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

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Running Title: Regulation of gbpA in *Vibrio vulnificus*

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**ABSTRACT**

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 to the wild type, the gbpA mutant was impaired in binding to mucin-agar and the mucin-secreting HT29-MTX 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 to the 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\(_2\)O\(_2\), 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 mucus layer, which is produced by specialized cells found throughout the entire intestinal tract (1). The mucus 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 mucus layer is composed of a variety of factors, but its characteristic physicochemical properties are attributable to the presence of mucins, which are complex linear polymorphic glycoproteins (1, 2). Mucins are highly glycosylated large glycoproteins (with molecular weights 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 mucus 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 towards mucin carbohydrates is still limited (8). The GlcNAc-binding protein A of Vibrio cholerae (VcGbpA) is a lectin-like mucus adhesin and is characterized at the molecular level (6, 9, 10, 11). VcGbpA is a common adhesin required for V. cholerae to adhere to chitinous and intestinal surfaces (6). VcGbpA plays an important role in the survival of V. cholerae by attachment to the surface of chitinous zooplankton in the aquatic ecosystem (6, 11). VcGbpA 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 VcGbpA 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. On the other hand, domains 2 and 3 anchor to the V. cholerae surfaces (11). It has been reported that VcGbpA expression was induced by mucin and negatively regulated by cyclic di-guanosine monophosphate (c-di-GMP) 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 adhesion molecules. The V. vulnificus 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 the present study, a V. vulnificus open reading frame (ORF) encoding a homologue of VcGbpA was identified. Construction of the gbpA mutant and evaluation of its phenotypes provided evidences that V. vulnificus GbpA (VvGbpA) 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. Since IscR (iron-sulfur (Fe-S) cluster regulator) (19), CRP (cyclic AMP receptor protein) (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 PgbpA. Deletion analyses of the upstream region of PgbpA and DNase I protection assays were performed to identify the binding sequences of IscR, CRP, and SmcR. Finally, the influence of hydrogen peroxide (H2O2) on the intracellular levels of IscR was examined to explain how IscR can mediate the induction of gbpA by oxidative stress.

**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% (wt/vol) NaCl (LBS) at 30°C, and their growth was monitored spectrophotometrically at 600 nm (A600). Anaerobic conditions were obtained by using an anaerobic chamber with an atmosphere of 90% N2, 5% CO2 and 5% H2 (Coy laboratory products, Grass Lake, MI). For anaerobic culture, the media were pre-incubated to remove dissolved O2 in the anaerobic chamber, which was verified by adding 0.00001% (wt/vol) resazurin salt (Sigma, St. Louis, MO) 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 GBPA02F and -R (for amplification of the 3' amplicon) were designed and used (Table 2). The gbpA gene with 318-bp deletion was amplified by PCR using the mixture of both amplicons as the template and GBPA01-F and GBPA02-R as primers. The resulting ΔgbpA was ligated into Spe-Sph-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, 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 ORF 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% (vol/vol) ethanol for 1 h, dried at 70°C for 24 h (33) and then added to the 1.5% agar (wt/vol) solution, which was autoclaved and cooled down to 60°C, to the final concentration of 3% (wt/vol). 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, approximately 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% (vol/vol) fetal bovine serum (MCF) were developed into mucin-secreting cells, named HT29-methotrexate (MTX) cells, as described previously (34). The HT29-MTX cells were fixed with para-formaldehyde and treated with DAPI (4', 6-diamidino-2-phenylindole). Mucin secretion of the HT29-MTX cells was detected with the anti-mucin5AC (MUC5AC) 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 (CLSM) (LSM710, Zeiss, Jena, Germany) (35, 36).

