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SmcR and Cyclic AMP Receptor Protein Coactivate *Vibrio vulnificus vvpE* Encoding Elastase through the RpoS-dependent Promoter in a Synergistic Manner*

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Hye Sook Jeong‡, Myoung Hee Lee‡, Kyu-Ho Lee§, Soon-Jung Park¶, and Sang Ho Choi‡‖

From the ‡Departments of Food Science and Technology and of Molecular Biotechnology, Biotechnology Research Institute, Chonnam National University, Kwang-Ju 500-757, the §Department of Environmental Science, Hankuk University of Foreign Studies, Yongin, Kyunggi-Do 449-791, and the ¶Department of Parasitology, Yonsei University, College of Medicine, Seoul 133-791, South Korea

The putative virulence factors of *Vibrio vulnificus* include an elastase, the gene product of *vvpE*. We previously demonstrated that *vvpE* expression is differentially directed by two different promoters in a growth phase-dependent manner. The activity of the stationary-phase promoter (promoter S (PS)) is dependent on RpoS and is also under the positive control of cyclic AMP receptor protein (CRP). In this study, primer extension analyses revealed that SmcR, the *Vibrio harveyi* LuxR homolog, is also involved in the regulation of *vvpE* transcription by activating PS. Although the influence of CRP on PS is mediated by SmcR, the level of PS activity observed when CRP and SmcR function together was found to be greater than the sum of the PS activities achieved by each activator alone. Western blot analyses demonstrated that the cellular levels of RpoS, CRP, and SmcR were not significantly affected by one other, indicating that CRP and SmcR function cooperatively to activate PS rather than sequentially in a regulatory cascade. The binding sites for CRP and SmcR were mapped based on a deletion analysis of the *vvpE* promoter region and confirmed by *in vitro* DNase I protection assays. The binding sites for CRP and SmcR were juxtapositioned and centered 220 and 198 bp upstream of the transcription start site of PS, respectively. Accordingly, these results reveal that CRP and SmcR function synergistically to coactivate the expression of *vvpE* by the RpoS-dependent promoter (PS) and that the activators exert their effect by directly binding to the promoter in the stationary phase.

The pathogenic marine bacterium *Vibrio vulnificus* is the causative agent of food-borne diseases, including life-threatening septicemia and possibly gastroenteritis, in individuals with underlying predisposed conditions such as liver damage, excess levels of iron, and immunocompromised conditions. Wound infections also result from exposure to seawater or from the handling of shellfish contaminated with *V. vulnificus*. The mortality from septicemia is very high (>50%), and death can occur within 1–2 days after the first signs of illness. Several potential virulence factors, including an endotoxin, polysaccharide capsule, iron-sequestering systems, cytolytic hemolysin, elastase, phospholipase A_2, and other exotoxins, have been identified in *V. vulnificus* (for recent reviews, see Refs. 1 and 2).

The elastase activity is from a neutral metalloprotease and represents the major proteolytic activity of *V. vulnificus* (3, 4). Our previous study revealed the existence of at least two proteases that are produced by *V. vulnificus* (5). Therefore, *vvpE* was designed to differentiate the elastase gene from other genes encoding other potential proteases of *V. vulnificus* (5). As such, a gene encoding the *V. vulnificus* elastase was recently cloned and sequenced by us (5) and by others (6). The deduced gene product was predicted to be a 609-amino acid polypeptide, and the mature elastase is a 45-kDa protein consisting of 413 amino acids generated by the deletion of the N-terminal 196 amino acids. Using the mature protease purified from recombinant *Escherichia coli*, two functional domains, a 35-kDa N-terminal domain required for catalytic activity and 10-kDa domain required for attachment to substrate, were identified (7).

The characteristics of elastase as a potential virulence factor have primarily been studied using the purified protein in animal models (reviewed in Ref. 8). The injection of purified elastase can reproduce many aspects of the diseases caused by *V. vulnificus*, including dermonecrosis, tissue destruction, edema, and ulceration. These diverse activities are believed to be caused by the proteolytic degradation or inactivation of biologically important host proteins and immune system components such as collagen, fibrin, and complement. Conversely, increased vascular permeability is stimulated by the direct activation of the Hageman factor and prekallikrein by elastase, leading to the production of bradykinin. In addition, the activity of elastase toward host iron-binding proteins is involved in the utilization of heme and iron.

Compared with the substantial number of reports on the characterization of purified elastase, there have been only a few studies on the regulatory mechanism used by the bacterium to modulate the expression of the *vvpE* gene (9, 10). In a previous report, we showed that transcription of the *vvpE* gene of *V. vulnificus* is initiated by two different types of promoter, promoter L (PL)[1] and promoter S (PS), in a growth phase-dependent manner (9). The basal level expression of *vvpE* is directed by PL, independent of RpoS and cyclic AMP receptor protein (CRP), and remains low throughout the log and stationary growth phases. In addition to this basal level, more *vvpE* expression is induced by PS in the stationary phase,

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‖ To whom correspondence should be addressed. Tel.: 82-62-530-2146; Fax: 82-62-530-2149; E-mail: shchoi@chonnam.ac.kr.

[1] The abbreviations used are: PL, promoter L; PS, promoter S; CRP, cyclic AMP receptor protein; IPTG, isopropyl-β-D-thiogalactopyranoside; RNAP, RNA polymerase; α-CTD, α-subunit C-terminal domain; IHF, integration host factor.

TABLE I
Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Ref. or source
Strains		
V. vulnificus		
ATCC29307	Clinical isolate	Laboratory collection
KP101	ATCC29307, Δ*rpoS*	[b]
DI0201	ATCC29307,Δ*crp*	[c]
KC74	ATCC29307, *crp*::*nptI*	9
HS03	ATCC29307, *smcR*::*nptI*	This study
HS04	ATCC29307, Δ*rpoS*, *smcR*::*nptI*	This study
DI0202	ATCC29307, Δ*crp*, *smcR*::*nptI*	This study
E. coli		
DH5α	*supE44* Δ*lacU169* (ϕ80 *lacZ* ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Laboratory collection
SM10 λpir	*Thi thr leu tonA lacY supE rec*A::RP4–2-Tc::Mu λ*pir*, *oriT* of RP4; Km^r; conjugational donor	14
BL21 (DE3)	F^-, *ompT*, *hsdS* (r_B^-, m_B^-), *gal*(DE3)	Laboratory collection
Plasmids		
pRK415	Broad host range vector, IncP *ori*, *oriT* of RK2; Tc^r	18
pCVD442	R6K γ *ori*, *sacB*, *oriT* of RP4; Ap^r	13
pKC980	pUC18 with *vvpE*; Ap^r	5
pHS0001	pRK415 with *rpoS*; Tc^r	9
pKC0004	pRK415 with *crp*; Tc^r	9
pKP001	pQE32 with *rpoS*; Ap^r	[b]
pHK0201	pRSET A with *crp*; Ap^r	16
pHS103	pUC18 with *smcR*; Ap^r	[c]
pHS1031	pHS103 with *nptI*; Ap^r, Km^r	This study
pHS1032	pCVD442 with *smcR*::*nptI*	This study
pHS104	pRSET C with *smcR*; Ap^r	This study
pHS105	pRK415 with *smcR*; Tc^r	This study
pHK0011	pRK415 with promoterless *luxAB*; Tc^r	9
pHS201	pHK0011 with 748-bp fragment of *vvpE* upstream region; Tc^r	This study
pHS202	pHK0011 with 634-bp fragment of *vvpE* upstream region; Tc^r	This study
pHS203	pHK0011 with 620-bp fragment of *vvpE* upstream region; Tc^r	This study
pHS204	pHK0011 with 527-bp fragment of *vvpE* upstream region; Tc^r	This study
pHS205	pHK0011 with 441-bp fragment of *vvpE* upstream region; Tc^r	This study

[a] Ap^r, ampicillin-resistant; Km^r, kanamycin-resistant; Tc^r, tetracycline-resistant.
[b] K.-H. Lee, manuscript in preparation.
[c] S. H. Choi, manuscript in preparation.

which is under the positive control of both CRP and RpoS. This differential utilization of two promoters can permit precise levels of elastase in response to modifications of the environment and growth stage. Meanwhile, it has been reported that SmcR, the *Vibrio harveyi* LuxR homolog, is also involved in the positive control of *vvpE* expression (10).

