Identification and characterization of *Vibrio vulnificus plpA* encoding a phospholipase A_2 essential for pathogenesis

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The marine bacterium *Vibrio vulnificus* causes food-borne diseases, which may lead to life-threatening septicemia in some individuals. Therefore, identifying virulence factors in *V. vulnificus* is of high priority. We performed a transcriptome analysis on *V. vulnificus* after infection of human intestinal HT29-methotrexate cells and found induction of *plpA*, encoding a putative phospholipase, *Vv*PlpA. Bioinformatics, biochemical, and genetic analyses demonstrated that *Vv*PlpA is a phospholipase A_2 secreted in a type II secretion system-dependent manner. Compared with the wild type, the *plpA* mutant exhibited reduced mortality, systemic infection, and inflammation in mice as well as low cytotoxicity toward the human epithelial INT-407 cells. Moreover, *plpA* mutation attenuated the release of actin and cytosolic cyclophilin A from INT-407 cells, indicating that *Vv*PlpA is a virulence factor essential for causing lysis and necrotic death of the epithelial cells. *plpA* transcription was growth phase– dependent, reaching maximum levels during the early stationary phase. Also, transcription factor HlyU and cAMP receptor protein (CRP) mediate additive activation and host-dependent induction of *plpA*. Molecular biological analyses revealed that *plpA* expression is controlled via the promoter, P_{plpA}, and that HlyU and CRP directly bind to P_{plpA} upstream sequences. Taken together, this study demonstrated that *Vv*PlpA is a type II secretion system-dependent secretory phospholipase A_2 regulated by HlyU and CRP and is essential for the pathogenicity of *V. vulnificus*.

The pathogenic marine bacterium *Vibrio vulnificus* is a causative agent of food-borne diseases such as gastroenteritis in healthy people and life-threatening septicemia in individuals with underlying predisposed conditions (for recent reviews, see Refs. 1 and 2). *V. vulnificus* infections are notable for their invasiveness, severe tissue destruction, and rapidly fulminating course of disease, leading to high mortality rates (>50%) and immediate death (within 1–2 days after the first symptoms appear). These pathological features of the disease indicate that the pathogenicity of *V. vulnificus* is a multifactorial and complex phenomenon that involves numerous virulence factors (3). The characterization of somatic and secreted products of *V. vulnificus* has yielded a large list of putative virulence factors, including a carbohydrate capsule, iron-sequestering systems, a cytolysin/hemolysin, an elastolytic metalloprotease, a multifunctional-autoprocessing repeats-in-toxin (MARTX)[3] toxin, a lipopolysaccharide, a lipase, pili, and flagella to account for the destructive nature of *V. vulnificus* infections (1, 3). Of these putative virulence factors, however, only a few, such as cytolysin/hemolysin and MARTX toxin, have been confirmed as virulence factors that contribute to the lysis and necrotic death of the host cells by using the molecular version of Koch's postulates (4–6). Therefore, extensive screening and characterization of more virulence factors are still required for understanding the molecular pathogenesis of the multifaceted host–pathogen interaction of *V. vulnificus*.

Phospholipases, lipolytic enzymes hydrolyzing one or more ester linkages in phospholipids, are found in diverse bacterial pathogens, implying that these enzymes play essential roles in the pathogenesis of bacteria (7). Phospholipases are classified into four major groups (A–D) based on their site of action on the phospholipid (7). Among them, phospholipase A (PLA) is able to hydrolyze a fatty acid from the glycerol backbone and is subdivided into PLA_1 and PLA_2, each hydrolyzing the fatty acid from the *sn*-1 and -2 position of the glycerol moiety, respectively (7). Accordingly, PLAs are considered to be bacterial vir-

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This article contains supplemental Tables S1 and S2.

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[3] The abbreviations used are: MARTX, multifunctional-autoprocessing repeat-in-toxin; PLA, phospholipase A; MTX, methotrexate; CRP, cAMP receptor protein; *Vv*PlpA, *V. vulnificus* PlpA; qPCR, quantitative PCR; T2SS, type II secretion system; *Va*PlpA, *V. anguillarum* PlpA; PC, phosphatidylcholine; rPlpA, recombinant *Vv*PlpA protein; MPXI, myeloperoxidase index; ALB, albumin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; MOI, multiplicity of infection; WGA, wheat germ agglutinin; CypA, cyclophilin A; MEM, minimal essential medium; RLU, relative light unit.

ulence factors disrupting the phospholipid membrane, thus leading to the lysis of human epithelial cells (7). In addition, the products generated by epithelial cell lysis, such as lysophosphatidylcholine, may further act as secondary messengers that induce apoptotic death of human endothelial cells (8). Nevertheless, very little is known about the regulatory mechanisms used by the bacteria to modulate the expression of PLAs (9).

To the best of our knowledge, no definitive analysis of the role of the PLAs in the pathogenesis of *V. vulnificus* has been reported until now. Furthermore, no genes encoding the PLAs have been identified from *V. vulnificus*, and thus the molecular mechanisms by which the bacterium modulates the expression of the genes have not yet been characterized. In the present study, we conducted a transcriptome analysis and identified the *plpA* gene, which is preferentially expressed in *V. vulnificus* cells exposed to human intestinal HT29-methotrexate (MTX) cells (10, 11). The characteristics of *V. vulnificus* PlpA (*Vv*PlpA), the product of *plpA*, were verified experimentally. Construction of the *plpA* mutant and evaluation of its phenotypes provided strong evidence that *Vv*PlpA contributes to the pathogenesis of *V. vulnificus*. To elucidate the regulation of the *plpA* expression, we compared the *plpA* transcript levels in the wild type and in the mutants lacking various transcription factors. Moreover, the transcriptional unit and promoter of *plpA* were determined, and the roles of HlyU, a transcription factor regulating virulence genes in *Vibrio* species (12–14), and cAMP receptor protein (CRP) (15) were analyzed at the molecular level.

Results

Identification and sequence analysis of VvPlpA

The transcriptomes of the *V. vulnificus* MO6-24/O harvested after either the infection of human intestinal HT29-MTX cells or grown with basal medium Eagle (Gibco-BRL) alone were compared as described under "Experimental procedures." Transcriptome analysis revealed 650 differentially expressed genes in the host cell-exposed *V. vulnificus* compared with the unexposed one; 319 genes were up-regulated, and 331 were down-regulated (supplemental Tables S1 and S2). Among the up-regulated, virulence-related genes, a gene VVMO6_03257 encoding a putative phospholipase showed the highest-fold change of expression level (20.5-fold, $p = 0.0001$). In contrast, the expressions of several other putative phospholipase genes (VVMO6_00112, 00550, 00661, 01752, 03024, 03759, and 04072) were not significantly induced (data not shown), suggesting that the VVMO6_03257 gene product plays an important role in host–microbe interaction, at least under our experimental conditions. Real-time quantitative PCR (qPCR) confirmed that the expression of VVMO6_03257 is increased about 17.8-fold ($p = 0.0005$) after exposure of *V. vulnificus* to the host cells (data not shown).

The amino acid sequence deduced from the VVMO6_03257 nucleotide sequence predicted a protein with an N-terminal signal peptide for the type II secretion system (T2SS) (Fig. 1*A*) (16). The mature protein is composed of 398 amino acids with a theoretical mass of 45.22 kDa and pI of 5.38. When aligned using the Clustal Omega program (http://www.ebi.ac.uk/Tools/msa/clustalo)[4] (66), the deduced amino acid sequence of the mature protein was 69% identical to that of the *Vibrio anguillarum* phospholipase (*Va*PlpA) (17), leading us to name the protein *Vv*PlpA. Amino acid sequence analysis further revealed that *Vv*PlpA possesses five conserved blocks for the SGNH hydrolases as well as a GDSL motif found in typical lipases (Fig. 1*A*) (18). Also, the predicted profile of the hydrophobicity was significantly similar to that of *Va*PlpA, indicating that *Vv*PlpA is a soluble protein like *Va*PlpA (data not shown) (17). Meanwhile, we found that the genetic environment of *plpA* in *V. vulnificus* is distinct from that in *V. anguillarum*. Unlike the *V. anguillarum plpA* located upstream of and sharing the promoter region with hemolysin gene, *vah1* (19), *V. vulnificus plpA* has its own promoter region, and presents at a different location from hemolysin gene, *vvhA*, or MARTX toxin gene, *rtxA1* (data not shown).

The secretion of *Vv*PlpA to the cell exterior through T2SS as predicted above was experimentally verified (Fig. 1*B*). To accomplish this, an isogenic mutant in which the *pilD* gene encoding the PilD component of T2SS was specifically deleted (20) was used, and the *Vv*PlpA secretion from the mutant was compared with that from the parental wild type. As shown in Fig. 1*B*, *Vv*PlpA was not detected from the culture supernatant of the *pilD* mutant. This lack of *Vv*PlpA secretion was restored by the introduction of pMS0908 (20), carrying a *pilD* gene, to the *pilD* mutant. These results confirmed that *Vv*PlpA is indeed an extracellular protein, and its secretion is T2SS-dependent.

The lipase activity of *Vv*PlpA, predicted from amino acid sequence analysis, was also experimentally verified. Fluorescence was emitted from the red/green BODIPY PC-A2 (Invitrogen), a fluorescently labeled phosphatidylcholine (PC), in the presence of 0.02–0.5 μg of recombinant *Vv*PlpA protein (rPlpA). The fluorescence emission intensity increased as greater amounts of rPlpA were added (Fig. 1*C*), suggesting that rPlpA contains the PLA_2 activity cleaving specifically the *sn*-2 bond of the red/green BODIPY PC-A2 substrate (Invitrogen). Based on the standard curve developed using the honey bee venom PLA_2 (21), the specific activity of rPlpA was calculated to be ~247 units/mg (data not shown) (1 unit = the amount of protein that cleaves 1 μmol of red/green BODIPY PC-A2 per min).

We further examined whether *Vv*PlpA indeed confers the PLA_2 activity to *V. vulnificus*. For this, an isogenic mutant KK131 lacking a functional *plpA* gene was constructed (Table 1). When culture supernatant of the wild type, *plpA* mutant, or complemented strain was added to the reaction as a source for the enzyme, significantly lower fluorescence was emitted from the sample containing that of the *plpA* mutant compared with the other two samples (Fig. 1*D*). Therefore, the combined results indicated that *Vv*PlpA is a T2SS-dependent secretory enzyme conferring the PLA_2 activity to *V. vulnificus*.