The 12-well culture dishes (Nunc) were seeded with the HT29-MTX cells (approximately 1 x 10$^7$ cells per well), infected with the *V. vulnificus* strains at a multiplicity of infection (MOI) 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 approximately 10$^9$ cells of either the wild type or the gbpA mutant. Mouse survivals were recorded for 24 h.
colonizes in small intestine and disseminates to other organs (37, 38). Therefore, colonization of each strain in mice small intestine was determined by competition assays as previously described (30). Briefly, four ICR female mice (7-weeks-old) were infected as described above for the 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 approximately 10⁶ CFU of each strain, was given intragastrically to the mice. The mice were sacrificed at 1 to 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 A₆₀₀ were isolated using an RNeasy® mini kit (Qiagen, Valencia, CA). When necessary, the strains grown anaerobically to an A₆₀₀ of 0.5 were exposed to various concentrations of H₂O₂ for 10 min and harvested to isolate total RNA. For the primer extension analysis, a 26-base primer GBPA05R (Table 2) complementary to the regulatory region of *gbpA* was end labeled with [γ-32P]ATP and added to the RNA. The primer was then extended with SuperScript II RNase H reverse transcriptase (Invitrogen, Carlsbad, CA). 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, 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 phosphorimage analyser (BAS1500; Fuji Photo Film Co., Ltd., Tokyo, Japan).

For quantitative real-time PCR (qRT-PCR), cDNA was synthesized using by the iScript™ cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA) and real-time PCR amplification of the cDNA was performed by using the Chromo 4 real-time PCR detection system (Bio-Rad Laboratories) with pairs of specific primers (Table 2), as described previously (32). Relative expression levels of the specific transcripts were calculated by using the 16S rRNA expression level as the internal reference for normalization.

For quantitative real-time PCR (qRT-PCR), the concentrations of total RNA from the strains were measured by using NanoVue Plus Spectrophotometer (GE Healthcare, Piscataway, NJ). cDNA was synthesized from 1 μg of the total RNA by using the iScript™ cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA) and real-time PCR amplification of the cDNA was performed by using the Chromo 4 real-time PCR detection system (Bio-Rad Laboratories) 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 His₆ 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 (22, 26, 41) (Table 1), 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 A₆₀₀ of 0.5, or anaerobically to an A₆₀₀ of 0.5 and then exposed to various concentrations of H₂O₂ 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-32P]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.

**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 GBPA05F (Table 2) as the primer. The gels were visualized as described above for the transcript analysis.

**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 VcGbpA singled out a protein, hereafter named VvGbpA. The amino acid sequence analysis predicted that pre-VvGbpA protein contains an N-terminal signal peptide for the type II secretion system, suggesting that VvGbpA is a secreted protein (data not shown; http://www.ebi.ac.uk/clustalw) (46). The deduced mature VvGbpA is comprised of 485 amino acids with a theoretical mass of 52.83 kDa and a pI of 4.75. The amino acid sequence of VvGbpA was 80% identical to that of VcGbpA (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 VcGbpA (11). The predicted profile of the hydrophobicity (http://web.expasy.org) was significantly similar to that of VcGbpA, indicating that VvGbpA is a soluble protein (data not shown) (11). All of this information suggested that VvGbpA is a secreted but cell surface-associated protein as is VcGbpA.

**Data analyses**—Averages and standard deviations (SD) 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/). 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.
virulence of *V. vulnificus*—To examine the function of *VvGbpA*, mucin binding ability of wild type and the *gbpA* mutant was examined. The number of the *gbpA* mutant 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. 1A). This indicated that the *gbpA* mutant was defective in mucin binding. In addition, the extracellular provision of purified *VvGbpA* 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 *VvGbpA* was comparable to that of the parental wild type, in terms of the bacterial numbers adherent to the mucin-agar (Fig. 1A).

