Transcription, Chromatin, and Epigenetics:

Vibrio vulnificus rpoS Expression Is Repressed by Direct Binding of cAMP-cAMP Receptor Protein Complex to Its Two Promoter Regions

Hyun-Jung Lee, Soon-Jung Park, Sang Ho Choi and Kyu-Ho Lee

J. Biol. Chem. 2008, 283:30438-30450.
doi: 10.1074/jbc.M802219200 originally published online August 18, 2008

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 283, NO. 45, pp. 30438–30450, November 7, 2008

Vibrio vulnificus rpoS Expression Is Repressed by Direct Binding of cAMP-cAMP Receptor Protein Complex to Its Two Promoter Regions*[S]

Received for publication, March 20, 2008, and in revised form, August 1, 2008 Published, JBC Papers in Press, August 18, 2008, DOI 10.1074/jbc.M802219200

Hyun-Jung Lee[‡], Soon-Jung Park[§], Sang Ho Choi[¶], and Kyu-Ho Lee[‡1]

From the [‡]Department of Environmental Science, Hankuk University of Foreign Studies, Yongin 449-791, the [§]Department of Environmental Medical Biology and Institute of Tropical Medicine, The Brain Korea 21 Project, Yonsei University College of Medicine, Seoul 120-752, and the [¶]National Research Laboratory of Molecular Microbiology and Toxicology and Department of Agricultural Biotechnology, Seoul National University, Seoul 151-921, South Korea

Vibrio vulnificus*, a septicemia-causing pathogenic bacterium, acquires resistance against various stresses and expresses virulence factors via an *rpoS* gene product. In this study, we investigated the transcriptional characteristics of this global regulator. Two distinct transcriptional initiation sites for the *rpoS* gene, the proximal promoter (P_p) and the distal promoter (P_d), were defined by primer extension experiments. Various *rpoS*::*luxAB* transcriptional fusions indicated that P_d is a major promoter of *rpoS* expression. Western blot analysis showed that RpoS levels were inversely correlated with intracellular levels of 3′,5′-cyclic AMP (cAMP). The expressions of both P_d and P_p were increased in *cya* and *crp* mutants. The exogenous addition of cAMP to the *cya* mutant resulted in repressed expression of *rpoS*. In addition, *rpoS* expression was significantly lowered in the *cpdA* mutant, in which the level of cAMP was elevated because of the absence of 3′,5′-cAMP phosphodiesterase. *In vitro* transcription assays using the *V. vulnificus* RNA polymerase showed that the transcripts from both promoters were reduced by addition of the cAMP-cAMP receptor protein (CRP). The cAMP-CRP was shown to bind to two *rpoS* promoters by electrophoretic mobility shift assays. The alteration of the putative CRP-binding site on each *rpoS* promoter, via site-directed mutagenesis, abolished the binding of cAMP-CRP as well as regulation by cAMP-CRP. Therefore, this study shows a relationship between the level of intracellular cAMP and the degree of *rpoS* expression and further demonstrates, for the first time, the direct binding of the cAMP-CRP complex to *rpoS* upstream regions, which results in repression of *rpoS* gene expression.

Global and immediate response to diverse environmental stimuli is one of the characteristics of bacterial adaptation and survival. Cellular responses to stressful conditions have been extensively studied in many bacterial species, most notably in *Escherichia coli* (1). To respond properly to diverse stresses, *E. coli* requires the *rpoS* gene product, which is a second principal σ factor, RpoS (σ^s), to endow it with the ability to mediate changes in bacterial physiology and structure. *Vibrio vulnificus*, a human pathogen causing a fatal septicemia with rapid pathogenic progression and a high mortality rate (2), has been shown to require RpoS for better survival under nutrient starvation, oxidative stress, UV irradiation, and acidic conditions (3, 4).

RpoS is also required for eliciting phenotypes related to virulence in many pathogenic bacteria belonging to the γ-subdivision of Proteobacteria (5). *V. vulnificus* has been shown to use RpoS to express its major virulence factors, such as an elastase (6) and other exoproteases (3), and for expression of the *fur* gene, which encodes a transcriptional regulator of virulence-associated iron-uptake systems (7, 8).

Thus, the intracellular level of RpoS needs to be finely adjusted depending on the environmental conditions, and it is modulated via transcriptional control of the *rpoS* gene, translational efficiency of *rpoS* mRNA, proteolysis of the RpoS protein, and the interaction of RpoS with RNA polymerase core subunits (1). Transcriptional regulation of *rpoS* expression has been shown to be mediated by diverse trans-acting proteins (9). A transcription factor, ArcA, represses *rpoS* expression by binding to the regulatory regions flanking the major promoter of the *E. coli rpoS* gene (10). The repressor Fis and the activator PsrA directly bind to the *rpoS* gene regulatory regions of *Salmonella enterica* and *Pseudomonas* spp., respectively (11, 12). In *Borrelia burdorferi*, another σ factor, RpoN, is involved in the expression of its *rpoS* gene (13).

The role of cAMP-CRP[2] complex as a negative transcriptional regulator in *rpoS* expression was shown genetically using *crp*, *cya*, and *crr* knock-out mutants of *E. coli* and other bacterial species (14, 15). However, whether control by cAMP-CRP complex is direct or indirect has not yet been determined, because the effects of cAMP-CRP on *rpoS* expression were shown only by transcriptional fusion assays, and direct evi-

* This work was supported by Grant R01-2007-000-10159-0 (to K.-H. L.) from KOSEF and a grant from the Extreme Molecular Genomics Research Program of the Ministry of Marine Affairs and Fisheries (to K.-H. L.), Republic of Korea. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY221025 and AY240931.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–3.

[1] To whom correspondence should be addressed: Dept. of Environmental Science, Hankuk University of Foreign Studies, Wangsan-Li, Mohyun-Myun, Yongin, Kyunggi-Do 449-791, Korea. Tel.: 82-31-330-4039; Fax: 82-31-330-4529; E-mail: khlee@hufs.ac.kr.

[2] The abbreviations used are: CRP, cAMP receptor protein; IC, initiation codon; ORF, open reading frame; RNAP, RNA polymerase; EMSA, electrophoretic mobility shift assay; RLU, relative light unit; mt, mutagenized.

TABLE 1
Bacterial strains and plasmids used in this study

Strain/plasmid	Genotype	Source/reference
E. coli		
DH5α	(Φ80 *lacZ* Δ*M15*) *recA1 endA1 gyrA96 relA1 thi-1 hsdR17*(r_K^- m_K^-) *supE44 deoR* Δ(*lacZYA-argF*)*U169*	Laboratory collection
SM10*λpir*	*thi-1 thr leu tonA lacY supE recA*:: Rp4-2-Tc::Mu*λpir*, OriT of RP4, Km^r	20
BL21(DE3)	*E. coli* B F⁻ *dcm ompT hsdS* (rB^- mB^-) *gal* λ(DE3)	Laboratory collection
V. vulnificus		
ATCC29307	Clinical isolate	Laboratory collection
AR	ATCC29307 but spontaneous rifampin-resistance	4
MO6-24/O	Clinical isolate	Laboratory collection
KC74	ATCC29307, *crp*::*nptI*	6
HY101	ATCC29307, Δ*cpdA*	This study
KP301	ATCC29307, Δ*cya*	This study
Plasmids		
pQE30	Expression vector, Ap^r	Qiagen
pQErpoS	pQE32 with *V. vulnificus rpoS*	4
pRSETA	Expression vector, Ap^r	Invitrogene
pHK0201	pRSETA with *V. vulnificus crp*	25
pET28a	Expression vector, Km^r	Novagen
pSH0505	pET28a with *V. vulnificus rpoD*	This study
pINE32	pUC19 with *V. vulnificus nlpD-rpoS-mutS*	4
pHK0011	pRK415, a promoterless *luxAB*, Tc^r	6
pKP-105	pHK0011 with *rpoS* promoter (-105 to +88)	This study
pKP-368	pHK0011 with *rpoS* promoter (-368 to +88)	This study
pKP-732	pHK0011 with *rpoS* promoter (-732 to +88)	This study
pKP-891	pHK0011 with *rpoS* promoter (-891 to +88)	This study
pKP-1315	pHK0011 with *rpoS* promoter (-1315 to +88)	This study
pKP-1640	pHK0011 with *rpoS* promoter (-1640 to +88)	This study
pGEM®-T Easy	Cloning vector, Ap^r	Promega
pGEMT-rpoS$_{mt1}$	pGEM®-T Easy containing 1,403-bp *V. vulnificus rpoS* upstream region of the mutated CRP-binding site I	This study
prpoSM1	pHK0011 with *rpoS*$_{mt1}$ promoter (-1315 to +88)	This study
pGEMT-rpoS$_{mt2}$	pGEM®-T Easy containing 1,403-bp *V. vulnificus rpoS* upstream region of the mutated CRP-binding site II	This study
prpoSM2	pHK0011 with *rpoS*$_{mt2}$ promoter (-368 to +88)	This study
pBluescript II SK(+)	Cloning vector; Ap^r	Stratagene
pVVcya	pBluescript II SK(+) with 3,245-bp *V. vulnificus cya*	This study
pVVΔcya	pVVcya, but with internal 1,973-bp NsiI-restriction fragment deleted	This study
pDM4	Suicide vector, *ori*R6K Cm^r	19
pDMΔcya	pDM4 containing 1,272-bp DNA of internally deleted *cya*	This study
pUC4K	*nptI*, Ap^r, Km^r	Pharmacia
pHY01	pDM4 with 762-bp *cpdA* upstream region	This study
pHY02	pHY01 with 449-bp *cpdA* internal region	This study
pHY03	pHY02 with 1.2-kb *nptI* gene	This study
pRLG770	pKM2 with *rrnB* P1 and terminator region	24
prpoS-P$_p$	pRLG with 160-bp rpoS P$_p$ promoter region	This study
prpoS-P$_d$	pRLG with 138-bp rpoS P$_d$ promoter region	This study

TABLE 2
Oligonucleotides used in this study[a]