However, until now, no molecular analysis of the role of CRP and SmcR in *vvpE* expression has been reported. Neither the promoter(s) of the *vvpE* gene activated by SmcR nor the sequences upstream of *vvpE* required for activation by CRP and SmcR have been previously identified. Furthermore, the question of whether the activators directly or indirectly affect *vvpE* expression has not yet been addressed. Accordingly, in an effort to elucidate the regulatory mechanism of *vvpE* expression at a molecular level, this study examined the influence of the mutation of *smcR* on the activity of *vvpE* promoters. The relationship between RpoS, CRP, and SmcR was also examined by determining the cellular level of the proteins in an *rpoS*, *crp*, and *smcR* background. As a result, it was demonstrated that CRP and SmcR coactivate PS of *vvpE* in a synergistic and seemingly growth phase-dependent manner. Finally, the binding of CRP and SmcR directly to the upstream portion of *vvpE* was demonstrated, and the binding sites for CRP and SmcR were determined.

EXPERIMENTAL PROCEDURES

Strains, Plasmids, and Culture Media—The strains and plasmids used in this study are listed in Table I. The *E. coli* strains used for plasmid DNA replication or conjugational transfer of plasmids were grown in LB broth with or without 1.5% (w/v) agar. Unless noted otherwise, the *V. vulnificus* strains were grown in LB medium supplemented with 2.0% (w/v) NaCl (LBS). When required, appropriate antibiotics were added to the media as follows: 100 μg/ml ampicillin, 50 μg/ml kanamycin, and 10 μg/ml tetracycline.

Measurement of Cell Growth and Elastase Activities—Cultures of the *V. vulnificus* strains were grown at 30 °C under aeration, and growth was monitored by measuring the A_{600} of the cultures. Cultures incubated for 12–16 h (A_{600} = 2.0) were harvested, and the elastase activities in the stationary phase were determined according to previously described procedures (5). The means ± S.E. were calculated from at least three independent experiments.

General Genetic Methods—Isolation of the plasmid DNA and genomic DNA and transformations were carried out according to the procedures described by Sambrook and Russell (11). The restriction and DNA-modifying enzymes (New England Biolabs Inc., Beverly, MA) were used as recommended by the manufacturer. The DNA fragments were purified from agarose gels using a Geneclean II kit (Bio 101, Inc., Vista, CA). The primary DNA cloning and manipulation were conducted in *E. coli* DH5α, and restriction mapping was used to confirm that the transformants contained the appropriate plasmids. PCR amplification of the DNA was performed using a GeneAmp PCR 2400 system (PerkinElmer Life Sciences) and standard protocols.

Generation of smcR::nptI Mutants—A 1.2-kb DNA fragment from *V. vulnificus* (ATCC29307) that carried the entire *smcR* coding region was cloned into pHS103 [2] (Table I). To inactivate *smcR in vitro*, a 1.2-kb *nptI* DNA conferring resistance to kanamycin (12) was inserted into a unique ClaI site present within the coding region of *smcR*. The 2.4-kb *smcR*::*nptI* cartridge was then liberated from the resulting construct (pHS1031) and ligated with SmaI-digested pCVD442 (13) to form pHS1032 (Table I). To generate the *smcR*::*nptI* mutant by homologous recombination, *E. coli* SM10 λ*pir*, *tra* (containing pHS1032) (14) was used as a conjugal donor to *V. vulnificus* ATCC29307 (see Fig. 1A). For construction of the *smcR rpoS* or *smcR crp* double mutant, isogenic mutants of ATCC29307, which lack either *rpoS* (KP101)[3] or *crp* (DI0201),[2] were used as the recipients. The conjugation and isolation of the transconjugants were conducted using previously described methods (5, 9, 15).

Overexpression and Purification of V. vulnificus SmcR, CRP, and RpoS—The coding region of *smcR* was amplified using the chromosomal

[2] S. H. Choi, manuscript in preparation.
[3] K.-H. Lee, manuscript in preparation.

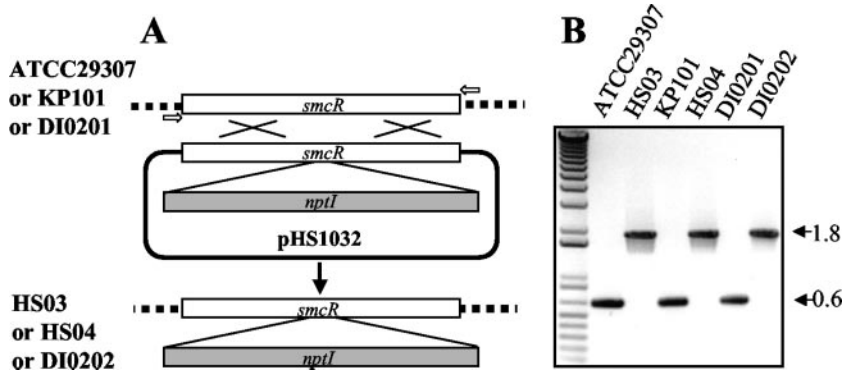

FIG. 1. **Diagram of allelic exchange and confirmation of *smcR::nptI* mutants.** *A*, homologous recombination between the chromosomal *smcR* gene from strain ATCC29307, KP101, or DI0201 and pHS1032. *Dashed lines*, chromosomal DNA; *solid line*, plasmid DNA; *open boxes*, the target *smcR* gene; *shaded boxes*, the *nptI* gene; *open arrows*, locations of the oligonucleotide primers used to confirm the *nptI* insert; ×, crossover. *B*, PCR analysis of ATCC29307 and isogenic mutants generated by allelic exchange. Molecular size markers (1-kb ladder, Invitrogen) and PCR products (in kb) are indicated.

DNA of *V. vulnificus* ATCC29307 as the template and oligonucleotide primers His-SmcR001 and His-SmcR002 (see Table II). The 0.6-kb PCR product was subcloned into a His_6 tagging expression vector, pRSET C (Invitrogen). The resulting plasmid, pHS104, encoded SmcR with a His_6 tag at the amino terminus. The His-tagged SmcR protein was then expressed in *E. coli* BL21(DE3), and the protein was purified by affinity chromatography according to the manufacturer's procedure (QIAGEN Inc., Valencia, CA). In a similar way, the expression and purification of His-tagged CRP and His-tagged RpoS were carried out using pHK0201 and pKP001,[3] carrying the *V. vulnificus crp* and *rpoS* genes, respectively, as described (16).

Western Blot Analysis of V. vulnificus SmcR, CRP, and RpoS Proteins—The purified His-tagged proteins were used to raise primary antibodies against SmcR, CRP, and RpoS of *V. vulnificus*. Polyclonal antibodies specific to each protein were made by immunizing Sprague-Dawley rats on three occasions at 3-week intervals with 200 μg of the protein for each immunization. Western immunoblotting was performed according to the procedure described previously by Jeong *et al.* (5). Briefly, the cellular proteins of the wild type and its isogenic mutants grown to the log and stationary phases were resolved by SDS-PAGE (17). The resolved proteins were then transferred to a nitrocellulose membrane (Bio-Rad) and probed with a 1:5000 dilution of the rat polyclonal antibodies. The bound antibodies were detected using goat anti-rat IgG conjugated with alkaline phosphatase (Sigma) and visualized by incubation with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate (Sigma) (5).

Transcript Analysis—Total cellular RNA was isolated from the *V. vulnificus* strains at different growth phases using a TRIzol reagent kit (Invitrogen) according to the manufacturer's specifications. For the primer extension experiments, an end-labeled 24-base primer (VVPE9905) complementary to a coding region of *vvpE* was added to the RNA and then extended with SuperScript II RNase H^- reverse transcriptase (Invitrogen) as described previously (9, 16). The cDNA products were then purified and resolved on a sequencing gel alongside sequencing ladders generated with the same primer used for the primer extension. The nucleotide sequence for the plasmid DNA of pKC980 (5) was determined using the dideoxy chain termination method with Top™ DNA polymerase (Bioneer, Seoul, Korea) following the manufacturer's protocols.