To further understand the role of *VvGbpA* 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 CLSM (Fig. 1C to F). The wild type and *gbpA* mutant were incubated with the HT29-MTX cells and the bacteria adherent to the cells were enumerated (Fig. 1B). 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 *VvGbpA* 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 *VvGbpA* in pathogenesis, mouse mortality and colonization activity of the *gbpA* mutant were evaluated. As shown in Fig. 2A, the survival of mice inoculated intragastrically with the *gbpA* mutant was consistently and significantly prolonged (P = 0.0174, log rank test) compared to 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. 2B). 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 *VvGbpA* is a virulence factor essential for the intestinal colonization of *V. vulnificus*. Taken together, it is apparent that *VvGbpA* 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 *VvGbpA* is influenced by growth phase, levels of the *gbpA* mRNA of the wild type culture were analyzed at various growth stages (Fig. 3A 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 which 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. 3C). 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. 3C). The cellular levels of *VvGbpA* determined by Western blot analyses also varied in the mutants (Fig. 3D). The magnitude of variation in the *VvGbpA* 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 *VvGbpA* protein that varied in the *iscR*, *crp*, and *smcR* mutant were restored to the levels comparable to those in the wild type by introducing pKK1403, pKK1404, and pKK1405 carrying recombinant *iscR*, *crp*, and *smcR*, respectively (Fig. 3E and F). Taken together, these results suggested that IscR and CRP activate whereas 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 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 gbpA transcript in KK142 (pKK1403) was only about 80% of 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 VvGbpA 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 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). In consistent with this, the level of VvGbpA 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. 3A 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-In order 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_{oo} of 0.5 (Fig. 6A). 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 subsequently designated +1 (Fig. 6B). 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. 6B).

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. 7A). The reporters were transferred into the wild type and isogenic mutants and culture luminescence was used to quantify the P_{gbpA} activity (Fig. 7B). The luminescence produced by pKK1407 carrying P_{gbpA} deleted up to -220 was \_6.0 \times 10^3\ \text{RLU in the wild type but significantly reduced in the iscR and crp mutants, supporting our previous observation that IscR and CRP activate P}_{gbpA}. The RLU of the smcR mutant containing pKK1407 increased, confirming the SmcR repression of P_{gbpA} (Fig. 7B). Deletion up to -106 significantly decreased the P_{gbpA} activity as determined based on the reduced luminescence of
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. Since 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. 8A). The binding of IscR was also specific, because assays were performed in the presence of 0.1 μg of poly (dl-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. 8A), confirming that IscR 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. 8B 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. 9A, 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. 9A). 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. 6B) (48). The CRP footprint extended from -82 to -54 (CRPB, centered at -68) (Fig. 9B) and the sequences of CRPB scored 86% identity to a consensus sequence for CRP binding (Fig. 6B) (49). These results suggested that both IscR and CRP may act as class I activators interacting with the C-terminal domains of RNA polymerase α subunits (αCTD) (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 ICRB and CRPB, SMCRB overlaps with the sequences of -35 region of P_gbpA (Fig. 6B) and thus 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. 9A, B and 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 (ROS)-Recently, it has been discovered that IscR senses ROS 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 VvGbpA protein in the strains grown anaerobically and exposed to a range of H₂O₂. The levels of the gbpA transcript and VvGbpA protein in the wild type were gradually elevated along with increasing concentrations of H₂O₂ (Fig. 10A and B). In contrast, the H₂O₂-dependent increase of the gbpA expression was not evident in the iscR mutant (Fig. 10A and B), indicating that the activation of P_gbpA in response to oxidative stress is mediated by IscR. Since the cellular level of IscR also increased by exposure to H₂O₂ (Fig. 10B), 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 H₂O₂ (Fig. 10B).

Previous reports that the [2Fe-2S] cluster in IscR is disrupted by oxidative stress to result in the apo-locked IscR motif (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 (32) (Table 1), was compared with those of the wild type and iscR mutant. When determined by qRT-PCR (Fig. 10C) and Western blot analyses (Fig. 10D), 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. Further, 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. 10D) (52). In contrast, the levels of CRP and SmcR did not significantly differ among the strains (Fig. 10D). 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 since apo-IscR derepresses its own expression (19, 52). Taken together, the results led us to propose a model in which IscR senses ROS 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.