VvPlpA is essential for virulence

Next we confirmed the induction of *plpA* expression under conditions closer to those *in vivo* rather than those under *in*

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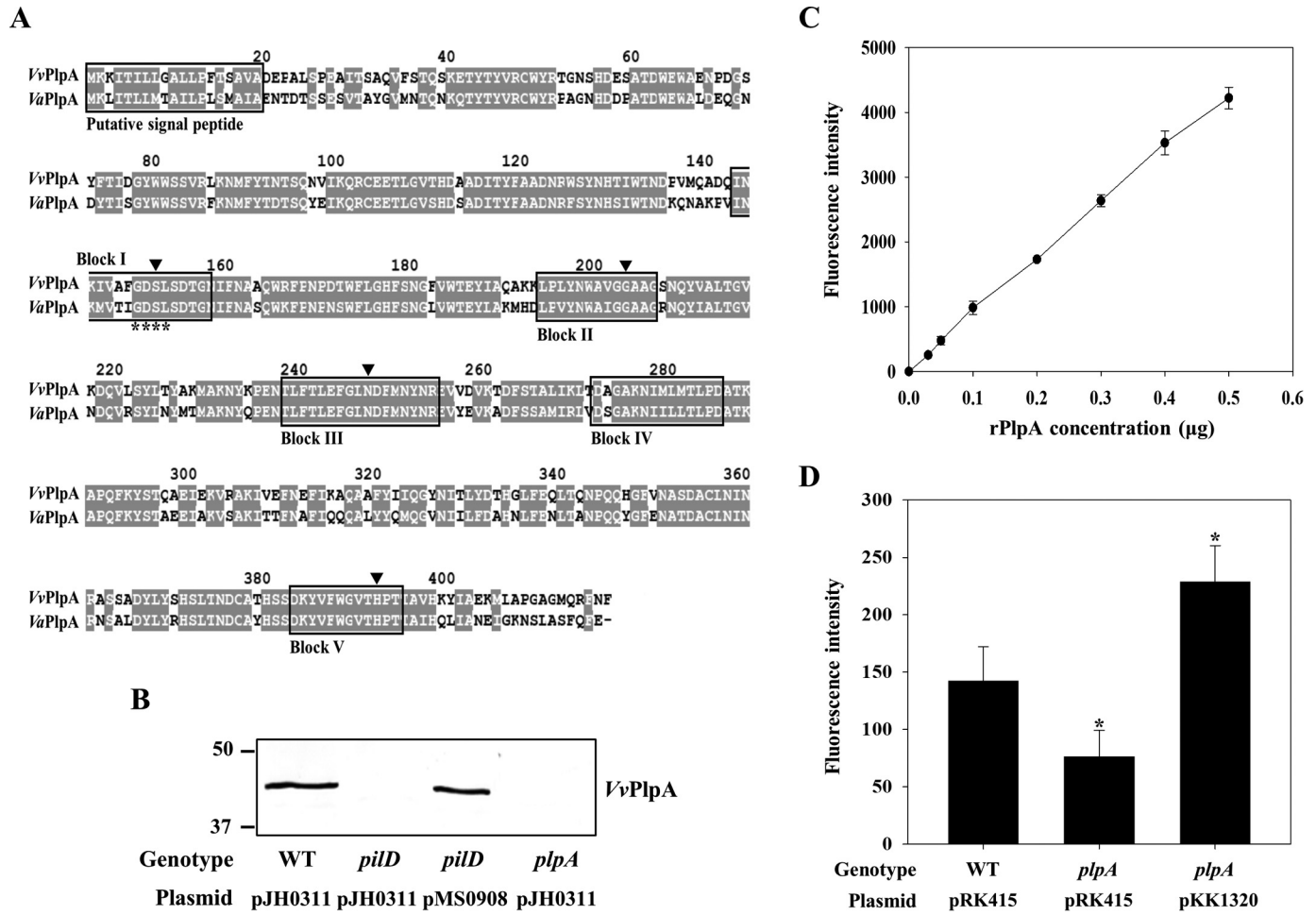

Figure 1. Analysis of amino acid sequence, secretion, and enzymatic activity of *Vv*PlpA. *A*, the amino acid sequences retrieved from the NCBI protein database (http://www.ncbi.nlm.nih.gov) (accession numbers ADV88279 for *Vv*PlpA and AAY26144 for *Va*PlpA) were aligned using the Clustal Omega and Genedoc program. Identical residues (*gray boxes*) and missing sequence (*dash*) are indicated. The putative signal peptides and consensus blocks for SGNH hydrolases are *boxed* by *a black line*. The GDSL motif is indicated by *asterisks*. The four residues conserved in the SGNH hydrolases are indicated by *black-filled inverse triangles*. *B*, *Vv*PlpA in the supernatants of the *V. vulnificus* strains grown to an A_{600} of 0.9 was detected by Western blot analysis. Molecular size markers (Bio-Rad) are shown in kDa. *C* and *D*, PLA_2 activity was examined by measuring fluorescence intensity emitted from the fluorogenic phosphatidylcholine substrate (red/green BODIPY PC-A2) after incubation with various amounts of the rPlpA (*C*) or supernatants of the *V. vulnificus* strains (*D*) for 0.5 h. *Error bars*, S.D. Statistical significance was determined by Student's *t* test. *, $p < 0.05$ relative to the wild type. *WT pJH0311* and *WT pRK415*, wild type; *pilD pJH0311*, *pilD* mutant; *plpA pJH0311* and *plpA pRK415*, *plpA* mutant; *pilD pMS0908* and *plpA pKK1320*, complemented strains.

vitro-cultured host cells. When *V. vulnificus* was inoculated into the blood of uninfected mice, the expression of *plpA* was dramatically elevated (Fig. 2*A*). This result suggested that *Vv*PlpA may be highly expressed *in vivo* and take part in the pathogenesis of *V. vulnificus*. To test this hypothesis, the virulence of wild type and *plpA* mutant KK131 was compared in mice. As shown in Fig. 2*B*, mice intragastrically infected with the wild type started to die at 5 h postinfection. In contrast, the survival time of mice infected with KK131 was consistently and significantly prolonged ($p = 0.0063$, log-rank test). At 20 h postinfection, the percentages of surviving mice were 28 and 57% for the wild type– and KK131–infected group, respectively (Fig. 2*B*).

To further elucidate the role of *Vv*PlpA during pathogenesis, we compared the number of *V. vulnificus* in the blood and various hematological indexes of either the wild type– or *plpA* mutant–infected mice. To deliver exactly the same number of *V. vulnificus* into the host without any colonization problems, we adopted an intraperitoneal infection model for these analyses. First, we confirmed the importance of *Vv*PlpA in this infection model by comparing the survival rates of mice infected with the wild type or *plpA* mutant (data not shown). When the bacteria in the blood of the infected mice were enumerated, about 4 times more wild-type cells, compared with the *plpA* mutant cells, were recovered (Fig. 2*C*). Consistent with this, hematological analyses revealed that the levels of myeloperoxidase index (MPXI) and albumin (ALB), the biomarkers for septicemia (in inverse correlation; see Refs. 22 and 23), were significantly lower in the wild type–infected mice compared with those in the *plpA* mutant–infected or PBS-treated mice (Fig. 2, *D* and *E*). Also, mice infected with the wild type exhibited significantly higher levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities, the indexes for liver damage (24), compared with those infected with the *plpA* mutant or treated with PBS (Fig. 2*F*). Combined with the fact that *plpA* mutation does not attenuate the bacterial fitness *in vitro* (data not shown), these results clearly demonstrated the crucial role of *Vv*PlpA in *V. vulnificus* systemic

Table 1
Plasmids and bacterial strains used in this study

Strain or plasmid	Relevant characteristics[a]	Reference or source
Bacterial strains		
V. vulnificus		
MO6-24/O	Wild type; clinical isolate; virulent	Ref. 65
KK131	MO6-24/O with Δ*plpA*	This study
ZW141	MO6-24/O with Δ*hlyU*	This study
DI0201	MO6-24/O with Δ*crp*	Ref. 49
KK151	MO6-24/O with Δ*hlyU*Δ*crp*	This study
MS023	MO6-24/O with Δ*pilD*	Ref. 20
E. coli		
S17-1λ*pir*	λ-*pir* lysogen; *thi pro hsdR hsdM*$^+$ *recA* RP4–2 Tc:: Mu-Km::Tn7;Tp^r Sm^r; host for π-requiring plasmids; conjugal donor	Ref. 55
BL21 (DE3)	F$^-$ *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm* (DE3)	Laboratory collection
Plasmids		
pDM4	R6K γ *ori sacB*; suicide vector; *oriT* of RP4; Cm^r	Ref. 54
pKK1301	pDM4 with Δ*plpA*; Cm^r	This study
pZW1401	pDM4 with Δ*hlyU*; Cm^r	This study
pBS0907	pDM4 with Δ*crp*; Cm^r	Ref. 56
pRK415	Broad-host-range vector; IncP *ori oriT* RK2; Tc^r	Ref. 57
pKK1320	pRK415 with *plpA*; Tc^r	This study
pJH0311	Broad-host-range vector; Ap^r, Cm^r	Ref. 58
pZW1510	pJH0311 with *hlyU*; Ap^r, Cm^r	This study
pKK1502	pJH0311 with *crp*; Ap^r, Cm^r	This study
pMS0908	pJH0311 with *pilD*; Ap^r, Cm^r	Ref. 20
pMBP parallel 1	MBP tag fusion expression vector; Ap^r	Ref. 59
pKK1503	pMBP parallel 1 with *plpA-his*$_6$; Ap^r	This study
pET28a(+)	His$_6$ tag fusion expression vector; Km^r	Novagen
pYU1317	pET28a (+) with *hlyU*; Km^r	This study
pKK1504	pET28a (+) with truncated *plpA*; Km^r	This study
pHK0201	pRSET A with *crp*; Ap^r	Ref. 49
pGEM-T Easy	PCR product cloning vector; Ap^r	Promega
pKK1505	pGEM-T Easy with 461-bp fragment of *plpA* upstream region; Ap^r	This study
pBBR-lux	Broad-host-range vector with *luxCDABE* operon; Cm^r	Ref. 64
pKK1514	pBBR-lux with 378-bp fragment of *plpA* upstream region; Cm^r	This study
pKK1515	pBBR-lux with 308-bp fragment of *plpA* upstream region; Cm^r	This study
pKK1516	pBBR-lux with 228-bp fragment of *plpA* upstream region; Cm^r	This study
pKK1517	pBBR-lux with 129-bp fragment of *plpA* upstream region; Cm^r	This study

[a] Tp^r, trimethoprim-resistant; Sm^r, streptomycin-resistant; Cm^r, chloramphenicol-resistant; Tc^r, tetracycline-resistance; Ap^r, ampicillin-resistant; Km^r, kanamycin-resistant.

infection, and the accompanying inflammation resulted in host death.

