

Differential Expression of *Vibrio vulnificus* Elastase Gene in a Growth Phase-dependent Manner by Two Different Types of Promoters*

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Elastase activity of *Vibrio vulnificus* was highly dependent on growth phase, reached a maximum during the stationary phase, and was regulated at the level of transcription. The stationary phase production of elastase in *crp* or *rpoS* mutants, which were constructed by allelic exchanges, decreased about 3- and 10-fold, respectively. However, the promoter activity of *vvpE* encoding elastase was unaffected by those mutations in the log phase when analyzed using a *vvpE-lux* fusion. A primer extension analysis revealed that the transcription of *vvpE* begins at two different sites, consisting of putative promoter L (PL) and promoter S (PS). The PL activity was constitutive through the log and stationary phases, lower than the PS activity, and unaffected by the *crp* or *rpoS* mutations. The transcription of PS, induced only in the stationary phase, was dependent on RpoS. The mutation in *crp* reduced the activity of PS; however, the additional inactivation of *crp* did not influence the PS activity in the *rpoS* mutant, indicating that CRP exerted its effects through PS requiring RpoS. These results demonstrate that *vvpE* expression is differentially directed by PL and PS depending on the growth phase and elevated by RpoS and CRP in the stationary phase.

The pathogenic marine bacterium *Vibrio vulnificus* is the causative agent of food-borne diseases, such as life-threatening septicemia and possibly gastroenteritis, in individuals with underlying predisposed conditions. The mortality from septicemia is very high (>50%), and death may occur within 1–2 days after the first signs of illness (1). Several potential virulence factors including an endotoxin, a polysaccharide capsule, iron-sequestering systems, a cytolytic hemolysin, an elastase, a phospholipase A2, and other exotoxins have been identified for *V. vulnificus* (for a recent review, see Ref. 2).

Among the putative virulence factors is an elastolytic metalloprotease. Elastase, with a broad substrate specificity including biologically important host molecules, has been suggested

to be an important virulence factor of various human pathogenic bacteria (3, 4). The characteristics of the elastase of *V. vulnificus* as a potential virulence factor have been studied primarily using the purified protein in animal models (5–7). Injection of purified elastase reproduced many of the observed aspects of disease caused by *V. vulnificus*, including dermonecrosis, destruction of tissues, edema, and ulceration. However, when the isogenic mutant deficient in the elastase was compared with the parental strain for virulence, it appeared that elastase is less important in the pathogenesis of *V. vulnificus* than would have been predicted from examining the effects of administering purified proteins to animals (4, 8). One possible explanation for this contradiction is that the expression of *vvpE* encoding elastase may not be sufficient at least under the conditions used; hence the effects of the inactivation of the *vvpE* on the virulence of the pathogen were not apparent. This possibility strongly underscores the necessity of understanding the regulation of *vvpE* expression. However, no promoter(s) of the *vvpE* gene have been identified, and the molecular mechanisms by which the bacterium modulates the expression of the *vvpE* gene have not yet been characterized. This lack of information on the regulatory characteristics of the elastase gene makes it difficult to understand how the expression pattern and level of elastase varies spatially and temporally during infection with viable *V. vulnificus*.

Accordingly, the influence of the growth phase on the expression of the *vvpE* of *V. vulnificus* was examined in the present study. *V. vulnificus* isogenic mutants, which lack either the cyclic AMP receptor protein (CRP,¹ a gene product of *crp*) or both CRP and RpoS (σ^s , a gene product of *rpoS*), were constructed, and the promoter activities of the *vvpE* in these mutants were then analyzed. The *vvpE* promoters were also mapped through a primer extension analysis of the *vvpE* transcripts in different growth phases.

EXPERIMENTAL PROCEDURES

Strains, Plasmids, and Culture Media—The strains and plasmids used in this study are listed in Table I. *Escherichia coli* strains used for plasmid DNA replication or conjugational transfer of plasmids were grown in Luria-Bertani (LB) broth or on LB broth containing 1.5% (w/v) agar. Unless noted otherwise, *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 μ g/ml ampicillin, 50 μ g/ml kanamycin, and 10 μ g/ml tetracycline.

Measurement of Cell Growth and Elastase Activities—Cultures of *V.*

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¹ The abbreviations used are: CRP, cyclic AMP receptor protein; PCR, polymerase chain reaction; kb, kilobase(s); PL, promoter L; PS, promoter S.