For Northern slot blot analysis, a series of reactions was performed according to standard procedures (11) with 20 μg of total RNA. A 1.2-kb HindIII-HindIII DNA probe representing the internal sequences of *vvpE* was labeled with [α-^{32}P]dCTP using a Prime-a-gene labeling system (Promega, Madison, WI) and used for hybridization as described previously (9). The primer extension products and Northern hybridization blots were visualized using a phosphorimage analyzer (Model BAS1500, Fuji Photo Film Co. Ltd., Tokyo, Japan).

Construction of a Set of vvpE-luxAB Transcriptional Fusions—A set of *vvpE-luxAB* transcriptional fusion reporters was created by subcloning a series of DNA fragments that overlapped the *vvpE* promoter region into pHK0011 (see Fig. 6*A*) that was carrying promoterless *luxAB* luciferase genes (9). The subcloned fragments were amplified by PCR using pKC980 (5) as the template. Primer VVPE006 (see Table II) included an XbaI restriction site followed by bases corresponding to the 5′-end of the *vvpE* coding region. VVPE006 was used in conjunction with one of the following primers to amplify the DNA upstream of *vvpE*: VVPE001 (for pHS201), VVPE002 (for pHS202), VVPE003 (for pHS203), VVPE004 (for pHS204), or VVPE005 (for pHS205) (see Table II). A KpnI restriction site was added to these primers to facilitate the cloning of the PCR products. The DNA fragments were digested with XbaI and KpnI and inserted into pHK0011 that had been digested with the same enzymes, thereby creating five *vvpE-luxAB* reporter constructs, as confirmed by DNA sequencing. The *vvpE-luxAB* reporters were then transferred into ATCC29307 and the isogenic mutants by conjugation. The cellular luminescence of the cultures was measured with a luminometer (Lumat Model 9501, Berthold, Wildbad, Germany) and expressed in arbitrary relative light units as described previously (9, 16).

Gel Mobility Shift Assay and DNase I Footprinting—The gel shift assays were performed according to standard procedures (11). The 200-bp upstream region of *vvpE*, extending from residues −300 to −101 with respect to the +1 transcription start site, was amplified by PCR using ^{32}P-labeled VVPE021 and unlabeled VVPE022 as the primers. The binding of CRP to the labeled DNA and electrophoretic analysis of the CRP·DNA complexes have already been described (16). The protein-DNA binding reactions with SmcR were the same as those with CRP, except that cAMP was omitted from the reaction buffer. For competition analyses, the same but unlabeled 200-bp DNA fragment was used as a competitor DNA.

For the DNase I protection assays, a 270-bp fragment of the *vvpE* promoter region was generated by PCR amplification using a combination of ^{32}P-labeled and unlabeled primers VVPE023 and VVPE024. The binding of CRP to the labeled DNA and DNase I digestion of the CRP·DNA complexes was carried out following the procedures described previously by Choi *et al.* (16). After precipitation with ethanol, the digested DNA products were resolved on a sequencing gel alongside sequencing ladders of pKC980 generated using either VVPE023 (for the coding strand) or VVPE024 (for the noncoding strand) as the primer. Similar experimental conditions were used with SmcR, except that cAMP was omitted from the reaction buffer for the SmcR·DNA complex formation. The gels were visualized as described for the primer extension analyses.

RESULTS

Construction and Confirmation of V. vulnificus smcR, smcR crp, and smcR rpoS Mutants—Previously, it has been suggested that SmcR positively regulates *vvpE* gene expression in *V. vulnificus* (10). Thus, to further examine the regulation of *vvpE* by SmcR, *V. vulnificus smcR* mutants were constructed by allelic exchange (Fig. 1*A*). A double crossover in which each wild-type *smcR* gene was replaced with an *smcR*::*nptI* allele was confirmed by PCR as shown in Fig. 1*B*. The PCR analysis of the genomic DNA from ATCC29307, KP101, and DI0201 using primers SmcR001 and SmcR002 (Table II) produced a 0.6-kb fragment (Fig. 1*B*); meanwhile, the genomic DNA from the *smcR*::*nptI* mutants resulted in an amplified DNA fragment ~1.8 kb in length. The 1.8-kb fragment was in agreement with the projected size of a DNA fragment containing the wild-type *smcR* (0.6 kb) and *nptI* (1.2 kb) genes. The mutants

TABLE II
Oligonucleotides used in this study

Oligonucleotide	Oligonucleotide sequence (5′–3′)[a]	Location[b]	Use
VVPE9905	GACGTTGATTGAGTTTCATTATCG	69–92	Primer extension
VVPE001	GTAGGTACCACTCAAGCTGACGAACTTGATC	−335 to −314	Promoter deletion
VVPE002	GTTGGTACCCTGTCCCATTTATCTTATTGATA	−221 to −199	Promoter deletion
VVPE003	CTTGGTACCTTATTGATAAATCTGCGTAAA	−207 to −187	Promoter deletion
VVPE004	CAGGTACCAGTGAGATGGATTCTTTGTATAACA	−114 to −90	Promoter deletion
VVPE005	GTTGGTACCATTTTCTGAACCATGGTTGTTG	−28 to −10	Promoter deletion
VVPE006	GAATCTAGACCGATACAGAAGGCAGATCGGC	392–413	Promoter deletion
VVPE021	AGAATGGCGATTTTCATAG	−300 to −282	Gel mobility shift assay
VVPE022	GAATCCATCTCACTGCGA	−118 to −101	Gel mobility shift assay
VVPE023	CGTCGAGAATGGCGATTTTC	−305 to −285	DNase I footprinting
VVPE024	AGCCAACTTCACCAAAAAA	−55 to −36	DNase I footprinting
SmcR001	ATGGACTCAATCGCAAAGA	Chromosomal DNA	Mutant construction
SmcR002	TAGGCGTGCTCGCGTTTA	Chromosomal DNA	Mutant construction
SmcR003	GAATCTAGAAAGGAAACAACCTATGGACTC	Chromosomal DNA	Complementation of *smcR*
SmcR004	GAAGAATTCCCTTGATATGAGGTTACTGG	Chromosomal DNA	Complementation of *smcR*
HIS-SmcR001	ATACTGCAGATGGACTCAATCGCAAAGAG	Chromosomal DNA	Amplification of *smcR*
HIS-SmcR002	TATGAATTCTATTCGTGCTCGCGTTTATAG	Chromosomal DNA	Amplification of *smcR*

[a] Regions of oligonucleotides not complementary to the corresponding genes are underlined.
[b] Shown are the oligonucleotide positions, where +1 is the transcription start site of *vvpE*.

chosen for further analysis were named HS03 for the s*mcR* mutant, HS04 for the *smcR rpoS* double mutant, and DI0202 for the *smcR crp* double mutant (Fig. 1*B* and Table I). To determine the stability of the insertional mutation, the mutant strains were grown overnight without kanamycin selection. The inserted *nptI* DNA was stably maintained as determined by the sustained kanamycin resistance (all of >500 colonies tested) and generation of the appropriately sized DNA fragment by PCR (data not shown).

Effect of smcR Mutation on Production of Elastase—When the *smcR* mutant HS03 was compared with its parental wild type during stationary growth, it produced much less elastase, and the level of elastase activity was almost 10-fold less than in the wild type (Fig. 2). The *vvpE* transcript was not apparent in the *smcR* mutant, indicating that the effect of SmcR on the expression of elastase is at the level of transcription. We examined whether the reintroduction of recombinant *smcR* could complement the decrease in elastase activity in HS03. For this purpose, plasmid pHS105 was constructed by subcloning the *smcR* coding region, which was amplified by PCR using primers SmcR003 and SmcR004 (Table II), into pRK415 under the control of an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter (18). When *smcR* was induced by IPTG, the elastase activity and *vvpE* transcript of HS03 (pHS105) in the stationary phase were restored to levels comparable to those in the wild type (Fig. 2). Therefore, the decreased elastase activity of HS03 apparently resulted from the inactivation of functional *smcR* rather than any polar effects on genes downstream of *smcR*.