Primer extension experiments	
PE+83	5'- ATCTCGATATCGTCGTTAAAATCATCATCC -3'
PE-368	5'- GATAGGCTGACCTCGCTGTCCTGCGATATC -3'
Construction of *rpoS::luxAB* fusions	
rpoS+88	5'- CATTAATCTAGATATCGTCG -3'
rpoS-105	5'- AATCAGTGGATCCAAAGCGC -3'
rpoS-368	5'- GCATGGATCCCGCAGGACAGCG -3'
rpoS-732	5'- TGGGATCCTGCTTATGTCGC -3'
rpoS-891	5'-GGGGATCCTGGAGCTATCG -3'
rpoS-1315	5'- TTACAGTGGGATCCAAAACG -3'
rpoS-1640	5'- ATGAGGGATCCTCAAGCAG -3'
rpoSM1-F[b]	5'-AAGAAAA*CACTG*AACCTGTCACTTCACCACCACCGGCAA-3'
rpoSM1-R	5'-GTGAAGTGACAGGTTC*AGTG*TTTTCTTTACCTTTTGAACC-3'
rpoSM2-F	5'-TAAAT*CGGCCTCAG*AACAACTTGCGACACAACTAGCGAC-3'
rpoSM2-R	5'-TTGTGTCGCAAGTTGTTC*TGAGGCC*GATTTACGGTAAATAGC-3'
Construction of *cya* mutant	
cya-F	5'-TTGACAAAAGGGCCCTTGCC-3'
cya-R	5'-CTAGGGTCAATCTAGAGTGTG-3'
Construction of *cpdA* mutant	
cpd5F	5'-TGCGACTAGTGGCGCAAGAAGAAG-3'
cpd5R	5'-CTTTAGATCTTATTGGGGTTCGG-3'
icc3F	5'-CGAGAGATCTACCTTCCCCCC-3'
icc3R	5'-ATTGGCATGCAGTGTTAATTC-3'
Electrophoretic mobility shift experiments (*gap* DNA)	
gap-F	5'- CATTAATCTAGATATCGTCG-3'
gap-R	5'- AATCAGTGGATCCAAAGCGC-3'
Construction of *rpoS in vitro* transcription plasmids	
rpoS-IV-1	5'-CGGAATTCAAGGGTATTGATCAATCC-3'
rpoS-IV-2	5'-CCCAAGCTTGATTACTCTCCCTTTTGTTGG-3'
rpoS-IV-3	5'-CGGAATTCAATTCGTTACCAAGGTAAATCAG-3'
rpoS-IV-5	5'-CCCAAGCTTTAACTTTGGTGACTGTGTTGC-3'
Construction of RpoD recombinant protein	
F-rpoDvv	5'-TTGGATACCGCATATGGATCATAATCCGCAGTCACAG-3'
R-rpoDvv	5'-CATTTCTCGAGTTACTCGTCAAGGAAGCTACGCAG-3

[a] Restriction sites are underlined and their usages in cloning experiments are described under "Experimental Procedures."
[b] Modified nucleotides for site-directed mutagenesis are italicized and the detailed information is described in the text.

dence for the binding of cAMP-CRP complex to *rpoS* promoter regions and the effect of cAMP-CRP on *rpoS* transcription have not been reported in any bacterial species (1, 16). In addition, the locations of the putative CRP-binding sites of *E. coli rpoS* were predicted to be the sites centered at −61.5 and +56.5 with respect to the major transcription start site. Because the CRP-binding sites usually overlap with the promoters of the genes repressed by the cAMP-CRP complex, those putative CRP-binding sites are not typical for a negative regulation; one (centered at −61.5) is usually an activating site and the other (centered at +56.5) is located at the downstream region of the *rpoS* initiation codon (IC) (1, 10). Thus, the molecular basis of how the cAMP-CRP complex modulates the transcription of the *rpoS* gene has not been completely defined.

We have previously isolated the *rpoS* homolog from *V. vulnificus* (17) and characterized the roles of RpoS in bacterial response to various environmental conditions, which bacteria might encounter within a host (4, 6, 7). Although the functions of RpoS are quite conserved in diverse bacteria, the regulatory modes of *rpoS* gene transcription are distinct in each bacterium (9). Therefore, we are interested in the transcriptional characteristics of *V. vulnificus rpoS*. In this study, we defined two distinct promoters for the *V. vulnificus rpoS* gene and demonstrated direct evidence for transcriptional regulation of *rpoS* via the interaction of the cAMP-CRP complex with these two promoters.

EXPERIMENTAL PROCEDURES

Strains, Plasmids, and Culture Cultivation—Bacterial strains and plasmids used for this study are listed in Table 1. *E. coli* strains used for plasmid DNA preparation and conjugational transfer were grown in Luria-Bertani (LB) broth or on LB plates containing 1.5% (w/v) agar. *V. vulnificus* strains were grown in LB medium supplemented with additional 2% (w/v) NaCl (LBS), unless stated otherwise. All medium components were purchased from Difco, and chemicals and antibiotics were purchased from Sigma.

Primer Extension Experiment—An oligonucleotide, PE+83 (Table 2), complementary to the open reading frame (ORF) of *rpoS* was end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase and then used for cDNA synthesis. RNA was converted to cDNA with SuperScript II reverse transcriptase (Invitrogen). The resultant cDNA products were purified and resolved on a sequencing gel alongside sequencing ladders generated with the same primer used for primer extension. The nucleotide sequence of pINE32 was determined using the dideoxy chain termination method with Top^{TM} DNA polymerase (Bioneer) as described previously (18). The sequencing gels were dried and then visualized upon exposure to a PhosphorImager (Personal Molecular Imager FX, Bio-Rad). Another oligonucleotide, PE-368 (Table 2), was used for synthesis of cDNA to examine *rpoS* expression derived from the second promoter as described above.

*Construction of the Δ*cpdA *V. vulnificus Strain, HY101*—A 762-bp *cpdA* upstream region was amplified using primers cpd5F and cpd5R (Table 2), which locate at −784 and −27 relative to the *cpdA* IC, respectively. The resultant PCR product was digested with the appropriate restriction enzymes, SpeI and BglII, and ligated to a suicide vector pDM4 treated with the same enzymes (19) to produce pHY01. A 449-bp DNA fragment was amplified using primers icc3F and icc3R (Table 2), which are located at +302 and +750 relative to the IC of *cpdA*, respectively. This PCR product was digested with BglII and SphI and cloned into pHY01, resulting in pHY02. Finally, a 1.2-kb kanamycin resistance gene, isolated by digesting pUC4K (GE Healthcare) with BamHI, was inserted into the BglII site of pHY02 to yield pHY03. Plasmid pHY03 in the SM10 λ*pir* (20) strain was mobilized to *V. vulnificus* AR, a rifampin-resistant derivative strain of *V. vulnificus* ATCC29307, and the conjugates were selected by plating the mixture of *E. coli* and *V. vulnificus* on LBS supplemented with 4 μg/ml chloramphenicol and 50 μg/ml rifampin. A colony with characteristics indicating a double homologous recombination event (resistance to 5% (w/v) sucrose, sensitivity to chloramphenicol, and resistance to kanamycin) was further confirmed by PCR, using primers cpd5F and icc3R, and named HY101.

*Construction of Δ*cya *V. vulnificus Strain, KP301*—A 3,245-bp DNA fragment containing the *cya* ORF and its upstream and downstream regions was amplified from the genomic DNA of *V. vulnificus* ATCC29307 using two primers, cya-F and cya-R (Table 2). The PCR product was digested with ApaI and XbaI and then cloned into pBluescript II SK(+) to produce pVVcya. A mutation in *cya* was created by deleting the NsiI fragment within the *cya* coding region in pVVcya, thus yielding pVVΔcya. A 1,973-bp DNA fragment of pVVΔcya digested with ApaI and XbaI was ligated into a suicide vector, pDM4, to generate pDMΔcya. The *E. coli* SM10 λ*pir* strain carrying pDMΔcya was conjugated to *V. vulnificus* ATCC29307, and the exconjugants were then selected on thiosulfate citrate bile sucrose medium supplemented with 4 μg/ml chloramphenicol. A colony indicating double homologous recombination was confirmed by PCR, using primers cya-F and cya-R, and named KP301.

Construction of rpoS luxAB Transcriptional Fusions—A set of *rpoS*::*luxAB* transcriptional fusions was made by subcloning a series of *rpoS* promoter DNA fragments into pHK0011 (6). Primer rpoS+88 (Table 2) contained an XbaI restriction site followed by bases corresponding to the 5′-end of the *rpoS* coding region. Primer rpoS+88 was used in conjunction with one of the following primers to amplify DNA upstream of *rpoS*: rpoS-105 (for pKP-105), rpoS-368 (for pKP-368), rpoS-732 (for pKP-732), rpoS-891 (for pKP-891), rpoS-1315 (for pKP-1315), and rpoS-1640 (for pKP-1640) (Tables 1 and 2). The PCR products were digested with BamHI and XbaI and then inserted into pHK0011, which had been digested with the same enzymes, to create six different *rpoS*::*luxAB* fusions.

Luciferase Assay—The *rpoS*::*luxAB* reporters were mobilized into wild type, Δ*cpdA*, *crp*, and Δ*cya V. vulnificus* mutants via conjugal transfer. Overnight (16–18 h) cultures of the bacterial cells containing one of these fusions were inoculated into fresh LBS medium containing tetracycline (3 μg/ml) and then grown to the stationary phase. At various time points of bacterial growth, a portion of the samples was taken from each culture and diluted 100-fold with LBS medium. The expression from various lengths of the *rpoS* promoter was measured by monitoring light production in the presence of 0.006% (v/v) *n*-decyl aldehyde using a luminometer (TD-20/20; Turners Designs). Light production was expressed in arbitrary relative light units (RLU), and the specific bioluminescence was calculated by normalizing RLU with cell mass (A_{595}), as described previously (7). cAMP at a concentration of 0.5 mM was added exogenously to the culture of Δ*cya* mutant *V. vulnificus* either with pKP-368 or pKP-1315, and the light emission from these cells was monitored at the various phases of bacterial growth.

Western Blot Analysis—The plasmid pQErpoS was constructed to express the recombinant RpoS protein as a histidine-tagged form, which was then used to produce polyclonal antibodies against *V. vulnificus* RpoS as described previously (4). Wild type *V. vulnificus* was harvested at various phases of bacterial growth in LBS broth, and bacterial extracts were then prepared by sonication in TNT buffer (10 mM Tris-HCl, 150 mM NaCl, and 0.05% (v/v) Tween 20, pH 8.0) (21). Forty micrograms of the extracts were fractionated by SDS-PAGE. After a transfer to a Hybond P membrane (Amersham Biosciences), Western blot analysis was performed by serially incubating the filter with anti-*V. vulnificus* RpoS antibodies (1:5,000) and alkaline phosphatase-conjugated rabbit anti-rat immunoglobulin G (1:5,000; Sigma). The RpoS protein of *V. vulnificus* was visualized using an nitro blue tetrazolium-5-bromo-4-chloro-3-indolyl phosphate system (Promega). To investigate the role of cAMP-CRP in RpoS formation, cell extracts of wild type, *crp*, Δ*cya,* and Δ*cpdA* mutant *V. vulnificus* were prepared and then examined by Western blot analysis with RpoS-specific antibodies. cAMP was added to the Δ*cya* mutant at a final concentration of 0.5 mM, and the bacterial extract was also prepared for Western blot analysis.

