Direct Interaction between Quorum-sensing Regulator SmcR and RNA Polymerase Is Mediated by Integration Host Factor to Activate \textit{vvpE} Encoding Elastase in \textit{Vibrio vulnificus}*

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It has been suggested that quorum sensing is an important signal transduction system regulating the expression of numerous virulence genes in bacterial pathogens. We previously revealed that SmcR, a LuxR homologue of \textit{Vibrio vulnificus}, activates promoter \textit{S}, an RpoS-dependent promoter of \textit{vvpE} encoding a potential virulence factor elastase and binds \textit{in vivo} to a binding site centered at −196.5. In this study, chromatin immunoprecipitation assays and promoter deletion analyses demonstrated that SmcR binds to the \textit{vvpE} regulatory region \textit{in vivo} and directly interacts with RNAP for activation of the \textit{vvpE} expression. A search for regulatory genes involved in the regulation of elastase production singled out \textit{ihfA}, which encodes for a subunit of integration host factor (IHF). Levels of both elastase activity and \textit{vvpE} transcript decreased significantly as a result of inactivation of \textit{ihfA}, and primer extension analyses demonstrated that IHF regulates the \textit{vvpE} transcription by activating PS. Direct binding of IHF to the two distinct binding sites centered at −174 and −131, respectively, was determined using an electrophoretic mobility shift assay and a DNase I protection assay. Chromatin immunoprecipitation assays revealed that the interaction of SmcR with RNAP \textit{in vivo} was mediated by IHF. Collectively, the results proposed a model whereby IHF positions SmcR to contact RNAP by looping the \textit{vvpE} regulatory DNA, thus allowing precise control of the expression level of VvpE during the pathogenesis of \textit{V. vulnificus}.

Many bacteria exchange diffusible signal molecules that accumulate extracellularly as a method to monitor their cell population densities (for recent reviews, see Refs. 1 and 2). This type of cell density-dependent regulation is termed quorum sensing and has been recognized as a global regulatory system controlling the expression of numerous genes in bacteria. The \textit{Vibrio harveyi} regulation of bioluminescence is frequently used as a model for quorum sensing. LuxR, a transcriptional activator of the luminescence operon, is a quorum-sensing master regulator in \textit{V. harveyi}, and its synthesis is controlled by the levels of three autoinducers: AI-1, AI-2, and CAI-1 (3). Thus far, homologues of LuxR, which are postulated to regulate virulence genes, have been identified in various pathogenic \textit{Vibrio} spp. (4–9). However, until now, only a few studies have addressed the molecular mechanism by which the LuxR homologues modulate the expression of virulence genes (10–12).

As a LuxR homologue, SmcR has been identified from \textit{Vibrio vulnificus}, a food-borne pathogenic bacterium (13). Upon analysis of the completed genome sequence, homologues of the genes required for sensing and responding to autoinducers, such as LuxO and LuxT, are also identified in \textit{V. vulnificus} (14). It seemed logical to consider SmcR as a quorum-sensing regulator of \textit{V. vulnificus} because of the similarities between the components of quorum-sensing systems in \textit{V. vulnificus} and \textit{V. harveyi}. Recent works demonstrated that SmcR regulates numerous genes contributing to pathogenesis as well as survival of \textit{V. vulnificus} (4, 10, 15–17). Among these SmcR target genes, \textit{vvpE} encodes a potential virulence factor, elastase (elastolytic metalloprotease), and its regulation by SmcR is the best characterized \textit{in vitro} (10, 18). We previously reported that the expression of \textit{vvpE} is initiated by two different types of promoter, PL and PS (Fig. 1), in a growth phase-dependent manner. Although the basal level expression of \textit{vvpE} is directed by PL, independent of SmcR, and remains low throughout the log and stationary growth phases, SmcR activates the RpoS-dependent promoter PS by directly binding to a SmcR-binding site in the stationary phase. The SmcR-binding site was determined using a DNase I protection assay \textit{in vitro} and was centered at 196.5 bp upstream of the transcription start site of PS (Fig. 1) (10, 17).

