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Expression of *Vibrio vulnificus* insulin-degrading enzyme is regulated by the cAMP–CRP complex

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Components of the bacterial phosphoenolpyruvate (PEP) : carbohydrate phosphortransferase system (PTS) have multiple regulatory roles in addition to PEP-dependent transport/phosphorylation of numerous carbohydrates. We have recently shown that, in an opportunistic human pathogen, *Vibrio vulnificus*, enzyme IIA^{Glc} ($EIIA^{Glc}$) interacts with a peptidase that has high sequence similarity to mammalian insulin-degrading enzymes, called *Vibrio* insulin-degrading enzyme (vIDE). Although the vIDE–$EIIA^{Glc}$ interaction is independent of the phosphorylation state of $EIIA^{Glc}$, vIDE shows no peptidase activity unless complexed with the unphosphorylated form of $EIIA^{Glc}$. A deletion mutant of *ideV*, the gene encoding vIDE, shows remarkably lower degrees of survival and virulence than the wild-type strain in mice, implying that vIDE is a virulence factor. In this study, we investigated regulation of *ideV* expression at the transcriptional level. Primer extension analysis identified two different transcriptional start sites of *ideV*: P_L for the longer transcript and P_S for the shorter transcript. We performed ligand fishing experiments by using the promoter region of *ideV* and found that the cAMP receptor protein (CRP) specifically binds to the promoter. DNase I footprinting experiments revealed that CRP binds to a region between the two promoters. *In vitro* transcription assays showed that CRP activates *ideV* P_S transcription in the presence of cAMP whose concentration is regulated by $EIIA^{Glc}$. These results suggest that $EIIA^{Glc}$ regulates the expression level of vIDE as well as its activity.

INTRODUCTION

Most, if not all, *Vibrio* species are found in marine or brackish water. Therefore, *Vibrio*-linked illnesses are usually associated with seafood or seawater and tend to occur in the coastal areas in summer and autumn when the waters are warm and *Vibrio* counts are high (Chuang *et al.*, 1992). *Vibrio vulnificus* is a motile, Gram-negative, curved rod-shaped pathogenic bacterium with a single polar flagellum. Under certain conditions, *V. vulnificus* has the ability to cause serious and often fatal infections. These include an invasive septicaemia usually contracted through the consumption of raw or undercooked shellfish, as well as wound infections acquired through contact with shellfish or marine waters where the organism is present (Strom & Paranjpye, 2000).

Bacteria utilize different transport mechanisms for the uptake of solutes: facilitated diffusion, active transport driven by ATP or ion gradients and group translocation. Among them, group translocation of carbohydrates is mediated by the bacterial phosphoenolpyruvate (PEP) : carbohydrate phosphortransferase system (PTS) (Kundig *et al.*, 1964). The PTS catalyses translocation with a concomitant phosphorylation of sugars and hexitols and regulates the metabolism in response to the availability of carbohydrates (Deutscher *et al.*, 2006). PTSs are ubiquitous in eubacteria but do not occur in archaea and eukaryotes. PTSs consist of two general proteins, enzyme I (EI) and HPr, and a variable number of sugar-specific transporters collectively known as enzymes II (EII). EI transfers phosphoryl groups from PEP to the phosphoryl carrier protein HPr. HPr then transfers the phosphoryl groups to EII. EII usually consists of three functional units, EIIA, EIIB and EIIC, which occur either as protein subunits in a complex or as domains of a single polypeptide chain. EIIA and EIIB sequentially transfer phosphoryl groups from HPr to the transported sugars.

Recently, the relationship between carbohydrate utilization and virulence has been studied in several human pathogenic bacteria such as *Listeria monocytogenes*, *Bacillus anthracis* and group A *Streptococcus* (Mertins *et al.*, 2007; Shelburne *et al.*, 2008; Tsvetanova *et al.*, 2007). In these species, the availability of certain carbohydrates in the environment acts as a signal to control their virulence gene

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Abbreviations: CRP, cAMP receptor protein; PEP, phosphoenolpyruvate; PTS, PEP:carbohydrate phosphotransferase system; TSS, transcription start site.

Three supplementary figures are available with the online version of this paper.

expression through the regulation of virulence transcription factors. Furthermore, glucose transport and metabolism via the carbohydrate PTS has been shown to be required for the successful infection of macrophages and mice by *Salmonella enterica* (Bowden *et al.*, 2009) and colonization of the mouse intestine by *Vibrio cholerae* (Houot *et al.*, 2010).

We also have recently shown that $EIIA^{Glc}$ regulates the activity of a protease homologous to mammalian insulin-degrading enzymes (IDE) by direct interaction in *V. vulnificus*. While the specific interaction with the IDE homologue [named *Vibrio* IDE (vIDE)] is independent of the phosphorylation state of $EIIA^{Glc}$, only unphosphorylated $EIIA^{Glc}$ can trigger the peptidase activity of vIDE. The *ideV* mutant shows no growth defect *in vitro*, but it shows significantly lower degrees of survival and virulence than the wild-type strain in mice (Kim *et al.*, 2010), suggesting that vIDE is a virulence factor. We believed that, in addition to activity, expression of this virulence factor should be precisely regulated. Therefore, we set out to search for a transcription factor(s) exhibiting high-affinity interaction with the *ideV* promoter. Consequently, we found that the cAMP receptor protein (CRP) specifically binds to this promoter and have investigated the regulation of *ideV* transcription by the cAMP–CRP complex in this study.

METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were cultured in Luria–Bertani (LB) medium at 37 °C and *V. vulnificus* strains were cultured in LB containing 2.5 % (w/v) NaCl (LBS) at 30 °C. When necessary, antibiotics were used at the following concentrations (μg ml^{-1}): for *E. coli*, ampicillin (Ap), 100; kanamycin (Km), 20; and tetracycline (Tc), 20; and for *V. vulnificus*, Tc, 2; and Km, 200.