Purification of V. vulnificus RNA Polymerase (RNAP) Coreenzyme and σ Factor 70 (RpoD)—*V. vulnificus* MO6–24/O was grown in LBS medium at 30 °C for 6 h. Cell pellet was resuspended in the cold grinding buffer (50 mM Tris, 5% (v/v) glycerol, 2 mM EDTA, 233 mM NaCl, 1 mg/ml lysozyme, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, and 7 mM β-mercaptoethanol, pH 8.0). After 20 min of incubation on ice, the bacterial cells were disrupted by sonication in the presence of 4% (w/v) sodium deoxycholate. The core RNAP was purified by an immunoaffinity chromatography using the polyol-responsive monoclonal antibody 8RB13 (NeoClone Biotechnology) as described (22, 23). Eluted core RNAP was then dialyzed with the storage buffer (50 mM Tris-HCl, 0.1 mM EDTA, 0.1 mM dithiothreitol, 50% (v/v) glycerol, pH 7.6). The coding region of *V. vulnificus rpoD* was amplified using oligonucleotide primers, F-rpoDvv and R-rpoDvv (Table 2). The 1.8-kb PCR product was digested with NdeI and XhoI and then ligated to the expression vector, pET28a (Novagen), which was digested with the same enzymes, to produce pSH0505. His-tagged recombinant RpoD protein was expressed in *E. coli* BL21 in the presence of 1.0 mM isopropyl thio-β-D-galactoside and then purified by a nickel-nitrilotriacetic acid affinity chromatography according to the manufacturer's procedure (Qiagen).

In Vitro Transcription Assay in the Presence of CRP—The plasmids containing the templates for RNAP were constructed by inserting the DNA fragments of *rpoS* promoter regions to pRLG770 (24). The insert DNA, including *rpoS* P_d, was amplified using the PCR primers rpoS-IV-1 and rpoS-IV-2. The insert DNA, including *rpoS* P_p, was amplified using rpoS-IV-3 and rpoS-IV-5 (Table 2). Amplified DNA were digested with EcoRI and HindIII and then ligated to EcoRI/HindIII-treated pRLG770. Purified CRP (0, 72, 144, 216, and 288 nM) and the plasmid (0.25 nM) were mixed in the reaction buffer (50 mM KCl, 20 mM Tris-HCl, 3 mM $MgCl_2$, 0.1 mM dithiothreitol, 0.1 mM EDTA, 0.5 mM cAMP, pH 8.0) and incubated at 37 °C for 40 min. The radiolabeled nucleotide (5 μCi of [α-^{32}P]UTP) and cold nucleotides (25 μM UTP and 500 μM each ATP, CTP, and GTP) were added to the reaction mixture, and the transcription was initiated by adding the *V. vulnificus* RNAP holoenzyme premixture of 10 nM core RNAP and 10 nM RpoD (supplemental Fig. 1). The *in vitro* transcription reactions were stopped with the stop buffer (95% formamide, 20 mM EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol, pH 8.0) after 15 min. The reaction mixtures were electrophoresed on a 6.5% polyacrylamide/bisacrylamide (19:1), 7 M urea denaturing gel. Transcripts were visualized and quantified using Personal Molecular Imager FX and Quantity One software, respectively (Bio-Rad).

Electrophoretic Mobility Shift Assay—*V. vulnificus* recombinant CRP protein was overexpressed in *E. coli* BL21 carrying pHK0201 (25), a pRSETA (Invitrogen)-based expression plasmid, and purified by a nickel-nitrilotriacetic acid affinity chromatography according to the manufacturer's procedure (Qiagen).The 394-bp upstream region of the *rpoS* gene, extending from residues −732 to −339 with respect to the IC of *rpo*S, was amplified by PCR using ^{32}P-labeled PE-368 and unlabeled rpoS-732 as primers (Table 2). The other *rpoS* promoter region used for binding assays was made with two primers, PE+83 and rpoS-368 (Table 2), which contained the 456-bp DNA fragment from −373 to +83 with respect to the IC of *rpo*S. Seven nanomolar of the labeled DNA fragment was incubated with varying concentrations of purified histidine-tagged CRP protein (150–600 nM) for 30 min at 37 °C in a 20-μl reaction mixture containing 1× binding buffer (26), including 500 μM cAMP (Sigma). Following the addition of 3 μl of loading buffer to each reaction, the samples were separated by electrophoresis on a 6% nondenaturing polyacrylamide gel. For competition analyses, the identical but unlabeled *rpoS* DNA fragment was included as a competitor DNA. Various amounts of competitor DNA (14–70 nM) were added to the reaction mixture containing 7 nM of the labeled DNA prior to the addition of 600 nM CRP. A 378-bp DNA fragment encompassing the promoter region of the *gap* gene encoding glyceraldehyde-3-phosphate dehydrogenase was amplified from genomic DNA of *V. vulnificus* using primers gap-F and gap-R (Table 2) and included as nonspecific DNA in the binding assay.

cAMP Assay—*V. vulnificus* cells grown in LBS were harvested, and the amounts of cAMP in the bacterial lysates were estimated according to the manufacturer's instructions (cAMP Biotrak EIA System, Amersham Biosciences).

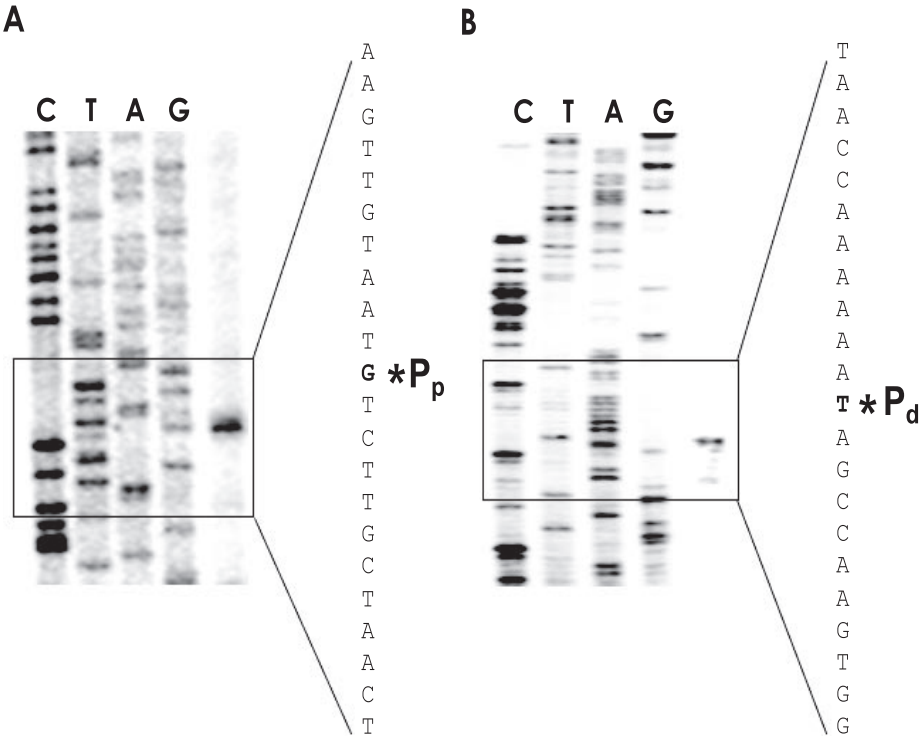
Site-directed Mutagenesis of the rpoS Promoters—The putative CRP-binding sites include the sequences homologous to the inverted repeat, $TGTGAN_6TCACA$. Inverted repeats, which are located at −26 to −47 relative to the distal transcription initiation site (CRP-binding site I) and −27 to −48 relative to the proximal transcription initiation site (CRP-binding site II) of the *rpoS* gene, were mutagenized using primers carrying the substituted nucleotides. To amplify the mutated CRP-binding site I, two sets of primers, rpoS-1315/rpoSM1-R and rpoSM1-F/rpoS+88 (Table 2), were utilized. Two PCR products (an 846-bp PCR product using rpoS-1315 and rpoSM1-R and a 584-bp PCR product using rpoSM1-F and rpoS+88) were used as template DNAs to produce the mutagenized *rpoS* DNA fragment, encompassing the segment from −1315 and +88 relative to the IC of *rpoS*, using primers rpoS-1315 and rpoS+88. The resultant mutagenized *rpoS* promoter DNA was cloned into the pGEM®-T Easy vector (Promega) to produce pGEMT-$rpoS_{mt1}$. To amplify the mutated CRP-binding site II, two primer sets, rpoS-368/rpoSM2-R and rpoSM2-F/rpoS+88, were used. Two PCR products (a 345-bp PCR product using rpoS-368 and rpoSM2-R, and a 142-bp PCR product using rpoSM2-F and rpoS+88) were used as template DNAs to produce the mutagenized *rpoS* DNA fragment, encompassing the segment from −368 and +88 relative to the IC of *rpoS*, using primers rpoS-368 and rpoS+88. The resultant mutagenized *rpoS* promoter DNA was cloned into the pGEM®-T Easy vector (Promega) to produce pGEMT-$rpoS_{mt2}$. The mutagenized nucleotide sequences of both pGEMT-$rpoS_{mt1}$ and pGEMT-$rpoS_{mt2}$ were confirmed by DNA sequencing. Then the insert DNA fragment of each plasmid, digested with BamHI and XbaI, was ligated to BamHI/XbaI-digested pHK0011, which contained the promoterless *luxAB* genes. The resultant plasmids, prpoSM1 and prpoSM2, were mobilized into *V. vulnificus* strains by conjugation, and the exconjugants were selected in thiosulfate citrate bile sucrose medium supplemented with 3 μg/ml tetracycline.

Statistical Analyses—Results were expressed as the means ± S.D. from at least three independent experiments. Statistical analysis was performed using the Student's *t* test (SYSTAT program, SigmaPlot version 9, Systat Software Inc.). Differences were considered significant at p values <0.01.

Nucleotide Sequence Accession Numbers—The nucleotide sequences of the *cpdA* and *cya* genes isolated from *V. vulnificus* ATCC29307 have been deposited in the GenBank™ data base under the accession numbers AY221025 and AY240931, respectively.

RESULTS

Identification of Two Transcription Start Sites of the V. vulnificus rpoS Gene—RNA prepared from the stationary phase cells was converted to cDNA using primer PE+83, which annealed to the nucleotide sequences between +54 and +83 relative to the IC of *rpoS*. Upon gel electrophoresis with the sequencing ladder of the *rpoS* gene with the same primer, a single signal was observed 29 bp upstream of the IC of RpoS (Fig. 1*A*), which is 52 bp downstream of the stop codon of the adjacent gene, *nlpD*. Putative −10 (TAAAGT) and −35 (TTGCGA) sequences were



C

```
atccagttaagggtattgatcaatccaaaccaaaggagtatgttggttcaaaaggtaaaga
aaatgttaaacctgtcacttcgccaccaccggcaaataacaaaaaaTagccaagtggcag
          -35                          -10          Pd
tggccaacaaaagggagagtaatcaaaaacttctctgcaggagaacaaggaaataaaggca
ttgatatcgcaggacagcgaggtcagcctatcgtttcaacggcaggaggtaccgtggttta
ttcgggtaacgcgctaagaggttatggcaatttaatcattggtaaacataacgataattac
ctcagtgcgtatgcgcataacgatcggttgttggtatcggaagggcaaagtgttaaagctg
ggcaacaaatagccacaatgggcagttctggagccaaaacagtgatgttgcacttcgaaat

tcgttaccaaggtaaatcagtgaatccaaagcgctatttaccgTAAatcaactttgaaaca
                                          stop codon of nlpD
acttgcgacacaactagcgacatttaaagttgtaatGtcttgctaactcgccatggggagg
  -35                          -10          Pp
cgttATGagtatcagcaacacagtcaccaaagttaaagatttcgatgttaatcagatggat
  initiation codon of rpoS
```

FIGURE 1. **Identification of the transcriptional start sites of the *rpoS* gene in *V. vulnificus*.** *A*, primer extension experiment using *V. vulnificus* RNA and the oligonucleotide primer PE+83 (annealing to the region from +54 to +83 relative to the IC of *rpoS*). *Lanes C, T, A,* and *G* represent the nucleotide sequencing ladders of pINE32. The *asterisk* indicates the site of transcriptional initiation for *rpoS*. *B*, primer extension experiment using the oligonucleotide primer PE-368 (annealing to the region from −368 to −339 relative to IC) as described above. *C*, two *rpoS* promoters in the *nlpD* open reading frame and *nlpD-rpoS* intergenic space. The promoter P_p is proximal to the IC of *rpoS,* and the promoter P_d is distal from the IC of *rpoS*. The promoters, −10 and −35 regions, are *underlined*, whereas the stop codon of *nlpD* and the start codon of *rpoS* are *capitalized*.

discernable, and this promoter was designated as P_p, a promoter proximal to the *rpoS* ORF (Fig. 1*C*).

