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Regulatory Characteristics of *Vibrio vulnificus gbpA* Gene Encoding a Mucin-binding Protein Essential for Pathogenesis*

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Kyung Ku Jang, So Yeon Gil, Jong Gyu Lim, and Sang Ho Choi[1]

From the National Research Laboratory of Molecular Microbiology and Toxicology, Department of Agricultural Biotechnology, Center for Food Safety and Toxicology, Seoul National University, Seoul 151-921, South Korea

Binding to mucin is the initial step for enteropathogens to establish pathogenesis. An open reading frame, *gbpA*, of *Vibrio vulnificus* was identified and characterized in this study. Compared with wild type, the *gbpA* mutant was impaired in binding to mucin-agar and the mucin-secreting HT29-methotrexate cells, and the impaired mucin binding was restored by the purified GbpA provided exogenously. The *gbpA* mutant had attenuated virulence and ability of intestinal colonization in a mouse model, indicating that GbpA is a mucin-binding protein and essential for pathogenesis of *V. vulnificus*. The *gbpA* transcription was growth phase-dependent, reaching a maximum during the exponential phase. The Fe-S cluster regulator (IscR) and the cyclic AMP receptor protein (CRP) coactivated, whereas SmcR, a LuxR homologue, repressed *gbpA*. The cellular levels of IscR, CRP, and SmcR were not significantly affected by one another, indicating that the regulator proteins function cooperatively to regulate *gbpA* rather than sequentially in a regulatory cascade. The regulatory proteins directly bind upstream of the *gbpA* promoter P_{gbpA}. DNase I protection assays, together with the deletion analyses of P_{gbpA}, demonstrated that IscR binds to two specific sequences centered at −164.5 and −106, and CRP and SmcR bind specifically to the sequences centered at −68 and −45, respectively. Furthermore, *gbpA* was induced by exposure to H_2O_2, and the induction appeared to be mediated by elevated intracellular levels of IscR. Consequently, the combined results indicated that IscR, CRP, and SmcR cooperate for precise regulation of *gbpA* during the *V. vulnificus* pathogenesis.

Epithelial surfaces of the intestine are the most common portals by which enteropathogenic bacteria enter the deeper tissues of a mammalian host. The epithelial surfaces are covered by a mucous layer, which is produced by specialized cells found throughout the entire intestinal tract (1). The mucous layer is the first barrier that the enteropathogens encounter, and it prevents the pathogens from reaching and persisting on the intestinal epithelial surfaces and thereby is a major component of innate immunity (1). The mucous layer is composed of a variety of factors, but its characteristic physico-chemical properties are attributable to the presence of mucins, which are complex linear polymorphic glycoproteins (1, 2). Mucins are highly glycosylated large glycoproteins (with a molecular mass ranging from 5×10^3 to 4×10^6 Da), and up to 85% of their dry weight is carbohydrates (3). Although mucin contains extensively different types of carbohydrates, the residue, *N*-acetyl-D-glucosamine (GlcNAc), is one of the most abundant sugars in the carbohydrate side chains (4).

Adhesion to the mucosal surfaces followed by colonization on the mucosal tissue is considered to constitute the first stages of the infectious process (5). Accordingly, mutants of enteropathogenic bacteria that have difficulty in adhesion to the mucous layer were substantially defective in intestinal colonization, leading to attenuated virulence (6, 7). Although numerous factors (known as adhesins) are involved in the adhesion of enteropathogens, information on the adhesins with specificity toward mucin carbohydrates is still limited (8). The GlcNAc-binding protein A of *Vibrio cholerae* (*Vc*GbpA) is a lectin-like mucus adhesin and is characterized at the molecular level (6, 9–11). *Vc*GbpA is a common adhesin required for *V. cholerae* to adhere to chitinous and intestinal surfaces (6). *Vc*GbpA plays an important role in the survival of *V. cholerae* by attachment to the surface of chitinous zooplankton in the aquatic ecosystem (6, 11). *Vc*GbpA is a mucin-binding protein that binds to GlcNAc residues of mucin and contributes to intestinal colonization and virulence in a mouse model (6, 11). Structural analysis demonstrated that *Vc*GbpA possesses a four-domain structure of which domains 1 and 4 interact with chitin and domain 1 is also crucial for mucin binding and intestinal colonization. In contrast, domains 2 and 3 anchor to the *V. cholerae* surfaces (11). It has been reported that *Vc*GbpA expression was induced by mucin and negatively regulated by cyclic di-guanosine monophosphate at the post-transcription level and by quorum sensing at the post-translation level (9, 10, 12). However, neither the promoter(s) of the *gbpA* gene nor any *trans*-acting regulatory protein(s) required for the transcription of *gbpA* has been identified previously.

Vibrio vulnificus is a Gram-negative bacterium commonly associated with human disease caused by ingestion of undercooked oysters or contact of the organism with an open wound (13). Like many other enteropathogenic bacteria, *V. vulnificus* also expresses diverse adhesin molecules. The *V. vulnificus*

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[1] To whom correspondence should be addressed: Dept. of Agricultural Biotechnology, Seoul National University, Seoul 151-921, South Korea. Tel.: 82-2-880-4857; Fax: 82-2-873-5095; E-mail: choish@snu.ac.kr.

TABLE 1
Plasmids and bacterial strains used in this study

Strain or plasmid	Relevant characteristics[a]	Ref. or source
Bacterial strains		
V. vulnificus		
MO6-24/O	Wild type, clinical isolate; virulent	61
MORR	MO6-24/O with spontaneous Rif^r mutation, virulent	Laboratory collection
MORSR	MO6-24/O with spontaneous Rif^r, Sm^r mutation, virulent	Laboratory collection
JK093	MO6-24/O with Δ*iscR*	26
JK128	MO6-24/O with $iscR_{3CA}$ encoding the apo-locked IscR, $IscR_{3CA}$	32
HS03	MO6-24/O with *smcR*::*nptI*; Km^r	22
DI0201	MO6-24/O with Δ*crp*	41
SO111	MO6-24/O with Δ*gbpA*	This study
KK141	MORSR Δ*gbpA*, Rif^r, Sm^r	This study
KK142	MO6-24/O with Δ*iscR*Δ*crp*	This study
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	29
BL21 (DE3)	F$^-$ *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm* (DE3)	Laboratory collection
Plasmids		
pGEM-T Easy	PCR product cloning vector; Ap^r	Promega
pKK1401	pGEM-T Easy with 390-bp fragment of *gbpA* upstream region; Ap^r	This study
pDM4	R6K γ *ori sacB*; suicide vector; *oriT* of RP4; Cm^r	28
pSO1101	pDM4 with Δ*gbpA* Cm^r	This study
pBS0907	pDM4 with Δ*crp*; Cm^r	31
pJK1113	pKS1101 with *nptI*; Ap^r, Km^r	32
pKK1402	pJK1113 with *gbpA*; Ap^r, Km^r	This study
pKK1403	pJK1113 with *iscR*; Ap^r, Km^r	This study
pKK1404	pJK1113 with *crp*; Ap^r, Km^r	This study
pKK1405	pJK1113 with *smcR*; Ap^r, Km^r	This study
pET22a(+)	His_6 tag fusion expression vector; Ap^r	Novagen
pSO1201	pET22a (+) with *gbpA;* Ap^r	This study
pJK0928	pET28a (+) with *iscR*; Ap^r	31
pHK0201	pRSET A with *crp*; Ap^r	41
pHS104	pRSET C with *SmcR*; Ap^r	22
pBBR-lux	Broad host range vector with *luxCDABE* operon; Cm^r	44
pKK1407	pBBR_lux with 555-bp fragment of *gbpA* upstream region; Cm^r	This study
pKK1408	pBBR_lux with 451-bp fragment of *gbpA* upstream region; Cm^r	This study
pKK1409	pBBR_lux with 411-bp fragment of *gbpA* upstream region; Cm^r	This study
pKK1410	pBBR_lux with 341-bp fragment of *gbpA* upstream region; Cm^r	This study

[a] The following abbreviations are used: Rif, rifampicin-resistant; Sm^r, streptomycin-resistant; Km^r, kanamycin-resistant; Tp^r, trimethoprim-resistant; Ap^r, ampicillin-resistant; Cm^r, chloramphenicol-resistant.

adhesins include a flagellum, a type IV pilus, a lipoprotein, and OmpU that are crucial for adhesion to human epithelial cells *in vitro* and virulence in mice (14–18). However, there is still no information about the factors responsible for the initial adhesion of the pathogen to mucin. In this study, a *V. vulnificus* open reading frame (ORF) encoding a homologue of *Vc*GbpA was identified. Construction of the *gbpA* mutant and evaluation of its phenotypes provided evidence that *V. vulnificus* GbpA (*Vv*GbpA) is also a mucin-binding protein and plays a crucial role in the pathogenesis of the organism. Efforts to understand the regulatory mechanisms of the *gbpA* expression were initiated by determining the *gbpA* mRNA levels in cells of different growth phases. Because IscR (iron-sulfur (Fe-S) cluster regulator) (19), cyclic AMP receptor protein (CRP)[2] (20), and SmcR (LuxR homologue) (21) were previously reported to affect the pathogenesis of *V. vulnificus* (22–26), influences of the global regulatory proteins on the expression of *gbpA* were also examined. Genetic and biochemical studies demonstrated that IscR and CRP coactivated and SmcR repressed *gbpA* in a growth phase-dependent manner. Furthermore, the three regulatory proteins regulate *gbpA* cooperatively rather than sequentially and exert their effects by directly binding to the *gbpA* promoter P_{gbpA}. Deletion analyses of the upstream region of P_{gbpA} and DNase I protection assays were performed to identify the binding sequences of IscR, CRP, and SmcR. Finally, the influence of hydrogen peroxide (H_2O_2) on the intracellular levels of IscR was examined to explain how IscR can mediate the induction of *gbpA* by oxidative stress.