RNA isolation. Cells were harvested and suspended in lysis buffer [20 mM sodium acetate, pH 5.5, 0.5 % (w/v) SDS, 1 mM EDTA] and mixed with an equal volume of acid phenol/chloroform (5 : 1, pH 4.5; Ambion). After incubation at 60 °C for 5 min with gentle shaking, the samples were centrifuged for 5 min at 10 000 ***g*** and the RNA-containing aqueous phases were collected. RNA samples were further purified using an RNeasy mini kit (Qiagen) according to the manufacturer's instructions.

Primer extension. The synthetic oligonucleotide primers used in this study are listed in Table 2. Oligonucleotide primers (10 pmol each) were incubated with 5 units T4 polynucleotide kinase (Fermentas) in the presence of [γ-^{32}P]ATP (6000 Ci mmol^{-1}; 222 TBq mmol^{-1}) at 37 °C for 1 h and precipitated with ethanol. RNA samples (50 µg each) were mixed with radioactive-isotope-labelled primers in hybridization buffer (40 mM PIPES, pH 6.5, 400 mM NaCl and 1 mM EDTA), denatured at 95 °C for 5 min and hybridized at 42 °C for 2 h. Reverse transcription was performed with M-MLV reverse transcriptase (Invitrogen) in the presence of RNaseOUT (Invitrogen) to prevent RNase contamination. Nucleic acids were collected through phenol extraction and ethanol precipitation. Co-precipitated RNAs were hydrolysed by the addition of 0.1 M NaOH and the samples were loaded on a sequencing gel. For the identification of the transcription start sites of *ideV*, a sequencing ladder was generated by using a Thermo Sequence Cycle Sequencing kit (USB) with the same primer according to the manufacturer's instructions.

Ligand fishing experiment and protein identification. To search for the protein(s) regulating *ideV* transcription, a DNA fragment covering the *ideV* promoter region was prepared to use as bait. A DNA fragment covering the *ideV* ORF region was used as the control. The DNA fragments were amplified by PCR using primers listed in

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
E. coli DH5α	*hsdR17 recA1 endA1 supE44 thi-1gyrA relA1* Δ(*lacZYA-argF*) *U169 deoR* ϕ*80dlac*Δ (*lacZ*) M15	Sambrook & Russell (2001)
E. coli BL21(DE3)	F$^-$ l$^-$ *ompT hsdS$_B$* (r_B^- m_B^-) *gal dcm met* (DE3)	Novagen
V. vulnificus MO6-24/O	Clinical isolate, wild-type strain	Wright *et al.* (1990)
V. vulnificus CMM988	MO6-24/O Δ*crp*, Km^r	Bang *et al.* (1999)
Plasmids		
pET3a	Expression vector, Ap^r	Novagen
pET3a-vCRP	Expression vector for *Vibrio* CRP, Ap^r	This study
pRK415	Broad-host-range plasmid, Tc^r	Keen *et al.* (1988)
pHK0011	pRK415 with promoterless *luxAB*, Tc^r	eong *et al.* (2001)
pVm_P*ideV*::*luxAB*	pHK0011-based plasmid for luciferase assay of transcriptional fusion of *ideV* promoter with *luxAB*	This study
pVm_P*ideV*mt1::*luxAB*	AGCGTT in the CRP binding site of the *ideV* promoter in pVm_P*ideV*::*luxAB* is mutated to GAATCC	This study
pVm_P*ideV*mt2::*luxAB*	TCTCAA in the CRP binding site of the *ideV* promoter in pVm_P*ideV*::*luxAB* is mutated to GAATCC	This study
pSA600	Template plasmid for *in vitro* transcription, Ap^r	Ryu & Garges (1994)
pTxn_wt	*ideV* promoter region in pSA600	This study
pTxn_mt	pTxn_wt with the mutated CRP binding site	This study

Table 2. Oligonucleotides used in this study

Engineered restriction sites and changed nucleotides are underlined.

Primer (purpose)	Sequence (5′–3′)
Primer extension	
P_PE83	CCCGACATTGACCGCAAGAGCCGCA
P_PE62	AGTGATGAGTGTCATTTGGACTTAAGTGCA
Ligand fishing	
P_IDEPFB	CACGTTGCGTCTCCGAAAATGTCAATATTG
P_IDEPR	CGATCCGCTTTGCGCTGCCATCACAGCACAATG
P_IDEOFB	CAAATCGAATCGGGCTGAAATCTCGCGTTG
P_IDEOR	AGACAGTCCACACGCGGTTTCTTCTGCGAAG
Protein expression	
PcCRPF	CATCGTGGATCCAAATTAACGAGTACCGTAAAC
PcCRPR	GTGAGATCATATGGTTCTAGGTAAACCTCA
DNase I footprinting	
P_FP65	GCTGATTTGGTGGCGTCTTCGGAGCTGATC
P_FPR	CGATCCGCTTTGCGCTGCCATCACAGCACAATG
Cloning of plasmids for *in vitro* transcription template	
PvIDEtxnF	AGCAAAACTGCAGACTGGTTATCGAGCGTCAAG
PvIDEtxnR	CGATCCGAATTCCGCTGCCATCACAGCACAATG
Cloning of plasmids for luciferase activity assay	
PidePluxF	CGATGCTGGCTCGGTACCAACTCCACACAC
PidePluxR	CCTTCTCGTTCGATAGGATCCTCAAAATGC
Site-directed mutagenesis of the CRP binding site	
P_QCR	GCTCAATCGACCGATTCGCCATGGAAATACCAC
P_QCF	GTCGATTGAGCCATCTTTGCGTACCTCTCTTTA
P_QCR2	CTATGGCGCTCGAGACCGATTCGCCATGGAAAT
P_QCF2	AGCGCCATAGGGATGTACCTCTCTTTAACAGATT