TABLE I
Plasmids and bacterial strains used in this study

Strain or plasmid	Relevant characteristics ^a	References
<i>E. coli</i>		
DH5 α	<i>supE44</i> Δ <i>lacU169</i> (ϕ 80 <i>lacZ</i> Δ M15) <i>hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Laboratory collection
SY327 λ pir	Δ (<i>lac pro</i>) <i>argE</i> (Am) <i>recA56 rpoB</i> λ pir; host for π -requiring plasmids	12
SM10 λ pir	<i>thi thr leu tonA lacY supE recA::RP4-2-Tc::Mu</i> λ pir, <i>oriT</i> of RP4, Km ^r ; conjugational donor	12
<i>V. vulnificus</i>		
ATCC29307	Clinical isolate	Laboratory collection
KC74	ATCC29307, <i>crp::nptI</i>	This study
KP101	ATCC29307, Δ <i>rpoS</i>	^b
KC84	ATCC29307, Δ <i>rpoS</i> , <i>crp::nptI</i>	This study
Plasmid		
pUC4K	pUC4 with <i>nptI</i> ; Ap ^r , Km ^r	Amersham Pharmacia Biotech
pRK415	IncP <i>ori</i> ; broad host range vector; <i>oriT</i> of RP4; Tc ^r	15
pCVD442	R6K γ <i>ori</i> , <i>sacB</i> , <i>oriT</i> of RP4; Ap ^r	11
pKC980	pUC18 with <i>vvpE</i> ; Ap ^r	
pKC9964	pCVD402 with <i>crp</i> ; Ap ^r	This study
pKC9965	pKC9964, <i>crp::nptI</i> ; Ap ^r , Km ^r	This study
pKC0004	pRK415 with <i>crp</i> ; Tc ^r	This study
pHS0001	pRK415 with <i>rpoS</i> ; Tc ^r	This study
pHK0011	pRK415 with promoterless <i>luxAB</i> ; Tc ^r	This study
pHS0011	pHK0011, <i>vvpE-luxAB</i> transcriptional fusion; Tc ^r	This study

^a Ap^r, ampicillin-resistant; Km^r, kanamycin-resistant; Tc^r, tetracycline-resistant.

^b K.-H. Lee, manuscript in preparation.

vulnificus strains grown at 30 °C under aeration and 5-ml samples were removed at the indicated times for determination of cell density and elastase activity. Growth was monitored by measuring the A_{600} of the cultures, and measurement of the A_{600} of the diluted cultures was done once the A was above 0.8. Cultures incubated for 12–16 h ($A_{600} = 1.2$) were harvested, and the enzyme activities in the stationary phase were determined according to the procedures described previously (4). Averages and S.E. values were calculated from at least three independent determinations.

General Genetic Methods—Procedures for the isolation of plasmid DNA and genomic DNA and for transformation were carried out as described by Sambrook *et al.* (9). Restriction and DNA-modifying enzymes were used as recommended by the manufacturer (New England Biolabs, Beverly, MA). DNA fragments were purified from agarose gels using the GeneClean II kit (Bio 101, Inc., Vista, CA). Primary DNA cloning and manipulation were conducted in *E. coli* DH5 α , and restriction mapping was used to confirm that transformants contained the appropriate plasmids. PCR amplification of DNA was performed using a GeneAmp PCR system 2400 (PerkinElmer Life Sciences) and standard protocols.

Construction of *crp::nptI* Cartridge—Plasmid pUC::*crp*, which carries a *V. vulnificus* chromosomal DNA fragment of 1.7 kb and contains the entire *crp* gene,² was inactivated *in vitro* by the insertion of *nptI* encoding for aminoglycoside 3'-phosphotransferase and conferring resistance to kanamycin (10). For this purpose, a *HindIII-SalI* fragment of the *crp* open reading frame from pUC::*crp* was subcloned into pCVD442 (11), forming pKC9964. The 1.2-kb DNA fragment carrying *nptI* was isolated from pUC4K (Amersham Pharmacia Biotech) and then inserted into a unique *SphI* site present within the open reading frame of *crp* in pKC9964. The resulting construct, pKC9965, is a derivative of pCVD442 and was maintained in *E. coli* SY327 λ pir (12).