The same RNA was hybridized with another primer PE-368, which contained complementary sequences to nucleotide sequences between −368 and −339, relative to the IC of *rpoS*. The initiation site of the resultant cDNA was mapped at −473 with respect to the IC of *rpoS* (Fig. 1*B*). This putative promoter resided within the ORF of the upstream gene, *nlpD*, with reasonable promoter sequences (CTGTCA and AATAAC as −35 and −10, respectively), and was designated as P_d, a distal promoter to the *rpoS* ORF (Fig. 1*C*).

Expressional Analysis of rpoS Promoters Using luxAB-transcriptional Fusions—A series of *luxAB*-transcriptional fusions were constructed by cloning various lengths of the *rpoS* promoter regions (Fig. 2*A*). Two *rpoS*::*luxAB* fusions, pKP-105 and pKP-368, contained only the P_p, whereas the remaining four fusions with longer promoter regions had both promoters P_d and P_p. During growth, the expressions of these diverse fusions were determined by measuring their luciferase activities (Fig. 2*B*). The basal and induced expression levels of pKP-105 were similar to those of pKP-368. The remaining four longer fusions (pKP-732, -891, -1315, and -1640) also showed similar levels of expression compared with each other, but significantly higher expression (over 200-fold) relative to the other two shorter fusions, including only P_p. When bacterial cells entered the stationary phase, the shorter fusions were induced about 3–4-fold, and the longer fusions were induced 12–15-fold. For further analyses, pKP-368 was chosen to represent the expression from P_p only, and pKP-1315 was chosen to represent the expression from both P_d and P_p.

Intracellular Levels of RpoS and cAMP during V. vulnificus Growth—Using polyclonal antibodies specific to *V. vulnificus* RpoS, intracellular levels of RpoS were measured at various time points along a growth curve (Fig. 3). Upon Western blot analysis with RpoS-specific antibodies, an immunoreactive band of ~40 kDa was observed in eight different bacterial extracts along the growth curve. The intensity of this band became stronger as the cells entered the stationary phase, as shown by the assay using *luxAB*-transcriptional fusions. Densitometric quantification of these immunoreactive bands indicates about a 5-fold increase in the RpoS level as bacterial cells entered the stationary phase in LBS medium.

The observation that transcription of the *rpoS* gene and the amount of RpoS increased at the stationary phase suggests the presence of some intracellular signal(s) that switched on the *rpoS*

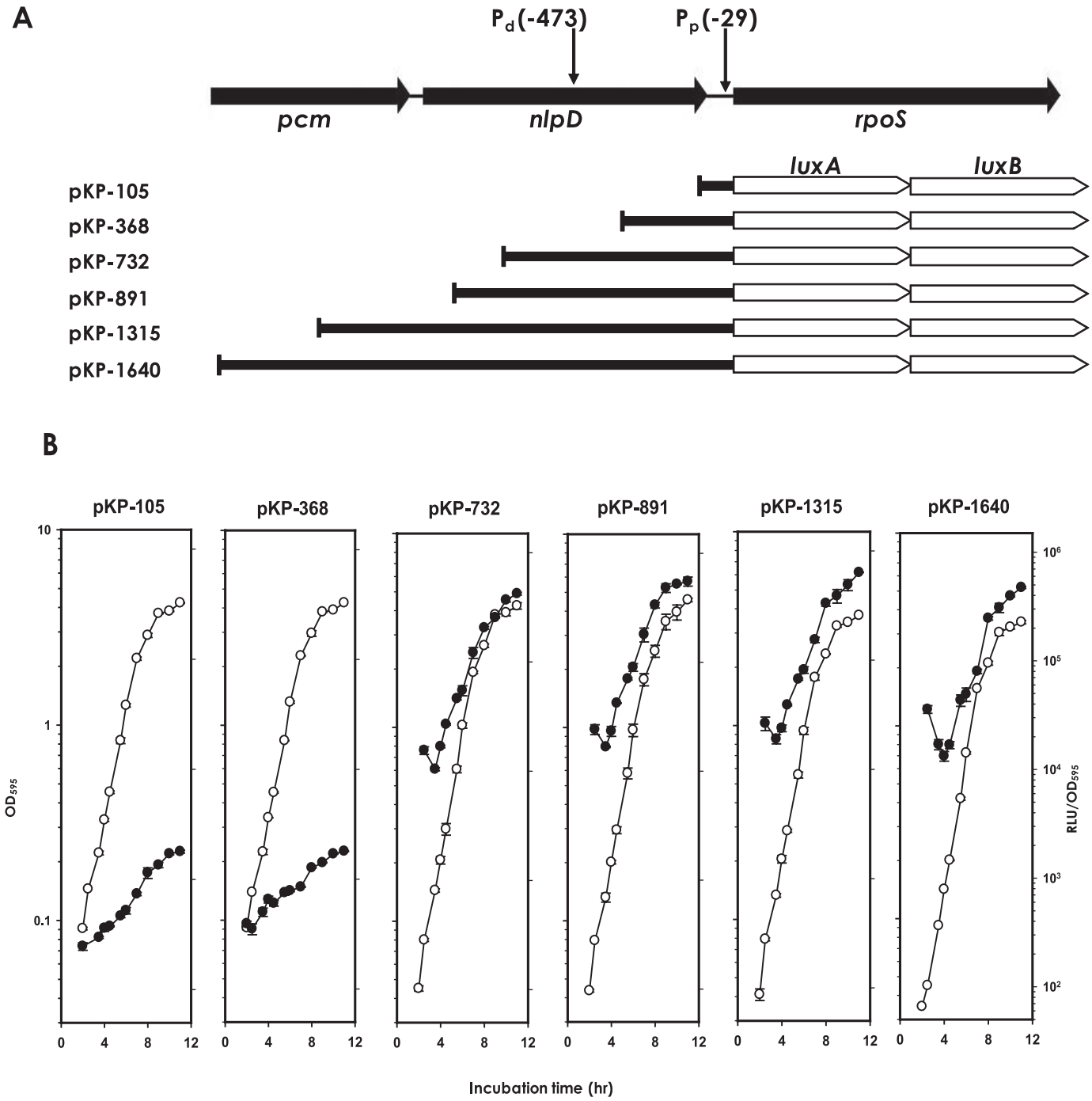


FIGURE 2. **Deletion analysis of the regulatory region of *rpoS*.** *A*, construction of *rpoS*::*luxAB* fusion plasmids. PCR fragments carrying different lengths of the upstream region of *rpoS* were cloned into pHK0011 to create various transcriptional reporter *luxAB* fusions. Two promoters, defined by primer extension analyses, are indicated as P_d and P_p. *B*, expression of *rpoS*::*luxAB* fusions. Wild type *V. vulnificus* carrying one of the six *rpoS*::*luxAB* fusions was grown in LBS medium supplemented with 3 μg/ml tetracycline and examined for luminescence as they grew (*open circles*). Luciferase activities are expressed as normalized values (*closed circles*): number of relative light units (*RLU*) divided by the A_{595} value of each sample.

expression system. The most probable signals related to the high cell density or stationary phase conditions are quorum-sensing signal molecules and cAMP, as suggested previously (27–29). The *luxS* mutant *V. vulnificus*, which is defective in production of the autoinducer-2 (30), showed similar levels of RpoS to the wild type (supplemental Fig. 2). cAMP levels were also estimated from the bacterial cells grown under the same incubation condition used for Western blot analysis (Fig. 3). *V. vulnificus* grown exponentially in the complex medium, such as salt-enriched LB broth, contained 20–50 fmol of cAMP/μg of bacterial protein. When *V. vulnificus* entered the stationary phase, its intracellular cAMP contents dramatically decreased to the levels less than 5 fmol of cAMP/μg of bacterial protein. This result suggests an inverse correlation between cAMP concentration and *rpoS* expression.

Effect of cAMP-CRP on rpoS Expression—The inverse relationship between cAMP contents and *rpoS* expression implied that cAMP and CRP might repress *rpoS* gene expression. To

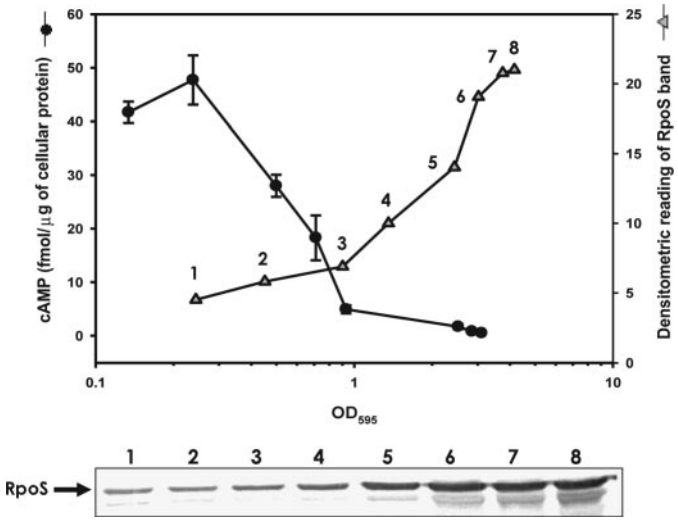


FIGURE 3. **Intracellular levels of cAMP and RpoS at various growth stages of *V. vulnificus*.** *V. vulnificus* cells grown in LBS were assayed for cAMP using the cAMP Biotrak enzyme immunoassay system (Amersham Biosciences), and presented as femtomoles of cAMP per μg of bacterial protein (*circles*). At various time points of bacterial growth (designated by numbers from 1 to 8), cells were harvested and then subjected to Western blot analysis (*lower panel*). The intensities of bands corresponding to RpoS were estimated by densitometry and are also presented in the plot (*triangles*).