Table 2 (P_IDEPFB and P_IDEPR for the promoter and P_IDEOFB and P_IDEOR for the ORF; primers P_IDEPFB and P_IDEOFB were biotinylated at their 5′ ends). Ligand fishing with the prepared DNA fragments and the following experiments to identify the protein band that specifically bound to the promoter DNA were carried out as described previously (Jeong *et al.*, 2004). Briefly, prepared DNA baits were mixed with 200 µl UltraLink Immobilized NeutrAvidin plus resin (Pierce) in immobilization buffer (20 mM phosphate buffered, pH 7.5, and 150 mM NaCl) and incubated for 30 min with gentle agitation. After the buffer was removed, the DNA-bound resin was equilibrated with the binding buffer B [40 mM HEPES-KOH, pH 7.9, 100 mM KCl, 10 mM $MgCl_2$, 2 mM DTT and 10 % (v/v) glycerol]. *V. vulnificus* cells were cultured in LBS until OD_{600} 1.0 and suspended in binding buffer B. The cells were disrupted by passing twice through a French press, and cell debris and insoluble fractions were removed by ultracentrifugation. The bait-bound resin and the crude extract were mixed and incubated with gentle agitation at 4 °C for 1 h. The crude extract was removed and the resin was washed with 500 µl binding buffer B three times. The proteins bound to the resins were eluted with 0.1 % (w/v) SDS solution and subjected to electrophoresis. The polyacrylamide gel was stained with Coomassie brilliant blue R, and the band of interest was excised from the gel. The polypeptide in the gel fragment was digested with 200 ng trypsin. The peptides digested with trypsin were eluted three times with 20 µl 5 % (v/v) trifluoroacetic acid containing 75 % (v/v) acetonitrile and concentrated to 5 µl in a vacuum for peptide mapping. MALDI-TOF mass spectra were acquired using a Voyager-DE STR (Applied Biosystems) MALDI-TOF mass spectrometer.

Purification of CRP. To construct an expression vector for CRP, the CRP-coding region was amplified from the chromosomal DNA of *V. vulnificus* MO6-24/O by PCR with primers PcCRPF and PcCRPR (Table 2) possessing the synthetic restriction enzyme sites. The PCR product digested with *Nde*I and *Bam*HI was inserted into the corresponding sites of pET3a vector (Novagen) to make pET3a-vCRP. The BL21 strain harbouring pET3a-vCRP was cultured in LB until OD_{600} 1.0, and expression of CRP was induced with 1 mM IPTG for 4 h. The cells were harvested and washed with 10 mM Tris/HCl containing 50 mM NaCl (pH 7.5). The cell pellet was suspended in binding buffer A (20 mM Tris/HCl, pH 7.5, 300 mM NaCl) and disrupted by passing twice through a French press at 69 000 kPa. After ultracentrifugation at 220 000 ***g*** for 30 min, the supernatant was collected and mixed with Talon metal affinity resin (Clontech) pre-equilibrated with the binding buffer A in a Poly-prep chromatography column (8 × 40 mm; Bio-Rad). The column was washed six times with one resin volume of the binding buffer A and the proteins bound to the resin were eluted with binding buffer A containing 150 mM imidazole. The eluted protein sample was concentrated to 2 ml and further purified by gel filtration chromatography using a Superose 12 column (GE healthcare Lifescience) pre-equilibrated in 20 mM Tris/HCl containing 100 mM KCl (pH 7.5).

DNase I footprinting. To determine the location of the CRP binding site(s) within the *ideV* promoter region, the DNA fragment carrying the *ideV* promoter region was amplified by PCR using the primer P_FP65 labelled with ^{32}P at the 5′-end by using polynucleotide kinase as described above and the reverse primer P_FPR (Table 2) and purified by using a PCR purification kit (Qiagen). The radiolabelled PCR product was incubated with purified CRP in 50 mM Tris/HCl, pH 7.5, 10 mM $MgCl_2$, 100 mM KCl, 10 % (v/v) glycerol, 2 mM EDTA, 1 mM DTT, 20 µg poly dI-dC ml^{-1} and 100 µM cAMP for 15 min. The addition of Ca^{2+}/Mg^{2+} (5 mM/10 mM) solution was

followed and the DNA fragment was digested with 0.3 units RQ1 RNase-Free DNase (Promega) for 70 s. The reaction was stopped by addition of the stop solution [200 mM NaCl, 30 mM EDTA and 1 % (w/v) SDS]. After phenol extraction and ethanol precipitation, the digested DNA was subjected to electrophoresis on a sequencing gel. A sequencing ladder generated with the same primer was loaded for the identification of the binding sites of CRP.

***In vitro* transcription assay.** To construct the template for *in vitro* transcription, the promoter region of *ideV*, from 240 bp upstream to 120 bp downstream with respect to the vIDE start codon, was amplified from the chromosomal DNA of *V. vulnificus* MO6-24/O by PCR with primers PvIDEtxnF and PvIDEtxnR possessing the synthetic restriction enzyme sites (Table 2). The PCR product digested with *Pst*I and *Eco*RI was inserted into the corresponding sites located in front of the *rpoC* terminator in pSA600 vector (Ryu & Garges, 1994). The template plasmid pTxn_wt (35 ng) was incubated with 0.4 units *E. coli* RNA polymerase holoenzyme (USB) in the reaction buffer (40 mM Tris/HCl, pH 8.0, 10 mM $MgCl_2$, 5 mM DTT, 50 mM KCl and 50 μg BSA ml^{-1}) for 15 min and cAMP and CRP were added. To protect mRNA products, 20 units of recombinant RNase inhibitor (RNaseOUT; Invitrogen) was added to each reaction. RNA polymerization was initiated by adding the NTP mix containing 1 mM ATP, 100 μM UTP, 100 μM GTP, 10 μM CTP and 0.375 μM [α-^{32}P]CTP (800 Ci $mmol^{-1}$; 29.6 TBq $mmol^{-1}$). The *in vitro* transcription reactions were stopped by adding 50 mM EDTA after 1 h. After the reaction, samples extracted with acid phenol/chloroform (5 : 1, pH 4.5; Ambion) and precipitated with ethanol were subjected to electrophoresis on a 6 % (w/v) polyacrylamide gel containing 6 M urea. After the gel was dried on Whatman 3MM paper, autoradiography was obtained by exposure on an imaging plate (Fuji film) and scanning with BAS-2500 (Fuji film). The 106/107 nt *rep* RNA from the plasmid was used as an internal control to compare the amount of transcripts.