Generation of *crp::nptI* Mutant or *crp::nptI* Δ *rpoS* Double Mutant by Allelic Exchange—To generate the *crp::nptI* mutants in *V. vulnificus* by homologous recombination (see Fig. 2A), *E. coli* SM10 λ pir, *tra* (11, 12) was transformed with pKC9965 and used as a conjugal donor to *V. vulnificus* ATCC29307. For the construction of the *crp rpoS* double mutant, *V. vulnificus* KP101, an isogenic *rpoS* mutant of ATCC29307, was used as a recipient. The KP101, in which two thirds of the *rpoS* open reading frame were deleted, was constructed by replacing *rpoS* on the chromosome with Δ *rpoS*.³

Conjugation was conducted using methods previously described (4, 13). The desired transconjugants that showed a green colony formation on thiosulfate citrate bile salts agar supplemented with kanamycin, maltose (0.5%, w/v), neutral red (30 μ g/ml), and sucrose (6%, w/v) were selected. The transconjugants that were ampicillin-sensitive due to the absence of the pKC9965, were confirmed for the presence of *nptI* in the

crp gene of the mutants by PCR using a pair of primers, CRP9901 (5'-TACCTACTGGCCATGATCGATG-3') and CRP9902 (5'-CG-GAATCTGAGAGGGTTTACTG-3') (see Fig. 2B). The *V. vulnificus* mutants chosen for further analysis were named KC74 for the *crp* mutant and KC84 for the *crp rpoS* double mutant.

Construction of a *vvpE-luxAB* Transcriptional Fusion—The parent plasmid, pHK0011, carries a 3.2-kb *HindIII-BamHI* fragment of promoterless *luxAB* from Mini-Tn5 *luxAB* (14) in the broad host range vector pRK415 (15) digested with *HindIII* (see Fig. 4A). The 697-base pair upstream regulatory region of the *vvpE* from pKC980 (4) was amplified by PCR using the two primers VVPE0002 (5'-GAGGTAC-CCCAAATGATTACTGATTTTCCC-3') and VVPE0003 (5'-CTTCTA-GAAGACGGACACCATTCTGCAGC-3') and inserted into pHK0011 digested with *KpnI* and *XbaI* to yield pHS0011.

Measurement of Cellular Luminescence—pHS0011 was transferred into ATCC29307 and the isogenic mutants by conjugation. Cultures were grown to log phase ($A_{600} = 0.6$) or stationary phase ($A_{600} = 1.2$), and then 1-ml samples taken from each culture were diluted 100-fold with phosphate-buffered saline (pH 7.4) and placed into cuvettes. A decanal stock solution (0.3%, v/v) was prepared by adding decanal to a 1:1 mixture of water and ethanol. After adding 10 μ l of the decanal stock solution to cuvettes with the cells, the cellular luminescences were measured with a Lumat model 9501 luminometer (Berthold, Wildbad, Germany) and expressed using the arbitrary relative light units of the instrument.

RNA Purification and Northern Dot Blot Analysis of *vvpE* Transcript—Total cellular RNAs were isolated in different growth phases using a Trizol reagent kit (Life Technologies, Inc.). For a Northern dot blot analysis, a series of reactions was performed according to standard procedures (9) with 20 μ g of total RNA. A 1.2-kb *HindIII-HindIII* DNA probe representing the internal sequences of the *vvpE* was labeled with [α -³²P]dCTP using the Prime-a-gene labeling system (Promega, Madison, WI) and used for hybridization as described previously (4). The blots were visualized and quantified using an image analyzer (BAS1500 model; Fuji Photo Film Co. Ltd., Tokyo, Japan) and the TINA (version 2.0) program.

Primer Extension Analysis—Primer extension experiments were carried out with SuperScript II RNase H⁻ reverse transcriptase (Life Technologies, Inc.) according to Sambrook *et al.* (9). The 24-base primer used was VVPE9905 (5'-GACGTTGATTGAGTTTCATTATCG-3') located within the open reading frame of *vvpE*. The primer was end-labeled with [γ -³²P]ATP using T4 polynucleotide kinase (Life Technologies, Inc.). The cDNA products were purified and resolved on a sequencing gel alongside sequencing ladders generated with the same primers used for the primer extension. The nucleotide sequence of the plasmid DNA of pKC980 (4) was determined using the dideoxy chain termination method with TopTM DNA polymerase (Bioneer, Seoul, Korea) following the manufacturer's protocols. The gels were then dried and exposed by the same procedure as used for Northern dot blots.

² J. H. Rhee, manuscript in preparation.

³ K.-H. Lee, manuscript in preparation.