The elastase activity in the *smcR rpoS* double mutant HS04 was present at ~2 units (Fig. 2), which was much lower than that reached by the wild type, yet indistinguishable from that in the *rpoS* single mutant KP101 (Fig. 2). The expression of *vvpE* remained low unless the functional gene product of *rpoS* was provided and was completely unaffected by the additional inactivation of *smcR*. Furthermore, it was noted that the repressed level of elastase in HS04 was not restored at all by the reintroduction of either SmcR (pHS105) or RpoS (pHS0001) (Fig. 2), indicating that SmcR is capable of activating the expression of *vvpE* only when RpoS is present and that SmcR exerts its effects on *vvpE* expression through a promoter whose activity depends on RpoS.

Effect of SmcR on vvpE Expression Is Mediated through the RpoS-dependent Promoter—A previous study suggested that the expression of *vvpE* is directed by two different types of promoters, PL and PS, in a growth phase-dependent manner (9). The activity of PS, induced only when the cells enter the stationary phase, was entirely dependent on RpoS and was also under the positive control of CRP (Fig. 3). As such, the presence of at least two promoters for *vvpE* raises the question of whether the regulation by SmcR is through the stationary phase-induced promoter (PS) or the constitutive promoter (PL). To answer this question, the activities of PL and PS in the wild type and isogenic *smcR* mutant were determined by primer extension analyses. As such, RNA was prepared from cultures grown to the log or stationary phase. PL activities were observed in the cells grown to both the log and stationary phases. In addition, when determined based on the intensity of the bands of the reverse transcripts, the PL activities were not significantly changed by the inactivation of *smcR*. These results are consistent with our previous observations that the activity of PL is constitutive regardless of the growth phase and is apparently independent of the regulatory factors required for the activation of PS (9). In contrast to PL, no band corresponding to PS activity was detected in the RNA from the *smcR* mutant. Accordingly, these results indicate that SmcR is involved in the regulation of *vvpE* transcription by activating the RpoS-dependent promoter (PS) (Fig. 3).

Effect of CRP on vvpE Expression Is Mediated through SmcR—When the *crp* mutant DI0201 was compared with its parental wild type during stationary growth, it produced less elastase, and the level of elastase activity was almost 4-fold less than in the wild type (Fig. 4). The levels of elastase activity and *vvpE* transcript in the *smcR crp* double mutant DI0202 were determined and found to be lower than those in the *crp* single mutant DI0201, yet indistinguishable from those in the *smcR* single mutant HS03. As such, these results indicated that the mutation of *crp* reduced the activity of PS; however, the additional inactivation of *crp* had no influence on the PS activity in the *smcR* mutant. For the complementation of DI0202, the plasmid pKC0004 was constructed by subcloning *crp* in a similar way to the construction of pHS105 (9). The transfer and induction of pKC0004 provided the *smcR crp* double mutant DI0202 with an increased level of CRP. Western blot analysis revealed that the level of CRP in the DI0202 (pKC0004) cells was relatively higher than that in the *smcR* single mutant HS03 cells, in which all CRP molecules are products of the *crp* gene carried on the chromosome and expressed by its own promoter (Fig. 4). However, the repressed levels of elastase activity and *vvpE* transcript were not recovered at all in DI0202 (pKC0004) (Fig. 4), and the levels were almost identical to those in HS03. Therefore, these combined results indicate

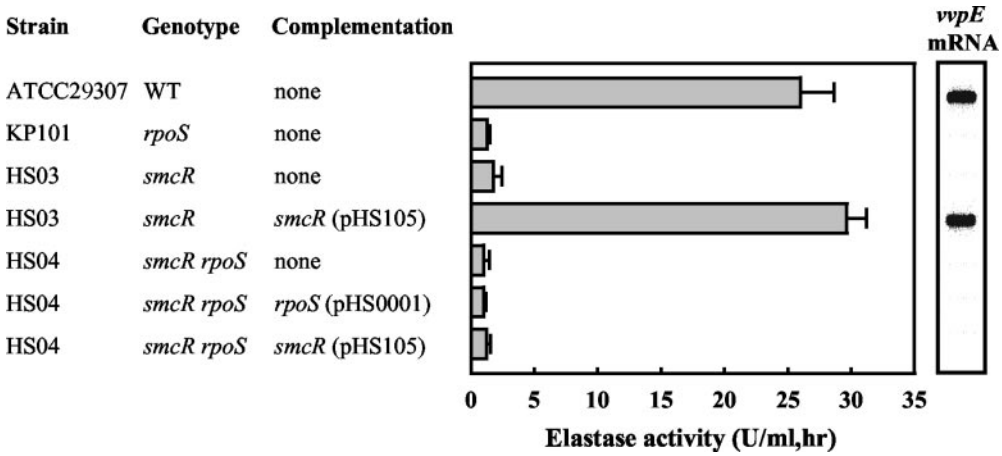

FIG. 2. **Dependence of elastase production on SmcR and RpoS.** Cultures of the wild type (*WT*) and isogenic mutants were grown in LBS, and then samples removed during the stationary phase (A_{600} = 2.0) were analyzed for their elastase activity and *vvpE* transcript levels. For a complementation test, when the cultures reached an A_{600} of 0.6, IPTG was added to a final concentration of 0.1 mM to induce the expression of recombinant *smcR* (*i.e.* on pHS105) or *rpoS* (*i.e.* on pHS0001) as indicated. Details for determining the elastase activity and a Northern blot of the *vvpE* transcript are given under "Experimental Procedures." *Error bars* represent S.E.

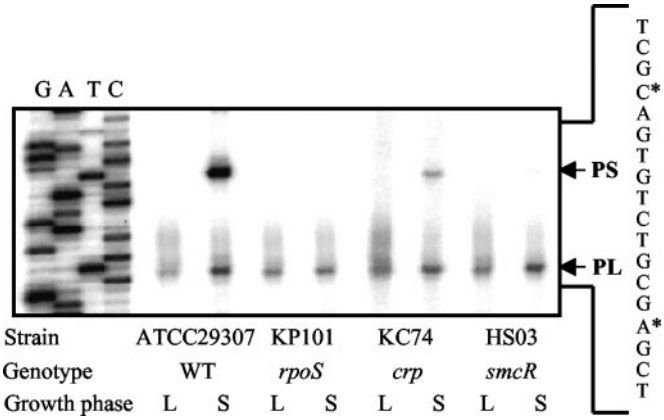

FIG. 3. **Activities of *vvpE* promoters PL and PS in *V. vulnificus* with different genetic backgrounds.** The PL and PS activities were determined separately by primer extension of the RNA derived from the wild type (*WT*) and isogenic mutants as indicated. Total RNA was prepared from the log phase (*L*; A_{600} = 0.6) and stationary phase (*S*; A_{600} = 2.0) of each culture. *G*, *A*, *T*, and *C* represent the nucleotide sequencing ladders of pKC980. *Asterisks* indicate the transcription start sites for PL (A) and PS (C).

that a variation in the cellular level of CRP did not affect the activity of PS unless SmcR was also present, thereby suggesting that the activation of PS by CRP is mediated through SmcR.

CRP and SmcR Coactivate vvpE Expression Synergistically—To determine whether an increased amount of SmcR would compensate for a lack of CRP in the activation of PS, the *smcR* expression plasmid pHS105 was introduced into the *smcR crp* double mutant DI0202. When *smcR* was induced by IPTG, the cellular level of SmcR in DI0202 (pHS105) was higher than that in the *crp* single mutant DI0201 (Fig. 4). However, the levels of elastase and *vvpE* transcript in DI0202 (pHS105) were only one-fourth of those in the wild type, yet were indistinguishable from the levels in DI0201 (Fig. 4), indicating that SmcR, even when overproduced, is unable to activate PS to the wild-type level in the absence of CRP. Thus, even though the effect of CRP on *vvpE* expression occurred through SmcR and was weaker than that of SmcR, overproduced SmcR was still unable to compensate for the lack of CRP.