VvPlpA is essential for the lysis and necrotic death of host cells

To investigate the role of *Vv*PlpA in the cytotoxicity toward host cells, the activities of lactate dehydrogenase (LDH) released from INT-407 cells infected with the wild type and *plpA* mutant KK131 were determined. As shown in Fig. 3*A*, KK131 exhibited significantly decreased LDH-releasing activity compared with that of the wild type, when the *V. vulnificus* strains were used to infect the INT-407 cells at multiplicity of infections (MOIs) of 1, 5, and 20 for 2 h. Similarly, INT-407 cells infected with KK131 at an MOI of 10 and then incubated for 1, 2, and 3 h released lower levels of LDH compared with those infected with the wild type (Fig. 3*B*), indicating that *Vv*PlpA plays an important role in *V. vulnificus* infecting and injuring host cells (Fig. 3, *A* and *B*). Complementation of the *plpA* mutation with a functional *plpA* gene (pKK1320) restored the LDH-releasing activity of KK131 to levels comparable with those of the wild type (Fig. 3, *A* and *B*). Therefore, the decreased cytotoxicity of KK131 resulted from the inactivation of *plpA* rather than the reduced expression of other genes downstream of *plpA*.

To further understand the effects of *Vv*PlpA on host cells, INT-407 cells were stained with Texas Red®-X– conjugated wheat germ agglutinin (WGA) (Thermo Fisher Scientific, San Jose, CA) and Hoechst® 33342 (Thermo Fisher Scientific), after which their morphological changes caused by infection with the *V. vulnificus* strains were examined. As shown in Fig. 3*C*, INT-407 cells exhibited typical morphology of surface-attached epithelial cells with regularly shaped cell margins and rounded nuclei in the absence of infection (PBS). Infection with the wild type injured INT-407 cells and resulted in severely shrunken cell morphology with irregular cell margins, presumably accompanied by the release of cellular materials (WT (pRK415)). Notably, however, infection with the *plpA* mutant KK131 resulted in cells with less severely shrunken and swollen morphology (*plpA* (pRK415)). The morphology of INT-407 cells infected with the complemented strain (*plpA* (pKK1320)) was similar to that infected with the wild type, suggesting that *Vv*PlpA is responsible for the injury and lysis of INT-407 cells.

To examine this possibility further, the release of actin protein, acting as a biomarker of cell lysis, was determined by Western blot analysis (Fig. 3*D*). A significant amount of actin was detected from the supernatant of INT-407 cells infected with the wild type and complemented strains of *V. vulnificus*. In contrast, the level of actin from the supernatant of the host cells infected with the *plpA* mutant KK131 was almost undetectable and comparable with that of the cells treated with PBS (Fig. 3*D*), confirming that *Vv*PlpA is responsible for host cell lysis. Furthermore, in addition to actin, cyclophilin A (CypA, a peptidyl-prolyl *cis-trans* isomerase), which is used as a biomarker of necrotic cell death (25), was also released from INT-407 cells infected with the wild type and complemented strains but not from the host cells infected with KK131 (Fig. 3*D*). These results

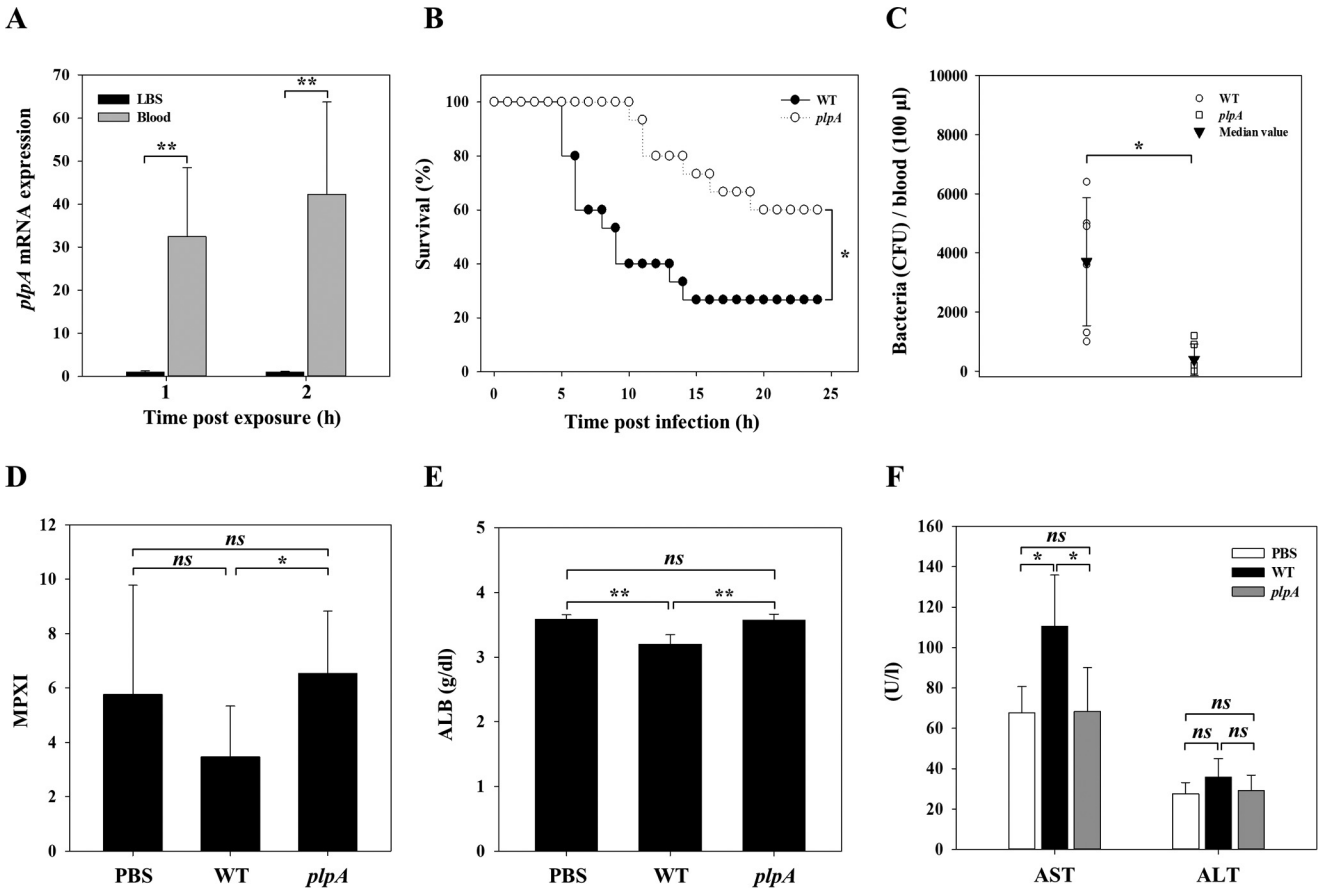

Figure 2. *Vv*PlpA is essential for pathogenesis of *V. vulnificus*. *A*, the wild type grown to an A_{600} of 0.5 was exposed to either LBS or blood of uninfected mice ($n = 3$) for 1 or 2 h and then used to isolate total RNAs. The *plpA* mRNA was determined by real-time qPCR analyses, and the *plpA* mRNA level in the bacteria exposed to LBS was set as 1. *B*, 7-week-old female ICR mice ($n = 15$ per group), injected intraperitoneally with iron-dextran, were intragastrically infected with the wild type and *plpA* mutant at doses of 10^8 cfu. *C–F*, the mice, without an iron-dextran administration, were intraperitoneally infected with the wild type ($n = 11$), *plpA* mutant ($n = 11$), or PBS ($n = 5$, control) at doses of 10^6 cfu and then sacrificed to obtain blood at 4 h postinfection. *C*, the number of either the wild type or *plpA* mutant in the blood of infected mice ($n = 6$ per group) was enumerated as cfu/100 μl of blood. Each *open symbol* represents an individual mouse. *Black-filled inverted triangles* indicate median values. *D–F*, the levels of MPXI (*D*), ALB (*E*), and AST/ALT (*F*) in the wild type– or *plpA* mutant–infected or PBS-treated mice ($n = 5$ per group) were determined by hematological analyses. *Error bars*, S.D. Statistical significance was determined by Student's *t* test for *A* and *C–F*, and by log-rank test for *B*. *, $p < 0.05$; **, $p < 0.005$; *ns*, not significant. *WT*, wild type; *plpA*, *plpA* mutant; *PBS*, control.

indicated that *Vv*PlpA mediates host cell lysis as well as necrotic death by releasing cytosolic components. Therefore, these results, combined with the previous results of decreased mouse mortality and LDH-releasing activity in the absence of *Vv*PlpA, indicated that *Vv*PlpA is a virulence factor essential for cell lysis and necrotic cell death, thereby contributing to the pathogenesis of *V. vulnificus*.

Transcription of plpA is growth phase–dependent and regulated by HlyU and CRP

To extend our understanding of *plpA* regulation, levels of the *plpA* transcript in the wild type grown under various conditions (oxidative stress, iron limitation, and different temperatures) were compared, but none of the tested conditions influenced *plpA* expression (data not shown). However, when the levels of *plpA* mRNA were analyzed at various growth phases, the *plpA* transcript level increased to maximum levels in the early stationary phase ($A_{600} = 1.0$) and then decreased in the late stationary phase ($A_{600} = 2.5$) (Fig. 4*A*), indicating growth phase–dependent expression of *plpA*.

To identify transcription factor(s) associated with the *plpA* regulation, the levels of *plpA* mRNA were compared in the wild type and several mutants lacking a transcription factor known to regulate the virulence of *V. vulnificus*. The transcription factor-lacking mutants consisted of *fur*, *hlyU*, *iscR*, *aphB*, *crp*, *leuO*, and *smcR* mutants (13, 26–30). Expression of *plpA* did not differ in the wild type or mutants lacking Fur, IscR, AphB, LeuO, or a quorum-sensing master regulator SmcR (27) (data not shown). This result suggested that *plpA* might not be regulated by iron availability or by quorum sensing. In contrast, the *hlyU* and *crp* mutants showed almost 2.5-fold lower levels of *plpA* transcript compared with those in the wild type (Fig. 4*B*), indicating that both HlyU and CRP may act as positive regulators. The levels of *Vv*PlpA determined by Western blot analyses also decreased in the culture supernatants of the mutants (Fig. 4*B*). The magnitudes of decrease in *Vv*PlpA did not significantly differ from those observed in the *plpA* transcript, indicating that the regulation of *plpA* expression occurs mostly at the transcriptional level. The decreased levels of the *plpA* transcript and *Vv*PlpA protein in the *hlyU* and *crp* mutants were restored to levels comparable with those in the wild type by introducing pZW1510 and pKK1502, each carrying the *hlyU* and *crp* gene, respectively (Fig. 4*C*). Taken together, these results suggested that both HlyU and CRP