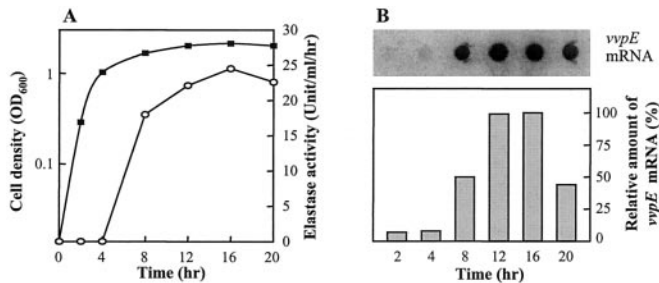


FIG. 1. Growth kinetics and growth phase-dependent expression of *vvpE*. Samples removed in different growth phases, as indicated, from a culture of strain ATCC29307 were analyzed for elastolytic protease activity (A) and for Northern dot blot analysis (B). Relative amounts of the *vvpE* transcript of each dot were presented using the amount of the *vvpE* transcript at 16 h as 100%. ■, cell density; ○, elastase activity.

RESULTS

Growth Phase-dependent Expression of *vvpE*—The elastase activities of the ATCC29307 culture were analyzed at the indicated time intervals (Fig. 1A). Elastase activity appeared in the exponential phase of growth and reached a maximum in the stationary phase. The level of elastase activity increased about 10-fold in the stationary phase. This growth phase variation of elastase possibly occurs either at the transcriptional level or post-transcriptional level of *vvpE* expression.

The relative levels of the *vvpE* mRNA in the same amount of total RNA isolated from ATCC29307 showed that the *vvpE* mRNA levels increased as the bacterial culture entered the stationary phase (Fig. 1B). This result suggests that the increase in the level of elastase activity in the stationary phase was correlated with the increase in the mRNA level of the *vvpE* gene. This increase in the *vvpE* mRNA level could be the result of an increase in the rate of mRNA transcription initiation or increased mRNA stability. However, when the total RNAs were separated by electrophoresis and hybridized with the *vvpE* DNA probe, only one band corresponding to the full size of the *vvpE* mRNA deduced from the DNA sequence (about 2.0 kb) appeared (data not shown). These results indicated that the increased level of transcription initiation plays the major, if not sole, role for the increased elastase activity on the entry of the *V. vulnificus* cells into the stationary phase.

Construction and Confirmation of *V. vulnificus* *crp* Mutant or *crp rpoS* Double Mutant—The *crp* mutants KC74 and KC84 were constructed by allelic exchange and confirmed by PCR (Fig. 2). The PCR analysis of the genomic DNA from ATCC 29307 or KP101 with primers CRP9901 and CRP9902 produced a 1.2-kb fragment (Fig. 2B), whereas the genomic DNA from the *crp::nptI* mutants resulted in an amplified DNA fragment approximately 2.4 kb in length. The 2.4-kb fragment is in agreement with the projected size of the DNA fragment containing wild-type *crp* (1.2 kb) and the *nptI* gene (1.2 kb). The mutants exhibited a slow growth and no fermentation of many sugars such as maltose and D-galactose, which is consistent with the phenotypes of a typical *crp* mutant. To determine the stability of the insertional mutation, KC74 or KC84 strains were grown overnight without kanamycin selection. The inserted *nptI* DNA was stably maintained, as determined by the maintenance of kanamycin resistance and by the generation of the appropriately sized DNA fragment by PCR (data not shown).

Effect of *crp* Mutation on Production of Elastase—ATCC29307 and the *crp* mutant KC74 were grown to the stationary phase, and the elastase activities of each culture were compared. Although the general pattern of elastase production was similar in the two strains, in the KC74 strain the level of

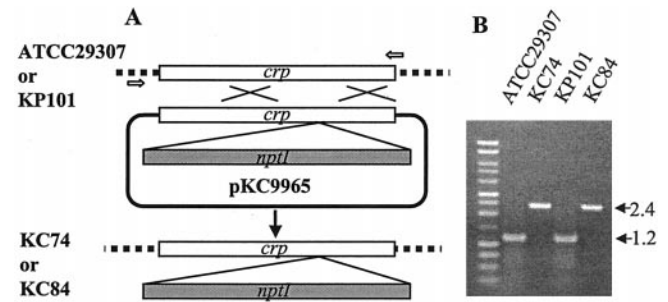


FIG. 2. Diagram of allelic exchange and confirmation of the *crp::nptI* mutants. A, homologous recombination between chromosomal *crp* gene from strain ATCC29307 or KP101 and pKC9965. Dashed lines, chromosomal DNA; solid line, plasmid DNA; open boxes, the target *crp* gene; shaded boxes, the *nptI* gene; open arrows, locations of the oligonucleotide primers used for confirmation of the *nptI* insert; X, crossover. B, PCR analysis of ATCC29307 and isogenic mutants generated by allelic exchange. Molecular size markers (1-kb ladder, Promega) and PCR products (in kb) are indicated.