**DISCUSSION**

There are several lines of evidence that *V. vulnificus* embed themselves in oyster tissues and form biofilms to persist in oyster as the primary route of infection (13, 53). Furthermore, biofilms are likely a form of pathogenic *V. vulnificus* 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, the present study identified GbpA required for adhesion to mucin and the mucin-secreting HT29-MTX cells (Fig. 1A 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 module 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 transcription 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 onto 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 are recognized by apo-IscR, whereas the Type 1 sequence are recognized exclusively by holo-IscR (47, 59, 60). The IscR binding sequences on PgbpA scored an 80% homology to the Type 2 sequences of *E. coli* (Fig. 6B) (48, 60). Furthermore, the H$_2$O$_2$-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 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 PgbpA. Two distinct IscR binding sequences centered at -164.5 and -106, a CRP binding sequence centered at -68, and a 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 enhances the overall success of *V. vulnificus* during pathogenesis.
Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions: KKJ, SYG, JGL, and SHC designed research; KKJ and SYG performed research; KKJ, SYG, JGL, and SHC analyzed data; and KKJ and SHC wrote the paper; and all authors reviewed the results and approved the final version of the manuscript.
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FOOTNOTES

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2The abbreviations used are: GlcNAc, N-acetyl-D-glucosamine; cyclic di-guanosine monophosphate, c-di-GMP; ORF, open reading frame; Fe-S, iron-sulfur; H₂O₂, hydrogen peroxide; MTX, methotrexate; CLSM, confocal laser scanning microscope; MOI, multiplicity of infection; qRT-PCR, quantitative real-time PCR; EMSA, electrophoretic mobility shift assay; RLU, relative light unit; αCTD, The C-terminal domain of the alpha subunit of RNA polymerase; ROS, reactive oxygen species.
FIGURE LEGENDS

FIGURE 1. Effect of GbpA on mucin binding and host cell adhesion of V. vulnificus. Upper panel, mucin-binding activities of the strains. A, the strains (approximately 10⁷ 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 incubation, the adherent bacterial cells were enumerated as CFU per well. WT, wild type; gbpA, gbpA mutant. B, the mucin-secreting HT29-MTX cells (approximately 10⁷ cells) seeded in each well of 12-well culture dishes were infected at an MOI of 10 with the strains as indicated. After 30 min incubation, the adherent bacterial cells were enumerated as CFU per well. Error bars represent the SD. *, 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, the merged image of C, D, and E. Images are visualized using a CLSM. Scale bar is 40 μm.

FIGURE 2. Mouse lethality and colonization activity of the V. vulnificus strains. A, seven-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 approximately 10⁷ CFU as indicated. Mouse survival was monitored for 24 hr. 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.

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 (A₆₀₀) and total RNAs were isolated from the cells harvested at different growth phases (from left, at A₆₀₀ of 0.5, 1.0, 1.5, 2.0 and 2.5) as indicated by arrows. B, the gbpA mRNA levels were determined by qRT-PCR analyses, and the gbpA mRNA level in the cells grown to an A₆₀₀ of 0.5 was set as 1. Error bars represent the SD. *, P < 0.05, and **, P < 0.005 relative to the wild type. WT (pJK1113, empty vector), wild type; gbpA (pJK1113), gbpA mutant; gbpA (pKK1403), and gbpA (pKK1404), complemented strains.

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 A₆₀₀ of 0.5 and analyzed to determine the gbpA mRNA and GbpA protein levels. (C and E) The 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. WT (pJK1113), wild type; iscR (pJK1113), iscR mutant; smcR (pJK1113), smcR mutant; crp (pJK1113), crp mutant; iscR (pKK1403), crp (pKK1404), and smcR (pKK1405), complemented strains.
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-\textit{V. vulnificus} IscR, anti-\textit{V. vulnificus} CRP, and anti-\textit{V. vulnificus} SmcR, anti-\textit{V. vulnificus} GbpA sera, respectively. WT, wild type; iscR, iscR mutant; crp, crp mutant; smcR, smcR mutant.