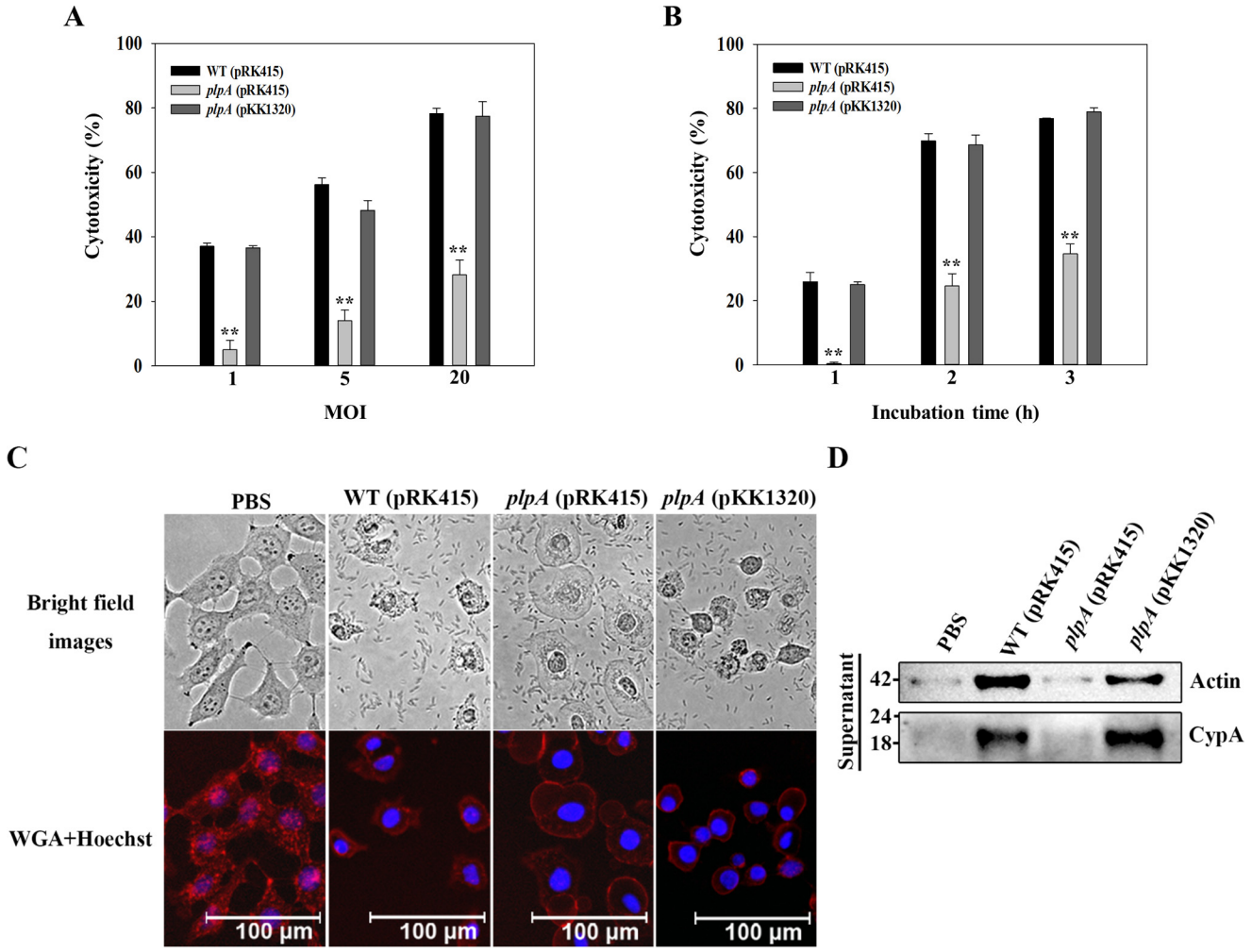

Figure 3. *Vv*PlpA is essential for necrotic cell death in tissue culture. *A* and *B*, INT-407 cells were infected with the *V. vulnificus* strains at various MOIs for 2 h (*A*) or at an MOI of 10 for various incubation times (*B*). The cytotoxicity was expressed using the total LDH activity of the cells completely lysed by 1% Triton X-100 as 100%. *Error bars*, S.D. Statistical significance was determined by Student's *t* test. **, $p < 0.005$ relative to groups infected with the wild type at each MOI or incubation time. *C*, INT-407 cells were infected with the *V. vulnificus* strains at an MOI of 10 for 1.5 h as indicated, stained using Texas Red®-X–conjugated WGA (for membrane, *red*) and Hoechst 33342 (for nucleus, *blue*), and then photographed using a fluorescence microscope. *D*, INT-407 cells were infected with the *V. vulnificus* strains at an MOI of 10 for 2 h, and actin and CypA released in the culture supernatants were analyzed by Western blot analysis. Molecular size markers (GenDEPOT, Barker, TX) are shown in kDa. *WT (pRK415)*, wild type; *plpA (pRK415)*, *plpA* mutant; *plpA (pKK1320)*, complemented strain; *PBS*, control (uninfected).

positively regulate *plpA* expression at the transcriptional level.

Additive activation of plpA by HlyU and CRP

To characterize the functions of HlyU and CRP in *plpA* expression, *hlyU crp* double mutant KK151 was constructed and used to determine *plpA* expression. Lack of both *hlyU* and *crp* in KK151 resulted in a significant reduction of *plpA* expression, as the transcript level was only one-quarter of that in the wild type (Fig. 5*A*). Furthermore, this residual *plpA* transcript level in KK151 was considerably lower than that in either the *hlyU* or *crp* single mutant (Fig. 5*A*), suggesting that both transcriptional regulators activate *plpA* additively. Indeed, the cellular level of HlyU in the *crp* mutant (or CRP in the *hlyU* mutant) was comparable with that in the wild type (Fig. 5*B*), confirming that neither CRP nor HlyU controls the expression of the other in a sequential regulatory cascade.

To further determine whether increasing the level of cellular HlyU could compensate for the lack of CRP in the activation of *plpA*, KK151 was complemented with pZW1510 expressing *hlyU* under the *lac* promoter. When *hlyU* was induced by isopropyl-β-D-thiogalactopyranoside, the HlyU level in KK151 (pZW1510) was significantly increased, exceeding the level in the wild type or *crp* single mutant (Fig. 5*B*). Nonetheless, the *plpA* transcript level in KK151 (pZW1510) was still lower than that in the wild type, corresponding to only about 60% of that in the wild type (Fig. 5*A*). This result suggested that if CRP is absent, overexpressed HlyU is insufficient in increasing *plpA* expression levels comparable with those in the wild type. Similarly, overproduced CRP (Fig. 5*B*) was unable to completely compensate for the lack of HlyU in the activation of *plpA* (Fig. 5*A*). Also, the changes in the *plpA* transcript were directly reflected on the secreted *Vv*PlpA protein levels (Fig. 5*B*).

Next, we verified whether HlyU and CRP are involved in the induction of *plpA* during exposure to host cells by comparing the *plpA* transcript levels in the *V. vulnificus* strains exposed to either minimal essential medium (MEM, control) or INT-407 cells for 1 h. As shown in Fig. 5*C*, the *plpA* transcript level in the

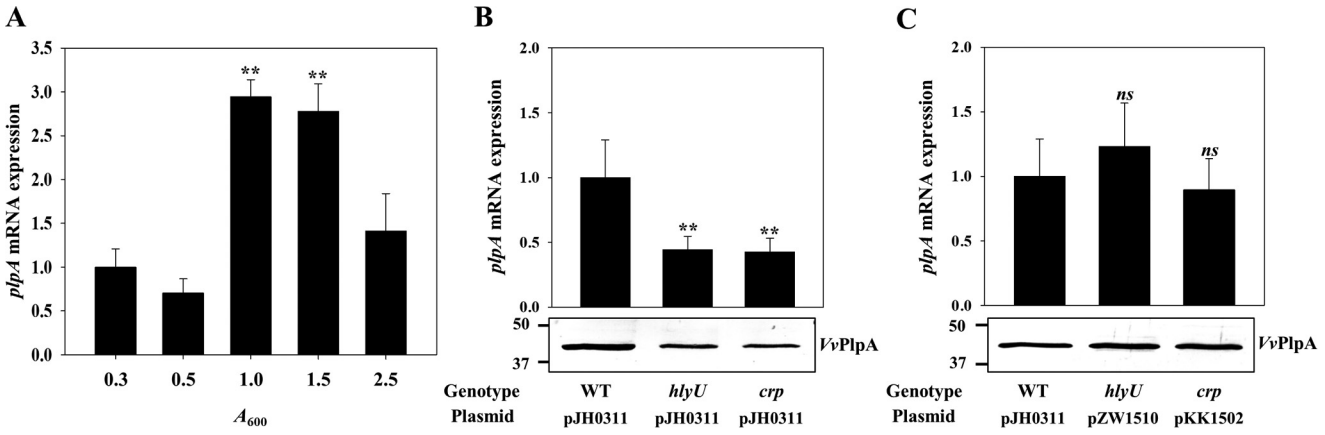

Figure 4. Expression of *plpA* in *V. vulnificus* under different growth phases (*A*) or with different genetic backgrounds (*B* and *C*). *A*, total RNAs from the *V. vulnificus* wild type under different growth phases were isolated, and the *plpA* mRNA levels were determined by real-time qPCR analyses. The *plpA* mRNA level in the cells grown to an A_{600} of 0.3 was set as 1. *B* and *C*, *V. vulnificus* strains were grown to an A_{600} of 0.9 and used to determine the *plpA* mRNA and *Vv*PlpA levels. The *plpA* mRNA levels in the total RNAs isolated from the cultures were determined by real-time qPCR analyses, and the *plpA* mRNA level in the wild type was set as 1. The *Vv*PlpA levels in the supernatants harvested form the cultures were determined by Western blot analyses. Molecular size markers (Bio-Rad) are shown in kDa. *Error bars*, S.D. Statistical significance was determined by Student's *t* test. **, $p < 0.005$ relative to the cells grown to an A_{600} of 0.3 (for *A*) or to the wild type (for *B* and *C*); *ns*, not significant. *WT pJH0311*, wild type; *hlyU pJH0311*, *hlyU* mutant; *crp pJH0311*, *crp* mutant; *hlyU pZW1510* and *crp pKK1502*, complemented strains.