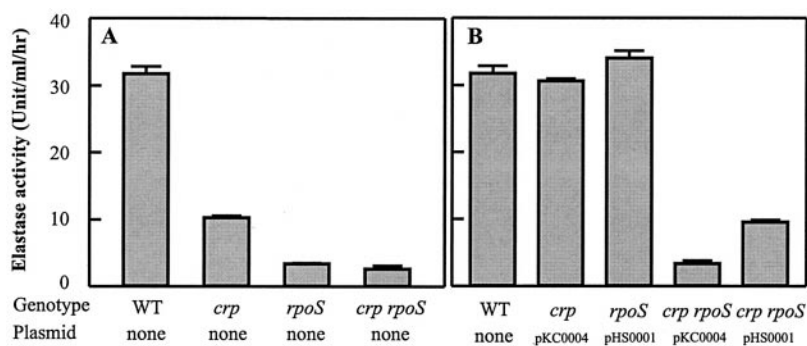
elastase was lower (Fig. 3A). Whereas elastase activity was present at about 30 units in the wild-type strain, the residual level of elastase activity in KC74 corresponded to approximately one-third of that in the wild type. The reduced production of elastase suggested that CRP acts as a positive regulator in *vvpE* expression. To characterize the role of CRP in more detail, the effects of glucose and cAMP exogenously added to the wild type were studied. During the stationary phase, the elastase activity in the wild-type culture supplemented with 0.5% glucose alone was reduced to the level of the KC74 but was restored by the addition of glucose and cAMP (1 mM) together (data not shown). These results indicate that the expression of *vvpE* in *V. vulnificus* is under the positive control of cAMP-CRP at least in the stationary phase.

Effect of *rpoS* Mutation on Production of Elastase—When the *rpoS* mutant KP101 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 that of the wild type (Fig. 3A). This result indicated that the major portion of elastase was produced by the RpoS-dependent promoter. Taking these results together led us to conclude that *vvpE* is under the positive control of both CRP and RpoS during stationary growth. However, it was apparent that *vvpE* was not completely repressed, even poorly expressed, in the absence of the active gene product of *rpoS*. This suggested the existence of at least one more promoter for *vvpE*, which is expressed in a RpoS-independent manner in the stationary phase.

Relationship between CRP and RpoS in Regulation of *vvpE*—To examine whether the activation by CRP is through the RpoS-dependent promoter or RpoS-independent promoter, the level of elastase activity in the *crp rpoS* double mutant was determined. The elastase activity in the *crp rpoS* double mutant KC84 was present at 4 units (Fig. 3A). This level of elastase activity was much lower than those reached by the wild type or *crp* mutant KC74 but was indistinguishable from that in the *rpoS* single mutant KP101 (Fig. 3A). The expression of *vvpE* remained low unless the functional gene product of *rpoS* was provided and was not significantly affected by the additional inactivation of *crp*. This observation indicated that activation of *vvpE* by CRP in the stationary phase is mediated through the promoter whose activity depends on RpoS.

Complementation of *crp* and *rpoS* Mutation—For the complementation test, plasmid pKC0004 or pHS0001 was constructed by subcloning *crp* or *rpoS*, respectively, into pRK415. The elastase activities of both KC74 (pKC0004) and KP101 (pHS0001) in the stationary phase were restored to levels comparable with the wild-type level of ATCC29307 (Fig. 3B). Therefore, the

FIG. 3. Dependence of elastase production of *V. vulnificus* in the stationary phase on CRP and RpoS. A, elastase activities were determined from ATCC29307 and each isogenic mutant as indicated. B, complementation of the mutants with functional *crp* (pKC0004) or *rpoS* (pHS0001) as indicated. For both panels, samples removed at A_{600} of 1.2 were analyzed for elastase activity on each bar. Error bars represent the S.E. WT, wild type.



decreased elastase activity of KC74 and KP101 resulted from the inactivation of functional *crp* or *rpoS* rather than any polar effects on any genes downstream of *crp* or *rpoS*.