[2] The abbreviations used are: CRP, cyclic AMP receptor protein; MTX, methotrexate; qRT-PCR, quantitative real time PCR; EMSA, electrophoretic mobility shift assay; RLU, relative light unit; F, forward; R, reverse.

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, wild type MO6-24/O and its mutants, were grown in Luria-Bertani (LB) medium supplemented with 2% (w/v) NaCl (LBS) at 30 °C, and their growth was monitored spectrophotometrically at 600 nm (A_{600}). Anaerobic conditions were obtained by using an anaerobic chamber with an atmosphere of 90% N_2, 5% CO_2, and 5% H_2 (Coy Laboratory Products, Grass Lake, MI). For anaerobic culture, the media were pre-incubated to remove dissolved O_2 in the anaerobic chamber, which was verified by adding 0.00001% (w/v) resazurin salt (Sigma) to the media (27).

Generation and Complementation of the gbpA and iscR crp Mutants—The *gbpA* gene was inactivated *in vitro* by deletion of the ORF of *gbpA* (318-bp of 1458-bp) using the PCR-mediated linker-scanning mutation method as described previously (27). Briefly, pairs of primers GBPA01F and -R (for amplification of the 5′-amplicon) or GBPA01-F and -R (for amplification of the 3′-amplicon) were designed and used (Table 2). The *gbpA* gene with a 318-bp deletion was amplified by PCR using the mixture

TABLE 2
Oligonucleotides used in this study

Name	Oligonucleotide Sequence (5′ → 3′)[a,b]	Use
For mutant construction		
GBPA01-F	GAGATGCACATCAGCAACGCG	Deletion of the *gbpA* ORF
GBPA01-R	AAAGGATCCAGCGAACTTACACAGTGT	
GBPA02-F	GCTGGATCCTTTGTCTTCCCAGATG	Deletion of the *gbpA* ORF
GBPA02-R	CGCAACAACGGAATCAAACGC	
For mutant complementation		
GBPA03-F	GGATCCGCCAAATAAAGTCAG	Amplification of the *gbpA* ORF
GBPA03-R	GGATCCTTACAGTTTGTCCCAC	
ISCR01-F	ATCCATGGCTATGAAACTGACATCTAAAGG	Amplification of the *iscR* ORF
ISCR01-R	ATTCTAGATTAAGAGCGGAAATTTACACCG	
CRP01-F	GAGATACCATGGTTCTAGGTAAACCTCA	Amplification of the *crp* ORF
CRP01-R	GTTAATTCTAGATTAACGAGTACCGTAAACAAC	
SMCR01-F	ATCCATGGACTCAATCGCAAAGAGAC	Amplification of the *smcR* ORF
SMCR02-R	ATTCTAGATTATTCGTGCTCGCGTTTATA	
For GbpA overexpression		
GBPA04-F	CCATGGCTAAAAAACAACCGCAAAAAACC	Amplification of the *gbpA* ORF
GBPA04-R	CTCGAGCAGTTTGTCCCACGCCATT	
For promoter deletion analysis		
GBPA003	GAGCTCTAAGTGCTCAATGACATAGTAAAG	Deletion of the *gbpA* regulatory region
GBPA004	GAGCTCTCACACTTTTTCGAGAAATTA	
GBPA005	GAGCTC ACATCTATAAATAACGCTTCTAAAT	
GBPA006	GAGCTCTTATGCCTGACATCACAC	
GBPA007	ACTAGTCACCATTTTCCACTGCAG	
For primer extension analysis, EMSA, and DNase I protection assay		
GBPA05-F	ATTGCCATAGCTGGTGGTTTTCA	Amplification of the *gbpA* upstream region
GBPA05-R	CCCCGCTATCTTGGGTATGGTAAAAA	Amplification of the *gbpA* upstream region, Extension of the *gbpA* transcript
For qRT-PCR		
GBPA_qRT-F	TGAAAGCCTGGGGTGAAGCA	Quantification of the *gbpA* expression
GBPA_qRT-R	ATCGCGTAGCGTTGAGAGCG	

[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.

of both amplicons as the template and GBPA01-F and GBPA02-R as primers. The resulting Δ*gbpA* was ligated into SpeI-SphI-digested pDM4 (28) to generate pSO1101 (Table 1). *Escherichia coli* S17-1 λ *pir, tra* strain (29) containing pSO1101 was used as a conjugal donor to *V. vulnificus* MO6-24/O and MORSR (MO6-24/O with rifampicin and streptomycin resistance, see Ref. 30) to generate the *gbpA* mutant SO111 and KK141, respectively (Table 1). Similarly, pBS0907, which was constructed previously to carry a mutant allele of *V. vulnificus crp* on pDM4 (Table 1) (31), was used to generate the *iscR crp* double mutant of *V. vulnificus*. *E. coli* S17-1 λ *pir, tra* containing pBS0907 was used as a conjugal donor in conjugation with the *iscR* mutant JK093 as a recipient (26). The resulting *iscR crp* double mutant was named KK142 (Table 1). The conjugation and isolation of the transconjugants were conducted using the method described previously (31).

To complement the *gbpA, iscR, crp,* and *smcR* (constructed previously (22)) mutations, each ORF of *gbpA*, *iscR*, *crp,* and *smcR* was amplified by PCR using a pair of specific primers as listed in Table 2. The amplified *gbpA, iscR*, *crp,* and *smcR* ORFs were cloned into pJK1113 under an arabinose-inducible promoter P_{BAD} (32) to create pKK1402, pKK1403, pKK1404, and pKK1405, 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, arabinose was added to a final concentration of 0.1 mM to induce the expression of the recombinant genes on the plasmids.

Mucin Binding Assay—Pig gastric mucin powder (Sigma) was sterilized by mixing with 95% (v/v) ethanol for 1 h, dried at 70 °C for 24 h (33), and then added to the 1.5% agar (w/v) solution, which was autoclaved and cooled down to 60 °C to the final concentration of 3% (w/v). The mucin-agar solution (2 ml) was solidified in each well of 12-well culture dishes (Nunc, Roskilde, Denmark). On the mucin-agar, *V. vulnificus* cultures (100 μl, ~10^7 colony-forming units (cfu)) were added to each well, and various amounts of purified GbpA were exogenously provided to the well when required. After incubation for 1 h at 30 °C, the nonadherent bacteria were removed by washing with 1 ml of phosphate-buffered saline (PBS) twice, and the adherent bacterial cells were recovered by treating with 200 μl of 0.1% Triton X-100 (Sigma) solution for 20 min and enumerated as cfu per well.

Development of the Mucin-secreting Cells and Adhesion Assay—The human colonic HT29 cells (ATCC®HTB-38™) (ATCC, Manassas, VA) in McCoy's 5A media (Gibco-BRL, Gaithersburg, MD) containing 1% (v/v) fetal bovine serum (MCF) were developed into mucin-secreting cells, named HT29-methotrexate (MTX), as described previously (34). The HT29-MTX cells were fixed with paraformaldehyde and treated with 4′, 6-diamidino-2-phenylindole (DAPI). Mucin secretion of the HT29-MTX cells was detected with the anti-mucin 5AC primary antibody (Merck Millipore, Darmstadt, Germany), labeled with the fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Abcam, Cambridge, UK), and visualized using a confocal laser scanning microscope (LSM710, Zeiss, Jena, Germany) (35, 36).

The 12-well culture dishes (Nunc) were seeded with the HT29-MTX cells (~1×10^7 cells per well), infected with the *V. vulnificus* strains at a multiplicity of infection of 10 for 30 min.

The culture dishes were washed two times with PBS to remove nonadherent bacteria and treated with 0.1% Triton X-100 for 20 min to recover adherent bacteria. The recovered bacterial cells were enumerated as cfu per well.

Mouse Lethality and Competition Assay—Mouse lethality of the wild type and *gbpA* mutant was compared as described previously (26). Groups of (n = 10) 7-week-old ICR female mice (specific pathogen-free; Seoul National University) were starved without food and water for 12 h until infection. Then the mice, without iron-dextran pretreatment, were intragastrically administered with 100 μl of the inoculum, representing $\sim 10^9$ cells of either the wild type or the *gbpA* mutant. Mouse survivals were recorded for 24 h.

Previous mouse colonization assays demonstrated that *V. vulnificus* initially and mostly colonizes in the small intestine and disseminates to other organs (37, 38). Therefore, colonization of each strain in the mouse small intestine was determined by competition assays as described previously (30). Briefly, four ICR female mice (7 weeks old) were infected as described above for mouse mortality, except that 100 μl of the inoculum, prepared by mixing MORR (MO6-24/O with rifampicin resistance (30)) and KK141 at a 1:1 ratio, representing $\sim 10^6$ cfu of each strain, was given intragastrically to the mice. The mice were sacrificed at 1–24 h postinfection, and their intestines were collected, washed, and homogenized. Equal amounts of the homogenates were spread on LBS agar containing either rifampicin (100 μg/ml) alone to enumerate the sum of the wild type and *gbpA* mutant cells or rifampicin and streptomycin (100 μg/ml) to specifically count the *gbpA* mutant cells. The ratio of cfu recovered from the intestines to the number of cfu inoculated is defined as a colonization index (39). All manipulations of mice were approved by the Animal Care and Use Committee of Seoul National University, and mice were humanely euthanized at the end point analysis.