Accordingly, it would seem that the activation of *vvpE* to the wild-type level requires both CRP and SmcR simultaneously. CRP alone had little effect on *vvpE* expression with PS, as the elastase activity of the cells carrying only CRP was ~2 units, which was indistinguishable from the basal level (*i.e.* without both activators). Cells carrying only SmcR expressed ~3 times more *vvpE* than the basal level (~6 units of elastase) (Fig. 4). The elastase activity in the wild type, in which both CRP and SmcR functioned together, was ~25 units, which was even greater than the sum of the *vvpE* expression achieved by each activator alone. Therefore, these combined results suggest that CRP and SmcR function synergistically to coactivate the expression of *vvpE* with the RpoS-dependent promoter (PS).

CRP and SmcR Function Cooperatively Rather than Sequentially to Activate vvpE Expression—Different mechanisms are possible for this coactivation of PS by CRP and SmcR. For example, multiple activators function sequentially in a regulatory cascade, where one activator influences the accumulation of another regulator(s), which in turn is directly responsible for the activation of PS. To test this possibility, the cellular levels of RpoS, CRP, and SmcR were determined in the same amount of total protein isolated from the wild type and its isogenic mutants (Fig. 5).

In the wild type, no RpoS was detected in the log phase, yet it appeared in stationary-phase cells. This result was consistent with the observation that the activity of the RpoS-dependent promoter (PS) appeared only in stationary-phase cells. Western blot analysis revealed that the cellular levels of RpoS in *crp* and *smcR* mutant stationary-phase cells were not significantly lower than those in the wild type, indicating that neither CRP nor SmcR influences the accumulation of RpoS, at least in stationary-phase cells. From this result, it is unlikely that CRP or SmcR indirectly activates the activity of PS by increasing the cellular level of RpoS, which is required for PS activity.

The level of SmcR was also higher in stationary-phase cells than in log-phase cells. In contrast to RpoS and SmcR, the cellular levels of CRP did not vary significantly in cells in different growth phases (Fig. 5). We noted that neither activator affected the cellular level of the other, *i.e.* compared with the wild type, the *smcR* mutant strain did not exhibit any significant changes in the cellular level of CRP and *vice versa*. Despite the observation that the activation of PS by CRP was mediated by SmcR, the above result indicates that the influence of CRP on PS is not the result of increasing the level of SmcR in cells. Consequently, it would appear that CRP and SmcR function cooperatively to activate PS activity rather than sequentially in a regulatory cascade.

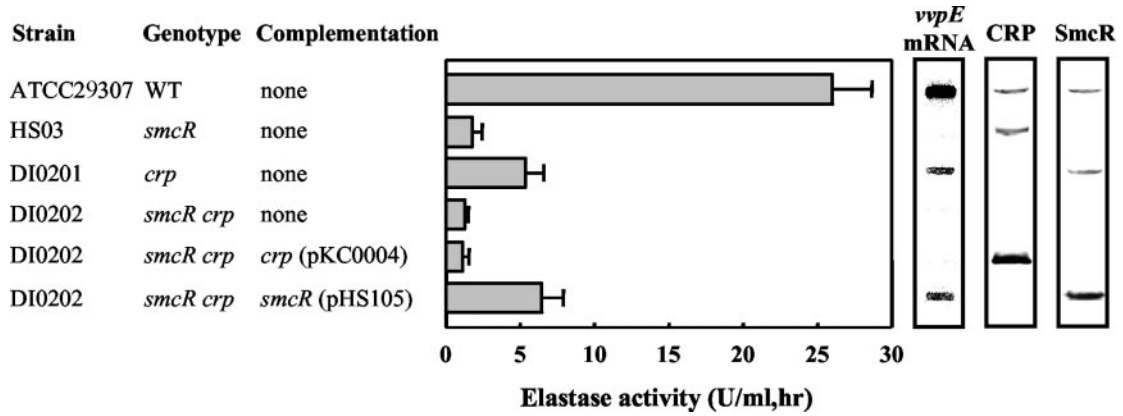

FIG. 4. **SmcR and CRP cooperate synergistically to control *vvpE*.** Samples were removed from cultures of the wild type (*WT*) and isogenic mutants grown to the stationary phase (A_{600} = 2.0). The samples were analyzed to determine elastase activity, *vvpE* transcript, and CRP or SmcR protein levels. Details for determining the elastase activity and a Northern blot of the *vvpE* transcript are described under "Experimental Procedures." The cellular levels of CRP and SmcR were determined by Western blot analyses using the IgG fractions of rat anti-*V. vulnificus* CRP and rat anti-*V. vulnificus* SmcR sera, respectively. For complementation tests, when the cultures reached an A_{600} of 0.6, IPTG was added to a final concentration of 0.1 mM to induce the expression of recombinant *crp* (*i.e.* on pKC0004) or *smcR* (pHS105) as indicated. *Error bars* represent the S.E.

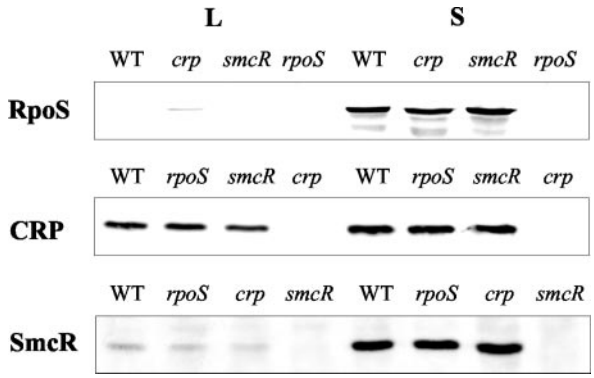

FIG. 5. **Cellular levels of SmcR, CRP, and RpoS are unaffected by one other.** The wild type (*WT*) and *rpoS*, *crp*, and *smcR* mutants of *V. vulnificus* strains were grown in LBS to A_{600} of ~0.6 (log phase (*L*)) and 2.0 (stationary phase (*S*)). The cells were then examined for the presence of RpoS, CRP, and SmcR proteins by Western blot analyses using the IgG fractions of rat anti-*V. vulnificus* RpoS, rat anti-*V. vulnificus* CRP, and rat anti-*V. vulnificus* SmcR sera, respectively.

Deletion Analysis of the vvpE Promoter Region—To delineate the *cis*-DNA sequences in the PS promoter region required for CRP and SmcR activation, transcriptional fusions of the putative *vvpE* regulatory region were made with the reporter gene *luxAB*. The pHS reporter fusions are shown in Fig. 6*A*. The reporter constructs were transferred into the wild-type strain, *crp* mutant KC74, and *smcR* mutant HS03. Culture luminescence was used to quantify the capacity of each *vvpE* upstream fragment to activate the transcription of *vvpE*.

For the wild-type strain containing pHS201, a plasmid carrying an intact regulatory region, the luminescence activity was ~4.5×10^6 relative light units (Fig. 6*B*). The light produced by both the *crp* and *smcR* mutants carrying pHS201 was significantly reduced, supporting the hypothesis that the expression of PS is dependent on CRP and SmcR. The luminescence was also reduced in the strains carrying pHS202. Moreover, the levels of luminescence in ATCC29307 (pHS202) and KC74 (pHS202) did not significantly differ, yet were higher than those in HS03 (pHS202). Similar results were observed when comparing the luminescence between the wild type and KC74 cells containing pHS203 (Fig. 6*B*). As such, these data indicate that the sequences necessary for the activation of PS by CRP are absent in the *vvpE* upstream regions carried in pHS202 and pHS203. Because the *vvpE* upstream regions in pHS202 and pHS203 were deleted up to −221 and −207 bp, respectively, it is reasonable to conclude that the important *cis*-acting element for the activation of PS by CRP ranges from 221 to 207 bp upstream of the PS transcription start site.