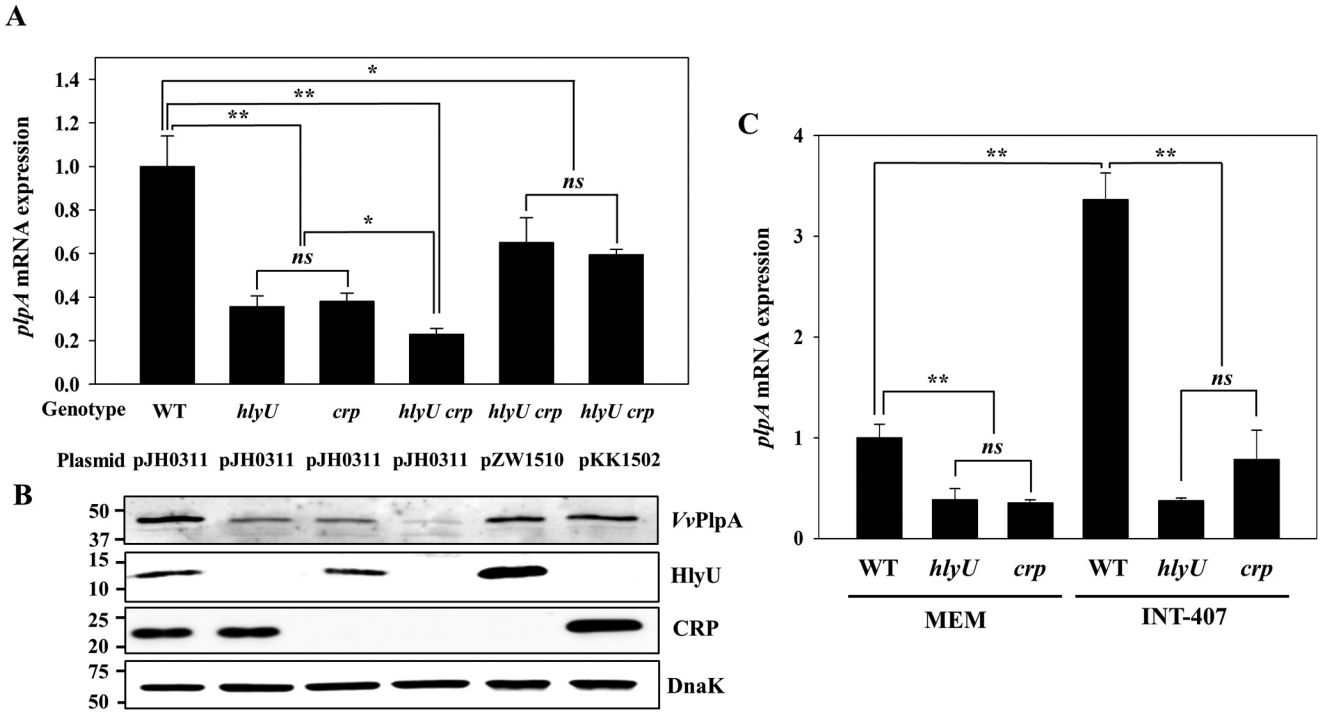

Figure 5. HlyU and CRP mediate additive activation and host-dependent induction of *plpA*. *A* and *B*, *V. vulnificus* strains were grown to an A_{600} of 0.9 and used to determine the *plpA* mRNA levels and *Vv*PlpA, HlyU, CRP, and DnaK protein levels. *A*, the *plpA* mRNA levels were determined by real-time qPCR analyses, and the *plpA* mRNA level in the wild type was set as 1. *B*, the secreted *Vv*PlpA and cellular HlyU, CRP, and DnaK (as an internal control) levels were determined by Western blot analyses. Molecular size markers (Bio-Rad) are shown in kDa. *C*, *V. vulnificus* strains were exposed to MEM (control) or INT-407 cells at an MOI of 10 for 1 h and then used to isolate total RNAs. The *plpA* mRNA levels were determined by real-time qPCR, and the *plpA* mRNA level in the wild type exposed to MEM was set as 1. *Error bars*, S.D. Statistical significance was determined by Student's *t* test. *, $p < 0.05$; **, $p < 0.005$; *ns*, not significant. *WT* and *WT pJH0311*, wild type; *hlyU* and *hlyU pJH0311*, *hlyU* mutant; *crp* and *crp pJH0311*, *crp* mutant; *hlyU crp pJH0311*, *hlyU crp* double mutant; *hlyU crp pZW1510* or *hlyU crp pKK1502*, *hlyU crp* double mutant overexpressing HlyU or CRP, respectively.

hlyU or *crp* mutant was significantly lower than that in the wild type, regardless of host cell exposure. Importantly, the host-dependent induction of *plpA* was evident in the wild type (3.36-fold induction; $p = 0.0002$), but not in either the *hlyU* or *crp* mutant ($p = 0.67$ or 0.054, respectively). Taken together, the results indicated that HlyU and CRP additively activate *plpA* expression, and thus both regulators are simultaneously required for the full activation of *plpA*. Furthermore, these regulators are essential for the induction of *plpA* in *V. vulnificus* during infection of host cells.

Determination and deletion analyses of the plpA promoter, P_{plpA}

To map the promoter of *plpA*, the transcription start site of *plpA* was determined by primer extension analysis. A single reverse transcript was produced from the primer extension of the RNA isolated from the wild type grown to an A_{600} of 0.9

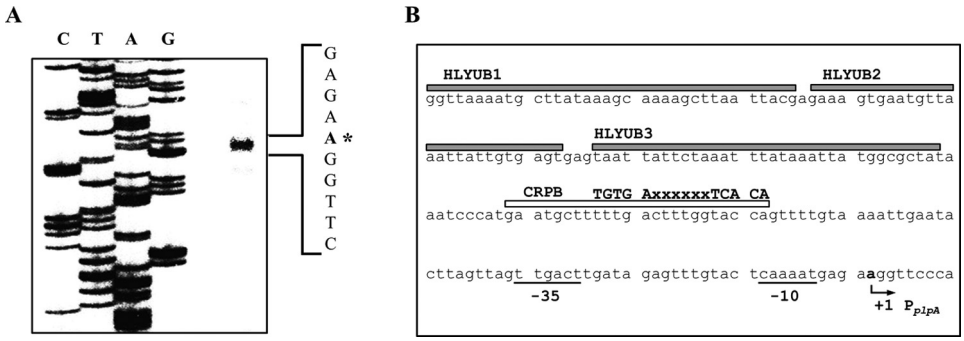

Figure 6. Transcription start site and sequences of the *plpA*-regulatory region. *A*, the transcription start site of *plpA* was determined by primer extension of the RNA isolated from the wild type grown to an A_{600} of 0.9. *Lanes C*, *T*, *A*, and *G*, nucleotide sequencing ladders of pKK1505. The *asterisk* indicates the transcription start site of *plpA*. *B*, the transcription start site of *plpA* is indicated by a *bent arrow*, and the positions of the putative −10 and −35 regions are *underlined*. The sequences for binding of HlyU (HLYUB1, HLYUB2, and HLYUB3; *gray boxes*) and CRP (CRPB; *white box*) were determined later in this study (Fig. 8, *C* and *D*). The consensus sequences for binding of CRP (32) are indicated *above* the *V. vulnificus* DNA sequence. *x*, any nucleotide.

(Fig. 6*A*). The 5′-end of the *plpA* transcript was located 65 bp upstream of the translational initiation codon of *plpA* and was subsequently designated +1 (Fig. 6*B*). The putative promoter constituting this transcription start site was named P_{plpA} to represent the *plpA* promoter, and the sequences for −10 and −35 regions of P_{plpA} were assigned on the basis of similarity to the consensus sequences of the *Escherichia coli* σ^{70} promoter (Fig. 6*B*). Several attempts to identify other transcription start sites using different sets of primers hybridizing to the *plpA* mRNA were not successful (data not shown), indicating that a single promoter, P_{plpA}, is used for *plpA* transcription.

To delineate the *cis*-DNA sequences in P_{plpA} required for HlyU- and CRP-mediated activation, pKK reporters carrying the P_{plpA} regulatory region, which was deleted up to different 5′-ends and fused transcriptionally to *luxCDABE*, were constructed (Fig. 7*A*). The reporters were transferred into the *V. vulnificus* strains, and cellular luminescence was used to quantify the ability of each P_{plpA} regulatory region to activate the promoter (Fig. 7*B*). The luminescence produced by pKK1514 carrying P_{plpA} deleted up to −227 was $\sim 1.1 \times 10^4$ relative light units (RLUs) in the wild type but significantly reduced in the *hlyU* and *crp* mutants, supporting our previous observation that HlyU and CRP coactivate *plpA* expression. Compared with pKK1514, pKK1515 carrying P_{plpA} deleted up to −157 produced significantly reduced RLUs in the wild type. Moreover, the RLUs of the wild type and the *hlyU* mutant containing pKK1515 did not significantly differ, indicating that the *cis*-DNA sequences required for HlyU to activate P_{plpA} are deleted in pKK1515. Similarly, the comparable levels of RLUs produced by pKK1516 in the wild type and *crp* mutant indicated that deletion of the P_{plpA} regulatory region up to −77 probably impairs CRP (in addition to HlyU) activation of P_{plpA}. The undetectable luminescence in the wild type (and the *hlyU* or *crp* mutant) containing pKK1517 indicated that deletion of the P_{plpA} regulatory region up to +22 results in a complete loss of P_{plpA} activity. These results implied that *cis*-DNA sequences essential for HlyU and CRP to activate P_{plpA} encompass the −157 and −77 regions, respectively.

HlyU and CRP regulate by binding directly to P_{plpA}

To examine whether HlyU and CRP bind directly to the promoter P_{plpA}, EMSAs were performed as shown in Fig. 8 (*A* and *B*). For this purpose, the 461-bp labeled DNA probe encompassing the P_{plpA} regulatory region (from −345 to +116) was incubated with increasing amounts of HlyU or CRP and then subjected to electrophoresis. EMSA revealed that the addition of HlyU to the DNA probe resulted in two retarded bands in a concentration-dependent manner, indicating that HlyU binds directly to at least two binding sites with different affinities (Fig. 8*A*). Furthermore, this binding of HlyU was specific because the EMSA was performed in the presence of a nonspecific competitor, poly(dI-dC) (0.1 μg; Sigma). In the second EMSA, the same but unlabeled 461-bp DNA probe was used as a self-competitor, which competed for the binding of HlyU in a dose-dependent manner (Fig. 8*A*), confirming that HlyU binds specifically as well as directly to P_{plpA}. Similarly, EMSAs demonstrated that CRP also binds directly and specifically to the P_{plpA} regulatory region (Fig. 8*B*). The combined results suggested that HlyU and CRP regulate *plpA* by directly binding to their specific regions within P_{plpA} rather than by modulating the cellular levels of unidentified *trans*-acting factor(s), which in turn binds directly to P_{plpA}.