The elastase activity in KC84 containing pHS0001 was restored to a level similar to that in KC74, whereas the repressed level of elastase in KC84 was not restored at all by the introduction of pKC0004 (Fig. 3B). This indicated that CRP was able to activate *vvpE* only when RpoS was present, supporting again our previous hypothesis that CRP exerts its effects on *vvpE* expression through the RpoS-dependent promoter.

Expression of *vvpE* in the Log Phase—In the log phase, the elastase activities of the wild type and its isogenic mutants were too low to be precisely compared. To ensure accurate sensitivity, a *vvpE-luxAB* transcriptional fusion was employed to determine the elastase activity of the cultures in the log phase. For the ATCC29307 strain containing pHS0011, luminescence activity was present at about 90,000 relative light units (Fig. 4B). After cells were introduced with pHS0011, the expression of luminescence in the *crp* or *rpoS* mutant and the *crp rpoS* double mutant cells did not differ significantly, and the levels of luminescence in the mutants were comparable with that in the wild type. Apparently, the promoter activity of *vvpE* in the log phase cells of *V. vulnificus* was not dependent on RpoS and not activated by CRP.

However, the levels of luminescence decreased in the *crp* or *rpoS* mutant and the *crp rpoS* double mutant in the stationary phase when compared with that in the wild type. The magnitude of the decrease in luminescence in the mutants was similar to the decrease in elastase activities, which were determined directly (Fig. 4C). The luminescence of the wild type is much higher in the stationary phase than in the log phase (Fig. 4, B and C). These results indicate that the upstream region of *vvpE* used for construction of the *vvpE-luxAB* fusion is sufficient for the stationary phase induction and the CRP and RpoS dependence of *vvpE*. These results also reconfirm the previous observation that *vvpE* expression is mainly regulated at the level of transcription.

Identification of Transcriptional Start Sites of *vvpE* with *crp*, *rpoS*, or *crp rpoS* Background—A single reverse transcript was identified from the RNA isolated from the log phase cells of both the wild type and all mutants tested (Fig. 5). This indicated that a transcriptional start site, P1, located 61 base pairs upstream of the translational initiation codon, was used for the transcription of *vvpE* in the log phase. The putative promoter that constitutes P1 was named PL to represent the log phase promoter. Apparently, the PL activities, determined based on the intensity of the bands of the reverse transcripts, were unaffected by the inactivation of *crp* and *rpoS*. This observation is consistent with the results obtained from the *vvpE* expression studies with a *vvpE-lux* fusion, as presented above.

The primer extension analysis performed with RNA prepared from stationary cells revealed two different transcriptional start sites; one was identical to P1, and the other, P2, was

3 base pairs apart from P1. The promoter S (PS), consisting of P2 and representing the stationary phase promoter, was not expressed in the log phase and was induced only when cells entered the stationary phase. In contrast to P1, the band corresponding to P2 was not detected with the RNA from the *rpoS* mutant or *crp rpoS* double mutant and was reduced in its intensity with the RNA from the *crp* mutant. This observation indicates that the induction of PS is entirely dependent on RpoS, and CRP activates PS only in the presence of functional RpoS. However, the activity of PL, which was much lower than that of PS, appeared to be constitutive throughout the log and stationary growth phases.

Consequently, the transcription of the *vvpE* of *V. vulnificus* was initiated by two different types of promoters, PL and PS, in a growth phase-dependent manner. The basal level of expression of *vvpE* was directed by PL, independent of RpoS and CRP, and remained low throughout the log and stationary growth phases. In addition to this basal level, more *vvpE* expression was induced by PS in the stationary phase, which was under the control of both CRP and RpoS. This differential utilization of two promoters may permit precise levels of elastase in response to modifications of the environment and the growth stage.

DISCUSSION

There have been several different lines of evidence leading to the hypothesis that elastase is an important, if not essential, component of virulence for *V. vulnificus*. Previously, the function of elastase during an infectious process, rather than the artificial system of injecting purified proteins, has been examined by constructing isogenic elastase mutants of *V. vulnificus* and applying the molecular version of Koch's postulates (4, 8). When the isogenic *vvpE* mutants were compared with the parental strain for virulence in animal and cell culture models, the mutants did not show any significant differences in any aspect of the disease process. The major problem to be addressed is the discrepancy between these infection experiments and the studies that primarily relied on injection of proteins into animals. However, it is important to note that the expression of *vvpE* encoding elastase of *V. vulnificus* remains at a low level under certain conditions, such as in the log phase of growth. Although other explanations are possible, the lack of significant difference in virulence between the *vvpE* mutant and wild-type parent could be related to this low level of elastase expression in the conditions used. This possibility, which remains to be proved, strongly underscores that the expression pattern and level of putative virulence attributes during an infectious process must be examined to identify their roles in pathogenesis.