FIGURE 6. Transcription start site and sequences of the gbpA regulatory region. A, 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, the 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 (ISCRib1 and ISCRib2, 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 \textit{V. vulnificus} DNA sequence. R, A or G; Y, C or T; W, A or T; x, any nucleotides.

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 (ISCRib1 and ISCRib2, white boxes), CRP (CRPB, the shaded box), and SmcR (SMcRB, the 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 SD. RLU, Relative luminescence units. WT, wild type; iscR, iscR mutant; crp, crp mutant; smcR, smcR mutant.

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.

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 probe DNA. The radiolabeled probe DNA (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$_2$O$_2$ 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, the gbpA mRNA levels were determined by qRT-PCR analyses, and the gbpA mRNA level in the wild type unexposed to H$_2$O$_2$ (A) or the wild type (C) was set to 1. Error bars represent the SD. **, $P < 0.005$ relative to the wild type unexposed to H$_2$O$_2$ (A) or to the wild type (C). B and D,
protein samples were resolved by SDS-PAGE, and IscR (or IscR<sub>3CA</sub>), CRP, SmcR, and GbpA were detected by Western blotting using the rabbit anti-<i>V. vulnificus</i> IscR, anti-<i>V. vulnificus</i> CRP, anti-<i>V. vulnificus</i> SmcR, and anti-<i>V. vulnificus</i> GbpA sera, respectively. WT, wild type; <i>iscR</i>, <i>iscR</i> mutant; <i>iscR<sub>3CA</sub></i>, a strain expressing apo-locked IscR<sub>3CA</sub>. 
TABLE 1
Plasmids and bacterial strains used in this study

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<td>This study</td>
</tr>
<tr>
<td>pKK1409</td>
<td>pBBR-_lux with 411-bp fragment of <em>gbpA</em> upstream region; Cm(^r)</td>
<td>This study</td>
</tr>
<tr>
<td>pKK1410</td>
<td>pBBR-_lux with 341-bp fragment of <em>gbpA</em> upstream region; Cm(^r)</td>
<td>This study</td>
</tr>
</tbody>
</table>