Site-directed mutagenesis of the CRP binding site. To introduce a mutation into the CRP binding site in the *ideV* promoter region of the plasmid pTxn_wt, PCR-based quick-change mutagenesis was performed as described previously (Weiner *et al.*, 1994). PCR with primers P_QCF and P_QCR (Table 2) changed 10 bp, and PCR with primers P_QCF2 and P_QCR2 further mutated the CRP binding sequence. Consequently the CRP binding site 5′-GCAAAGCGTTA-TCAATCTCAATA-3′ was mutated to 5′-ATCCCTATGGCGCT-CGAGACCGA-3′. The mutations in the plasmid pTxn_mt were confirmed by sequencing.

Luciferase activity assay. To construct the transcriptional fusion plasmids (Table 1), the DNA fragment covering from 750 bp upstream to 155 bp downstream with respect to the vIDE start codon was amplified with primers PidePluxF and PidePluxR possessing the synthetic restriction enzyme sites (Table 2). To generate pVm_P*ideV*::*luxAB*, the PCR product digested with *Kpn*I and *Bam*HI was inserted into the corresponding sites located in front of *luxAB* in the pHK0011 vector (Jeong *et al.*, 2001). pVm_P*ideV*mt1::*luxAB* and pVm_P*ideV*mt2::*luxAB* were also constructed where parts (AGCGTT and TCTCAA, respectively) of the CRP binding site in the *ideV* promoter in pVm_P*ideV*::*luxAB* were changed to GAATCC by site-directed mutagenesis. *V. vulnificus* cells harbouring these transcriptional fusion plasmids were grown at 30 °C in LBS medium. Aliquots were taken from cultures at several time points and luciferase activities were measured in triplicate in the presence of 0.006 % (v/v) decanal using a Luminometer (MicroLumat Plus, Berthold Technologies). *ideV* expression levels were calculated by normalizing relative activity with respect to the OD_{600}.

Determination of the cAMP concentration. The amount of cAMP in the bacterial cell culture medium was estimated using the cAMP Biotrak enzyme immunoassay system according to the manufacturer's instructions (GE Healthcare Lifescience). Aliquots of the cell culture medium were taken at various time points and boiled for 5 min; the amount of cAMP was measured in duplicate in assay buffer [50 mM acetate, pH 5.8, 0.02 % (w/v) BSA and 0.01 % (w/v) preservative].

RESULTS

Identification of two transcription start sites of the *V. vulnificus ideV* gene

To identify the transcription start site(s) (TSS) of *ideV*, primer extension experiment was performed with total cellular RNA prepared from stationary-phase cells and the 5′ ^{32}P-labelled primer, P_PE62 (Table 2). Upon electrophoresis, two bands corresponding to positions −23 and −116 with respect to the vIDE start codon were detected (Fig. 1a). Because of several uncertain signals between the two bands, primer extension was performed with another primer P_PE83 that annealed 95 bp downstream from the binding site of P_PE62. Two obvious bands appeared at the same positions as those signals obtained with the primer P_PE62 (Fig. 1b). From these results, the TSSs of *ideV* were determined to be 23 bp and 116 bp upstream of the vIDE start codon. These promoters were designated P_L for the distal promoter from the start codon and P_S for the proximal promoter. Putative −10 (CATAAT) and −35 (TTTGCA) sequences of P_S and those of P_L [−10 (AACAAT) and −35 (TTGCTA)] were mapped (Fig. 1c).

CRP specifically binds to the *ideV* promoter

To search for a regulator(s) influencing *ideV* transcription, a ligand fishing strategy (Jeong *et al.*, 2004) was employed with the promoter region of *ideV* (see Methods). The ORF region was used as a control. The size of the DNA fragments was 245 bp covering from +29 to −216 with respect to the P_S for the promoter region and from +1518 to +1762 for the ORF region (see Fig. S1, available with the online version of this paper). Through several independent trials with the crude extract of the wild-type *V. vulnificus* MO6-24/O strain, we found that a distinct protein band of about 23 kDa always co-elutes with the promoter DNA but not the ORF (Fig. 2). Tryptic in-gel digestion and the peptide mass fingerprinting acquired by MALDI-TOF mass analysis identified the protein band as the cAMP receptor protein (CRP) encoded at the gene locus *VVM_00509* of the recently sequenced *V. vulnificus* MO6-24/O genome (Park *et al.*, 2011). To confirm the specific binding of CRP to the promoter region of *ideV*, the ligand fishing experiment was also performed with the crude extract of a *crp* deletion mutant of *V. vulnificus* (Bang *et al.*, 1999). While the protein band of about 23 kDa bound to the promoter DNA was apparent in the wild-type crude extract, we could not detect the protein in the *crp* mutant crude extract (see Fig. S2), supporting the hypothesis that the protein specifically bound to the *ideV* promoter DNA corresponds to CRP.