RNA Purification and Transcript Analysis—Total RNA from the *V. vulnificus* strains grown aerobically to various levels of A_{600} were isolated using an RNeasy® mini kit (Qiagen, Valencia, CA). When necessary, the strains grown anaerobically to an A_{600} of 0.5 were exposed to various concentrations of H_2O_2 for 10 min and harvested to isolate total RNA. For the primer extension analysis, a 26-base primer GBPA05-R (Table 2) complementary to the regulatory region of *gbpA* 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 product was purified and resolved on a sequencing gel alongside ladders generated from pKK1401 with the same primer. The plasmid pKK1401 was constructed by cloning the 390-bp *gbpA* upstream region extending from −301 to +88 and amplified by PCR using a pair of primers GBPA05-F and -R (Table 2) into pGEM-T Easy (Promega, Madison, WI). The primer extension product was visualized using a phosphorimager analyzer (BAS1500; Fuji Photo Film Co., Ltd., Tokyo, Japan).

For qRT-PCR, the concentrations of total RNA from the strains were measured by using a NanoVue 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 a pair of specific primers (Table 2), as described previously (40). Relative expression levels of the *gbpA* mRNA in the same amounts of total RNA were calculated by using the 16S rRNA expression level as the internal reference for normalization.

Protein Purification and Western Blot Analysis—The ORF of *gbpA* was amplified by PCR using a pair of primer GBPA04-F and -R (Table 2) and cloned into a His_6 tag expression vector, pET-22a(+) (Novagen, Madison, WI), to result in pSO1201 (Table 1). The His-tagged GbpA protein was expressed in *E. coli* BL21 (DE3) and purified by affinity chromatography according to the manufacturer's procedure (Qiagen). In a similar way, pJK0928, pHK0201, and pHS104, which were constructed previously (Table 1) (22, 26, 41), were used to overexpress and purify the His-tagged IscR, CRP, and SmcR, respectively. The purified His-tagged proteins were used to raise rabbit polyclonal antibodies against GbpA, IscR, CRP, and SmcR of *V. vulnificus*, respectively (AB Frontier, Seoul, South Korea). For Western blot analyses, total proteins were isolated from the strains grown aerobically to an A_{600} of 0.5 or anaerobically to an A_{600} of 0.5 and then exposed to various concentrations of H_2O_2 for 10 min. The concentrations of the total proteins were determined by using Bradford method (42). The same amounts of the total proteins (10 μg) were resolved on SDS-PAGE and immunoblotted as described previously (27).

Electrophoretic Mobility Shift Assay (EMSA)—The 390-bp *gbpA* upstream region extending from −301 to +88 was amplified by PCR using [γ-^{32}P]ATP-labeled GBPA05-F and unlabeled GBPA05-R as primers (Table 2). The labeled 390-bp DNA (5 nM) probe was incubated with various concentrations of purified IscR for 30 min at 30 °C in a 20-μl reaction mixture containing 1× binding buffer (43) and 0.1 μg of poly(dI-dC) (Sigma). The protein-DNA binding reactions with purified CRP or SmcR were the same as those with IscR, except that the CRP or SmcR-binding buffer was used as a 1× binding buffer (41, 22). Electrophoretic analyses of the DNA-protein complexes were performed, as described previously (32), and visualized as described above for the transcript analysis.

Construction of a Set of gbpA-luxCDABE Transcriptional Fusions—The primer GBPA007 (Table 2), including an SpeI restriction site followed by bases corresponding to the 5′ end of the *gbpA* coding region, was used in conjunction with one of the following primers to amplify the DNA upstream of *gbpA*: GBPA003 (for pKK1407), GBPA004 (for pKK1408), GBPA005 (for pKK1409), or GBPA006 (for pKK1410) (Table 2). The primers were designed to amplify the *gbpA* regulatory region extending up to −220, −106, −76, and −6 bp, respectively. A SacI restriction site was added to these primers to facilitate cloning of the PCR products. The DNA fragments were inserted into the SpeI-SacI-digested pBBR-lux (44) carrying promoterless *luxCDABE* genes, thereby creating four *gbpA-lux* reporter constructs. All constructions were confirmed by DNA sequencing. The *gbpA-lux* reporters were then transferred into the *V. vulnificus* strains by conjugation. The cellular luminescence of the cultures grown to an A_{600} of 0.5 were measured with a luminometer (Tecan Infinite M200 reader, Männedorf,

Switzerland) and expressed in arbitrary relative light units (RLU) as described previously (22).

DNase I Protection Assay—The same labeled 390-bp DNA probe developed for EMSA was used for DNase I protection assays. The binding of IscR, CRP, or SmcR to the labeled DNA (25 nM) was performed as described above for EMSA, and DNase I digestion of the DNA-protein complexes followed the procedures described previously (31). After precipitation with ethanol, the digested DNA products were resolved on a sequencing gel alongside sequencing ladders of pKK1401 generated using GBPA05-F (Table 2) as the primer. The gels were visualized as described above for the transcript analysis.

Data Analyses—Averages and standard deviations (S.D.) were calculated from at least three independent experiments. Mouse mortality was evaluated using the log rank test program. All other data were analyzed by Student's *t* tests with the SAS program (SAS software, SAS Institute Inc.). Significance of differences between experimental groups was accepted at a *p* value of <0.05.

Results

Identification and Sequence Analysis of GbpA—A search of the *V. vulnificus* MO6-24/O genome sequence database (GenBank™ CP002469 and CP002470) (45) for homology to the amino acid sequences deduced from *Vc*GbpA singled out a protein, hereafter named *Vv*GbpA. The amino acid sequence analysis predicted that pre-*Vv*GbpA protein contains an N-terminal signal peptide for the type II secretion system, suggesting that *Vv*GPA is a secreted protein (data not shown) (46). The deduced mature *Vv*GbpA is composed of 485 amino acids with a theoretical mass of 52.83 kDa and a pI of 4.75. The amino acid sequence of *Vv*GbpA was 80% identical to that of *Vc*GbpA (data not shown) and exhibited a four domain modular structure consisting of two chitin binding domains and two bacterial surface binding domains as observed in *Vc*GbpA (11). The predicted profile of the hydrophobicity was significantly similar to that of *Vc*GbpA, indicating that *Vv*GbpA is a soluble protein (data not shown) (11). All of this information suggested that *Vv*GbpA is a secreted but cell surface-associated protein as is *Vc*GbpA.

GbpA Is Essential for Mucin Binding and Virulence of V. vulnificus—To examine the function of *Vv*GbpA, the mucin binding ability of wild type and the *gbpA* mutant was examined. The number of the *gbpA* mutants that adhered to the mucin-agar in a well of 12-well culture dishes was significantly lower than that of the parental wild type (Fig. 1*A*). This indicated that the *gbpA* mutant was defective in mucin binding. In addition, the extracellular provision of purified *Vv*GbpA was able to rescue the defect of the *gbpA* mutant in mucin binding ability in a dose-dependent manner. The mucin binding ability of the *gbpA* mutant incubated in the presence of 5 μM *Vv*GbpA was comparable with that of the parental wild type, in terms of the bacterial numbers adherent to the mucin-agar (Fig. 1*A*).

To further understand the role of *Vv*GbpA in binding to mucin, the HT29-MTX cells, monolayers of which mimic human intestinal epithelial cells that produce and secrete mucins (34), were developed. Mucin secretion of the HT29-MTX cells was confirmed using a confocal laser scanning microscope (Fig. 1, *C–F*). The wild type and *gbpA* mutant were incubated with the HT29-MTX cells, and the bacterial adherents to the cells were enumerated (Fig. 1*B*). The results revealed that adhesion of the *gbpA* mutant to the HT29-MTX cells was about 2-fold lower than that of the wild type. The impaired adhesion of the *gbpA* mutant was restored to the wild type level by complementation with a functional *gbpA* gene (pKK1402). These results indicated that *Vv*GbpA is a mucin-binding protein and plays an important role in the adhesion to the mucin-secreting human epithelial cells.

To experimentally examine the role of *Vv*GbpA in pathogenesis, mouse mortality and colonization activity of the *gbpA* mutant were evaluated. As shown in Fig. 2*A*, the survival of mice inoculated intragastrically with the *gbpA* mutant was consistently and significantly prolonged ($p = 0.0174$, log rank test) compared with that of mice inoculated with the parental wild type. Therefore, for the mouse model infected intragastrically, the *gbpA* mutant appeared to be significantly less virulent than its parental wild type. Mice were also coinoculated intragastrically with MORR (wild type) and KK141 (*gbpA* mutant), and both strains colonized on the small intestine were recovered and enumerated (Fig. 2*B*). The colonization index of the KK141 ranged from 10^{-3} to 10^{-2} and was consistently and significantly (about 100-fold) lower than that of MORR, demonstrating that the MORR clearly outcompeted the KK141 in the small intestine. These results indicated that *Vv*GbpA is a virulence factor essential for the intestinal colonization of *V. vulnificus*. Taken together, it is apparent that *Vv*GbpA is a mucin-binding protein contributing to the pathogenesis of *V. vulnificus*.