\(^a\) Rif, Rifampicin resistant; Sm\(^r\), streptomycin resistant; Km\(^r\), kanamycin resistant; Tp\(^r\), trimethoprim resistant; Ap\(^r\), ampicillin resistant; Cm\(^r\), chloramphenicol resistant.
### TABLE 2
Oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Oligonucleotide Sequence (5' → 3')</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>For mutant construction</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GBPA01-F</td>
<td>GAGATGCACATCAGCAACCGCG</td>
<td>Deletion of the gbpA ORF</td>
</tr>
<tr>
<td>GBPA01-R</td>
<td>AAAGGATCCAGCGAATTACATACAGTGT</td>
<td></td>
</tr>
<tr>
<td>GBPA02-F</td>
<td>GCTGGATCCTTTGTCTTCCAGATG</td>
<td>Deletion of the gbpA ORF</td>
</tr>
<tr>
<td>GBPA02-R</td>
<td>CGCAAACGGAATCAAACGC</td>
<td></td>
</tr>
<tr>
<td><strong>For mutant complementation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GBPA03-F</td>
<td>GGATCCGGCAAATAAAGTCAG</td>
<td>Amplification of the gbpA ORF</td>
</tr>
<tr>
<td>GBPA03-R</td>
<td>GGATCCCTACAGTGGTGCTCCAC</td>
<td></td>
</tr>
<tr>
<td>ISCR01-F</td>
<td>ATCCATGGCTATGAAACTGACCATCTAAAGG</td>
<td>Amplification of the iscR ORF</td>
</tr>
<tr>
<td>ISCR01-R</td>
<td>ATTCTAGATTAAGACGGAAATTTACACCG</td>
<td></td>
</tr>
<tr>
<td>CRP01-F</td>
<td>GAGATACCATGGTTCTAGGTAACCTCA</td>
<td>Amplification of the crp ORF</td>
</tr>
<tr>
<td>CRP01-R</td>
<td>GTTAAATTCTAGATTAACGAGTACCGTAAACAC</td>
<td></td>
</tr>
<tr>
<td>SMCR01-F</td>
<td>ATCCATGGACTCTAATCGAAAGAGAC</td>
<td>Amplification of the smcR ORF</td>
</tr>
<tr>
<td>SMCR02-R</td>
<td>ATTCTAGATTATTCGTGCTCGGGTTTATA</td>
<td></td>
</tr>
<tr>
<td><strong>For GbpA overexpression</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GBPA04-F</td>
<td>CCAATGGCTAAAAACAAACCCGAAAAAACC</td>
<td>Amplification of the gbpA ORF</td>
</tr>
<tr>
<td>GBPA04-R</td>
<td>CTCGACGAGTGTGCTCCACGCCATT</td>
<td></td>
</tr>
<tr>
<td><strong>For promoter deletion analysis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GBPA003</td>
<td>GAGCTCTAAGTGCTCAATGACATAGTAAAG</td>
<td>Deletion of the gbpA regulatory region</td>
</tr>
<tr>
<td>GBPA004</td>
<td>GAGCTCTACATCTTTTTCGAGAAATTA</td>
<td></td>
</tr>
<tr>
<td>GBPA005</td>
<td>GAGCTC ACATCTATAAAATAACGCTTCTAAAT</td>
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</tr>
<tr>
<td>GBPA006</td>
<td>GAGCTCTTATGCTGACTACACTACAC</td>
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</tr>
<tr>
<td>GBPA007</td>
<td>ACTAGTCCACCATTTCCTCCACTGAG</td>
<td></td>
</tr>
<tr>
<td><strong>For Primer extension analysis, EMSA, and DNase I protection assay</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GBPA05-F</td>
<td>ATGGCCATAGCTGCTGTTTTCCA</td>
<td>Amplification of the gbpA upstream region</td>
</tr>
<tr>
<td>GBPA05-R</td>
<td>CCCCGCTATCTTTGGTGATGAAAAA</td>
<td>Amplification of the gbpA upstream region, Extension of the gbpA transcript</td>
</tr>
<tr>
<td><strong>For qRT-PCR</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GBPA_qRT-F</td>
<td>TGAAGCCCTGGGGTGAAAAGAC</td>
<td>Quantification of the gbpA expression</td>
</tr>
<tr>
<td>GBPA_qRT-R</td>
<td>ATCCGCTAGGCTGAGGCG</td>
<td></td>
</tr>
</tbody>
</table>

The oligonucleotides were designed using the *V. vulnificus* MO6-24/O genomic sequence (GenBank™ accession number CP002469 and CP002470, www.ncbi.nlm.nih.gov).

Regions of oligonucleotides not complementary to the corresponding genes are underlined.
Fig. 1

A

Bacteria (CFU) / well (mucin agar)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>WT</th>
<th>gbp.A</th>
<th>gbp.A</th>
<th>gbp.A</th>
</tr>
</thead>
<tbody>
<tr>
<td>GbpA (µM)</td>
<td>0</td>
<td>0</td>
<td>2.5</td>
<td>5</td>
</tr>
</tbody>
</table>

B

Bacteria (CFU) / well (HT29-MTX)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>WT</th>
<th>gbp.A</th>
<th>gbp.A</th>
<th>gbp.A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid</td>
<td>pJK1113</td>
<td>pJK1113</td>
<td>pKK1402</td>
<td></td>
</tr>
</tbody>
</table>

C

D

E

F
Fig. 3

A

$A_{600}$

Time (h)