TABLE 3
Expression of *rpoS*::*luxAB* fusions in various genetic backgrounds

Genetic background	Intracellular cAMP level[a]	Expression of *luxAB*-transcriptional fusion (RLU/OD)[b]	
		pKP-368	pKP-1315
	fmol/μg protein		
Wild type	8.4	1,320 ± 86	267,780 ± 1,450
crp	421	3,430 ± 95	739,960 ± 87,700
Δ*cya*	Not detectable[c]	3,130 ± 26	632,590 ± 17,900
Δ*cya* + cAMP (0.5 mM)	nd[d]	1,110 ± 9	219,540 ± 9,080
Δ*cpdA*	24	590 ± 16	119,480 ± 4,610

[a] cAMP contents were measured from the stationary phase *V. vulnificus*, and its levels were expressed as moles of cAMP in the unit mass of bacterial cells (determined by protein amount in lysate).
[b] RLU per cell mass determined by A_{595}.
[c] cAMP concentration was below the detection limit of the assay used in this study.
[d] ND means not determined.

test this hypothesis, two representative *rpoS*::*luxAB* fusions (pKP-368 and pKP-1315) were transferred to two mutant *V. vulnificus* strains, deficient in either *cya* (coding for the cAMP-synthesizing enzyme, adenylate cyclase) or *crp* (coding for cAMP-receptor protein). Both fusions in *crp* or Δ*cya* mutant strains showed about a 3-fold increase in their expression over that of the wild type strain (Table 3). Exogenous addition of cAMP (0.5 mM) to the Δ*cya* mutant reduced the expression of both fusions to wild type levels. In addition, another *V. vulnificus* mutant, which is deficient in the *cpdA* gene encoding a 3′,5′-cAMP phosphodiesterase, showed an even lower expression of both fusions compared with the wild type strain.

Cellular levels of RpoS proteins in *crp* and Δ*cya V. vulnificus* mutants were also compared with that of the wild type isogenic strain (Fig. 4). In Western blot analysis using polyclonal antibodies against recombinant RpoS of *V. vulnificus*, more RpoS proteins were observed in both *crp* and Δ*cya* strains than in the wild type. The Δ*cya* strain grown in the presence of exogenously added cAMP demonstrated a decreased level of RpoS.

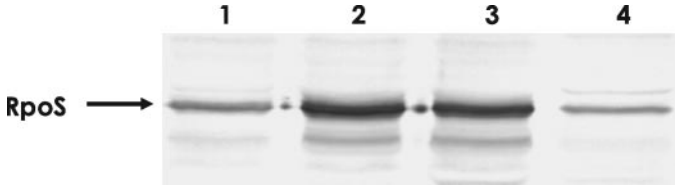


FIGURE 4. **Effect of cAMP and CRP on the intracellular level of RpoS protein.** Extracts of various *V. vulnificus* strains, which were grown to the early stationary phase (A_{595} of 1.0–1.5), were subjected to Western blot analysis using polyclonal antibodies against *V. vulnificus* RpoS. *Lane 1*, wild type; *lane 2*, *crp* mutant; *lane 3*, Δ*cya* mutant; and *lane 4*, Δ*cya* mutant grown in the presence of exogenously added cAMP (0.5 mM).

We then reconstituted the CRP-dependent repression of two *rpoS* promoters *in vitro*. The upstream regions of two *rpoS* promoters, P_d and P_p, were cloned into pRLG770 to produce prpoS-P_d and prpoS-P_p, respectively. *In vitro* transcription reaction with each plasmid DNA in the presence of core RNAP and RpoD of *V. vulnificus* produced two discrete transcripts, the plasmid-encoded RNA-1 (108 nucleotides long) and the longer transcript (188 nucleotides long RNA from prpoS-P_d or 210 nucleotides long RNA from prpoS-P_p) (Fig. 5). The results of *in vitro* transcription assays showed that cAMP-CRP has little or no effect on the synthesis of RNA-1 but repressed syntheses of the transcripts from the P_d and P_p promoters (Fig. 5, *A* and *B*). Effect of cAMP-CRP complex on transcription repression of P_p was more pronounced than that of P_d, because the slope of the decrease in relative intensities of *rpoS* P_p transcript was steeper than that of *rpoS* P_d transcript over the CRP concentration range used (Fig. 5, *C* and *D*). *rpoS* P_p transcript was not produced in the presence of more than 200 nM CRP. On the other hand, production of *rpoS* P_d transcript was less sensitive to CRP concentration. About 50% of the *rpoS* P_d transcript production was repressed in the presence of >200 nM CRP.

Specific Binding of cAMP-CRP Complex to the rpoS Promoters—This study clearly demonstrated that cAMP and CRP negatively affect the expression of *rpoS*. To determine whether the cAMP-CRP complex acts directly by binding to the *rpoS* promoter regions, we performed an electrophoretic mobility shift assay (EMSA) using *V. vulnificus* CRP protein and two different DNA fragments containing P_d or P_p. As shown in Fig. 6*A*, the addition of CRP and cAMP resulted in a shift of the 456-bp P_p DNA fragments to a band with slower mobility. Binding of cAMP-CRP to the P_p DNA was specific, because formation of the slower moving band was abolished by including excess unlabeled P_p DNA in the reaction mixture, but it retained its retarded mobility in the presence of a DNA fragment unrelated to the *rpoS* promoter sequence, such as the *V. vulnficus gap* promoter.

For another set of EMSA with cAMP-CRP, a 394-bp DNA containing P_d was used as a probe (Fig. 6*B*). When the labeled P_d DNA was incubated with cAMP-CRP, formation of the cAMP-CRP-DNA complex was detected as a slower moving DNA band. The specificity of cAMP-CRP binding to this DNA was also confirmed by competition experiments, in which excess unlabeled 394-bp P_d DNA competed out the binding of cAMP-CRP to the ^{32}P-labeled P_d DNA in a dose-dependent manner. In contrast, inclusion of nonspecific *gap* promoter DNA in the binding assays did not disrupt the CRP-P_d DNA interaction.

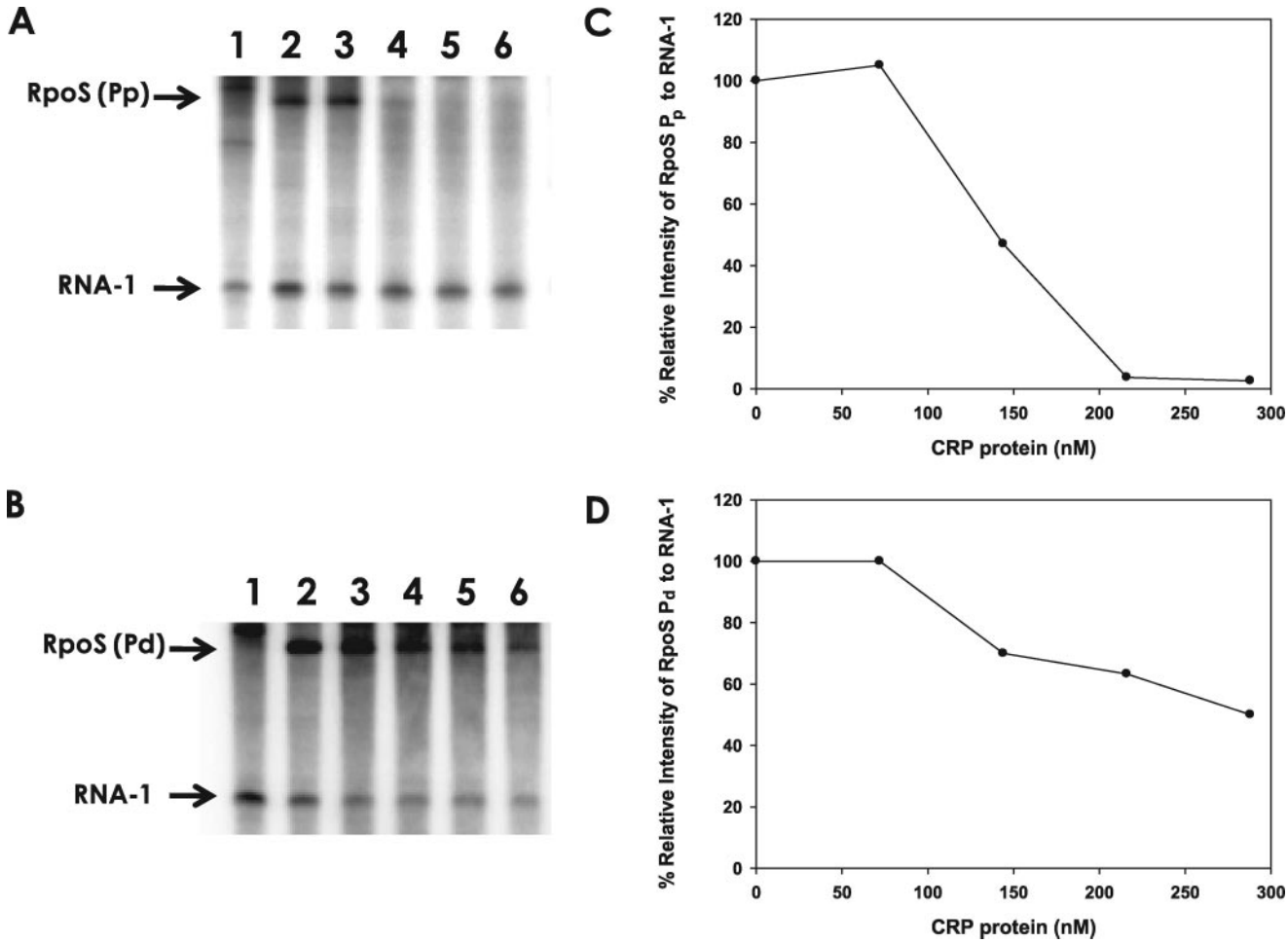


FIGURE 5. **Repression of *rpoS* transcription by the cAMP-CRP complex.** *A*, *in vitro* transcription at the *rpoS* promoter P_p in the presence of cAMP-CRP. The pRLG770 (24)-based plasmid carrying the *rpoS* P_p (prpoS-P_p) was used for a template for core RNAP, RpoD, and CRP derived from *V. vulnificus* (supplemental Fig. 1). Produced ^{32}P-labeled transcripts were separated on 6.5% polyacrylamide/bisacrylamide (19:1), 7 M urea denaturing gel showing two RNA bands (the control RNA of 108-nucleotide-long RNA-1 and the 210-nucleotide-long *rpoS* RNA) on each lane. *Lane 1*, pRLG770 (no *rpoS* promoter); *lanes 2–6*, prpoS-P_p incubated with 0, 72, 144, 216, or 288 nM of CRP. *B, in vitro* transcription at the *rpoS* promoter P_d in the presence of cAMP-CRP. The pRLG770 (24)-based plasmid carrying the *rpoS* P_d (prpoS-P_d) was used for an assay, which resulted in production of RNA-1 and 188-nucleotide-long *rpoS* RNA. *Lane 1*, pRLG770; *lanes 2–6*, prpoS-P_d incubated with 0, 72, 144, 216, or 288 nM of CRP. *C* and *D*, plots illustrating the relative intensity of *rpoS* transcript. The intensities of two RNA bands on each lane, the 108-base RNA-1 transcript, and the longer transcript starting from one of the *rpoS* promoters were estimated by densitometric reading (the plot *C* is from *A*, and the plot *D* is from the *B*), and then each *rpoS* transcript was normalized to RNA-1 on the same lane. Relative intensity of the normalized *rpoS* transcript was indicated as the percentage of that in the absence of CRP.