Expression of gbpA Is Growth Phase-dependent and Regulated by IscR, CRP, and SmcR—To examine whether the production of *Vv*GbpA is influenced by growth phase, levels of the *gbpA* mRNA of the wild type culture were analyzed at various growth stages (Fig. 3, *A* and *B*). The *gbpA* transcript reached maximum levels in the exponential phase and then decreased in the stationary phase, indicating that expression of *gbpA* is growth phase-dependent. To extend our understanding on the regulation of *gbpA*, the levels of the *gbpA* transcript in the wild type and mutants that lack transcription factors IscR, CRP, and SmcR (Table 1) were compared. The level of the *gbpA* transcript decreased in the *iscR* mutant and *crp* mutant (Fig. 3*C*). The result indicated that both IscR and CRP act as positive regulators for the *gbpA* expression. In contrast, the *gbpA* expression of the *smcR* mutant was greater than that of the wild type, indicating that SmcR negatively regulates *gbpA* (Fig. 3*C*). The cellular levels of *Vv*GbpA determined by Western blot analyses also varied in the mutants (Fig. 3*D*). The magnitude of variation in the *Vv*GbpA proteins did not significantly differ from that observed in the *gbpA* transcripts, indicating that the regulation of the *gbpA* expression occurs mostly at the transcription level. The levels of the *gbpA* transcript and *Vv*GbpA protein that varied in the *iscR*, *crp*, and *smcR* mutant were restored to the levels comparable with those in the wild type by introducing pKK1403, pKK1404, and pKK1405 carrying

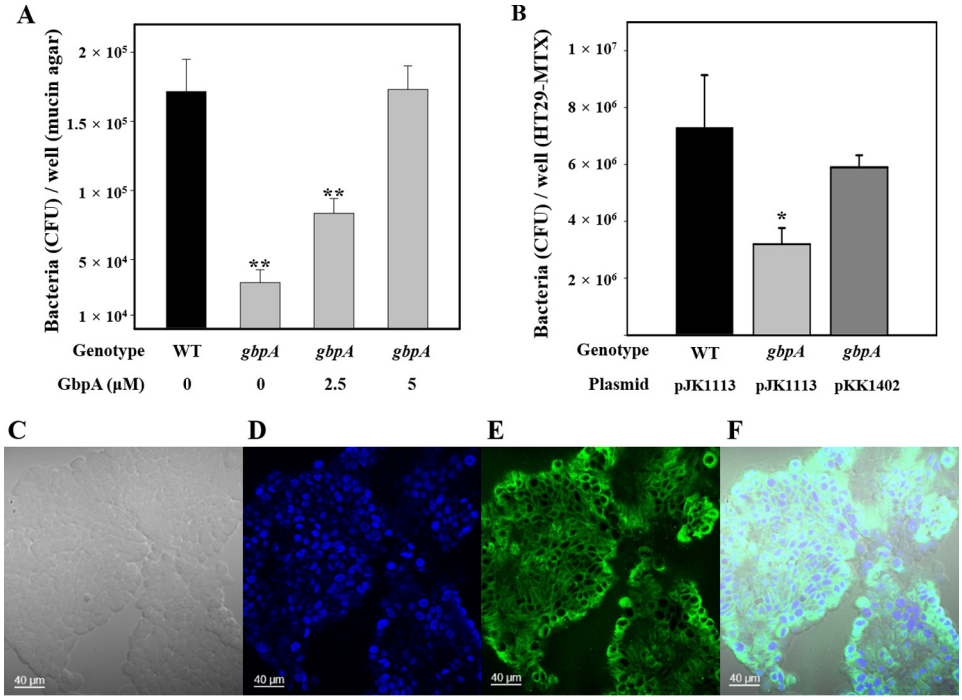

FIGURE 1. **Effect of GbpA on mucin binding and host cell adhesion of *V. vulnificus*.** *Upper panel,* mucin binding activities of the strains. *A*, strains (~10^7 cfu) were added to each well of 12-well culture dishes containing the mucin-agar and various amounts of GbpA provided exogenously as indicated. After 1 h of incubation, the adherent bacterial cells were enumerated as cfu per well. *WT,* wild type; *gbpA*, *gbpA* mutant. *B*, mucin-secreting HT29-MTX cells (~10^7 cells) seeded in each well of 12-well culture dishes were infected at a multiplicity of infection of 10 with the strains as indicated. After a 30-min incubation, the adherent bacterial cells were enumerated as cfu per well. *Error bars* represent the S.D. *, $p < 0.05$, and **, $p < 0.005$ relative to the wild type. *WT* (pJK1113, empty vector), wild type; *gbpA* (pJK1113), *gbpA* mutant; *gbpA* (pKK1402), complemented strain. *Lower panel,* development of the mucin-secreting HT29-MTX cells. *C*, bright field image of HT29-MTX cells. *D*, nucleus of HT29-MTX cells was stained *blue* with DAPI. *E*, mucin of HT29-MTX cells was stained *green* with the anti-MUC5AC primary antibody and then labeled with FITC-conjugated secondary antibody. *F*, merged image of *C–E*. Images are visualized using a confocal laser scanning microscope. *Scale bar,* 40 μm.

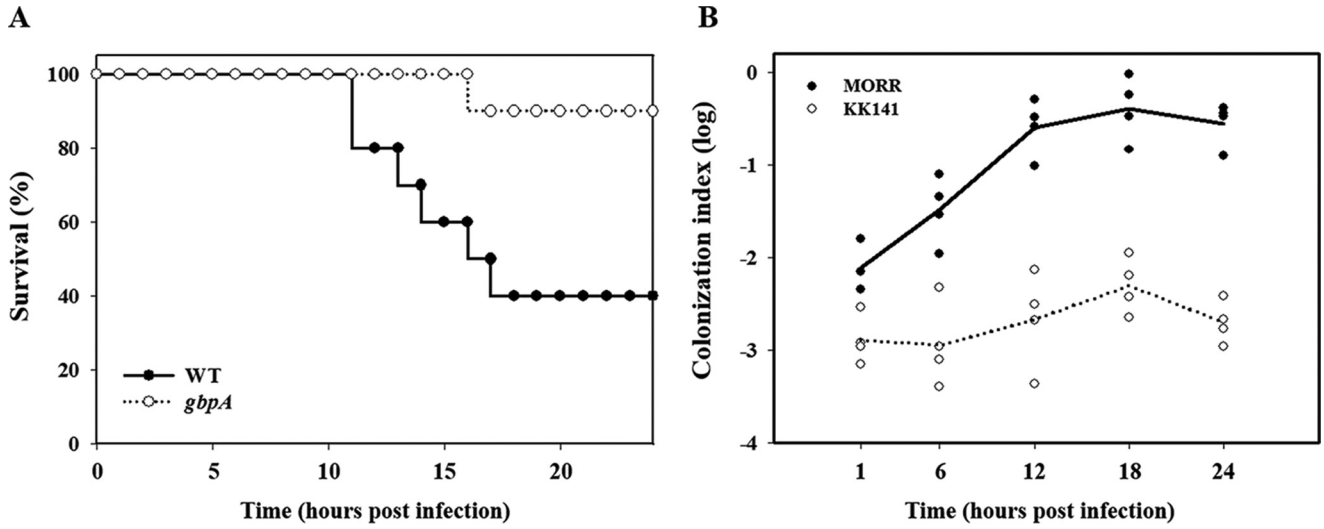

FIGURE 2. **Mouse lethality and colonization activity of the *V. vulnificus* strains.** *A*, 7-week-old specific pathogen-free female ICR mice were intragastrically infected with the wild type (*WT*) or the *gbpA* mutant (*gbpA*) (Table 1) at doses of ~10^9 cfu as indicated. Mouse survival was monitored for 24 h. *B*, four mice were intragastrically infected with an inoculum prepared by mixing equal numbers of MORR and KK141 (Table 1), and then the bacterial cells colonized on the small intestine were enumerated as cfu at the indicated time intervals. Each *circle* corresponds to the ratio of cfu recovered from the intestines to the cfu inoculated (the colonization index) for an individual mouse. The median values are displayed as a *solid line* (MORR) or *dashed line* (KK141) on the graph. *WT*, wild type; *gbpA*, *gbpA* mutant; *MORR*, wild type with rifampicin resistance; *KK141*, *gbpA* mutant with rifampicin and streptomycin resistance.

recombinant *iscR*, *crp*, and *smcR*, respectively (Fig. 3, *E* and *F*). Taken together, these results suggested that IscR and CRP activate and SmcR represses the *gbpA* transcription.

IscR and CRP Coactivate gbpA Additively—To further examine the roles of IscR and CRP in the *gbpA* expression, the *iscR crp* double mutant KK142 was constructed, and *gbpA* expression was determined. Inactivation of both *crp* and *iscR* resulted in significant reduction of the *gbpA* expression, and the residual *gbpA* mRNA level in the *iscR crp* double mutant corresponded to only one-tenth of that in the wild type (Fig. 4). The presence of either IscR (*crp* mutant) or CRP (*iscR* mutant) alone increased the *gbpA* expression, but their *gbpA* transcript levels