When transformed with pHS204, the levels of luminescence in the wild-type and *smcR* mutant cells were comparable. The magnitude of the decrease in luminescence from pHS201 in the *smcR* mutant was similar to that observed in cells carrying pHS204, indicating that the *vvpE* upstream region in pHS204 does not harbor any *cis*-acting elements essential for the activation of PS by SmcR. Because the 5′-end of the *vvpE* upstream region was shortened to −114 bp in pHS204, this suggests that the upstream region extending from −207 to −114 bp from the transcription start site of PS is required for SmcR activation of PS. The levels of luminescence in the strains containing pHS205 with further shortening in the 5′-end of the PS upstream region to −28 bp resulted in a complete loss of the activity of PS. Therefore, these results reveal that the functional promoter region extending from −221 to −114 bp contains consecutively the *cis*-elements necessary for CRP and SmcR to activate PS.

CRP and SmcR Specifically Bind the vvpE Promoter—It was clear that CRP and SmcR affect the expression of *vvpE* and that the sequences located between about −221 and −114 bp upstream of the transcription start site of PS are required for CRP and SmcR to activate PS. However, there are still several possible ways for CRP and SmcR to affect the activity of PS. One is by binding directly to the upstream region of PS to stimulate PS activity, whereas another is for CRP and SmcR to either increase or decrease the cellular level of an unidentified *trans*-acting factor(s), which in turn binds directly to the *vvpE* regulatory region.

To determine whether SmcR and CRP directly bind the *vvpE* promoter, a 200-bp DNA fragment encompassing residues −221 and −114 (extending from residues −300 to −101) was labeled, incubated with increasing amounts of CRP, and then subjected to electrophoresis. As shown in Fig. 7*A*, the addition of CRP at 100 nM resulted in a shift of the 200-bp DNA fragment to a single band with slower mobility. The binding of CRP was also specific because assays were performed in the presence of 1 μg of poly(dI-dC) as a nonspecific competitor (Fig. 7*A*, *first* through *fifth lanes*). In a second gel mobility shift assay, an additional unlabeled 200-bp DNA fragment was used as a self-competitor DNA to confirm the specific binding of CRP to the *vvpE* promoter (Fig. 7*A*, *sixth* through *ninth lanes*). The unlabeled 200-bp DNA was found to compete for binding with

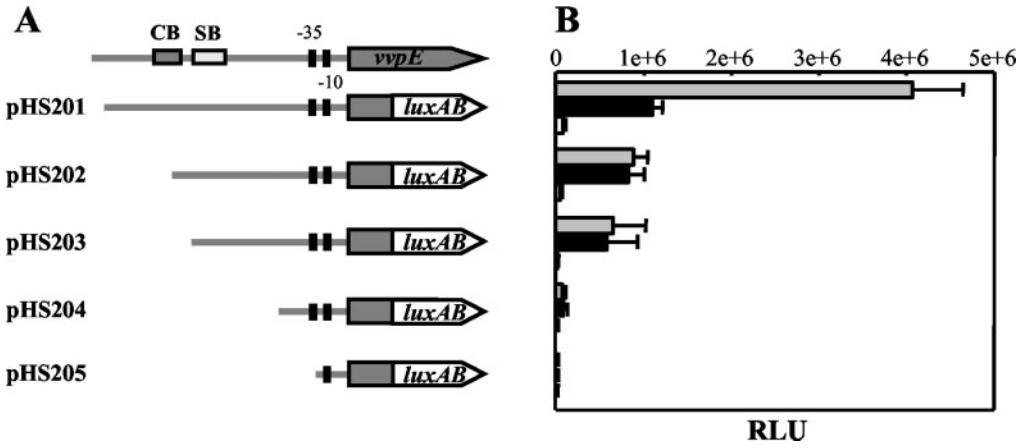

FIG. 6. **Localization of binding sites for CRP and SmcR in the *vvpE* promoter.** *A*, construction of *vvpE-lux* fusion pHS plasmids. PCR fragments carrying the regulatory region of *vvpE* with deletions were subcloned into pHK0011 (9) to create each pHS reporter. *Solid lines*, the upstream region of *vvpE*; *shaded blocks*, the *vvpE* coding region; *open blocks*, the *luxAB* DNA. The wild-type *vvpE* regulatory region is shown on top with the proposed −10 and −35 regions and the binding sites for CRP (*CB*) and SmcR (*SB*). *B*, cellular luminescence determined from the wild type (*shaded bars*), isogenic *crp* mutant (*closed bars*), and isogenic *smcR* mutant (*open bars*) of *V. vulnificus* containing each pHS reporter as indicated. Cultures in the stationary phase of growth (A_{600} = 2.0) were used to measure the cellular luminescence. *Error bars* represent the S.E. *RLU*, arbitrary relative light units.

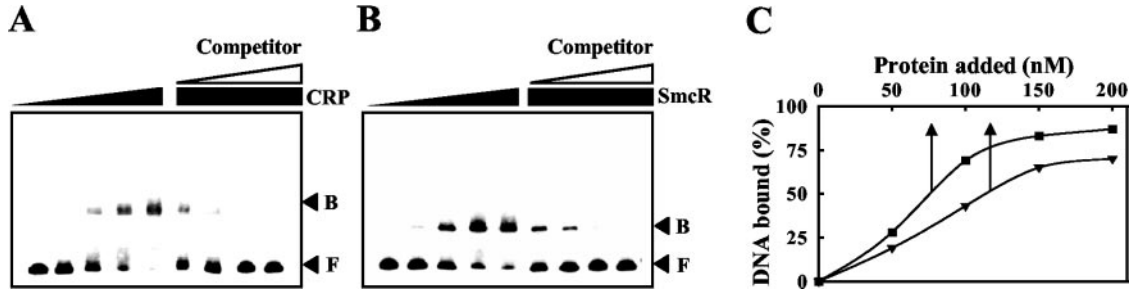

FIG. 7. **Gel mobility shift assay for binding of SmcR and CRP to the *vvpE* regulatory region.** For *A* and *B*, a 200-bp DNA fragment of the upstream region of *vvpE* was radioactively labeled and then used as a DNA probe. The radiolabeled fragments (7 nM) were mixed with increasing amounts (0, 50, 100, 150, and 200 nM in the first through *fifth lanes*, respectively) of CRP (*A*) or SmcR (*B*) and then resolved on a 5% polyacrylamide gel. For the binding of CRP, cAMP was included in all of the reaction mixtures at a final concentration of 1 mM. For competition analysis, the same but unlabeled 200-bp DNA fragment was used as a self-competitor DNA. Various amounts of the self-competitor DNA were added to the reaction mixture containing the labeled DNA (7 nM) prior to the addition of 200 nM CRP (*A*) or SmcR (*B*). The DNA probe was incubated with 35, 70, 175, and 350 nM competitor DNA (*sixth* through *ninth lanes*, respectively). *B*, bound DNA; *F*, free DNA. For *C*, the relative affinities of CRP and SmcR for the upstream region of *vvpE* were compared using the data from *A* and *B*, respectively. The concentration of bound DNA was calculated and plotted against the concentration of the proteins added. Each *arrow* points to the position of half-maximal binding corresponding to the K_d. ▼, CRP; ■, SmcR.

CRP in a dose-dependent manner, indicating that CRP binds specifically to the *vvpE* regulatory region.

In similar DNA binding assays, SmcR also displayed specific binding to the *vvpE* promoter (Fig. 7*B*). Based on the concentration of SmcR that was required to retard 50% of the labeled probe, it was estimated that the dissociation binding constant (K_d) for SmcR was ~80 nM (Fig. 7*C*). This K_d for SmcR was lower than the K_d for CRP, which was ~120 nM, determined in the same way. This result suggested that the affinity of SmcR for the *vvpE* promoter DNA was higher than that of CRP. In both gel mobility shift assays, the *vvpE* promoter region did not form any intermediate bands that were chased away to a slower migrating band at higher concentrations of the proteins. This pattern of migration suggests that a single, if not identical, binding site for CRP and SmcR is present in the *vvpE* promoter region.

Although their binding affinities were different, it was obvious that both CRP and SmcR were able to bind to the *vvpE* promoter region by themselves in the absence of the other, *i.e.* in an independent manner. To further determine whether there is cooperative binding between CRP and SmcR with the *vvpE* promoter DNA, binding assays were conducted with either one or both of the proteins. When both CRP and SmcR were present in the reaction, most of each band representing DNA bound with either CRP or SmcR disappeared, whereas a slower moving band representing DNA bound with both of the proteins was observed (Fig. 8). These results indicate that CRP and SmcR can bind simultaneously to the *vvpE* promoter region and that there is some cooperative interaction in their binding to the DNA.