The apparent affinity of cAMP-CRP complex to each *rpoS* promoter was compared by extrapolating the CRP concentration required for 50% binding of the labeled DNA (Fig. 6*C*). The dissociation binding constants (K_d) for CRP to P_p and P_d were ~250 and 410 nM, respectively. Thus, the results from both the *in vitro* transcription assays (Fig. 5) and EMSA (Fig. 6) suggest that the promoter P_p has higher affinity to cAMP-CRP complex.

Effect of Mutation of the Putative CRP-binding Sites on Binding of CRP and Expression of rpoS—The nucleotide sequences of the two *rpoS* promoters were analyzed to determine whether they include the putative cAMP-CRP binding sequence. Nucleotide sequences of both promoters showed considerable homology to the consensus sequence for the *E. coli* cAMP-CRP-binding site (AAATGTGATCTAGATCACATTT; see Ref. 31) at the upstream regions including their −35 regions (Fig. 7*A*). To verify if cAMP-CRP binding to these putative sites occurs and to determine whether these interactions are important in *rpoS* transcription from P_d and P_p, the putative binding sites were modified by site-directed mutagenesis. The DNA fragments containing the mutagenized nucleotide sequences (mt probes) were then used for electrophoretic mobility shift assays. No binding of cAMP-CRP to mt probes was observed (Fig. 7, *B* and *C, lanes 3–6*), although the amount of CRP added to the binding assay was two times (up to 765 nM CRP) more than that of the original nucleotide sequences (wild type probes) (Fig. 7, *B* and *C, lane 2*).

In addition, the mutagenized DNAs were used to construct the *luxAB* -transcriptional fusions, pKP-368mt and pKP-1315mt, which are basically the same as pKP-368 and pKP-1315, but include the mutated CRP-binding sites. These mutant fusions in the wild type strain showed derepressed levels of expression (Fig. 7, *D* and *E*), which were similar to the expression levels of pKP-368 and pKP-1315 in the *crp* and Δ*cya* strains. These results suggest that *rpoS* expression from P_d and P_p is repressed by direct binding of cAMP-CRP to the regions of

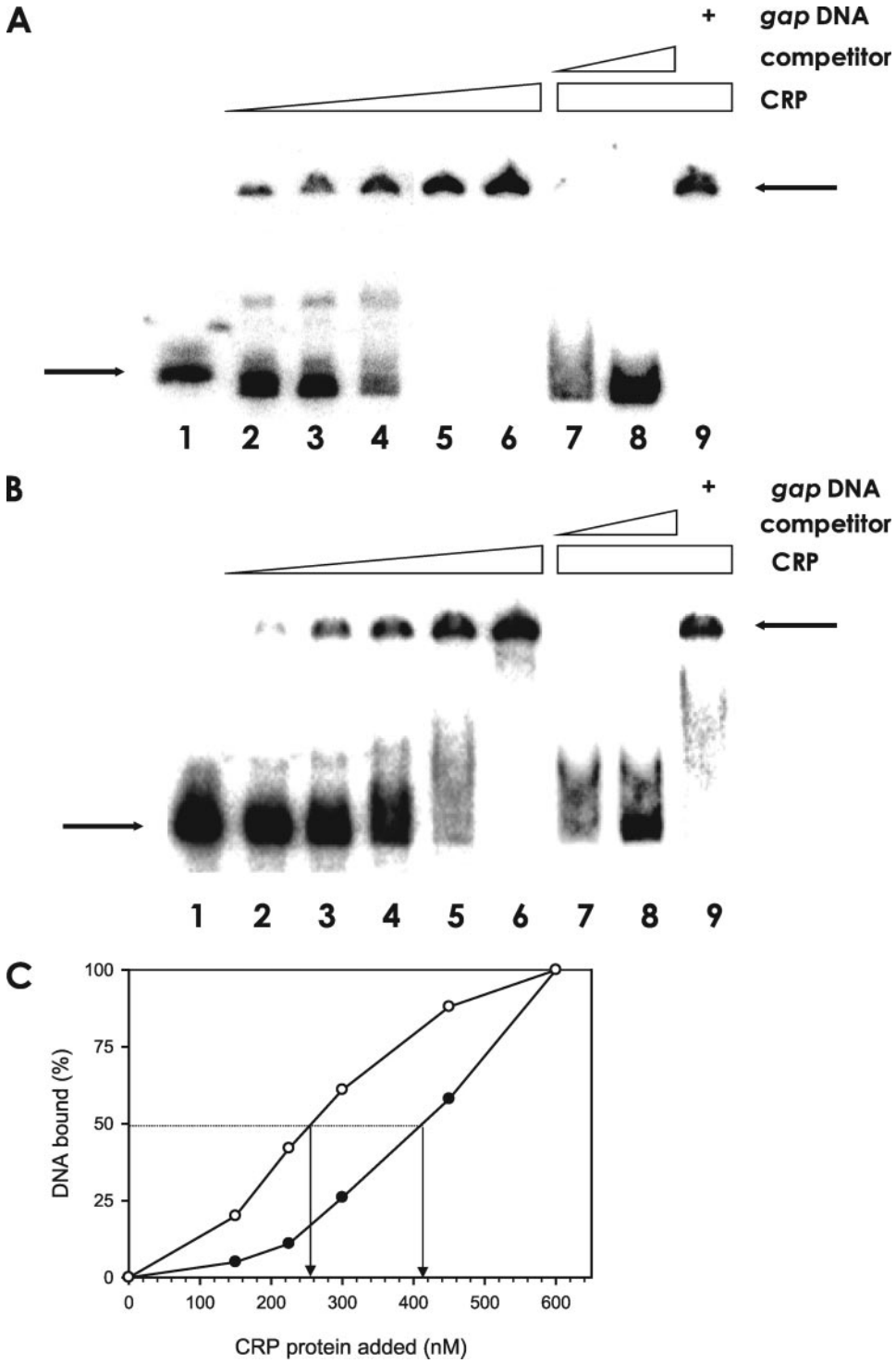


FIGURE 6. **Specific binding of the cAMP-CRP complex to the *rpoS* promoters.** *A,* binding assay of CRP to the *rpoS* promoter carrying P_p. The 456-bp DNA fragment of the *rpoS* upstream region (P_p) was radiolabeled and incubated, with increasing amounts of CRP up to 600 nM. The identical but unlabeled competitor DNA fragment was included in the reaction mixtures in various amounts. *Lane 1*, probe DNA alone; *lanes 2–6*, probe DNA incubated with 150, 225, 300, 450, or 600 nM CRP, respectively; *lane 7*, probe DNA incubated with 600 nM CRP and 14 nM of the identical but unlabeled DNA; *lane 8*, probe DNA incubated with 600 nM CRP and 70 nM of the identical but unlabeled DNA; and *lane 9*, probe DNA incubated with 600 nM CRP and 70 nM *gap* promoter DNA. The *arrows* on the *left side* indicate the unbound DNA probe, whereas the *arrows* on the *right side* indicate DNA bound to CRP. *B,* binding assay of CRP to the *rpoS* promoter carrying P_d. Labeled P_d DNA was incubated with increasing amounts of CRP. For competition analysis, the identical but unlabeled 394-bp DNA fragment was included in the binding reactions containing 600 nM CRP. *Lane 1*, probe DNA alone; *lanes* 2–6, probe DNA incubated with 150, 225, 300, 450, or 600 nM CRP, respectively; *lane 7*, probe DNA incubated with 600 nM CRP and 14 nM of the identical but unlabeled DNA; *lane 8*, probe DNA incubated with 600 nM CRP and 70 nM of the identical but unlabeled DNA; and *lane 9*, probe DNA incubated with 600 nM CRP and 70 nM nonspecific *gap* promoter DNA. The *arrows* on the *left side* indicate unbound DNA probe, whereas the *arrows* on the *right side* indicate the DNA bound to CRP. *C,* plot showing the affinity of cAMP-CRP to each promoter of *rpoS*. The intensities of bound DNA fragments, P_p (*open circle*) and P_d (*closed circle*), were estimated by densitometer and plotted against the CRP concentrations. *Arrows* indicate the concentrations of CRP causing half-maximal binding corresponding to the K_d.