0.01

0.1

1

10

0

2

4

6

8

10

12

14

16

B

$gfpA$ mRNA expression

0.0

0.2

0.4

0.6

0.8

1.0

1.2

1.4

0.5

1.0

1.5

2.0

2.5

A$_{600}$

C

$gfpA$ mRNA expression

0

1

2

3

4

5

0

**

**

D

Genotype

WT
 iscR
 crp
 smcR

Plasmid
 pJK1113
 pJK1113
 pJK1113
 pJK1113

E

$gfpA$ mRNA expression

0

1

2

3

4

5

0

**

**

F

Genotype

WT
 iscR
 crp
 smcR

Plasmid
 pJK1113
 pKK1403
 pKK1404
 pKK1405

GbpA

GbpA
Fig. 4

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Complementation</th>
<th>IscR</th>
<th>CRP</th>
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<tr>
<td>WT</td>
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</tr>
<tr>
<td><em>iscR</em></td>
<td>none</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>crp</em></td>
<td>none</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>iscR crp</em></td>
<td>none</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>iscR crp</em></td>
<td><em>iscR</em> (pKK1403)</td>
<td></td>
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<tr>
<td><em>iscR crp</em></td>
<td><em>crp</em> (pKK1404)</td>
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</tbody>
</table>

*gbpA mRNA expression*
Fig. 5

<table>
<thead>
<tr>
<th></th>
<th>L</th>
<th>S</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT  crp  smcR  iscR  WT  crp  smcR  iscR  WT  crp  smcR  iscR  WT  crp  smcR  iscR  WT  crp  smcR  iscR  WT  crp  smcR  iscR</td>
<td></td>
</tr>
<tr>
<td>IscR</td>
<td><img src="image" alt="Image of IscR expression" /></td>
<td><img src="image" alt="Image of IscR expression" /></td>
</tr>
<tr>
<td>CRP</td>
<td><img src="image" alt="Image of CRP expression" /></td>
<td><img src="image" alt="Image of CRP expression" /></td>
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<tr>
<td>SmcR</td>
<td><img src="image" alt="Image of SmcR expression" /></td>
<td><img src="image" alt="Image of SmcR expression" /></td>
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<tr>
<td>GbpA</td>
<td><img src="image" alt="Image of GbpA expression" /></td>
<td><img src="image" alt="Image of GbpA expression" /></td>
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</table>
Fig. 9

A

<table>
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<tr>
<th>IscR (nM)</th>
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</thead>
<tbody>
<tr>
<td>C T A G</td>
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<td>0 100 150 200 250 300</td>
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</table>

Coding strand

B

<table>
<thead>
<tr>
<th>CRP (nM)</th>
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</thead>
<tbody>
<tr>
<td>C T A G</td>
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<tr>
<td>0 100 200 300 400 500</td>
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</table>

Coding strand

C

<table>
<thead>
<tr>
<th>SmcR (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C T A G</td>
</tr>
<tr>
<td>0 100 200 300 400 500</td>
</tr>
</tbody>
</table>

Coding strand

ISCRB1

ISCRB2

CRPB

SMCRB
Fig. 10

A

**gfpA mRNA expression**

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>iscR</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.5</td>
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<tr>
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<td>1.5</td>
</tr>
<tr>
<td>100</td>
<td>2.5</td>
<td>2.0</td>
</tr>
</tbody>
</table>

B

**H₂O₂ (µM)**

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>50</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>IscR</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>CRP</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>SmcR</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>GbpA</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

C

**gfpA mRNA expression**

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>iscR</th>
<th>iscR&lt;sub&gt;3CA&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>50</td>
<td><strong>2.5</strong></td>
<td>2.0</td>
<td>3.0</td>
</tr>
<tr>
<td>100</td>
<td>2.5</td>
<td>3.0</td>
<td><strong>3.5</strong></td>
</tr>
</tbody>
</table>

D

**IscR or IscR<sub>3CA</sub>**

- CRP
- SmcR
- GbpA
Regulatory Characteristics of \textit{Vibrio vulnificus} \textit{gbpA} Encoding a Mucin-Binding Protein Essential for Pathogenesis
Kyung Ku Jang, So Yeon Gil, Jong Gyu Lim and Sang Ho Choi

\textit{J. Biol. Chem.} published online January 11, 2016

Access the most updated version of this article at doi: 10.1074/jbc.M115.685321

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