Casamino Acids, 10 mM potassium phosphate, 1 mM l-arginine, 1% [vol/vol] succinate, pH 7.5) (41) supplemented with 3 μg/ml tetracycline. The freshly growing cells of V. vulnificus at an early exponential phase, in which the OD595 of cultures had reached approximately 0.1, were seeded to media for measuring the growth and light production. The light produced by V. vulnificus harboring the reporter fusion plasmid was measured in the presence of 0.006% (vol/vol) n-decyl aldehyde using a luminometer (TD-20/20 luminometer; Turners Designs). Specific bioluminescence was calculated by normalizing the relative light units (RLU) with respect to cell mass (OD595) as previously described (32).

Primer extension analysis. Total RNA was prepared from the wild-type strain (V. vulnificus MO6-24/O [42]) and its isogenic smcR mutant using an RNasey minikit (Qiagen) according to the manufacturer’s instructions. A primer, PEnvPm-R (5′-GGTGGTTTTGAAAGCTGAAACGCTCC-3′), was designed to be complementary to positions +15 to +42 with respect to the IC of the vvpM gene. The primer, labeled

FIG 7 Regulatory mechanisms of expression of three major extracellular proteases of V. vulnificus. (A) Genetic organization of vvpE (VVMO6_04367) and its regulatory regions. Two TISs, the α-dependent promoter Pα and αβ-dependent promoter Pαβ, and the corresponding promoters (sites at −10 and −35) are indicated with arrows and dots, respectively (32). The positions (relative to the TIS for Pα) of each binding site are designated by vertical lines for two transcription activators (CRP [−229 to −211] and SmcR [−207 to −186]) and a transcription repressor (Fur [−57 to +80]) (16, 17, 32). (B) Genetic organization of vvpS (VVMO6_01684) and its regulatory regions. The TIS and tentative αβ-dependent promoter are indicated with arrows and dots, respectively (9). The binding sites (relative to the vvpS TIS) for the transcription activators SmcR and CRP are designated in regions −179 to −154 and −149 to −126, respectively. Two binding sites for transcription repressor LeuO are in the regions −189 to −146 and −142 to −116 (9). (C) Genetic organization of vvpM (VVMO6_03483) and its regulatory regions. The TIS and the αβ-dependent promoter (sites at −10 and −35) are indicated with arrows and dots, respectively. The SmcR-binding site centered at position +9.5 and the CRP-binding site centered at position +16.5 (relative to the TIS) are designated by vertical lines. The other CRP-binding site (−78 to +94) showing the lower binding affinity was not included in this figure. The regulatory function of Fur in vvpM expression is known to be indirect via regulating smcR expression (17, 49).
were performed with the total RNA of reactions using Moloney murine leukemia virus reverse transcriptase and random 6-mer primer (TaKaRa).

GelDoc 1000/2000 and Quantity One software (Bio-Rad).

Two primers, PvvpM-F (5' -GGACTTGGATGCGCGTTGG-3') and PvvpM-R (5' -CATCAATCCTTGTCATCTATCCG-3'), were used to amplify a 328-bp DNA fragment containing the regulatory region for the vvpM gene (Fig. 4A and 5A). A resultant DNA fragment was labeled with (γ-32P]ATP using T4 polynucleotide kinase (TaKaRa Bio), was annealed to 100 μM total RNA in a hybridization buffer (10 mM Tris-HCl, 1 mM EDTA, 1.25 M KCl [pH 8.0]) for 3 min at 65°C (43). RNA was converted to cDNA with SuperScript II reverse transcriptase (Promega). The resultant cDNA products were precipitated and resolved on a sequencing gel beside the sequencing ladders generated with the same primer. The plasmid pML_VvpM416 was used as a template to produce sequencing products.