In the present study, it was found that the expression of *vvpE* encoding elastase of *V. vulnificus* is regulated as a function of the growth phase and reaches a maximum in the stationary phase. It has been previously reported that Enterococcus

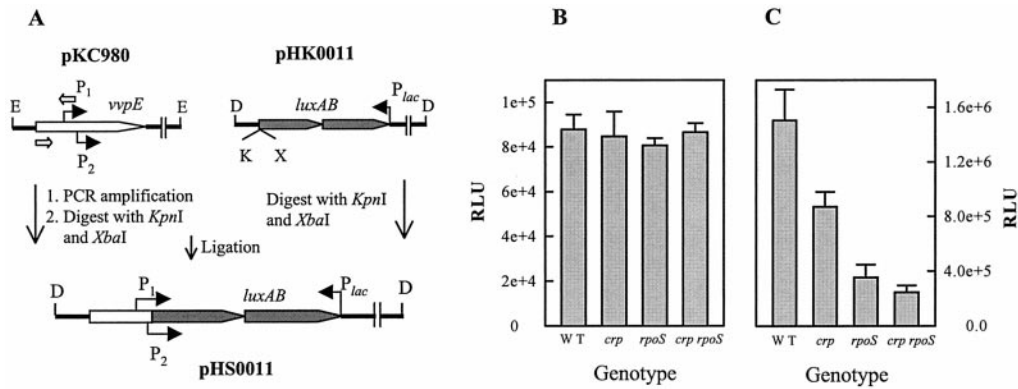


FIG. 4. Promoter activity of *vvpE* in the log phase assessed using a *vvpE-luxAB* transcriptional fusion. A, construction of *vvpE-lux* fusion plasmid pHS0011. Filled blocks, the *lux* DNA; open blocks, the *vvpE* DNA; solid lines, the vector DNA used. Hybridizing locations of the oligonucleotide primers used for the PCR are depicted by open arrows. Transcription start sites determined by the primer extension analysis are presented as P₁ and P₂. D, *Dra*I; E, *Eco*RI; K, *Kpn*I; X, *Xba*I. B and C, cellular luminescences were determined from ATCC29307 and the isogenic mutants as indicated. Cultures in log (B) and stationary (C) phases of growth were used to measure luminescences. Error bars represent the S.E. obtained from at least three independent determinations. WT, wild type; RLU, relative light units.

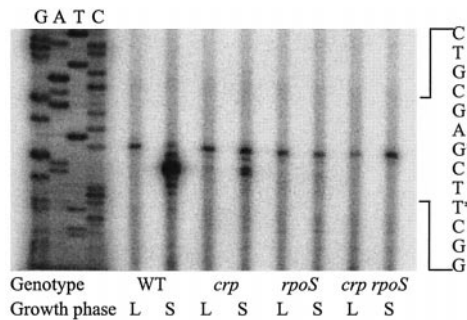


FIG. 5. Identification of transcription start sites of *vvpE*. The transcription start sites were determined by the primer extension of the RNA derived from ATCC29307 and the isogenic mutants as indicated. Total RNAs were prepared in the log phase (L; $A_{600} = 0.6$) and stationary phase (S; $A_{600} = 1.2$) of each culture. Lanes G, A, T, and C represent the nucleotide sequencing ladders of pKC980. Asterisks indicate the sites of the transcription start for P₁ (G) and P₂ (T), respectively. WT, wild type.

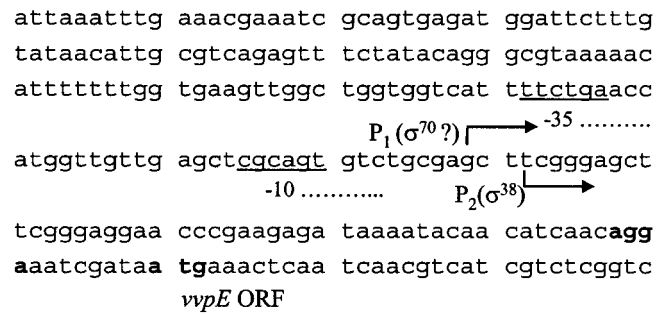


FIG. 6. Sequence of *vvpE* upstream region. Transcription start sites of the log phase (P₁) and stationary phase (P₂) are indicated by bent arrows. The positions of the putative -10 and -35 regions are underlined with continuous and broken lines for the promoters PL and PS, respectively. The ATG translation initiation codon and putative ribosome binding site (AGGA) are indicated in boldface. ORF, open reading frame.