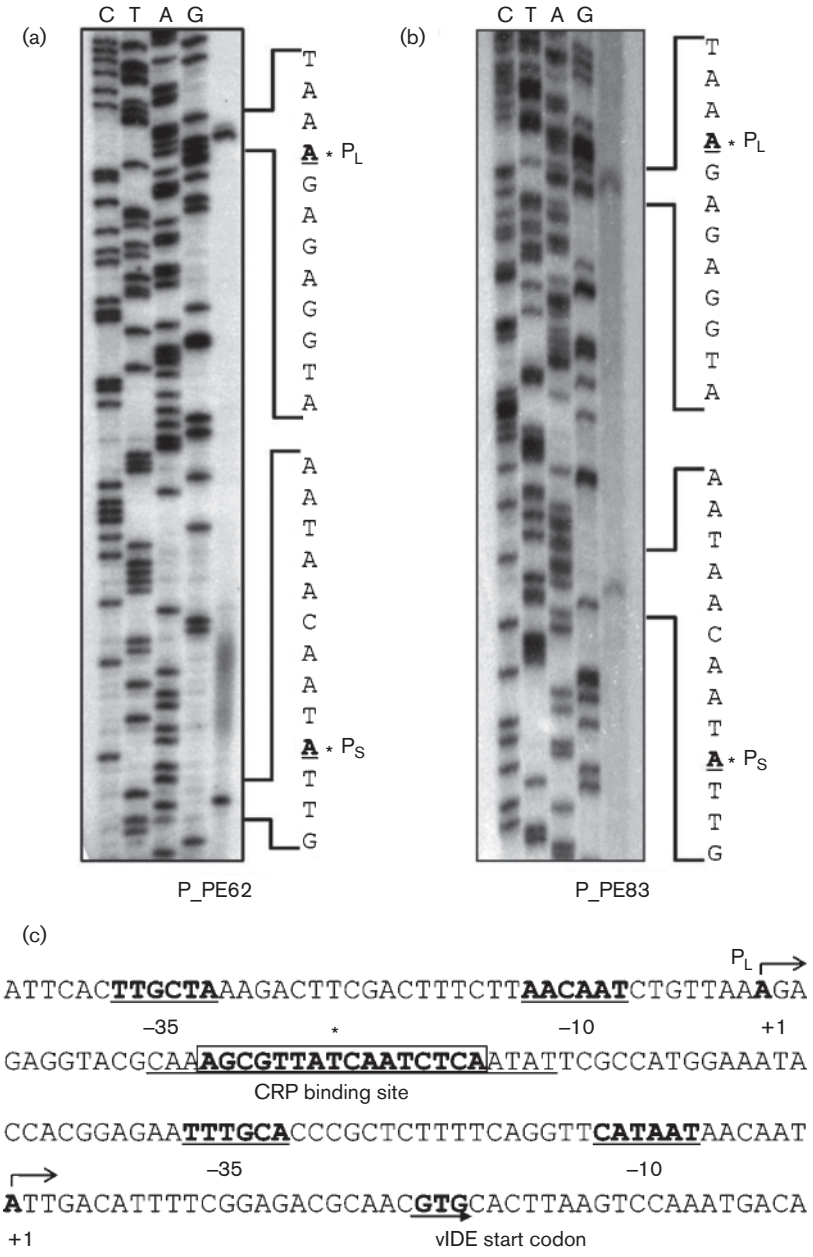

Fig. 1. Identification of the transcription start sites of the *V. vulnificus ideV* gene. Primer extension analyses were carried out using *V. vulnificus* total RNA and ^{32}P-labelled oligonucleotide primers P_PE62, which anneals to the region from +2 to +31 (a), and P_PE83, which anneals to the region from +101 to +125 with respect to the translation start site (b). Lanes C, T, A and G represent the nucleotide sequencing ladders generated with the same primer. The asterisks indicate the transcription start sites of the *ideV* gene. (c) Organization of the *ideV* promoter region. The vIDE translation start codon and the TSSs of P_L and P_S are indicated by arrows and the potential −35 and −10 boxes are underlined. The region protected by CRP from DNase I is underlined and the putative CRP binding sequence is indicated with a box. The centre of the CRP binding site, located at −71.5 with respect to P_S, is indicated by an asterisk.

To identify the binding site of CRP in the *ideV* promoter, electrophoretic mobility shift assays (EMSAs) were performed using purified CRP and a series of ^{32}P-labelled probes together encompassing the region from −157 to +54 with respect to the P_S. Incubation of all probes except those missing from −97 to −59 with CRP showed retardation in EMSA experiments (compare EMSAs of the probes GSA4′F and GSA6 with those of others in Fig. S3). From these results, the CRP binding site is proposed to be located between −97 and −59 bp with respect to the P_S of the *ideV* promoter. To confirm the results from EMSA and to determine the precise location and sequence of the CRP binding site, DNase I footprinting assays were performed. The pair of primers ^{32}P-labelled P_FP65 and P_FPR (Table 2) were used to produce the 5′-^{32}P-labelled DNA fragment of the *ideV* promoter covering from +124 to −129 with respect to P_S. This fragment was incubated with increasing amounts of purified CRP ranging from 0 to 8 μM in the presence of 100 μM cAMP and then treated with DNase I. After the enzymic digestion, the reaction mixtures were subjected to electrophoresis on a denaturing polyacrylamide gel containing 6 M urea with the sequencing ladder generated with the same labelled primer. The DNase I-digested patterns show that the region between −83 and −60 with respect to the P_S was protected by CRP (Fig. 3). From these results, we concluded that there is only one CRP binding site between P_L and P_S and we found that this site is similar to the consensus CRP binding sequence

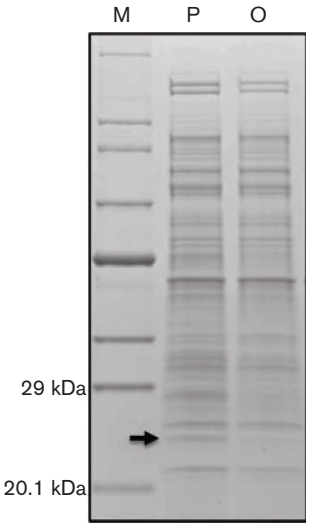

Fig. 2. Specific binding of CRP to the promoter region of the *ideV* gene. A ligand fishing experiment was performed to search for a protein(s) interacting with the promoter region of the *ideV* gene. Crude extract prepared from wild-type *V. vulnificus* MO6-24/O was mixed with the promoter region (lane P) or the ORF region (lane O) of *ideV* as indicated, and treated as described in Methods. In-gel digestion followed by MALDI-TOF analysis revealed that the protein band (marked with an arrow) specifically bound to the promoter DNA corresponded to CRP. EZ protein-blue MW marker (KOMA biotech) was used as molecular mass ladder (lane M).