DNase I protection assays were performed using the same 461-bp DNA probe to identify the HlyU-binding sequences in the P_{plpA} regulatory region (Fig. 8*C*). When HlyU was used up to 50 nM, the HlyU footprint extended from −191 to −157 (HLYUB1, centered at −174) (Fig. 8*C*). Upon increasing HlyU levels, two additional sequences extending from −151 to −128 (HLYUB2, centered at −139.5) and −126 to −93 (HLYUB3, centered at −109.5) were protected from DNase I digestion (Fig. 8*C*). Inspection of the HLYUBs revealed AT-rich sequences with an (imperfect) inverted repeat (Fig. 6*B*), indicating that HlyU binds to the sequences in the form of a dimer, as proposed previously (13, 31). Similarly, DNase I protection assays revealed the sequences of the region protected by CRP binding (Fig. 8*D*). The sequences of the protected region extended from −82 to −57 (CRPB, centered at −69.5) and scored 81% similarity to a consensus sequence for the binding of CRP (the TGTGAx^{6-8}TCACA; see Ref. 32) (Fig. 6*B*). The positioning of HLYUBs and CRPB suggested that both HlyU and CRP may act as class I activators interacting with the C-terminal domain of RNA polymerase α subunits (33). Taken together, these results demonstrated that HlyU and CRP regulate *plpA* directly by binding to the specific sequences of P_{plpA}.

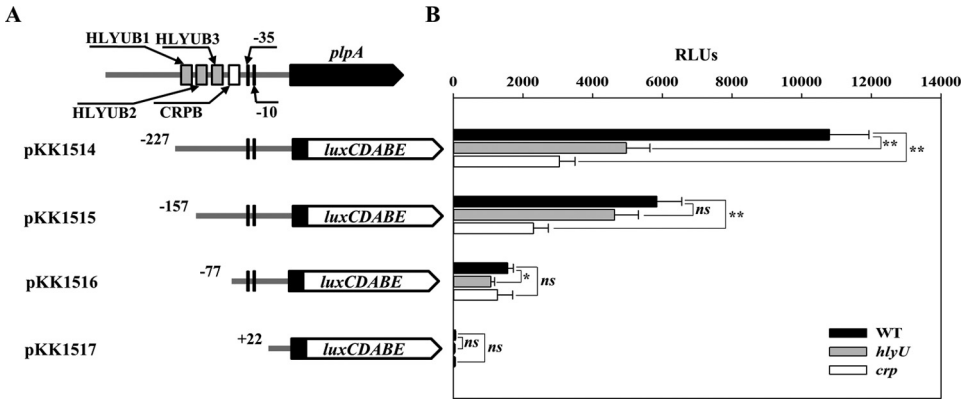

Figure 7. Deletion analysis of the P_{plpA} regulatory region. *A*, construction of *plpA*-lux fusion pKK reporters. PCR fragments carrying the *plpA*-regulatory region with 5′-end deletions were subcloned into pBBR-lux (64) to create each pKK reporter. *Solid lines*, the upstream region of *plpA*; *black blocks*, the *plpA* coding region; *white blocks*, *luxCDABE*. The wild-type *plpA*-regulatory region is shown on *top* with the proposed −10 and −35 regions, and the binding sites for HlyU (HLYUB1, HLYUB2, and HLYUB3; *gray boxes*) and CRP (CRPB; *white box*) were determined later in this study (Fig. 8, *C* and *D*). *B*, cellular luminescence was determined from the wild type (*black bars*), *hlyU* mutant (*gray bars*), and *crp* mutant (*white bars*) containing each pKK reporter as indicated. Cultures grown to an A_{600} of 0.9 were used to measure the cellular luminescence. *Error bars*, S.D. Statistical significance was determined by Student's *t* test. *, $p < 0.05$; **, $p < 0.005$; *ns*, not significant. *WT*, wild type; *hlyU*, *hlyU* mutant; *crp*, *crp* mutant.

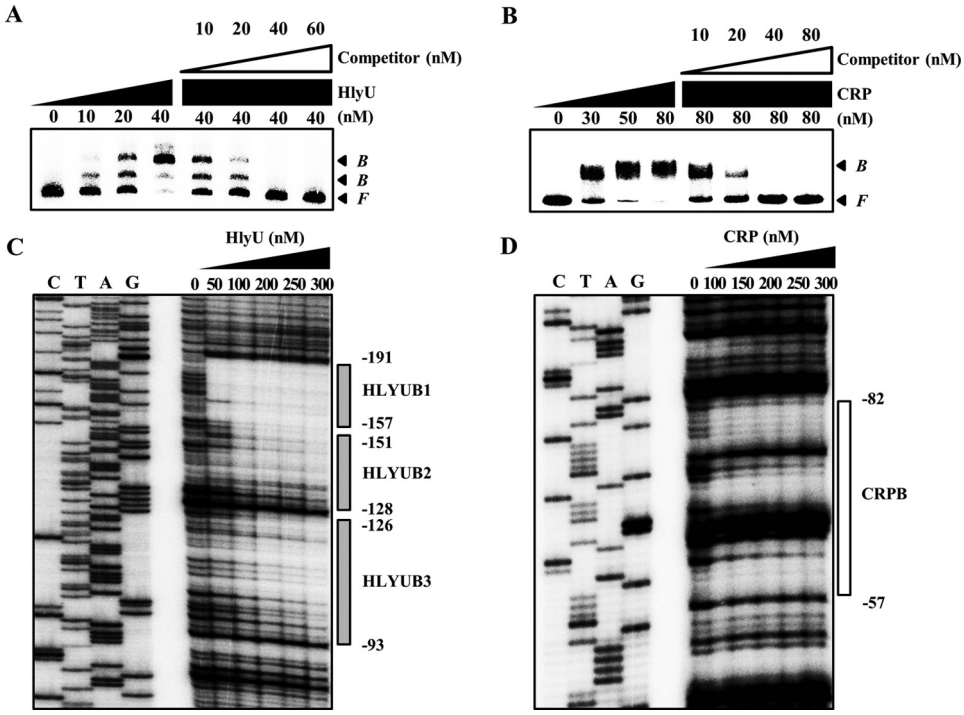

Figure 8. Specific bindings of HlyU and CRP to P_{plpA}. A 461-bp DNA fragment of the *plpA*-regulatory region was radioactively labeled and then used as a probe DNA. *A* and *B*, the radiolabeled probe DNA (5 nM) was incubated with increasing amounts of HlyU (*A*) and CRP (*B*) as indicated. For competition analysis, the same but unlabeled 461-bp DNA fragment was used as a self-competitor DNA. Various amounts of the self-competitor DNA were added to a reaction mixture containing the 5 nM labeled DNA before the addition of 40 nM HlyU (*A*) and 80 nM CRP (*B*) as indicated. *B*, bound DNA; *F*, free DNA. *C* and *D*, the same radiolabeled probe DNA (25 nM) was reacted with increasing amounts of HlyU (*C*) and CRP (*D*) as indicated. The regions protected from DNase I cleavage by HlyU and CRP are indicated by *gray boxes* (HLYUB1, HLYUB2, and HLYUB3) and *a white box* (CRPB), respectively. *Lanes C*, *T*, *A*, and *G* represent the nucleotide sequencing ladders of pKK1505. *Nucleotide numbers* shown are relative to the transcription start site of *plpA*.

Discussion

The epithelial surface of the gastrointestinal tracts is the major site of entry for *V. vulnificus*, an enteropathogen, to invade the host. Several methods, such as *in vivo*-induced antigen technology and *in vivo* expression technology, have been developed and adopted to discover *V. vulnificus* genes expressed preferentially in the host (34, 35). Although previous transcriptome analyses identified *V. vulnificus* genes induced specifically during infection, functional and regulatory features of the induced genes have been barely characterized (36, 37). In this study, *plpA* encoding a PLA_2 of *V. vulnificus*, *Vv*PlpA, was identified among the transcriptome profile expressed preferentially during infection of human intestine HT29-MTX cells (supplemental Tables S1 and S2) and then further characterized at molecular levels. As a part of the results, the ability of *Vv*PlpA to cleave the *sn*-2 bond in the fluorescently labeled PC was demonstrated (Fig. 1*C*). Consistent with this, rPlpA showed hemolytic activity on human and horse erythrocyte containing about 29 and 38% PC in its membrane (38, 39), respectively, but not to PC-deficient sheep erythrocyte (38)

(data not shown). These results suggested that PC is a preferential substrate for *Vv*PlpA, as is the case for *Va*PlpA (17). Moreover, compared with the wild type, the *plpA* mutant, KK131, showed significantly attenuated virulence; it showed reduced systemic infection, liver damage, and mortality in mice (Fig. 2, *B–F*). These results led us to conclude that *Vv*PlpA, which is secreted in a T2SS-dependent manner, is an essential virulence factor contributing to *V. vulnificus* pathogenesis along with other toxins, such as cytolysin/hemolysin and MARTX toxin (5, 40, 41). Indeed, the expression of neither the cytolysin/hemolysin gene, *vvhA*, nor the MARTX toxin gene, *rtxA1*, was affected by *plpA* mutation in *V. vulnificus* (data not shown). Furthermore, we found that the genes encoding *Vv*PlpA homologues are highly conserved and expressed well in various clinical and environmental isolates of *V. vulnificus* (data not shown), further supporting its crucial role in the adaption of *V. vulnificus* to variety of environments, including the host interior. One such role of *Vv*PlpA during infection is that this lipolytic enzyme disrupts membranes of intestine epithelial cells and thereby allows the pathogen to invade other tissues (or organs), such as blood vessels, where the pathogen most likely secretes additional toxins.

It has been reported that many pathogenic bacteria, including *Salmonella* spp. and *Yersinia enterocolitica*, induce necrotic death of the host epithelial barrier and immune cells to survive and spread easily in the host (42, 43). Consistent with this, *V. vulnificus* infection also manifests robust necrotic cell death and tissue destruction, which are caused by toxins such as MARTX, resulting in skin lesions (6, 44, 45). This study found that *Vv*PlpA, in addition to the MARTX toxin, also contributes to the induction of necrotic death of INT-407 epithelial cells (Fig. 3*D*). Therefore, the combined results suggested that *Vv*PlpA is essential for the necrotic death of human epithelial cells (Fig. 3).

Many pathogenic bacteria continually encounter environmental changes and cope with harsh conditions in the host. Therefore, the ability to constantly sense and respond to the host environment is important for the pathogens to establish successful infection (46, 47). Accordingly, pathogens have evolved sophisticated mechanisms to extensively regulate their gene expressions, which consequently plays a key role in the adaptation and pathogenesis of the bacteria to the host (46, 47). This study revealed that *V. vulnificus* regulates the expression of *plpA* in a growth phase–dependent manner and to the maximum level in the early stationary phase (Fig. 4*A*). Because this growth-phase dependent *plpA* expression was further confirmed by using a P_{plpA}-*luxCDABE* transcriptional fusion reporter (data not shown), it seems that the regulation of *plpA* expression occurred at a transcriptional level rather than a post-transcriptional level. Consistent with this, HlyU and CRP were found to directly activate *plpA* transcription both *in vitro* and *ex vivo* (Figs. 5 and 8). Because the expression of HlyU is induced specifically during the infection (34), the positive control of *Vv*PlpA by HlyU could enable *V. vulnificus* to express this critical virulence factor at the right place (*i.e.* inside the host). In addition, CRP, which is a central regulator of energy (catabolic) metabolism (15) may recognize host environments by sensing the starvation of specific nutrients imposed by the host cells and endogenous bacterial flora (47). Accordingly, CRP might contribute to the production of *Vv*PlpA at the appropriate time when *V. vulnificus* encounters such nutrient starvation, probably leading to immune deficiency in the host. Taken together, *V. vulnificus* could express *plpA* in a spatially and temporally regulated fashion to obtain maximum effectiveness during pathogenesis.