bacteriaceae undergoes a global modification of their gene expression pattern at the onset of the stationary phase (16, 17). As a result, the bacteria acquire tolerance to a number of chemical and physical stresses, such as extreme temperatures, oxidative agents, hyperosmotic tension, and nutritional starvation. The key regulator for the expression of the genes responsible for this increased tolerance is RpoS (σ^S or σ^{38}), a stationary phase-specific sigma factor (18, 19). Recently, a direct role for RpoS in regulating the expression of numerous virulence factors has been established for a number of pathogenic Enterobacteriaceae (20–22). From a standpoint of bacterial pathogenesis, the finding that numerous virulence factors are regulated by RpoS would be not surprising. When bacteria invade the human body, the scarcity of specific nutrients and increased stresses imposed by the host immune defense system would be encountered. As such, the bacteria must survive these stresses to multiply and finally result in local damage and systemic disease. This survival often involves coordinate expression of sets of assorted genes (23), and many of these genes are probably regulated by RpoS.

This report has shown that the expression of the *V. vulnificus* elastase gene is dependent on CRP. Besides regulating the synthesis of many catabolic enzymes, CRP regulation has also been observed in the synthesis of the toxin proteins of several pathogenic bacteria (24–26). It was found that CRP affects the RpoS-dependent expression of *vvpE* at PS. There are several possible ways for CRP to affect PS. One is by binding directly to

PS to stimulate open complex formation by the RpoS-RNA polymerase holoenzyme. Because purified *V. vulnificus* CRP is unavailable, *in vivo* footprinting was performed to identify the CRP binding region in the *vvpE* upstream region (27). Dimethylsulfate methylation-protection footprinting revealed no difference in the protection pattern in the upstream region of *vvpE* between KC74 and its parental wild type (data not shown). In agreement with this, no convincing consensus sequences for CRP binding are identified in the upstream region of *vvpE* (Fig. 6.). These observations suggest the possibility that CRP activates PS indirectly by increasing the cellular level of either RpoS or an unidentified third component(s) in the stationary phase. The unidentified component(s) may then modulate the level of RpoS or bind directly upstream of the *vvpE* to activate the PS.

In *E. coli*, the cellular levels of RpoS increase at the onset of the stationary phase, but the regulation mechanisms that modulate the level of the RpoS protein appear to be complicated and are not clearly understood (for a recent review, see Ref. 28). The cellular content of RpoS is regulated at the level of *rpoS* transcription, translation, and RpoS proteolysis in *E. coli*. Although some contradictions have also been observed, it has been proposed that the transcriptional control of *rpoS* involves the CRP-cAMP complex as a negative regulator in *E. coli*. However, it has not yet been established whether CRP is involved in the modulation of the cellular level of RpoS in *V. vulnificus*. Experiments to further examine the effects of CRP on the level of the expression of *rpoS* and on the level of RpoS in *V. vulnificus* are now underway.

Because the expression of *vvpE* from PL is constitutive through the log and stationary phases, PL would be transcribed most likely by the RNA polymerase holoenzyme with the homolog of RpoD (σ^{70}) that is the major housekeeping sigma factor in *E. coli*. However, the exact type of sigma factor associated with the RNA polymerase for the transcription of PL has not yet been determined. Several hundred base pairs upstream of *vvpE* were sequenced and analyzed by comparing them with putative promoter sequences suggested previously on the basis of homology to a consensus from *E. coli* (Fig. 6). A Shine-Dalgarno site (AGGA) and potential promoter sequences consisting of -10 and -35 segments separated by 17 nucleotides have been assigned. The assigned sequences for -35 (TTCTGA or TGAACC) scored a 50% homology to the -35 consensus sequences (TTGACA) of the promoters recognized by the RNA polymerase with RpoD. However, no sequence of the -10 regions assigned with respect to either P1 or P2 revealed a reasonable homology to -10 consensus sequences. It has been reported that the sequences for promoters transcribed by RNA polymerase with RpoS are notoriously weakly conserved. It is also noteworthy that the expression level of *vvpE* from PL is very low, and the weak homology of the promoter sequences to the consensus sequences may explain this low level of expression of *vvpE* from PL. However, additional work is needed to clarify whether these regions really act as RNA polymerase recognition sites.

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