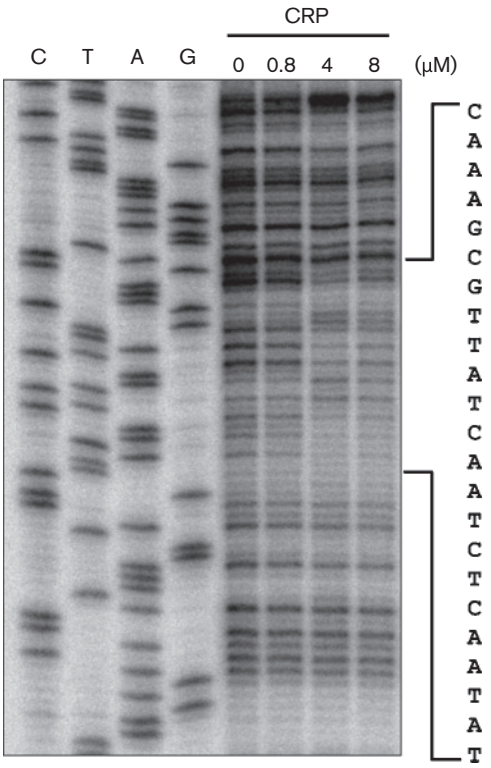

Fig. 3. Mapping of the CRP binding site on the promoter region of the *ideV* gene. The precise CRP binding site on the *ideV* promoter was determined by DNase I footprinting analysis. End-labelled *ideV* promoter DNA fragment was incubated with the indicated amounts of CRP in the presence of 100 μM cAMP and treated as described in Methods. The products were resolved on a 6 % (w/v) polyacrylamide gel containing 6 M urea. The region protected by CRP is indicated on the right side of the figure.

in *E. coli* (TGTGA-N^6-TCACA) with an imperfect 5 bp palindrome (see Fig. 1c).

The cAMP–CRP complex activates *ideV* P_S transcription *in vitro*

Since the binding site of CRP is determined in the *ideV* promoter, we then investigated the direct effect of CRP binding on *ideV* transcription. First, the synthetic promoter region of *ideV* was inserted into the *in vitro* transcription template plasmid pSA600 (Ryu & Garges, 1994) to produce pTxn_wt. In the presence of 100 μM cAMP, an increment of *ideV* transcription from P_S was observed with increasing amounts of CRP (Fig. 4a). The relative intensity of P_S transcript was increased about 15 times in the presence of 0.4 μM CRP and about 28 times with 1 μM CRP while the level of the *ideV* P_L transcript remained unchanged (Fig. 4c). This result indicates that CRP activates P_S but not P_L transcription. To confirm the effect of CRP on the P_S, the whole sequence encompassing the CRP binding site of pTxn_wt was mutated by quick change mutagenesis (see 'Methods'). When the mutated template pTxn_mt was used for *in vitro* transcription assays, the *ideV* transcription from P_S was hardly detectable even in the presence of 0.8 μM CRP (Fig. 4b, d).

Similarly, to determine the dependence of P_S transcription on the cAMP concentration, we performed *in vitro* transcription assays in the presence of 0.2 μM CRP with the increasing amounts of cAMP. The P_S transcription was increased as the concentration of cAMP was increased, reaching a plateau at about 2 μM (Fig. 5). This result supports the hypothesis that the *ideV* P_S promoter is under the control of the cAMP–CRP complex.

ideV expression is dependent on CRP, cAMP and glucose *in vivo*

To determine the *in vivo* effect of CRP on the *ideV* transcription, we constructed the *ideV*::*luxAB* transcriptional fusion plasmid pVm_P*ideV*::*luxAB*. This plasmid was transformed into wild-type and *crp* mutant *V. vulnificus* cells and the levels of *ideV* expression were determined by measuring luciferase activity (Jeong *et al.*, 2001). It should be noted that the growth of the *crp* mutant in LBS medium was somewhat slower than that of the wild-type *V. vulnificus* cells (data not shown). Both strains showed similarly low levels of *ideV* expression at the early stages of growth. However, as the cell density increases,

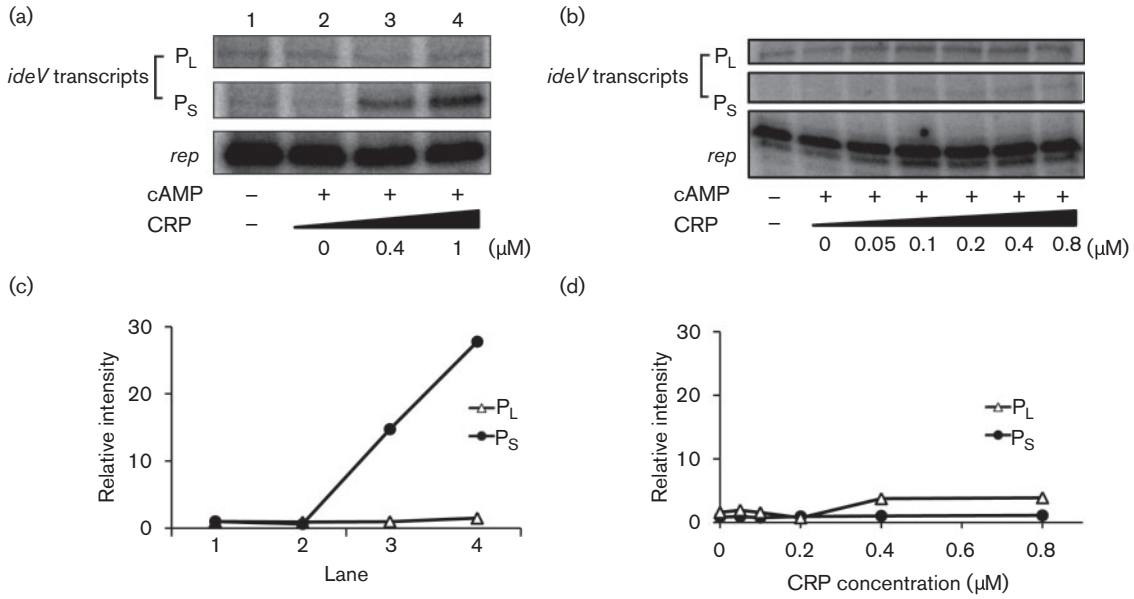

Fig. 4. *In vitro* transcription assays of the *ideV* gene with increasing amounts of CRP. *In vitro* transcription assays were performed with the synthetic promoter region of *ideV* inserted into the pSA600 plasmid in the presence of 100 μM cAMP. The reactions were performed with (a) pTxn_wt containing the wild-type CRP binding site and (b) pTxn_mt containing the mutated CRP binding site. The CRP concentration in each reaction mixture is indicated at the bottom of each figure. (c) and (d) Band densities in (a) and (b), respectively, were read by using BAS-2500 (Fuji film) and the relative intensities were calculated with respect to the *rep* transcripts.