The same labeled DNA (2 nM) was incubated in a CRP reaction mixture (20 mM Tris-HCl [pH 8.0], 100 mM KCl, 2 mM dithiothreitol [DTT], and 0.2 mM EDTA [18]) with rSmcR (50 to 300 nM). The same labeled DNA (2 nM) was incubated in a CRP reaction mixture (20 mM Tris-HCl [pH 8.0], 50 mM KCl, 3 mM MgCl2, 0.1 mM DTT, 0.1 mM EDTA, and 500 μM cAMP [32]) with rCRP (50 to ~600 nM).
After addition of 3 μl of loading buffer, the samples were separated on a 5% nondenaturing polyacrylamide gel. For competition analysis, the same but unlabeled DNA was added to the binding reaction in 10- to 30-fold molar excess of the labeled probe. A 378-bp segment of DNA of the gap (glyceraldehyde-3-phosphate dehydrogenase gene) promoter region was amplified from V. vulnificus with primers gap-F (5’-GGGGATCCGAATTCATTTATGGTATTTATCGTTCAAGAGTGT-5’) and gap-R (5’-GGGAATTCCATGGTCTATTCCCTAATG-3’). The CRP-1 probe covering from +1 to +22 (relative to TIS for vvpM) was synthesized (top, 5’-GAAGAAATGTCATCTTGGTGGAGCCTGCA-3’; bottom, 3’-CTTGTTTTACCTGAAGTGAAACCTCCGCAAGT-5’). The CRP-2 probe covering from +23 to +101 (relative to TIS for vvpM) was synthesized (top, 5’-GGGAATTCCATGGTGCTTTTCTGTTCAAGAGTGT-5’) (bottom, 3’-CTTGTTTTACCTGAAGTGAAACCTCCGCAAGT-5’). The underlines indicate the altered nucleotides). Each oligonucleotide pair was labeled with 32P, 50 nM probes were incubated in a CRP reaction mixture with rCRP (100 to –900 nM), and then the reaction mixtures were resolved on 8% nondenaturing polyacrylamide gel.

Site-directed mutagenesis of the regulatory regions. To amplify the mutant probe (I), including the altered upstream region (–2 to approximately +20 relative to the TIS of vvpM), two sets of primers, vvpM-EcoRI/F (5’-GGAAGAAATGTCAGCTTGGTGGAGCCTGCA-3’; 5’-GGGAATTCCATGGTGCTTTTCTATTCCCTAATG-3’), and vvpM-Smt-R (5’-GGGAATTCCATGGTGCTTTTCTGTTCAAGAGTGT-3’; 5’-GGGAATTCCATGGTCTATTCCCTAATG-3’), were used to amplify the vvpM promoter region. The underlines indicate the altered nucleotides. The CRP-1 and CRP-2 probes were designed using the 30-fold molar excess of the labeled probe. A 378-bp segment of DNA of the gap (glyceraldehyde-3-phosphate dehydrogenase gene) promoter region was amplified from V. vulnificus with primers gap-F and gap-R (5’-GGGAATTCCATGGTGCTTTTCTATTCCCTAATG-5’; 5’-GGGAATTCCATGGTCTATTCCCTAATG-3’). The CRP-1 probe covering from +1 to +22 (relative to TIS for vvpM) was synthesized (top, 5’-GAAGAAATGTCATCTTGGTGGAGCCTGCA-3’; bottom, 3’-CTTGTTTTACCTGAAGTGAAACCTCCGCAAGT-5’). The CRP-2 probe covering from +23 to +101 (relative to TIS for vvpM) was synthesized (top, 5’-GGGAATTCCATGGTGCTTTTCTGTTCAAGAGTGT-5’) (bottom, 3’-CTTGTTTTACCTGAAGTGAAACCTCCGCAAGT-5’). The underlines indicate the altered nucleotides. Each oligonucleotide pair was labeled with 32P, 50 nM probes were incubated in a CRP reaction mixture with rCRP (100 to –900 nM), and then the reaction mixtures were resolved on 8% nondenaturing polyacrylamide gel.

DNase I footprinting assay. The DNA probe (3 nM) used for the gel shift assay was incubated with rSmcR (0.2 to –3 μM) or rCRP (0.5 to –10 μM) at 37°C for 30 min. The reaction mixture then was treated with DNase I for 1 min and terminated with stop buffer (200 mM NaCl, 20 mM EDTA, 1% [wt/vol] SDS, and 250 μg/ml tRNA). After precipitation with ethanol, the digested DNA products were resolved on a 6% polyacrylamide sequencing gel alongside sequencing ladders (43). The sequencing reactions were performed on pML/VvpM416 using a 32P-labeled primer, vvpM-FR. The mutant DNA fragment with mutagenized SmcR-binding site was cloned into the pTOP Blunt V2 vector (Enzymomics) to produce pTOP_probe (I). The mutagenized nucleotide sequences were confirmed by DNA sequencing. The plasmid was used as the template DNA to amplify the mutant probe I for the gel shift assay as described above.

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Statistical analyses. Results were expressed as means ± standard deviations. Statistical analysis was performed using Student’s t test (SYSTAT and SigmaPlot version 9; Systat Software, Inc.). P values of >0.01 were designated nonsignificant.

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We have no conflicts of interest to declare.

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