Identification of Binding Sites for CRP and SmcR Using DNase I Protection Analysis—To determine the precise location of the CRP-binding site in the *vvpE* regulatory region, a DNase I footprinting experiment was performed using a 270-bp DNA fragment extending from −305 to −36 bp. As shown in Fig. 9*A*, the DNase I footprinting revealed a clear protection pattern by CRP in the upstream region of *vvpE* between −229 and −210 bp (Figs. 9*A* and 10). Several nucleotides revealed an enhanced cleavage, which is frequently observed in DNase I protection analyses of CRP-binding sites (19). The protected region overlapped with a consensus sequence for CRP binding extending from −228 to −213 bp (Figs. 9*A* and 10).

The sequences for binding of SmcR to the *vvpE* promoter were also mapped using the same 270-bp fragment by DNase I footprinting (Fig. 9*B*). The SmcR footprint extended from approximately −207 to −189 bp in the coding strand and from approximately −209 to −187 bp in the noncoding strand (Figs. 9*B* and 10). No other protected regions were observed in this

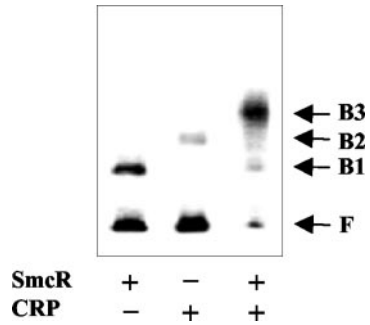

FIG. 8. **Binding of SmcR and CRP to the *vvpE* regulatory region by cooperative interaction.** Gel mobility shift assays were performed under the same conditions as described in the legend to Fig. 7, except that 100 nM SmcR, 100 nM CRP, or a mixture of 100 nM SmcR and 100 nM CRP was added to the radiolabeled fragments as indicated. The positions of the unbound fragments (*F*) and the positions of the fragments retarded by SmcR (*B1*), CRP (*B2*), or mixture of SmcR and CRP (*B3*) are indicated by *arrows*.

fragment, supporting the previous assumption that SmcR binds to only one site in the *vvpE* promoter. Inspection of the sequence protected by SmcR (Fig. 10) revealed neither inverted nor tandem repeats, which are often found in binding sites for other transcriptional activators. These observations confirm that CRP and SmcR activate PS directly by binding to the upstream region of *vvpE* and that the proteins bind to two distinct sites. Although the binding sites did not overlap each other, they were close enough to suggest a possible interaction between the two proteins present at the binding sites.

In summary, it is apparent that CRP and SmcR collaborate synergistically to activate the expression of *vvpE* with the RpoS-dependent promoter (PS). In addition, CRP and SmcR seem to function cooperatively to stimulate PS activity rather than sequentially in a regulatory cascade. Finally, CRP and SmcR exert their effect on PS activity by directly binding to two distinct sites centered −220 and −198 bp upstream of the transcription start site, respectively.

DISCUSSION

The diseases resulting from infection with *V. vulnificus* are remarkable as regards their invasive nature, ensuing severe tissue damage, and rapidly fulminating course. Therefore, understanding the molecular pathogenesis of this pathogen is critical in the development of improved treatment and prevention, as well as in the elucidation of how certain bacteria can cause such extensive damage. Among the putative virulence factors that have been proposed to account for the destructive nature of *V. vulnificus* infections is an elastolytic metalloprotease. Several different lines of evidence have led to the hypothesis that elastase is an important, if not essential, virulence factor for *V. vulnificus*. Nonetheless, to further verify the role of elastase in pathogenesis, it is essential to understand the mechanism whereby the expression pattern and level of elastase are modulated during infection. We previously demonstrated that *vvpE* expression is induced by the RpoS-dependent promoter (PS) in the stationary phase, which is under the control of CRP (9). Recently, it was reported that the SmcR of *V. vulnificus* is also involved in the regulation of *vvpE* (10). However, no definitive analysis of the roles the two regulatory proteins in the activation of *vvpE* expression has been reported in previous studies.

As a result of this study, it is apparent that SmcR activates the expression of the *V. vulnificus* elastase gene by binding directly to PS. SmcR is a homolog of *V. harveyi* LuxR, which is known to regulate the *V. harveyi lux* operon in a cell density-dependent manner (quorum-sensing regulatory system) (20). Recently, increasing numbers of LuxR homologs have been identified in different *Vibrio* spp. such as *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio anguillarum* (21–24). Although LuxR homologs from *Vibrio* spp. exhibit high levels of identity (72–92% in amino acid sequences) (20), the specific features of their functions clearly differ, such that the regulatory proteins act as repressors with some promoters and as activators with others. Null mutations of LuxR homologs result in a broad range of pleiotropic phenotype changes, indicating that LuxR homologs are apparently novel global regulators whereby the transcription of a set of different genes is regulated. However, the greatest limitation has been to assess direct binding of LuxR homologs to DNA *in vitro*, and thus, a consensus sequence(s) for the binding of the proteins has not yet been identified. HapR is known to bind to the *V. cholerae aphA* promoter region spanning from −85 to −58 bp (22). Our report of the sequence of the upstream region of *vvpE* for SmcR binding is the first known report of the DNA sequence for the binding of SmcR. The SmcR-binding site in the *vvpE* promoter does not appear to be conserved with these binding sites. Nonetheless, it has been noted that the sequences for the SmcR-binding site are highly AT-rich, as commonly observed in LuxR- and HapR-binding sites (Fig. 10) (22, 25).

This study has shown that the expression of the *V. vulnificus* elastase gene is dependent on CRP. The CRP-binding site is centered 220 bp upstream of the transcription start site of PS. However, 220 bp upstream of the transcription start site is unusually distant for direct activation by CRP (26). To our knowledge, a CRP role in activation by direct binding this far upstream of the promoter has not been previously reported. The binding sites for CRP found for any previously characterized promoters, including *galP1*, *lacP1*, *malTp*, *ansB*, and *uhpTp*, are centered not farther than −121.5 bp upstream of their transcription start sites (27–30). For activation of *papBA* in *E. coli*, where CRP binding at −215.5 bp is the most exceptional distant binding reported, CRP without Lrp (leucine-responsive regulatory protein) provides little activation. In this example, Lrp binds to multiple sites extended between CRP- and RNA polymerase (RNAP)-binding sites. Furthermore, Lrp is proposed to bring CRP into contact with the C-terminal domain of the α-subunit (α-CTD) of RNAP by bending (forming a DNA loop) the promoter DNA (31). But for *vvpE*, only one SmcR-binding site at −198 bp has been detected between CRP- and RNAP-binding sites (Fig. 10), and SmcR is not able to bend the *vvpE* promoter DNA (data not shown).