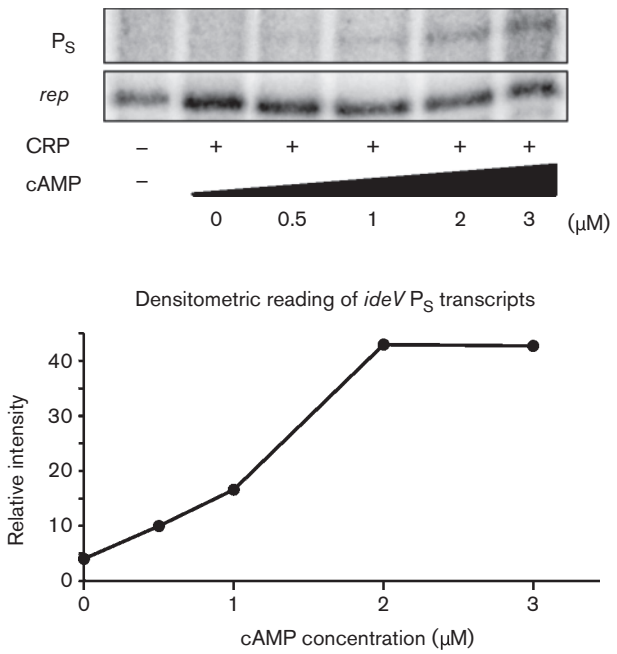

Fig. 5. *In vitro* transcription assays of the *ideV* P_S with increasing amounts of cAMP. *In vitro* transcription assays were performed with pTxn_wt in the presence of 0.2 μM CRP. The cAMP concentration was increased from 0 to 3 μM. The densities of P_S bands were read by using BAS-2500 (Fuji film) and the relative intensities were calculated with respect to the *rep* transcripts.

expression of *ideV* significantly increased in the wild-type strain, while this increase in the *ideV* expression level was significantly impaired in the *crp* mutant, to a level about four times less than that observed in the wild-type strain at stationary phase (Fig. 6a). It is known that the concentration of cAMP in the medium is proportional to the total amount of cAMP in the cell (Epstein *et al.*, 1975). Therefore, we measured the amount of cAMP in the culture medium at various time points to see whether the *ideV* expression level is influenced by the cAMP level *in vivo*. The increase of *ideV* expression exactly correlated with the increase of the cAMP concentration in the culture medium, suggesting that the cAMP–CRP complex is responsible for the transcriptional activation of *ideV*. To test the effect of mutation in the CRP binding site on *ideV* expression *in vivo*, we also modified pVm_P*ideV*::*luxAB* to construct pVm_P*ideV*mt1::*luxAB* and pVm_P*ideV*mt2::*luxAB* where parts (AGCGTT and TCTCAA, respectively) of the CRP binding site in the *ideV* promoter were changed to GAATCC. Measurement of luciferase activity in *V. vulnificus* cells harbouring these fusion plasmids indicates that the introduction of mutations in the CRP binding site results in remarkably decreased *ideV* expression (Fig. 6b), confirming that CRP activates *ideV* expression *in vivo*. Since it is generally known that the cellular level of cAMP decreases in the presence of glucose in Gram-negative bacteria, we tested the effect of glucose on *ideV* expression. As shown in Fig. 6(c), *ideV* expression was decreased in the presence of glucose, although the effect of glucose was not as remarkable as that of the introduction of mutations in the CRP binding site.