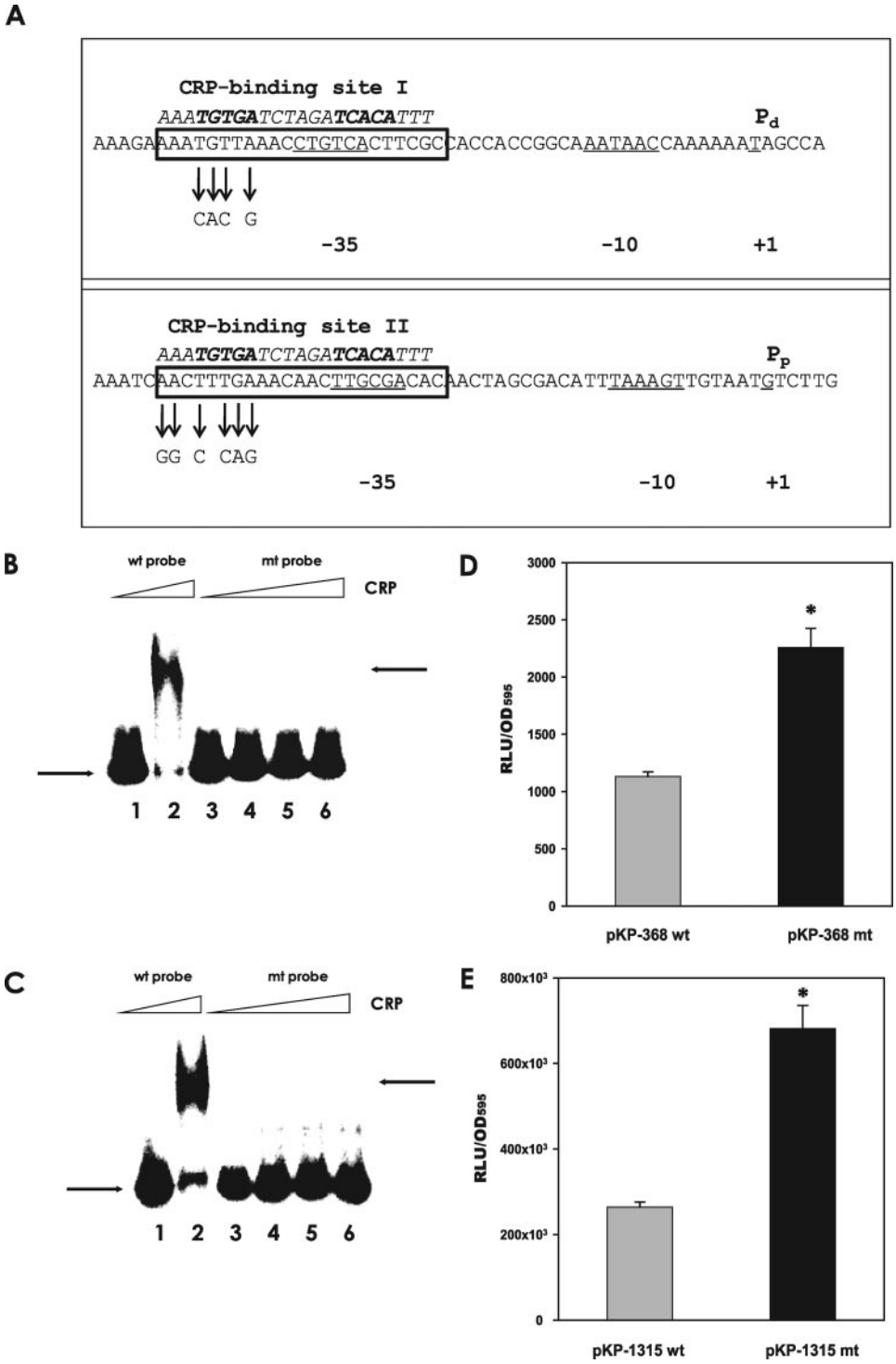


FIGURE 7. **Effect of mutation of the putative CRP-binding sites on CRP binding and *rpoS* regulation.** *A*, presence of the putative CRP-binding sites in each promoter region of the *rpoS* gene. Two promoters for the *rpoS* gene are indicated as P_p and P_d along with their −10 and −35 regions (CRP-binding site I in the P_d promoter and CRP-binding site II in the P_p promoter). Putative CRP-binding sites are presented in the *boxes* implied from the conserved nucleotide sequences for CRP binding indicated by *italicized letters* above the *V. vulnificus* DNA sequence. Site-directed mutagenized nucleotides in the mutant *rpoS* promoters are indicated with *arrows*. *B,* binding assay of CRP to the *rpoS* promoter carrying P_d and mutagenized P_d. Labeled P_d (wild type probe) and mutagenized P_d DNA (mt probe) were incubated with CRP, as described in Fig. 5. *Lane 1*, P_d DNA without CRP; *lane 2*, P_d with 340 nM CRP; *lane 3*, mutagenized P_d without CRP; *lanes 4–6*, mutagenized P_d with 340, 510, and 765 nM CRP, respectively. The *arrows* on the *left side* indicate the unbound DNA probe, whereas the *arrows* on the *right side* indicate the DNA bound to CRP. *C,* binding assay of CRP to the *rpoS* promoter carrying P_p and mutagenized P_p. Labeled P_p (wild type probe) and mutagenized P_p DNA (mt probe) were incubated with increasing amounts of CRP. *Lane 1*, P_p DNA without CRP; *lane 2*, P_p with 340 nM CRP; *lane 3*, mutagenized P_p without CRP; *lanes 4–6*, mutagenized P_p with 340, 510, and 765 nM, CRP, respectively. The *arrows* on the *left side* indicate unbound DNA probe, whereas the *arrows* on the *right side* indicate the DNA bound to CRP. *D* and *E,* effect of mutation on the expression of *rpoS*::*luxAB* transcriptional fusions. Wild type *V. vulnificus* carrying pKP-368 or pKP-368mt (pKP-368 including the mutated CRP-binding site II) and wild type carrying pKP-1315 or pKP-1315mt (pKP-1315, including the mutated CRP-binding site I) were grown in LBS medium supplemented with 3 μg/ml tetracycline, and their luciferase activities were measured. Luciferase activities are expressed as normalized values: the number of RLU divided by the A_{595} value of each sample. Data with *p* values of <0.01 are indicated with an *asterisk*.

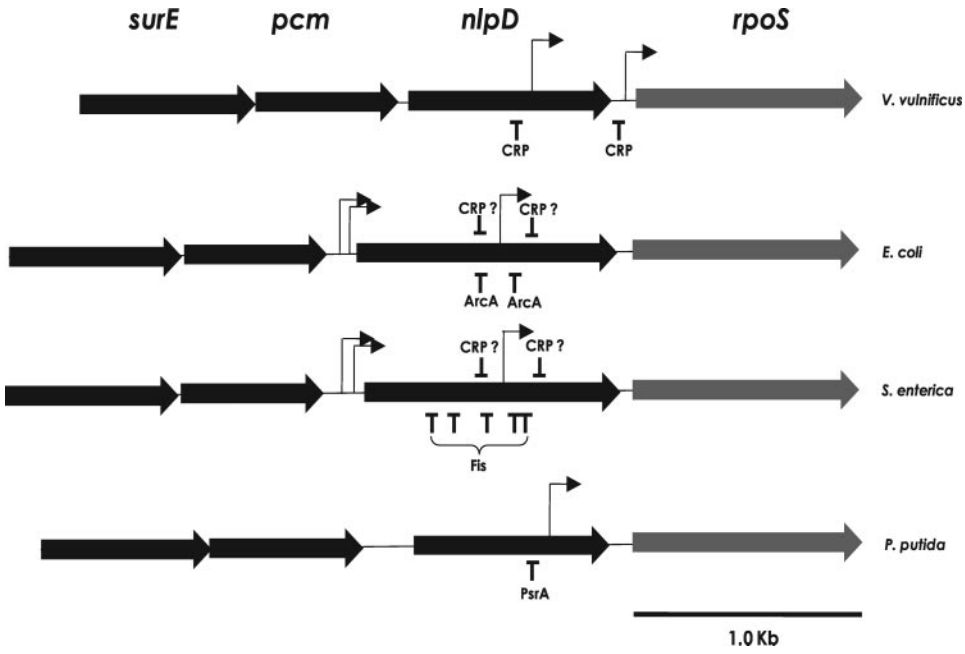


FIGURE 8. **Genetic organization and expression of *rpoS* genes in some Gram-negative bacteria.** The genetic organization of *rpoS* and its upstream genes (*nlpD*, *pcm*, and *surE*) of *V. vulnificus* were compared with those of *E. coli* (14), *S. enterica* serovar Typhimurium (11), and *P. putida* (12). The transcription start sites for each *rpoS* gene are designated with *arrows*, and the binding site locations for transcription factors are designated by *vertical lines* with the name of each transcription factor. In the case of *E. coli* and *S. enterica*, CRP-binding sites are putative and are thus designated with *question marks*.

−26 to −47 relative to P_d (CRP-binding site I) and −27 to −48 relative to P_p (CRP-binding site II) (Fig. 7*A*).

DISCUSSION

V. vulnificus is a normal inhabitant of marine estuarine environments and can be delivered into humans via ingestion of seafood or contact with seawater, whereupon it may cause fatal septicemia or gastroenteritis (2). Thus, this bacterium is expected to use efficient survival strategies to sense fluctuations in its surrounding conditions and to express the necessary defense elements against given stresses. In a previous investigation, we identified RpoS as a key regulator mediating the survival of *V. vulnificus* (4), as found in other bacteria. Variation of the intracellular levels of this global transcription factor, under certain conditions, is critical for modulating the expression of target genes in a finely coordinated manner.

Modulation of the amount of RpoS, which has been extensively investigated, is achieved by several levels of controls, including the regulation of transcription, translation, and proteolysis (1). In *E. coli*, one of the major regulatory mechanisms modulating the level of RpoS operates at the post-translational level via an RpoS-specific chaperone/protease system, ClpXP (32). Under glucose-starved conditions or in the exponential stage, a recognition factor, RssB, binds to the RpoS protein and enhances RpoS degradation by recruiting ClpXP to the RssB-RpoS complex (33). Translation of *E. coli rpoS* mRNA is regulated by small noncoding RNAs (34). A specific secondary structure in the *rpoS* mRNA, which inhibits efficient translational progress, is disrupted by being paired with DsrA RNA with the help of an RNA chaperon, Hfq, resulting in increased translation of *rpoS* mRNA (35). Similarly, *V. vulnificus* also utilizes some of the above regulatory mechanisms, both at the post-transcriptional and the post-translational levels via Hfq and ClpX, respectively. For example, the levels of RpoS, determined by Western blot analysis, were significantly reduced in the Δ*hfq V. vulnificus* mutant (36) and highly elevated in the Δ*clpX V. vulnificus* mutant (supplemental Fig. 3).

The regulation of *E. coli rpoS* expression at the transcriptional level includes complex mechanisms. Trans-acting factors involved in *rpoS* transcription respond to intracellular signals related to the cessation of growth caused by environmental stresses. The involvement of the intracellular molecule, guanosine 3′,5′-bispyrophosphate (ppGpp), as a positive signal of *rpoS* expression has been reported (37). The *barA* gene product, first identified as a regulator of *ompR* (38), has been demonstrated to induce *rpoS* transcription (39). A two-component regulatory system for anaerobiosis, ArcA/ArcB, is involved in modulation of *rpoS* expression (10). The phospho-ArcA represses transcription of the *rpoS* gene by directly binding to the *rpoS* promoter region.

Although the importance of CRP in the regulation of *rpoS* genes has been reported in *E. coli* and *S. enterica* serovar Typhimurium (14, 11), the regulatory mechanism of *rpoS* transcription by the cAMP-CRP complex requires further study. The role of cAMP-CRP complex as an important negative transcriptional regulator was proven only genetically via *rpoS*-fusion assays using knock-out mutants unable to produce CRP or cAMP (14, 16). Repression of *rpoS* transcription by cAMP-CRP was also confirmed by the phenotype of a *crr* knock-out mutant (15). $EIIA^{glc}$, which is a *crr* gene product and a component of the glucose uptake system, acts indirectly on the repression of *rpoS* expression by modulating the activity of adenylate cyclase. However, the molecular basis of transcriptional modulation of the *rpoS* gene by cAMP-CRP has not yet been identified in any bacterial system.

Because *rpoS* transcription is quite different among bacterial species (9), we examined the expression of the *rpoS* gene in *V. vulnificus* at the level of transcription. We preliminarily analyzed the transcription units of the *rpoS* gene and found two transcripts for *rpoS*,[3] as occurred in *E. coli* (40). However, the sizes of the *V. vulnificus rpoS* transcripts were distinct from those of *E. coli* and other bacterial species (Fig. 8). In *E. coli* and *S. enterica*, the *rpoS* gene is expressed as a polycistronic *nlpD-rpoS* mRNA and an ~2.0-kb-long *rpoS* monocistronic mRNA. Polycistronic *nlpD-rpoS* mRNA originates from two closely

[3] K.-J. Park and K.-H. Lee, unpublished data.

spaced promoters upstream of the *nlpD* gene. The second promoter responsible for monocistronic *rpoS* mRNA is located within the *nlpD* gene, which had been identified as a major *rpoS* promoter (41). In *E. coli* growing in LB medium, the polycistronic *nlpD-rpoS* mRNA was constitutively expressed, but the monocistronic *rpoS* mRNA was induced only at the stationary phase (14). In the case of *P. putida rpoS*, a single promoter located within the *nlpD* ORF was identified (Fig. 8).