Although transcriptional synergy by multiple activators is often found in eukaryotes, relatively few examples of synergy in prokaryotic organisms have been reported (30, 32). Specially, synergistic coactivation with CRP and LuxR homologs has not yet been reported. In this study, CRP did not activate PS by itself, but only in conjunction with SmcR. SmcR that bound to the *vvpE* promoter just downstream of the CRP-binding site activated PS as a solitary regulator, albeit not to the wild-type level. It was obvious that activation of PS to the wild-type level required both CRP and SmcR. There are several possible roles for CRP in this synergistic coactivation of PS. One involves enhancing the ability of SmcR to bind to its recognition site. Cooperative binding between SmcR and CRP with the *vvpE* promoter DNA was observed, thereby potentially supporting this hypothesis (Fig. 8). However, as observed in Fig. 4, overproduced SmcR failed to compensate at all for a lack of CRP. Although the mechanism for the coactivation of PS by CRP and SmcR still remains unclear, this indicates that the role of CRP is more than simply enhancing SmcR binding to PS. For example, CRP may stabilize the interaction, if any, between SmcR and RNAP. Experiments to further examine the

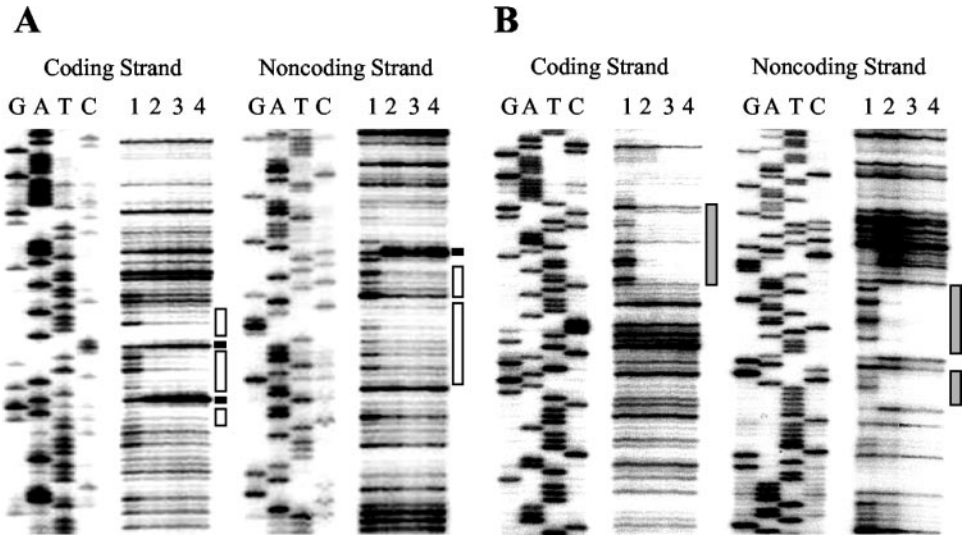

FIG. 9. **Sequences for binding of CRP and SmcR to the *vvpE* promoter.** *A*, shown are the results from DNase I protection analysis of CRP binding to the *vvpE* regulatory region. The labeling of the *vvpE* regulatory region was performed on both the coding and noncoding strands as indicated. ^{32}P-Labeled 270-bp fragments were incubated with increasing amounts of CRP and then digested with DNase I. *Lane 1*, no CRP added; *lanes 2–4*, CRP at 200, 400, and 600 nM, respectively. The nucleotides showing an enhanced cleavage in the presence of CRP are indicated by *black boxes*, whereas the regions protected by CRP are indicated by *open boxes*. *B*, to analyze the SmcR binding to the *vvpE* promoter using DNase I footprinting, the same experimental conditions were used, except that cAMP was omitted. *Lane 1*, no SmcR added; *lanes 2–4*, SmcR at 200, 400, and 600 nM, respectively. Protection by SmcR is indicated by *shaded boxes*. For both *A* and *B*, *G*, *A*, *T*, and *C* represent the nucleotide sequencing ladders of pKC980.

FIG. 10. **Sequence analysis of the *vvpE* upstream region.** The transcription start sites of the log (PL) and stationary (PS) phases are indicated by *bent arrows*. The positions of the putative −10 and −35 regions are *underlined* for PS. The sequences proposed for the binding sites of CRP (*CB*) and SmcR (*SB*) are represented by *lines* and *shaded boxes*, respectively. The conserved nucleotide sequences for the binding of CRP, IHF, and the α-CTD of RNAP are indicated above the *V. vulnificus* DNA sequence in *uppercase letters*. The ATG translation initiation codon and the putative ribosome-binding site (AGGA) are indicated in *boldface*. *W*, A or T; *R*, A or G; *N*, any base.

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acgaacttgatcttggtagtcgtcgagaatggcgattttcatagttatttcctacatgcatgtaacatta
tgcttgaactagaaccatcagcagctcttaccgctaaaagtatcaataaaggatgtacgtacattgtaat
                                  CB                  SB
                          (CRP) TGTGANNNNNNTCACA
ataaatcaaatttgtaactttatttctgagatgactgtcccatttatcttattgataaatctgcgtaaaa
tatttagtttaaacattgaaataaagactctactgacagggtaaatagaataactatttagacgcatttt

 (IHF) WATCAANNNNTTR                (IHF) WATCAANNNNTTR
aagaaaaagcacaatttattactagtgtttatcgctatgatttataaaattaaatttgaaacgaaatcg
ttctttttcgtgttaaaataatgatcacaaatagcgatactaaatattttaatttaaactttgctttagc

                                                  (α-CTD) NNAAAWWTWTTTTNNA
cagtgagatggattctttgtataacattgcgtcagagtttctatacagggcgtaaaaacatttttttggt
gtcactctacctaagaaacatattgtaacgcagtctcaaagatatgtcccgcatttttgtaaaaaaacca

AAANNN    -35              -10          PS      PL
gaagttggctggtggtcattttctgaccatggttgttgagctcgcagtgtctgcgagcttcgggagcttc
cttcaaccgaccaccagtaaaagactggtaccaacaactcgagcgtcacagacgctcgaagccctcgaag

gggaggaacccgaagagataaaatacaacatcaacaggaaatcgataatgaaactcaatc
ccctccttgggcttctctattttatgttgtagttgtcctttagctattactttgagttag
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exact role of CRP in the coactivation of PS by SmcR are now under way.

The multifaceted nature of the host-pathogen interaction indicates that more than one virulence factor is typically involved in pathogenesis (33). Most of these virulence factors act cooperatively to obtain maximum effectiveness in the pathogenesis, and their expression is coordinately controlled by a common regulatory system in response to environmental signals (34). It is likely that multiple global regulators may provide the additional levels of control for precisely coordinated expression of the virulence factors. CRP, which is a central regulator of energy (catabolic) metabolism, would make expression of virulence factors metabolically coordinated, such that the energy and resources in cells should be used in the most efficient way possible during pathogenesis. Indeed, CRP regulation has been observed in the synthesis of the virulence factors of several pathogenic bacteria (16, 35–37). Recently, quorum sensing has been also implicated as an important global regulator controlling the expression of numerous virulence genes in bacterial pathogens (for a recent review, see Ref. 38). It is not yet clear whether SmcR acts in a cell density-dependent manner or not; however, LuxR homologs from *Vibrio* spp. are proposed to sense the place where their cell densities reach higher than critical levels (38). Although further understanding of the implications of the collaboration between CRP and SmcR in terms of pathogenesis of *V. vulnificus* will await additional works, this metabolically and spatially coordinated regulation would facilitate cooperation of the virulence factors and would be crucial for the overall success of the organism during pathogenesis.

The sequences of the intervening DNA between the SmcR- and RNAP-binding sites (−35 and −10 regions) of PS were analyzed (Fig. 10). A UP element for the binding of the α-subunit of RNAP was present at an appropriate distance from the transcription start site (Fig. 10). The assigned sequences for the UP element (AAAACATTTTTTTGGTGAAGTT) scored an 86% homology to the UP element consensus sequences of the promoters recognized by the α-CTD of the RNAP in *E. coli* (39). It has been suggested that class I activators stimulate transcription with promoters by contacting the α-subunit of RNAP (40). However, it seems likely that 220 and 198 bp upstream of the transcription start site are still unusually large distances for class I activation of promoters. One possible way that CRP and/or SmcR (binding at approximately −220 and −198 bp,

respectively) directly contacts the α-CTD of RNAP is by forming a loop to bring the activators close to the RNAP. Because we did not observe that SmcR induced a significant DNA bending of the *vvpE* promoter region (data not shown), this implies that another factor(s), as yet unidentified, may be involved in activation of PS to convey the remote effects of CRP and SmcR to RNAP. Consistent with this, the two sequences of the intervening region revealed reasonable homologies to the integration host factor (IHF)-binding consensus sequences (Fig. 10) (41). IHF is an asymmetric histone-like protein that binds and bends the DNA at specific sequences. IHF-induced bending facilitates a protein-protein interaction between an upstream activator and RNAP (41). However, additional works are needed to clarify whether these regions really act as IHF recognition sites and whether IHF is really involved in the activation of PS by forming a DNA loop between CRP and/or SmcR and RNAP.

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