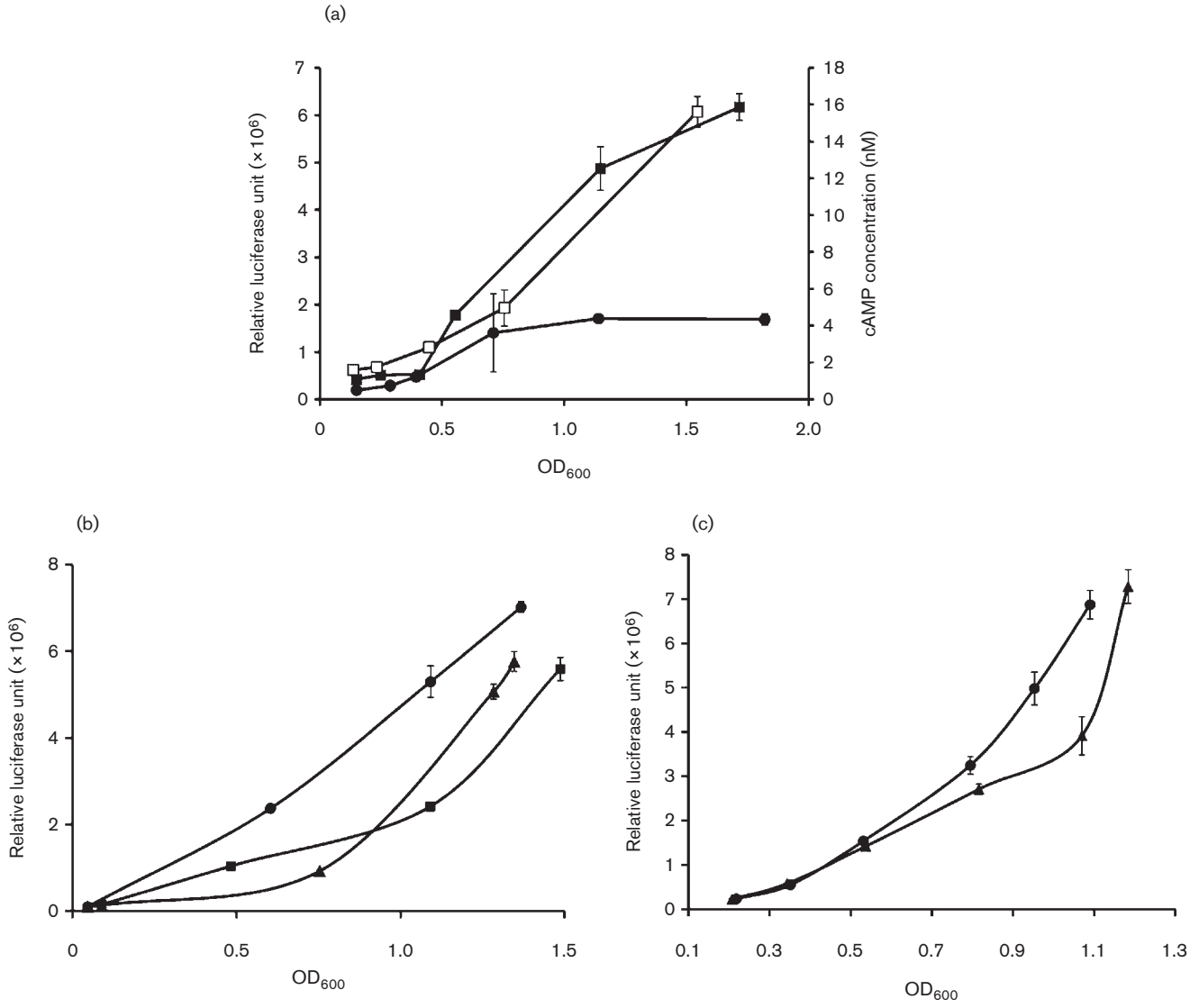

Fig. 6. The *ideV* expression level is dependent on CRP, cAMP and glucose *in vivo*. *V. vulnificus* MO6-24/O cells were grown at 30 °C in LBS medium and sampled at various times throughout growth (OD_{600}), and luciferase activities were measured in triplicate in the presence of 0.006 % (v/v) decanal using a luminometer (MicroLumat Plus, Berthold Technologies). Data are shown as mean ± SD (error bars). (a) Effect of CRP and cAMP levels on *ideV* expression. Luciferase activities were compared between wild-type and the *crp* mutant cells harbouring the *ideV*::*luxAB* transcriptional fusion plasmid pVm_P*ideV*::*luxAB* (Table 1). ■, Luciferase assay in wild-type *V. vulnificus* MO6-24/O; ●, luciferase assay in CMM988 (*crp* mutant); □, cAMP concentration in the culture medium of wild-type cells. The cAMP concentration was measured in duplicate using the cAMP Biotrak enzyme immunoassay system (GE Healthcare Lifescience). (b) Effect of mutations in the CRP binding site on the *ideV* expression. Luciferase activities were compared between wild-type *V. vulnificus* cells harbouring pVm_P*ideV*::*luxAB* (●) and those harbouring pVm_P*ideV*mt1::*luxAB* (▲) and pVm_P*ideV*mt2::*luxAB* (■) mutated at the CRP binding site (see 'Methods' and Table 1). (c) Effect of glucose in the culture medium on *ideV* expression. Luciferase activities were measured in wild-type cells harbouring pVm_P*ideV*::*luxAB* grown in LBS medium with (▲) and without (●) 0.4 % glucose added when OD_{600} reached about 0.3.

DISCUSSION

A common theme in bacterial pathogenesis is a sensory transduction mechanism that precisely regulates expression and activity of virulence factors in response to the external environment of the bacteria. Pathogenic bacteria are known to exploit physical and chemical parameters that distinguish the host from the external environment as signals for the expression and activation of virulence determinants. We recently found that *V. vulnificus* uses

glucose as such a chemical parameter that distinguishes the glucose-rich host from glucose-lacking estuarine seawaters, which are natural habitats for this pathogen, and that the PTS is such a signal transduction system that regulates activity of the virulence factor vIDE depending on the presence of glucose (Kim *et al.*, 2010). In this study, we tried to elucidate the mechanism regulating expression of vIDE. To this aim, we searched for the TSS of *ideV* first by employing primer extension experiments and found that there are two TSSs 92 bp apart from each other (Fig. 1). Based on this information, we identified CRP as the factor specifically binding to the region between the two TSSs (Figs 2 and 3) and thereby activating the transcription of *ideV* from the proximal TSS (P_S), but not the transcription from the distal TSS (P_L), in the presence of cAMP (Figs 4 and 5). P_S seems to belong to the class I CRP-dependent promoter group (Busby & Ebright, 1994) considering that it has a single DNA-binding site centred near position −72 (Fig. 3 and Fig. S3).

Data in Fig. 6 confirm that the cAMP–CRP complex activates *ideV* expression *in vivo* while glucose shows the opposite effect. The intracellular concentration of the cAMP–CRP complex is known to be dependent on the phosphorylation state of $EIIA^{Glc}$ in *E. coli* and other Gram-negative bacteria (Deutscher *et al.*, 2006; Park *et al.*, 2006). Concomitant transport and phosphorylation of glucose decrease the extent of phosphorylation of $EIIA^{Glc}$, and thus lower the activity of adenylyl cyclase, catalysing biosynthesis of cAMP from ATP, whereas growth on non-PTS carbohydrates, particularly poor carbon sources like acetate and succinate, results in the increased level of phosphorylated $EIIA^{Glc}$, which activates adenylyl cyclase. In this regard, it is intriguing that *ideV* transcription is activated by the cAMP–CRP complex, the level of which is increased by phosphorylated $EIIA^{Glc}$, while vIDE activity is strictly dependent on dephosphorylated $EIIA^{Glc}$ (Kim *et al.*, 2010).

When *V. vulnificus* inhabits the glucose-depleted estuarine environment, the expression level of vIDE would be high but with little activity due to depletion of the dephosphorylated form of $EIIA^{Glc}$. When this pathogen infects a host; however, glucose in the host will fully dephosphorylate $EIIA^{Glc}$, and thus the total activity of vIDE will reach the maximum level. As time goes on, the dephosphorylated $EIIA^{Glc}$ will decrease the cAMP–CRP level and thus gradually decrease expression of vIDE to an appropriate level by inhibiting one of the two promoters of *ideV*. Taken together, our data demonstrate that $EIIA^{Glc}$, as the sensor of glucose that distinguishes the host from the natural habitat of *V. vulnificus*, can intricately regulate both the expression level and the activity of the virulence factor vIDE.

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