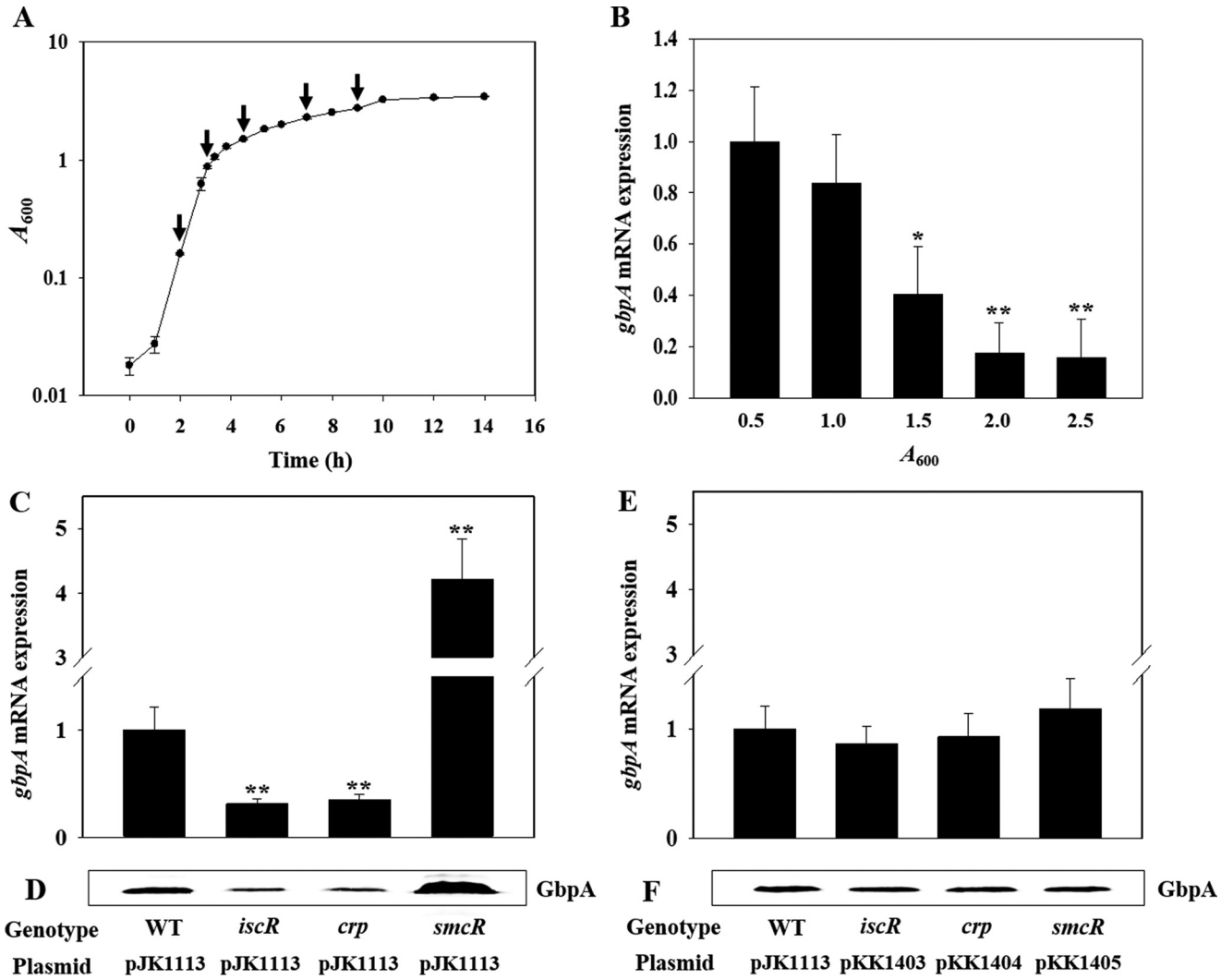

FIGURE 3. **Effects of growth phases and global regulatory proteins on the *gbpA* expression.** *Upper panel,* growth kinetics of *V. vulnificus* and growth phase-dependent expression of *gbpA*. *A*, growth of the wild type culture in LBS was monitored spectrophotometrically at 600 nm (A_{600}), and total RNAs were isolated from the cells harvested at different growth phases (from left, at A_{600} of 0.5, 1.0, 1.5, 2.0, and 2.5) as indicated by *arrows*. *B*, *gbpA* mRNA levels were determined by qRT-PCR analyses, and the *gbpA* mRNA level in the cells grown to an A_{600} of 0.5 was set as 1. *Error bars* represent the S.D. *, $p < 0.05$, and **, $p < 0.005$ relative to the cells grown to an A_{600} of 0.5. *Lower panel*, expression of *gbpA* in *V. vulnificus* with different genetic backgrounds. Samples were harvested from the cultures of the wild type (*WT*) and isogenic mutants grown aerobically to an A_{600} of 0.5 and analyzed to determine the *gbpA* mRNA and GbpA protein levels. *C* and *E, gbpA* mRNA levels were determined by qRT-PCR analyses, and the *gbpA* mRNA level in the wild type was set as 1. **, $p < 0.005$ relative to the wild type. *D* and *F,* protein samples were resolved by SDS-PAGE, and GbpA was detected by Western blotting using the rabbit anti-*V. vulnificus* GbpA serum. (pJK1113), wild type; *iscR* (pJK1113), *iscR* mutant; *smcR* (pJK1113), *smcR* mutant; *crp* (pJK1113), *crp* mutant; *iscR* (pKK1403), *crp* (pKK1404), and *smcR* (pKK1405), complemented strains.

were much lower than that obtained by IscR and CRP together (wild type), indicating that IscR and CRP coactivate the *gbpA* expression additively (Fig. 4). To determine whether an increased amount of IscR would compensate for a lack of CRP in the activation of *gbpA*, the *iscR* expression plasmid pKK1403 was introduced into the *iscR crp* double mutant KK142. When *iscR* was induced by arabinose, the cellular level of IscR in KK142 (pKK1403) was higher than that in the *crp* single mutant (Fig. 4). However, the level of the *gbpA* transcript in KK142 (pKK1403) was only about 80% that in the wild type (Fig. 4), indicating that IscR, even when overproduced, is unable to activate *gbpA* to the wild type level in the absence of CRP. Similarly, overproduced CRP was unable to completely compensate for the lack of IscR in the activation of *gbpA* (Fig. 4). The results indicated that both IscR and CRP are required simultaneously to activate *gbpA* to the wild type level.

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

Western blot analysis revealed that neither activator affected the cellular level of the other, *i.e.* compared with the wild type the *iscR* mutant strain did not exhibit any significant changes in the cellular level of CRP and vice versa (Fig. 5). From these results, it is unlikely that IscR (or CRP) indirectly activates *gbpA* by increasing the cellular level of CRP (or IscR), which directly

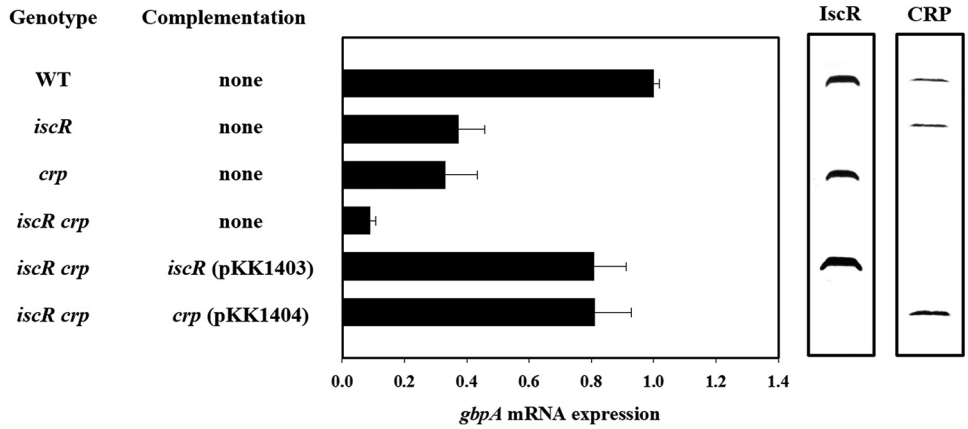

FIGURE 4. **IscR and CRP coactivate *gbpA* additively.** Samples were harvested from the cultures of the wild type (*WT*) and isogenic mutants grown aerobically to an A_{600} of 0.5 and analyzed to determine the *gbpA* mRNA levels and IscR and CRP protein levels. The *gbpA* mRNA levels were determined by qRT-PCR analyses, and the *gbpA* mRNA level in the wild type was set as 1. *Error bars* represent the S.D. The cellular levels of IscR and CRP were determined by Western blot analyses using the rabbit anti-*V. vulnificus* IscR and anti-*V. vulnificus* CRP sera, respectively. *WT,* wild type; *iscR*, *iscR* mutant; *crp*, *crp* mutant; *iscR crp*, *iscR crp* double mutant.

activates *gbpA*. Similarly, neither IscR nor CRP influences the cellular levels of SmcR, indicating that the activation of *gbpA* by IscR and CRP is not the result of decreasing cellular levels of SmcR, which directly represses *gbpA*. Consequently, it appears that IscR, CRP, and SmcR function cooperatively to regulate *gbpA* rather than sequentially in a regulatory cascade. It is noteworthy that the level of IscR, which activates *gbpA,* was higher in the log phase cells than in the stationary phase cells. Although levels of CRP did not vary significantly in cells of different growth phases, SmcR, which represses *gbpA,* accumulated more in cells of stationary phase (Fig. 5). Consistent with this, the level of *Vv*GbpA was higher in the log phase cells than the stationary phase cells of the wild type (Fig. 5). Therefore, it was postulated that the growth phase-dependent expression of *gbpA* (Fig. 3, *A* and *B*) is attributed to this variation in the levels of IscR and SmcR in cells of different growth phase.

Mapping the Regulatory Region of gbpA—To map the promoter of *gbpA*, a transcription start site of *gbpA* was determined by a primer extension analysis. A single reverse transcript was produced from primer extension of RNA isolated from the wild type grown aerobically to an A_{600} of 0.5 (Fig. 6*A*). Several attempts to identify other transcription start sites using different sets of primers were not successful (data not shown). The 5′ end of the *gbpA* transcript was located 235-bp upstream of the translational initiation codon of *gbpA* and was subsequently designated +1 (Fig. 6*B*). The putative promoter constituting this transcription start site was named P_{gbpA}, and the sequences for −10 and −35 regions of P_{gbpA} were assigned on the basis of similarity to consensus sequences of the *E. coli* σ^{70} promoter (Fig. 6*B*).