Primer extension experiments identified a transcription initiation site 29 bp upstream of the *rpoS* ORF and another initiation site for a larger transcript at −473 with respect to the IC of *V. vulnificus rpoS* (Fig. 1, *A* and *B*). Thus, a shorter *rpoS* transcript of 1,021 bp was detected in addition to a 1,518-bp monocistronic *rpoS* mRNA in *V. vulnificus*. The series of *rpoS*::*luxAB* fusions indicated that the promoter located within the *nlpD* gene (P_d) is a major promoter, as shown in other bacteria (Fig. 2). In contrast, the promoter located just upstream of the *rpoS* (P_p) is a minor factor for *rpoS* expression. Expression of both promoters was induced as the cells entered the stationary phase in LBS medium. However, the affinity of each promoter to the cAMP-CRP complex was quite different (Fig. 5, *C* and *D,* and Fig. 6*C*), which might suggest each promoter has a differential role in *V. vulnificus* in response to specific stresses. Therefore, further investigation of the roles of each promoter is required to understand its relative contribution to *rpoS* gene expression under various conditions. Unlike *E. coli*, the upstream region of *nlpD* is not involved in *rpoS* expression, because luciferase activities of two fusions containing the upstream regions of *nlpD* (pKP-1315 and pKP-1640) were similar to those of two fusions without the *nlpD* upstream region (pKP-732 and pKP-891) (Fig. 2). This further implies that *nlpD* transcription in *V. vulnificus* may start at the further upstream region of the *pcm* or *surE* genes.

RpoS levels of *V. vulnificus* were inversely correlated to the intracellular concentrations of cAMP; for example, stationary phase cells grown in complex media, such as LBS medium, contained less cAMP (<5 fmol of cAMP/μg of bacterial protein) than exponential phase cells (~50 fmol of cAMP/μg of bacterial protein) (Fig. 3). This pattern of intracellular cAMP fluctuation was similar to that shown in *E. coli* cells grown in LB medium (42). The role of cAMP-CRP in *rpoS* transcription was confirmed in various *V. vulnificus* strains, in which CRP was knocked out or the synthesis/degradation of cAMP was altered (Table 3). Expression of *rpoS* fusions was decreased in the Δ*cpdA* mutant that has an intracellular cAMP level approximately twice that estimated in its isogenic wild type strain. *rpoS* expression was highly increased in the *crp* and Δ*cya* mutants but decreased in the Δ*cya* mutant in the presence of exogenously added cAMP (Table 3 and Fig. 4). Therefore, *rpoS* expression is tightly dependent upon the cAMP-CRP complex at the transcriptional level in *V. vulnificus*.

V. vulnificus growing in a glucose-based minimal medium, such as artificial seawater supplemented with glucose as a sole carbon source, contained significant cAMP levels when cells entered the stationary phase because of carbon source depletion.[4] In addition, *E. coli* cells grown in a minimal medium, such as M9 supplemented with glucose, did not show induction of *rpoS* transcription during the stationary phase; cellular cAMP levels were also highly increased during this phase (14). Thus, *rpoS* transcription is highly repressed by cAMP-CRP complex under this condition, and thus regulation at the post-transcriptional level is important in the stationary phase induction of RpoS.

In this study, we further confirmed the role of the cAMP-CRP complex in *rpoS* expression by demonstrating the repression of *rpoS* expression by addition of CRP to the *in vitro* transcription reaction (Fig. 5) and the direct interaction of both *rpoS* promoters with recombinant CRP protein (Fig. 6). The interactions between the *rpoS* promoters and cAMP-CRP complex are mediated by DNA sequences homologous to the CRP-binding consensus sequence (Fig. 7*A*). Alteration of these putative CRP-binding sites resulted in the disappearance of specific interaction with the cAMP-CRP complex *in vitro* (Fig. 7, *B* and *C*) and abolishment of transcriptional repression by the cAMP-CRP complex *in vivo* (Fig. 7, *D* and *E*). This is the first study showing the direct interaction of the cAMP-CRP complex with the *rpoS* promoters.

The data presented in this study do not exclude the possibility that other transcriptional factors are involved in *rpoS* expression in *V. vulnificus*. The transcriptional fusions of *rpoS* in Δ*cya* and *crp* mutants still showed the induction of expression at the stationary phase (data not shown). This suggests that the induction of *rpoS* transcription is partly controlled by another factor, which is independent of cAMP and CRP. *In silico* screening of the *V. vulnificus* genome suggests the absence of a gene homologous to the transcriptional activator PsrA found in *Pseudomonas putida* (12). Therefore, there must be other transcriptional factors in *V. vulnificus* that remain to be elucidated in future studies.

Acknowledgments—We thank Kyung-Je Park for the initial investigation of the rpoS fusions; Sung-Min Kim for the construction of Δcpd A mutant and the measurement of cAMP contents; and Mi-Ae Lee for the careful proofreading of this manuscript.

REFERENCES

1. Hengge-Aronis, R. (2002) *Microbiol. Mol. Biol. Rev.* **66,** 373–395
2. Strom, M. S., and Paranjpye, R. N. (2000) *Microbes Infect.* **2,** 177–188
3. Hülsmann, A., Rosche, T. M., Kong, I.-S., Hassan, H. M., Beam, D. M., and Oliver, J. D. (2003) *Appl. Environ. Microbiol.* **69,** 6114–6120
4. Park, K.-J., Kang, M.-J., Kim, S. H., Lee, H.-J., Lim, J.-K., Choi, S. H., Park, S.-J., and Lee, K.-H. (2004) *J. Bacteriol.* **186,** 3304–3312
5. Hengge-Aronis, R. (2000) in *The General Stress Response in Escherichia coli* (Storz, G., and R. Hengge-Aronis, R., eds) pp. 161–178, ASM Press, Washington, D. C.
6. Jeong, H. S., Jeong, K. C., Choi, H. K., Park, K.-J., Lee, K.-H., Rhee, J. H., and Choi, S. H. (2001) *J. Biol. Chem.* **278,** 45072–45081
7. Lee, H.-J., Park, K.-J., Lee, A. Y., Park, S. G., Park, B. C., Lee, K.-H., and Park, S.-J. (2003) *J. Bacteriol.* **185,** 5891–5896
8. Litwin, C. M., and Quackenbush, J. (2001) *Microb. Pathog.* **31,** 295–307
9. Venturi, V. (2003) *Mol. Microbiol.* **49,** 1–9
10. Mika, F., and Hengge, R. (2005) *Genes Dev.* **19,** 2770–2781
11. Hirsch, M., and Elliott, T. (2005) *J. Bacteriol.* **187,** 1568–1580
12. Kojic, M., Aguilar, C., and Venturi, V. (2002) *J. Bacteriol.* **184,** 2324–2330
13. Burtnick, M. N., Downey, J. S., Brett, P. J., Boylan, J. A., Frye, J. G., Hoover, T. R., and Gherardini, F. C. (2007) *Mol. Microbiol.* **65,** 277–293

[4] S.-M. Kim and K.-H. Lee, unpublished data.

14. Lange, R., and Hengge-Aronis, R. (1994) *Genes Dev.* **8,** 1600–1612
15. Ueguchi, C., Misonou, N., and Mizuno, T. (2001) *J. Bacteriol.* **183,** 520–527
16. McCann, M. P., Fraley, C. D., and Matin, A. (1993) *J. Bacteriol.* **175,** 2143–2149
17. Park, K.-J., Kim, S. H., Kang, M.-J., Chung, D. H., Ha, S.-D., Kim, K.-S., Jahng, D., and Lee, K.-H. (2004) *J. Microbiol. Biotechnol.* **12,** 1063–1066
18. Roh, J.-B., Lee, M.-A., Lee, H.-J., Kim, S.-M., Cho, Y., Kim, Y.-J., Seok, Y.-J., Park, S.-J., and Lee, K.-H. (2006) *J. Biol. Chem.* **281,** 34775–34784
19. Milton, D. L., O'Toole, R., Hörstedt, P., and Wolf-Watz, H. (1996) *J. Bacteriol.* **178,** 1310–1319
20. Miller, V. L., and Mekalanos, J. J. (1988) *J. Bacteriol.* **170,** 2575–2583
21. Sambrook, J., and Russell, D. W. (2001) *Molecular Cloning: A Laboratory Manual,* 3rd Ed., pp. 17.1–17.17, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
22. Bergendahl, V., Thompson, N. E., Foley, K. M., Olson, B. M., and Burgess, R. L. (2003) *Protein Expression Purif.* **31,** 155–160
23. Thompson, N. E., Hanger, D. A., and Burgess, R. R. (1992) *Biochemistry* **31,** 7003–7008
24. Newlands, J. T., Gaal, T., Mecsas, J., and Gourse, R. L. (1993) *J. Bacteriol.* **175,** 661–668
25. Jeong, H. S., Lee, M. H., Lee, K.-H., Park, S.-J., and Choi, S. H. (2003) *J. Biol. Chem.* **276,** 13875–13880
26. González-Gil, G., Kahmann, R., and Muskhelishvili, G. (1998) *EMBO J.* **17,** 2877–2885
27. Milton, D. L. (2006) *Int. J. Med. Microbiol.* **296,** 61–71
28. Botsford, J. L. (1981) *Microbiol. Rev.* **45,** 620–642
29. Latifi, A., Foglino, M., Tanaka, K., Williams, P., and Lazdunski, A. (1996) *Mol. Microbiol.* **21,** 1137–1146
30. Kim, Y. R., and Rhee, R. H. (2003) *Biochem. Biophys. Res. Commun.* **304,** 405–410
31. Busby, S., and Ebright, R. H. (1999) *J. Mol. Biol.* **293,** 199–213
32. Schweder, T., Lee, K.-H., Lomovskaya, O., and Matin, A. (1996) *J. Bacteriol.* **178,** 470–476
33. Studemann, A., Noirclerc-Savoye, M., Klauck, E., Becker, G., Schneider, D., and Hengge-Aronis, R. (2003) *EMBO J.* **22,** 4111–4120
34. Gottesman, S. (2004) *Annu. Rev. Microbiol.* **58,** 303–328
35. Majdalani, N., Cunning, C., Sledjeski, D., Elliott, T., and Gottesman, S. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 12462–12467
36. Lee, H.-J., Bang, S. H., Lee, K.-H., and Park, S.-J. (2007) *J. Bacteriol.* **189,** 2629–2636
37. Gentry, D. R., Hernandez, V. J., Nguyen, L. H., Jensen, D. B., and Cashel, M. (1993) *J. Bacteriol.* **175,** 7982–7989
38. Nagasawa, S., Tokishiba, S., Aiba, H., and Mizno, T. (1992) *Mol. Microbiol.* **6,** 799–807
39. Mukhopadhyay, S., Audia, J. P., Roy, R. N., and Schellhorn, H. E. (2000) *Mol. Microbiol.* **37,** 371–381
40. Arnqvist, A., Olsén, A., and Normark, S. (1994) *Mol. Microbiol.* **13,** 1021–1032
41. Lange, R., Fischer, D., and Hengge-Aronis, R. (1995) *J. Bacteriol.* **177,** 4676–4680
42. Ma, Z., Richard, H., and Foster, J. W. (2003) *J. Bacteriol.* **185,** 6852–6859