Although a few examples of transcriptional coactivation of bacterial virulence genes by multiple activators have been reported (11, 48), coactivation by HlyU and CRP has not yet been reported. Each HlyU and CRP binds to the upstream sequence of P_{plpA} and activates the promoter as a solitary regulator, albeit not to levels comparable with that of the wild type (Figs. 5 and 8). Overproduced HlyU (or CRP) failed to fully compensate for a lack of CRP (or HlyU), indicating that both HlyU and CRP are required for the expression of *Vv*PlpA to the wild-type level (Fig. 5, *A* and *B*). Obviously, this coactivation of P_{plpA} by both HlyU and CRP, which probably sense and incorporate different signals from the environment, as mentioned above, could allow the tight regulation and fine tuning of *Vv*PlpA expression during pathogenesis. It is also noteworthy that HlyU and/or CRP are involved in the activation of the expression of other virulence factors, such as the MARTX toxin, cytolysin/hemolysin, VvpE (an elastolytic protease), GbpA (a mucin-binding protein), and HupA (a heme receptor protein), in addition to *Vv*PlpA (11, 13, 48–50). It is likely that regulation by common regulatory proteins provides precisely coordinated expression of multiple virulence factors, thereby facilitating the cooperation of the virulence factors crucial for the overall success of the organism during pathogenesis.

In summary, a transcriptome analysis identified *V. vulnificus plpA* among the genes, which are specifically induced when the pathogen is exposed to human intestine HT29-MTX cells. *Vv*PlpA is a T2SS-dependent secretory PLA_2 and promotes lysis and necrotic death of host cells, thereby contributing to the pathogenesis of *V. vulnificus*, as determined in the mouse infection model. The expression of *plpA*, which reaches maximum levels at the early stationary growth phase, is activated by the global regulators HlyU and CRP at the transcriptional level. HlyU and CRP mediate the host-dependent induction of *plpA* and exert their effects by directly binding to the regulatory region of P_{plpA}. The collaborative regulation of *plpA* by the global regulators could facilitate cooperation of *Vv*PlpA with other virulence factors to obtain maximum effectiveness, thereby enhancing the overall success of *V. vulnificus* during infection.

Experimental procedures

Strains, plasmids, and culture conditions

The strains and plasmids used in this study are listed in Table 1. Unless otherwise noted, the *V. vulnificus* strains were grown aerobically in LB medium supplemented with 2% (w/v) NaCl (LBS) at 30 °C, and their growth was monitored spectrophotometrically at 600 nm (A_{600}).

Transcriptome analyses

V. vulnificus MO6-24/O (wild type) cells were used either to infect HT29-MTX intestinal epithelial cells at an MOI of 10 or

Table 2
Oligonucleotides used in this study

Name	Oligonucleotide sequence (5′ → 3′)[a,b]	Use
For mutant construction		
PLPA01-F	ATGAAGAAGATAACTATTCTGTTGGGTGC	Deletion of *plpA* ORF
PLPA01-R	GTAGAATTCACAACGTTGCTTAATCACG	
PLPA02-F	GTGAATTCTACATCATTCAGGGTTACAACATC	Deletion of *plpA* ORF
PLPA02-R	TGAGCTGTGAGTGGCACAATCG	
HLYU01-F	CTTGTTTTGGTCGCGATGG	Deletion of *hlyU* ORF
HLYU01-R	CAGAATTCTTTATTGCGAAGAATAATGC	
HLYU02-F	AAAGAATTCTGCTCCATATCTTTTAAGTTC	Deletion of *hlyU* ORF
HLYU02-R	CGACACAAAGTCCCGGCC	
For mutant complementation		
PLPA03-F	GGTACCAATAATCAAAACATAAGAAGATAG	Amplification of *plpA* ORF
PLPA03-R	GGTACCCTAAAAATTAAAGCG	
HLYU03-F	GAGCTCTCTCAAAGAAGAGTATTACGTG	Amplification of *hlyU* ORF
HLYU03-R	GGTACCTTACACCGATCCTAGACC	
CRP01-F	GAGCTCACACCCTTGTGGTGCC	Amplification of *crp* ORF
CRP01-R	GGTACCTTAACGAGTACCGTAAACAACG	
For protein overexpression		
PLPA04-F	CATATGGAGCCAGCTCTCTCTCCTGAAGC	Amplification of *plpA-his*$_6$ ORF
PLPA04-R	ACTAGTCTAGTGATGGTGATGGTGATGAAAATTAAAGCGTTG	
PLPA05-F	CCATGGAGCCAGCTCTCTCTCC	Amplification of truncated *plpA* ORF
PLPA05-R	GTCGACGTTGCCGGTATCAGAG	
HLYU04-F	GGATCCATGAACTTAAAAGATATGGAGC	Amplification of *hlyU* ORF
HLYU04-R	CTCGAGTTATTCTTCGCAATAAAGA	
For real-time qPCR		
PLPA-qRT-F	TTGTTGGTATCGAACGGGCA	
PLPA-qRT-R	CGAGCTCCACCAATAACCGT	
16S-qRT-F	CGGCAGCACAGAGAAACTTG	Quantification of the16S rRNA expression
16S-qRT-R	CCGTAGGCATCATGCGGTAT	
For primer extension analysis, EMSA, and DNase I protection assay		
PLPA06-F	GCGTAAATGCGCAATGAAAAG	Amplification of *plpA* upstream region
PLPA06-R	CTGCAGATGTGAAGGGCAACAGA	Amplification of *plpA* upstream region, extension of *plpA* transcript
For promoter deletion analysis		
PLPAR001	GAGCTCTAAAATGCCAAAAAAATCGC	Deletion of *plpA* regulatory region
PLPAR002	GAGCTCGAGAAAGTGAATGTTAAATTATTG	
PLPAR003	GAGCTCTTTTTGACTTTGGTACCAGTTTT	
PLPAR004	GAGCTCCAACTGGTGATTAATAATCAAAAC	
PLPAR005	ACTAGTGTGATCGCTTCAGGAGAGAGA	

[a] The oligonucleotides were designed using the *V. vulnificus* MO6-24/O genomic sequence (GenBank™ accession number CP002469 and CP002470; www.ncbi.nlm.nih.gov).
[b] Regions of oligonucleotides not complementary to the corresponding genes are underlined.

to grow in the basal medium Eagle (Gibco-BRL) alone (as a negative control) for 2 h and were then harvested. Total RNAs were isolated from the bacteria using RNAprotect® bacteria reagent and the miRNeasy® minikit (Qiagen, Valencia, CA), fragmented, and used to synthesize double-stranded cDNA, as described previously (TruSeq® Stranded mRNA Sample Prep Kit, Illumina, San Diego, CA) (51). The cDNA library was amplified by PCR, sequenced, and then counted as the numbers of reads per kilobase of transcript per million mapped reads to quantify the expression levels of specific genes as described previously (52). These values, normalized using the quantile normalization procedure, were statistically analyzed by *t* tests to identify the genes expressed differentially (>4-fold change with $p \leq 0.05$) when exposed to the HT-29 MTX cells.

Generation and complementation of the plpA, hlyU, and hlyU crp mutants

The *plpA* gene was inactivated *in vitro* by deletion of the ORF of *plpA* (353 of 1254 bp) using the PCR-mediated linker-scanning mutation method as described previously (53). Briefly, pairs of primers PLPA01-F and -R (for amplification of the 5′ amplicon) or PLPA02-F and -R (for amplification of the 3′ amplicon) were designed and used (Table 2). The resulting Δ*plpA* was amplified by PCR using the mixture of both amplicons as the template and PLPA01-F and PLPA02-R as primers. Similar experimental procedures were adopted for amplification of the Δ*hlyU in vitro*, except that primers HLYU01-F, HLYU01-R, HLYU02-F, and HLYU02-R (for 249-bp deleted *hlyU*) were used as indicated in Table 2. The resulting Δ*plpA* and Δ*hlyU* were ligated into SpeI-SphI– digested pDM4 (54) to generate pKK1301 and pZW1401, respectively (Table 1). *E. coli* S17-1 λ*pir, tra* strain (55) containing pKK1301 or pZW1401 was used as a conjugal donor to *V. vulnificus* MO6-24/O to generate either the *plpA* mutant KK131 or the *hlyU* mutant ZW141, respectively (Table 1). Similarly, *E. coli* S17−1 λ*pir, tra* containing pBS0907, which was constructed previously to carry a mutant allele of *V. vulnificus crp* on pDM4 (Table 1) (56), was used as a conjugal donor to ZW141 to generate the *hlyU crp* double mutant KK151 (Table 1). The conjugation and isolation of the transconjugants were conducted using the method described previously (56).

To complement the *plpA*, *hlyU*, *crp*, and *pilD* (constructed previously (20)) mutations, each ORF of *plpA*, *hlyU*, *crp*, and *pilD* was amplified by PCR using a pair of specific primers, as listed in Table 2. The amplified *plpA*, *hlyU*, *crp*, and *pilD* ORFs were cloned into the broad-host-range vector pRK415 (for

plpA) (57) and pJH0311 (for *hlyU*, *crp*, and *pilD*) (58) under the *lac* promoter to create pKK1320, pZW1510, pKK1502, and pMS0908, respectively (Table 1). The plasmids were transferred into the appropriate mutants by conjugation as described above. For complementation tests, when the cultures reached an A_{600} of 0.3, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.1 mM to induce the expression of the recombinant genes on the plasmids.

Protein purification and Western blot analysis

The ORF of *plpA* with a His_6 tag was amplified by PCR using a pair of primers, PLPA04-F and -R (Table 2), digested with NcoI and SalI, and ligated into pMBP parallel 1 (59), resulting in pKK1503 (Table 1). The maltose-binding protein-PlpA-His_6 protein was expressed in *E. coli* BL21 (DE3), the maltose-binding protein portion was removed, and the His_6-tagged rPlpA was further purified using a HiLoad Superdex 200 gel filtration column according to the manufacturer's procedures (GE Healthcare). The purified rPlpA was used to perform a PLA_2 activity assay. To raise a polyclonal antibody against PlpA, an N-terminal region of PlpA (amino acids 1–150) was subcloned into pET-28a(+) (Novagen, Madison, WI) using primers PLPA05-F and -R (Table 2), resulting in pKK1504 (Table 1). The ORF of *hlyU* was subcloned into pET-28a(+) using a pair of primers HLYU04-F and -R (Table 2), resulting in pYU1317 (Table 1). The plasmid pHK0201 containing an ORF for His_6-tagged CRP was previously constructed (Table 1) (49). The His_6-tagged truncated PlpA, HlyU, and CRP were expressed in *E. coli* BL21 (DE3) and purified by affinity chromatography (Qiagen).