The pKK reporters carrying the upstream regulatory region of P_{gbpA}, which was deleted up to different 5′ ends and fused transcriptionally to *luxCDABE,* were constructed (Fig. 7*A*). The reporters were transferred into the wild type and isogenic mutants, and culture luminescence was used to quantify the P_{gbpA} activity (Fig. 7*B*). The luminescence produced by pKK1407 carrying P_{gbpA} deleted up to −220 was ~6.0×10^3 RLU in the wild type but significantly reduced in the *iscR* and *crp* mutants, supporting our previous observation that IscR and

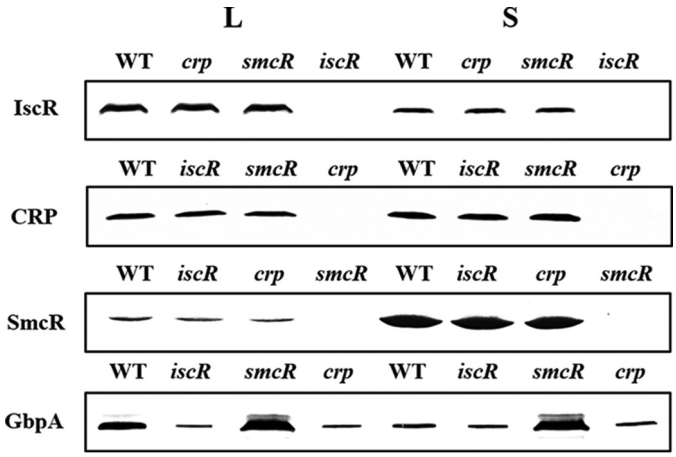

FIGURE 5. **Cellular levels of IscR, CRP, and SmcR are unaffected by one another.** The wild type and isogenic mutants were grown aerobically to an A_{600} of 0.5 (log phase, *L*) and 2.0 (stationary phase, *S*). The cells were then examined for the presence of IscR, CRP, SmcR, and GbpA proteins by Western blot analyses using the rabbit anti-*V. vulnificus* IscR, anti-*V. vulnificus* CRP, and anti-*V. vulnificus* SmcR, anti-*V. vulnificus* GbpA sera, respectively. *WT*, wild type; *iscR*, *iscR* mutant; *crp*, *crp* mutant; *smcR*, *smcR* mutant.

CRP activate P_{gbpA}. The RLU of the *smcR* mutant containing pKK1407 increased, confirming the SmcR repression of P_{gbpA} (Fig. 7*B*). Deletion up to −106 significantly decreased the P_{gbpA} activity as determined based on the reduced luminescence of the strains containing pKK1408. Interestingly, the RLU of pKK1408 in the *iscR* mutant was indistinguishable from that in the wild type, indicating the absence of the region(s) necessary for IscR to activate P_{gbpA} in pKK1408. Similarly, the comparable RLU produced by pKK1409 in the *crp* mutant and wild type indicated that the region upstream from −76 is probably required for CRP activation of P_{gbpA}. In contrast, the *smcR* mutant containing pKK1409 (and pKK1408) produced still greater luminescence compared with the wild type, indicating that the region downstream from −76 is essential for SmcR repression of P_{gbpA}. The undetectable luminescence produced by pKK1410 indicates that deletion up to −6 completely impaired the P_{gbpA} activity. Taken together, the results suggested that the regulatory region extending from −220 to −6

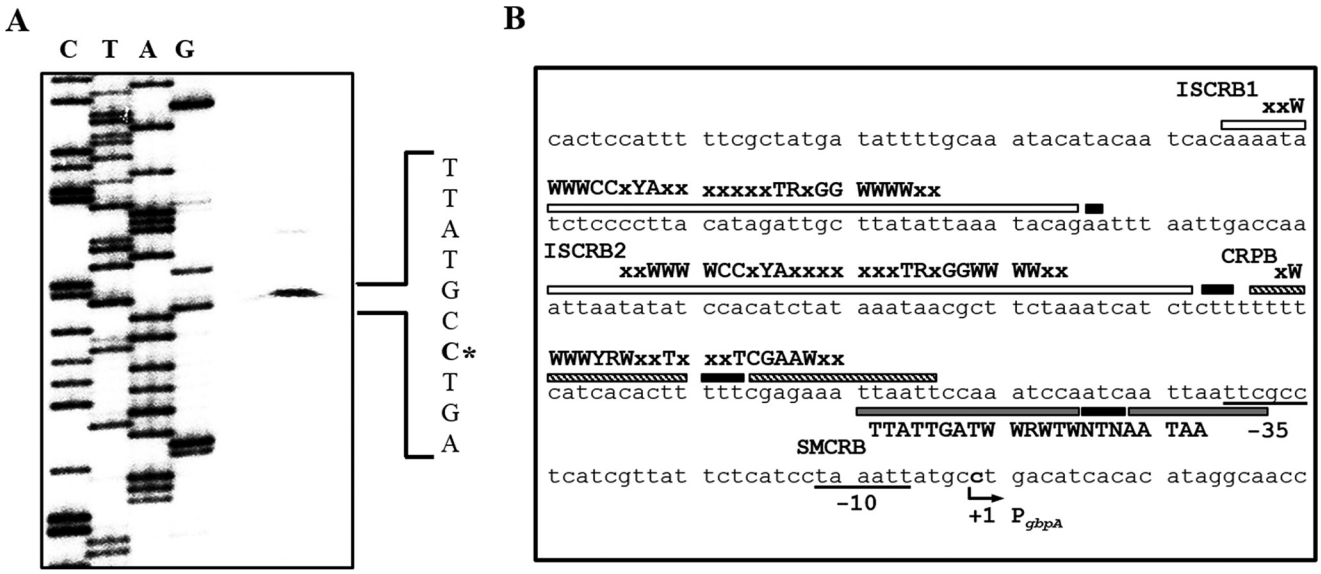

FIGURE 6. **Transcription start site and sequences of the *gbpA* regulatory region.** *A*, transcription start site of *gbpA* was determined by primer extension of the RNA isolated from the wild type grown aerobically to an A_{600} of 0.5. *Lanes C, T, A,* and *G* represent the nucleotide sequencing ladders of pKK1401. The *asterisk* indicates the transcription start site of *gbpA*. *B,* transcription start site of *gbpA* is indicated by a *bent arrow*, and the positions of the putative −10 and −35 regions are *underlined*. The sequences for binding of IscR (ISCRB1 and ISCRB2, *white boxes*), CRP (CRPB, *shaded boxes*), and SmcR (SMCRB, *gray boxes*) were determined later in this study (Fig. 10). The nucleotides showing enhanced cleavage are indicated by *black boxes*. The consensus sequences for binding of IscR (type 2), CRP, and SmcR are, respectively, indicated *above* the *V. vulnificus* DNA sequence. *R*, A or G; *Y*, C or T; *W*, A or T; *x,* any nucleotide.

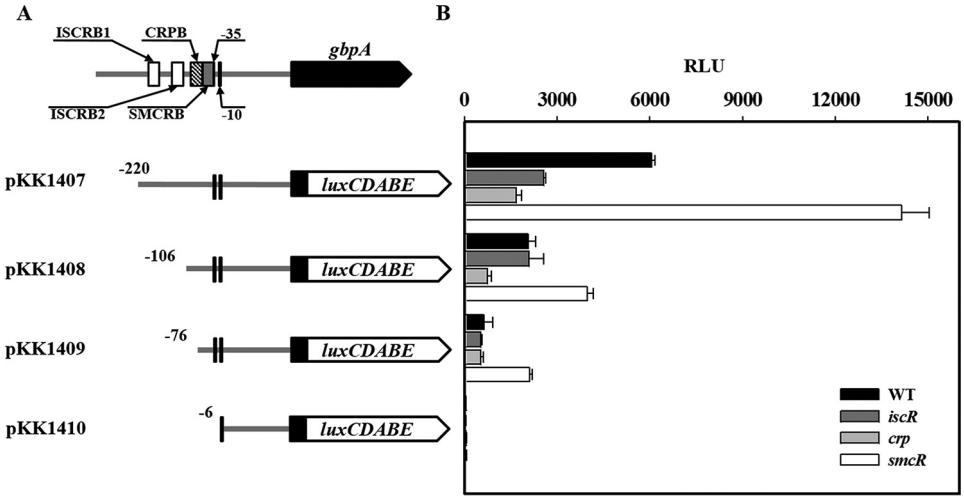

FIGURE 7. **Deletion analysis of the P_{gbpA} regulatory region.** *A*, construction of *gbpA-lux* fusion pKK reporters. PCR fragments carrying the *gbpA* regulatory region with 5′ end deletions were subcloned into pBBR-lux (44) to create each pKK reporter. *Solid lines*, the upstream region of *gbpA*; *black blocks*, the *gbpA* coding region; *open blocks*, *luxCDABE*. The wild type *gbpA* regulatory region is shown on *top* with the proposed −10 and −35 regions, and the binding sites for IscR (*ISCRB1* and *ISCRB2*, *white boxes*), CRP (*CRPB*, *shaded box*), and SmcR (*SMCRB*, *gray box*) were determined later in this study (Fig. 10). *B*, cellular luminescence determined from the wild type (*black bars*), *iscR* mutant (*dark gray bars*), *crp* mutant (*gray bars*), and *smcR* mutant (*open bars*) containing each pKK reporter as indicated. Cultures grown aerobically to an A_{600} of 0.5 were used to measure the cellular luminescence. *Error bars* represent the S.D. *WT,* wild type; *iscR*, *iscR* mutant; *crp*, *crp* mutant; *smcR*, *smcR* mutant.

consecutively harbors the *cis*-elements necessary for IscR, CRP, and SmcR regulation of P_{gbpA}.

IscR, CRP, and SmcR Regulate gbpA by Directly Binding to P_{gbpA}—There are still several possible ways for IscR, CRP, and SmcR to affect P_{gbpA} activity. One is by binding directly to the P_{gbpA} regulatory region to regulate the promoter, whereas another is by modulating the cellular level of unidentified *trans*-acting factor(s), which in turn binds directly to the P_{gbpA} regulatory region. To distinguish these two possibilities, the 390-bp labeled DNA probe encompassing the P_{gbpA} regulatory region (from −301 to +88) was incubated with increasing amounts of IscR and then subjected to electrophoresis. Because IscR was isolated, purified, and used under aerobic conditions, most of the purified IscR would be in the Fe-S clusterless apo-form (32, 47). The addition of IscR resulted in two retarded bands in a concentration-dependent manner, indicating that at least two binding sites for IscR are present in the P_{gbpA} regulatory region (Fig. 8*A*). The binding of IscR was also specific, because assays were performed in the presence of 0.1 μg of poly(dI-dC) as a nonspecific competitor. In a second EMSA, the same but unlabeled 390-bp DNA fragment was used as a self-competitor to confirm the specific binding of IscR. The unlabeled 390-bp DNA competed for the binding of IscR in a dose-dependent manner (Fig. 8*A*), confirming that IscR

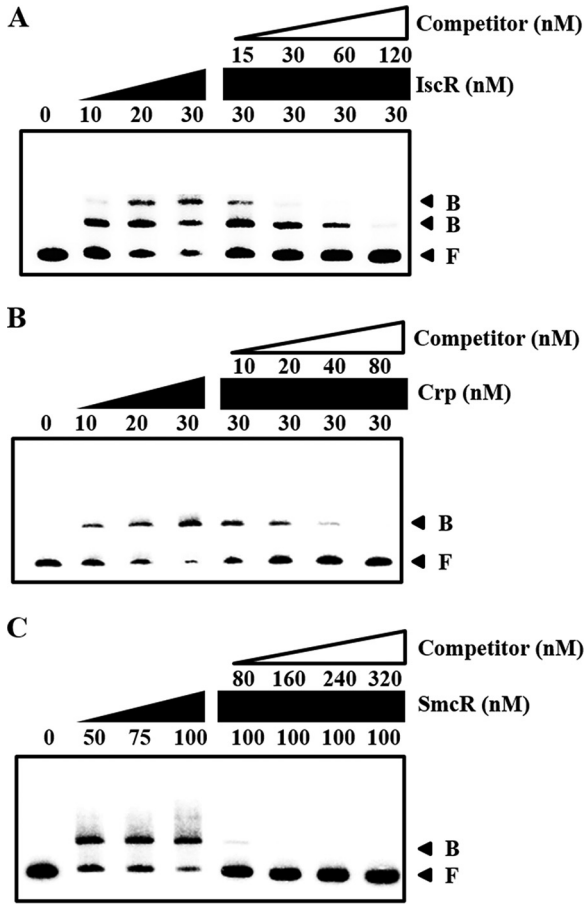

FIGURE 8. **Specific bindings of IscR, CRP, and SmcR to P_{gbpA}.** A 390-bp DNA fragment of the *gbpA* regulatory region was radioactively labeled and then used as a probe DNA. The radiolabeled probe DNA (5 nM) was mixed with increasing amounts IscR (*A*), CRP (*B*), and SmcR (*C*) as indicated. For competition analysis, the same but unlabeled 390-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 prior to the addition of 30 nM IscR (*A*), 30 nM CRP (*B*), and 100 nM SmcR (*C*). *B*, bound DNA; *F*, free DNA.

binds specifically to the P_{gbpA} regulatory region. In similar DNA binding assays, CRP and SmcR each displayed specific binding to the P_{gbpA} regulatory region (Fig. 8, *B* and *C*). These results suggested that IscR, CRP, and SmcR regulate *gbpA* by specifically binding to P_{gbpA}.

Identification of Binding Sites for IscR, CRP, and SmcR—To determine the precise location of the IscR-, CRP-, and SmcR-binding sites in the P_{gbpA} regulatory region, DNase I protection assays were performed using the same 390-bp DNA probe used for DNA binding assays. As shown in Fig. 9*A*, two regions extending from −184 to −145 (ISCRB1, centered at −164.5) and −124 to −88 (ISCRB2, centered at −106) were clearly protected by IscR (Fig. 9*A*). Both sequences were equally protected by the same level of IscR, indicating that IscR bound to the two sites with a comparable affinity. The sequences of ISCRB1 and ISCRB2 revealed a 29-bp imperfect palindrome and scored about 83 and 79% identity to a consensus sequence of the type 2 IscR-binding site, respectively, to which the apo-form of IscR most likely binds in *E. coli* (Fig. 6*B*) (48). The CRP footprint extended from −82 to −54 (CRPB, centered at −68) (Fig. 9*B*), and the sequences of CRPB scored 86% identity to a consensus sequence for CRP binding (Fig. 6*B*) (49). The positioning of ISCRB and CRPB suggested that both IscR and CRP may act as class I activators interacting with the C-terminal domains of RNA polymerase α subunits (50). The sequences protected by SmcR extended from −58 to −32 (SMCRB, centered at −45) and revealed 73% identity to a consensus sequence for SmcR binding (51). In contrast to ISCRB and CRPB, SMCRB overlaps with the sequences of the −35 region of P_{gbpA} (Fig. 6*B*), and thus the bound SmcR could prevent RNA polymerase binding, supporting the SmcR repression of P_{gbpA}. Several nucleotides also showed enhanced cleavages, indicating that binding of the regulators altered the configuration of the DNA of P_{gbpA} (Fig. 9, *A*–*C*). These results confirmed that IscR, CRP, and SmcR regulate *gbpA* by binding to specific sequences of P_{gbpA}.

IscR Activates P_{gbpA} by Sensing Reactive Oxygen Species—Recently, it has been discovered that IscR senses reactive oxygen species and activates the expression of numerous virulence genes (26, 32). This prompted us to examine the effect of oxidative stress on the *gbpA* expression by measuring the levels of *gbpA* transcript and *Vv*GbpA protein in the strains grown anaerobically and exposed to a range of H_2O_2. The levels of the *gbpA* transcript and *Vv*GbpA protein in the wild type were gradually elevated along with increasing concentrations of H_2O_2 (Fig. 10, *A* and *B*). In contrast, the H_2O_2-dependent increase of the *gbpA* expression was not evident in the *iscR* mutant (Fig. 10, *A* and *B*), indicating that the activation of P_{gbpA} in response to oxidative stress is mediated by IscR. Because the cellular level of IscR also increased by exposure to H_2O_2 (Fig. 10*B*), the increased activity of P_{gbpA} by oxidative stress was possibly attributed to the increased level of IscR. The levels of CRP and SmcR were not significantly changed in the wild type and *iscR* mutant exposed to H_2O_2 (Fig. 10*B*).

Previous reports that the [2Fe-2S] cluster in IscR is disrupted by oxidative stress to result in the clusterless apo-form (19, 32, 48, 52) led us to examine whether apo-IscR indeed activates P_{gbpA} *in vivo*. The level of *gbpA* transcript in the $iscR_{3CA}$ mutant, of which the *iscR* coding region on the chromosome was replaced with $iscR_{3CA}$ encoding an apo-locked $IscR_{3CA}$ (Table 1) (32), was compared with those of the wild type and *iscR* mutant. When determined by qRT-PCR (Fig. 10*C*) and Western blot analyses (Fig. 10*D*), the P_{gbpA} activity of the $iscR_{3CA}$ mutant was almost 3- and 13-fold greater than that of the wild type and *iscR* mutant, respectively, indicating that apo-IscR is able to activate P_{gbpA} *in vivo*. Furthermore, it was noted that the $IscR_{3CA}$ level of the $iscR_{3CA}$ mutant was significantly greater than the IscR level of the wild type, in which both holo- and apo-IscR coexist (Fig. 10*D*) (52). In contrast, the levels of CRP and SmcR did not significantly differ among the strains (Fig. 10*D*). These results indicated that the increased activity of P_{gbpA} in the $iscR_{3CA}$ mutant was possibly attributed to the elevated cellular level of $IscR_{3CA}$. This elevated $IscR_{3CA}$ level in the $iscR_{3CA}$ mutant was perhaps not surprising because apo-IscR de-represses its own expression (19, 52). Taken together, the results led us to propose a model in which IscR senses reactive oxygen species and shifts to the apo-form, leading to the de-repression of the *isc* operon, elevating apo-IscR protein levels, and accordingly, activating P_{gbpA}.

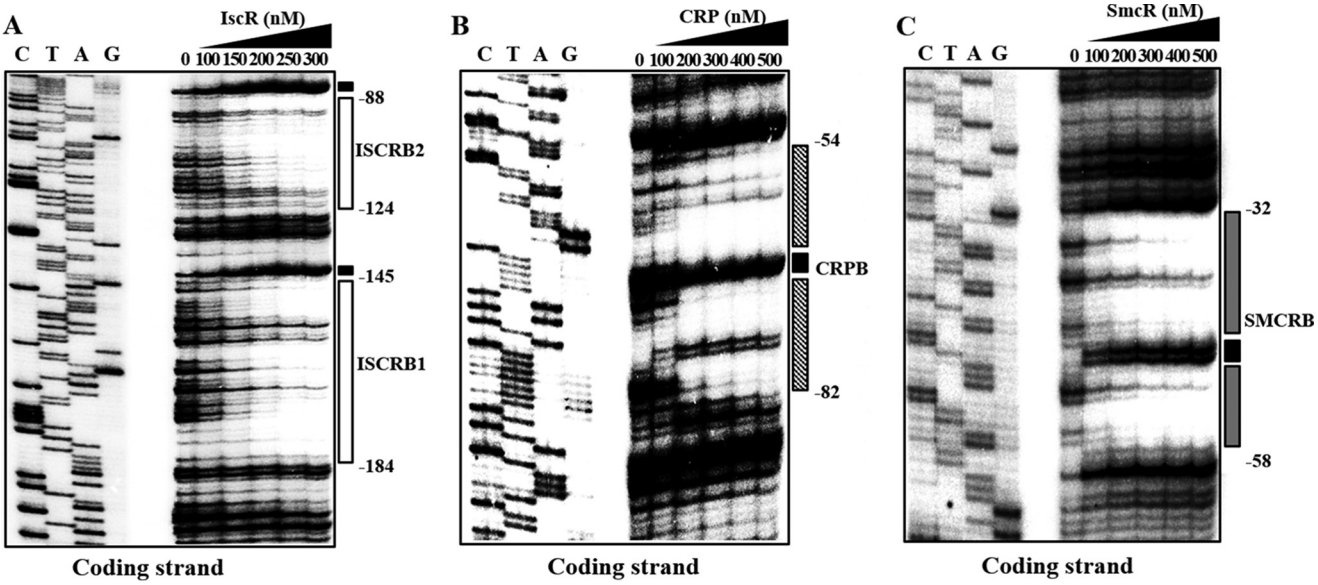

FIGURE 9. **Sequences for binding of IscR, CRP, and SmcR to P_{gbpA}.** A 390-bp DNA fragment of the *gbpA* regulatory region was radioactively labeled and then used as a DNA probe. The radiolabeled DNA probe (25 nM) was incubated with increasing amounts of IscR (*A*), CRP (*B*), and SmcR (*C*) as indicated. The regions protected by IscR, CRP, and SmcR are indicated by *white boxes, shaded boxes,* and *gray boxes,* respectively. The nucleotides showing enhanced cleavage are indicated by *black boxes. Lanes C, T, A,* and *G* represent the nucleotide sequencing ladders of pKK1401.