The purified His_6-tagged truncated PlpA, HlyU, and CRP proteins were used to raise rabbit polyclonal antibodies to PlpA, HlyU, and CRP of *V. vulnificus*, respectively (AB Frontier, Seoul, South Korea). A mouse antibody to *E. coli* DnaK was purchased (Enzo Lifescience, Farmingdale, NY) and used to detect *V. vulnificus* DnaK as a loading control (31). The *V. vulnificus* cultures grown to various A_{600} values were harvested and fractionated into cells and supernatants by centrifugation. The cells were lysed using cOmplete™ Lysis-M EDTA-free buffer (Roche, Mannheim, Germany) for 10 min, and residual cell debris was removed by centrifugation to obtain clear lysates. The supernatants were filtered through a Puradisc™ 25-mm syringe filter (pore size 0.2 μm; GE Healthcare) and concentrated using Amicon Ultra-15 (cut-off 10 kDa; Millipore, Temecula, CA). HlyU, CRP, and DnaK in the clear cell lysates (equivalent to 10 μg of total protein) or *Vv*PlpA in the supernatant concentrates (equivalent to 2 μg of total protein) were determined by Western blot analyses, as described previously (60). Similarly, culture medium of INT-407 (ATCC® CCL-6) human epithelial cells, infected with the *V. vulnificus* strains at an MOI of 10 for 2 h, were 5-fold concentrated and resolved by SDS-PAGE, and then the actin and CypA were detected by Western blot analyses as described previously (61).

Proteins of interest were detected with the following antibodies and reagent. Rabbit anti-*Vv*PlpA (1:1,000), rabbit anti-*V. vulnificus* HlyU (1:1,000), rabbit anti-*V. vulnificus* CRP (1:5,000), mouse anti-*E. coli* DnaK (1:10,000; Enzo Lifescience), rabbit anti-CypA (1:1,000; Cell Signaling Technology, Beverly, MA), rabbit anti-actin (1:10,000; Cell Signaling Technology), HRP-conjugated goat anti-rabbit IgG (1:10,000; Sigma), and HRP-conjugated rabbit anti-mouse IgG (1:10,000, Sigma) antibodies and ECL Select™ Western blotting detection reagent (GE Healthcare).

PLA_2 activity assay

PLA_2 activity was measured using the EnzChek® phospholipase A_2 assay kit (Invitrogen) that consists of a fluorogenic PC substrate (red/green BODIPY PC-A2) specific to PLA_2 (21). Briefly, the substrate/liposome mix was prepared by mixing 25 μl of 1 mM red/green BODIPY PC-A2 substrate in dimethyl sulfoxide, 25 μl of 10 mM 1,2-dioleoyl-*sn*-glycero-3-phosphocoline, and 25 μl of 1,2-dioleoyl-*sn*-glycero-3-phospho-(1′-rac-glycerol) (sodium salt) in 5 ml of PLA_2 assay buffer (Invitrogen). Various amounts of rPlpA were added to 50 μl of the substrate/liposome mix and incubated at room temperature for 0.5 h. When necessary, the culture supernatants of the *V. vulnificus* strains grown to an A_{600} of 0.9 were filtered and concentrated as described above and then added to the reaction mixture instead of rPlpA (equivalent to 2 μg of total protein). The cleavage of the *sn*-2 bond of the red/green BODIPY PC-A2 by the PLA_2 results in an increase of fluorescence emission, which was measured by using a microplate reader with an excitation at 480 nm and emission at 515 nm (Tecan Infinite M200 reader, Männedorf, Switzerland). Data were normalized by subtracting the fluorescence emissions from appropriate negative control samples (mixed with rPlpA storage buffer or blank medium concentrate). The activity of the rPlpA was determined using the standard curve developed based on the fluorescence emission by the various units of honey bee venom PLA_2.

RNA purification and transcript analysis

Total RNAs from the *V. vulnificus* strains grown to various A_{600} values were isolated using an RNeasy® minikit (Qiagen). For real-time qPCR, the concentrations of total RNAs from the strains were measured by using a Nano-Vue Plus spectrophotometer (GE Healthcare). cDNA was synthesized from 1 μg of the total RNA by using the iScript™ cDNA synthesis kit (Bio-Rad), and real-time PCR amplification of the cDNA was performed by using the Chromo 4 real-time PCR detection system (Bio-Rad) with pairs of specific primers (Table 2), as described previously (62). Relative expression levels of the *plpA* mRNA were calculated by using the 16S rRNA expression level as the internal reference for normalization (50).

If required, the *V. vulnificus* strains were exposed to blood of uninfected mice ($n = 3$), MEM, or INT-407 cells for various incubation times. The mixture of *V. vulnificus* and the mouse blood (or *V. vulnificus* and the host cells) was centrifuged at 250 × *g* for 10 min to precipitate eukaryotic cells and debris in the mouse blood (or the host cells), and then the *V. vulnificus* cells were harvested from the supernatant and used to isolate total RNAs, as described previously (29).

For primer extension analysis, a 23-base primer PLPA06-R (Table 2) complementary to the coding region of *plpA* was end-labeled with [γ-^{32}P]ATP and added to the RNA. The primer was then extended with SuperScript II RNase H^- reverse transcriptase (Invitrogen). The cDNA products were purified and

resolved on a sequencing gel alongside sequencing ladders generated from pKK1505 with the same primer. The plasmid pKK1505 was constructed by cloning the 461-bp *plpA* upstream region extending from −345 to +116, amplified by PCR using a pair of primers PLPA06-F and -R (Table 2), into pGEM-T Easy (Promega, Madison, WI). The primer extension product was visualized using a phosphor image analyzer (BAS1500, Fuji Photo Film Co., Ltd., Tokyo, Japan).

Mouse infection experiments

Three different experiments were conducted using female ICR mice (7-week-old specific-pathogen free; Seoul National University). For this purpose, the *V. vulnificus* strains grown to an A_{600} of 0.5 were harvested and suspended in PBS to 10^8 or 10^6 cfu/100 μl for intrasgastric or intraperitoneal infection, respectively. Mouse lethality of the *V. vulnificus* strains was compared as described previously ($n = 15$ per group) (11). The mice were intraperitoneally injected with 30 μg of iron-dextran for each gram of body weight, immediately after which they were administered with 100 μl of the inoculum intragastrically. Survival of the mice was recorded for 24 h.

To determine the number of *V. vulnificus* strains in the blood of infected mice and to conduct hematological analyses, the mice, without an iron-dextran pretreatment, were intraperitoneally infected with 100 μl of the inoculum ($n = 11$ for each wild type and *plpA* mutant and $n = 5$ for PBS control). The mice were sacrificed at 4 h postinfection to obtain blood samples. Equal amounts of diluted blood samples of wild type– and *plpA* mutant–infected mice ($n = 6$) were spread on LBS agar containing polymyxin B (100 units/ml) to specifically count the *V. vulnificus* cells. The recovered bacterial cells were enumerated as cfu/100 μl of blood. The remaining blood samples were used for hematological analyses, as described previously (63). Briefly, MPXI was analyzed by using a hematological autoanalyzer (AVIDA 2120, Bayer Diagnostics, Giessen, Germany). The serum levels for ALB, AST, and ALT were measured by using a biochemistry autoanalyzer (Hitachi 7180 autoanalyzer, High-Technologies Corp., Tokyo, Japan). All manipulations of mice were approved by the Animal Care and Use Committee at Seoul National University.

Cytotoxicity and cell morphology change

Two different assays were performed using INT-407 cells. To determine cytotoxicity, the monolayers of INT-407 cells were prepared and infected with the *V. vulnificus* strains in a 96-well tissue culture plate (Nunc, Roskilde, Denmark), and LDH activities in the supernatant were measured as described previously (29). Morphological changes were also examined by staining the INT-407 cells, which were seeded onto 6-well culture dishes (Nunc) and infected with the *V. vulnificus* strains at an MOI of 10 for 1.5 h. Briefly, INT-407 cell membranes and nuclei were stained with Texas Red®-X–conjugated WGA (final 5 μg/ml; Thermo Fisher Scientific) and with Hoechst® 33342 (final 5 μg/ml; Thermo Fisher Scientific), respectively, for 10 min and then photographed using a fluorescence microscope (FLoid® cell imaging station, Thermo Fisher Scientific).

Construction of a set of plpA-luxCDABE transcriptional fusions

The primer PLPAR005 (Table 2) carrying an SpeI restriction site was used in conjunction with one of the primers carrying an SacI restriction site, PLPAR001, PLPAR002, PLPAR003, or PLPAR004, to amplify the DNA of *plpA* extending up to −227, −157, −77, and +22 bp, respectively. The amplified DNA fragments were inserted into the SpeI-SacI–digested pBBR-lux carrying promoterless *luxCDABE* genes (64) to create four *plpA*-lux reporter constructs: pKK1514, pKK1515, pKK1516, and pKK1517. The constructs were then transferred into the *V. vulnificus* strains by conjugation. The cellular luminescences of the cultures grown to an A_{600} of 0.9 were measured using a microplate reader (Tecan Infinite M200 reader) and expressed in arbitrary RLUs as described previously (48).

EMSA and DNase I protection assay

The 461-bp *plpA* upstream region, extending from −345 to +116, was amplified by PCR using unlabeled PLPA06-F and [γ-^{32}P]ATP-labeled PLPA06-R and as primers (Table 2). The binding of HlyU to the labeled DNA and electrophoretic analysis of the HlyU–DNA complexes have already been described (13, 56). The protein–DNA binding reactions with CRP were performed in the same manner as those with HlyU, except that the 1× CRP-binding buffer was used (49).

The same labeled 461-bp DNA was used for DNase I protection assays. The binding of HlyU or CRP to the labeled DNA and DNase I digestion of the protein–DNA complexes followed the procedures described previously (56). The digested DNA products were precipitated with ethanol and then resolved on a sequencing gel beside the sequencing ladders generated as described above. The gels were visualized using a phosphor image analyzer (BAS1500).

Data analyses

Averages and S.D. values were calculated from at least three independent experiments. Mouse mortality was evaluated using the Log Rank Test program (http://bioinf.wehi.edu.au/software/russell/logrank/).[4] All other data were analyzed by Student's *t* tests using the SAS program (SAS Institute Inc.). Significance of differences between experimental groups was accepted at a p value of <0.05.

Transcriptome data accession number

All raw transcriptome data were deposited in ArrayExpress (http://www.ebi.ac.uk/arrayexpress/)[4] (67) under accession number E-MTAB-5629.

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