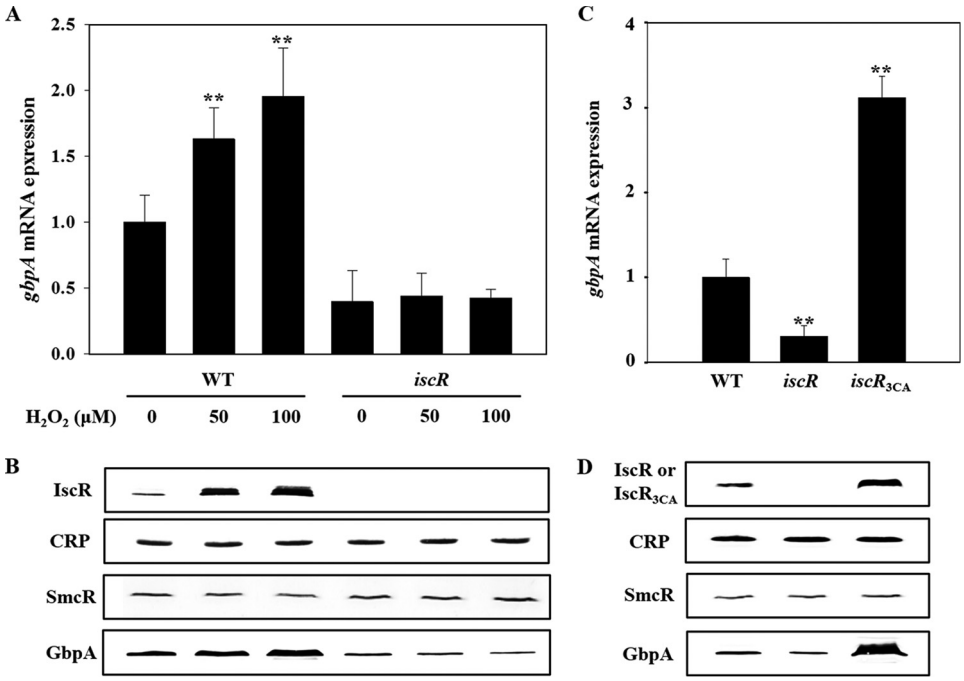

FIGURE 10. **Effects of oxidative stress and apo-IscR on the activity of P_{gbpA}.** Total RNAs and proteins were isolated either from the cultures grown anaerobically to an A_{600} of 0.5 and then exposed to various concentrations of H_2O_2 for 10 min as indicated (*A* and *B*) or from the cultures grown aerobically to an A_{600} of 0.5 (*C* and *D*). *A* and *C*, *gbpA* mRNA levels were determined by qRT-PCR analyses, and the *gbpA* mRNA level in the wild type unexposed to H_2O_2 (*A*) or the wild type (*C*) was set to 1. *Error bars* represent the S.D. **, $p < 0.005$ relative to the wild type unexposed to H_2O_2 (*A*) or to the wild type (*C*). *B* and *D*, protein samples were resolved by SDS-PAGE, and IscR (or $IscR_{3CA}$), CRP, SmcR, and GbpA were detected by Western blotting using the rabbit anti-*V. vulnificus* IscR, anti-*V. vulnificus* CRP, anti-*V. vulnificus* SmcR, and anti-*V. vulnificus* GbpA sera, respectively. *WT,* wild type; *iscR*, *iscR* mutant; $iscR_{3CA}$, a strain expressing apo-locked $IscR_{3CA}$.

Discussion

There are several lines of evidence that *V. vulnificus* cells embed themselves in oyster tissues and form biofilms to persist in the oyster as the primary route of infection (13, 53). Furthermore, biofilms are likely a form of pathogenic *V. vulnificus* cells and an important source for new outbreaks as they provide a means to reach a concentrated infective dose consumed by humans (25). Therefore, it is a reasonable hypothesis that cells of the *V. vulnificus* biofilms entering the host intestine might be detached at first and become free-living planktonic cells that disperse to epithelial surfaces for adhesion (25). Adhesion to the intestinal epithelia is a prerequisite step for the establishment of a successful infection, and thus interference with the adhesion is an efficient way to prevent or treat infections of *V. vulnificus*. Efforts to develop the anti-adhesion therapies were initiated by identification and characterization of the adhesins

of *V. vulnificus*. Previous studies demonstrated that *V. vulnificus* expresses different types of adhesin molecules essential for adhesion to human cell lines (14–18). Additionally, this study identified GbpA required for adhesion to mucin and the mucin-secreting HT29-MTX cells (Fig. 1, *A* and *B*). These observations suggested that *V. vulnificus* adheres to epithelial surfaces through multiple adhesive interactions as observed in other pathogens (5).

Nevertheless, little is known about the regulatory mechanisms adopted by *V. vulnificus* to modulate the expression of the adhesins. No information on the expression pattern or level of the adhesins during infection of the pathogen has been reported in previous studies. As a result of this study, the expression of *gbpA* is growth phase-dependent and decreases in the stationary phase cells (Fig. 3). SmcR, a master regulator of the *V. vulnificus* quorum sensing (21, 22, 51, 54), represses *gbpA* at the transcriptional level (Fig. 3). This result, along with elevated levels of SmcR in the stationary phase cells (Fig. 5), indicated that the decrease of *gbpA* expression in the stationary phase cells attributes most likely to SmcR repression. It is noteworthy that most individual cells in biofilms are close to stationary phase physiology with reduced growth rates and increased resistance to stress (55–57). Considering the *V. vulnificus* infection in the form of biofilms, the repression of *gbpA* by the elevated SmcR in the biofilm (stationary phase) cells could save the limited nutrients that can alternatively be used for expression of the genes responsible for the detachment of the biofilms. Consistent with this, SmcR appears to enhance the detachment of *V. vulnificus* biofilms entering the host intestine and thereby promote the dispersal of the pathogen to establish a new infectious cycle on the intestinal surfaces (25). Furthermore, GbpA is probably surplus upon establishing colonization and may be even detrimental to the detachment of individual cells from the established colony. SmcR, a cell density-dependent regulator, is believed to sense cell densities higher than critical levels in the colony and then render *V. vulnificus* to leave the congested colony for new colonization loci by repressing *gbpA*, which is crucial for pathogenesis.

Upon arrival onto new loci, CRP and IscR activate the *gbpA* expression to facilitate adhesion that is generally accompanied by the onset of accelerated (perhaps exponential) growth to colonize (Fig. 3). CRP, which is a central regulator of energy (catabolic) metabolism (20), may recognize host environments by sensing the starvation of specific nutrients imposed by the host cells and endogenous bacterial flora. IscR increases at the exponential phase (Fig. 5), indicating that the maximum expression of *gbpA* at the exponential phase attributes possibly to increased IscR (Fig. 3). The [2Fe-2S] cluster in IscR is disrupted by oxidative stress, and the resulting clusterless IscR (apo-IscR) increases the cellular level of IscR, most likely in its apo-form (19, 52, 58). There are two IscR-binding sequences, type 1 and type 2, and the type 2 sequence is recognized by apo-IscR, whereas the type 1 sequence is recognized exclusively by holo-IscR (47, 59, 60). The IscR binding sequences on P_{gbpA} scored an 80% homology to the type 2 sequences of *E. coli* (Fig. 6*B*) (48, 60). Furthermore, the H_2O_2 induction of *gbpA* was mediated by apo-IscR (Fig. 10). The combined results suggested that IscR senses the oxidative stress imposed by the host defense system, turns into the apo-form, and activates the *gbpA* expression, leading to improved adhesion to the host intestinal surfaces.

In summary, *gbpA* encoding a mucin-binding protein essential for pathogenesis of *V. vulnificus* was identified in this study. The *gbpA* expression was in a growth phase-dependent manner and was regulated positively by IscR and CRP but negatively by SmcR. The regulatory proteins regulate the *gbpA* expression cooperatively rather than sequentially and exerted their effects by directly binding to the regulatory region of P_{gbpA}. Two distinct IscR-binding sequences centered at −164.5 and −106, a CRP-binding sequence centered at −68, and an SmcR-binding sequence centered at −45 were identified. The *gbpA* expression was induced by exposure to oxidative stress, and the induction was mediated by the elevated intracellular levels of, most probably, apo-IscR. It is still difficult to define the implications of the collaboration between IscR, CRP, and SmcR in terms of pathogenesis of *V. vulnificus*. However, it is likely that the collaboration allows more precise tuning of the *gbpA* expression by integrating the signals presumably encountered in the host intestine, such as oxidative stress, starvation of specific nutrients, and increased cell density, and thereby it enhances the overall success of *V. vulnificus* during pathogenesis.

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Regulatory Characteristics of *Vibrio vulnificus gbpA* Gene Encoding a Mucin-binding Protein Essential for Pathogenesis

Kyung Ku Jang, So Yeon Gil, Jong Gyu Lim and Sang Ho Choi

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