In general, activators binding this far upstream of the promoter cooperate and interact with additional transcriptional regulator(s) on the promoter DNA because the activators are not able to activate RNA polymerase (RNAP) directly (19, 20). Therefore, the additional regulatory proteins that convey the signal of the activator to RNAP and/or induce structural changes of the DNA (form-

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Regulation of V. vulnificus Elastase Gene

The elastase gene (vvpE) plays a crucial role in the virulence of V. vulnificus by catalyzing the degradation of extracellular proteins. In this study, we investigated the regulatory mechanisms of the vvpE gene expression.

**EXPERIMENTAL PROCEDURES**

**Strains, Plasmids, and Culture Conditions**—The strains and plasmids used in this study are listed in Table 1. Unless noted otherwise, the V. vulnificus strains were grown in Luria-Bertani medium supplemented with 2.0% (w/v) NaCl (LBS). All of the media components were purchased from Difco, and the chemicals were purchased from Sigma.

**ChIP Assay**—The ChIP experiments were performed using formaldehyde cross-linking as described by Rhee et al. (22). Briefly, the cross-linked chromatin in the wild type, smcR, or ihfA mutant cells (Table 1) was fragmented by sonication to result in sheared chromatin with an average length of 300 bp. When required, the average lengths of the sheared chromatin were reduced by further treatment with DNase I (at a final concentration of 5 μg/ml) (Sigma). One-half of the clarified supernatant was saved as the total input sheared chromatin (positive control) prior to the reaction with the anti-SmcR antibody (10), whereas the sheared chromatin (100 μl) from the other half of the supernatant was reacted with 10 μl of the anti-SmcR antibody overnight at 4 °C. The resulting chromatin-antibody complex was specifically precipitated with protein A-Sepharose and washed, and the sheared chromatin were eluted using the method described elsewhere (22). The cross-linkings were reversed, and DNAs were purified and analyzed by PCR using a pair of the primers specific to the vvpE promoter region as listed in Table 2.

**Generation of Mutation in the vvpE Upstream Region**—A set of mutant vvpE upstream sequences were developed by substituting a 10-bp wild type sequence with the mutant sequence (5’-GTGGATCCTC-3’) using the PCR-mediated linker-scanning mutation method as described previously (17, 23).

**TABLE 1**

| Strain or plasmid | Relevant characteristics | Reference or source |
|-------------------|--------------------------|---------------------|
| Strains: V. vulnificus | ATCC29307 | Clinical isolate |
| E. coli SM10 apr | ATCC29307, smcR::nptI | Laboratory collection |
| E. coli BL21(DE3) | ATCC29307, Δi hfA | This study |
| Plasmids | | |
| pRK415 | Clinical isolate, IncP ori, oriT of RK2; Tc' | 34 |
| pDM4 | R6Kγ ori (requires π), oriT of RP4; Cm' | 43 |
| pHK0011 | pUC18 with vvpE; Ap' | 18 |
| pCK980 | pHK0011 with 748-bp fragment of vvpE upstream region; Tc' | 32 |
| pH8201 | pH S201 with −206/-197 substitution of vvpE upstream region; Tc' | This study |
| pH8176 | pH S201 with −176/-167 substitution of vvpE upstream region; Tc' | This study |
| pH8136 | pH S201 with −136/-127 substitution of vvpE upstream region; Tc' | This study |
| pH8106 | pH S201 with −106/-97 substitution of vvpE upstream region; Tc' | This study |
| pH856 | pH S201 with −56/-47 substitution of vvpE upstream region; Tc' | This study |
| pH8221 | pH S201 with −101/-97 deletion of vvpE upstream region; Tc' | This study |
| pH8222 | pH S201 with −106/-97 deletion of vvpE upstream region; Tc' | This study |
| pH8301 | pGEM T with i hfA; Ap' | This study |
| pH8302 | pGEM T with Δi hfA; Ap' | This study |
| pH83021 | pDM4 with Δi hfA; Cm' | This study |
| pH8303 | pKK415 with i hfA; Tc' | This study |
| pH8304 | pRSET C with i hfA; Ap' | This study |
| pH8305 | pRSET C with i hfB; Ap' | This study |

*Ap’, ampicillin-resistant; Cm’, chloramphenicol-resistant; Km’, kanamycin-resistant; Tc’, tetracycline-resistant.*
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**TABLE 2**

Oligonucleotides used in this study

| Oligonucleotide* | Oligonucleotide sequence (5'–3')b | Locationc | Use(s)                  |
|------------------|----------------------------------|-----------|-------------------------|
| Hybridized to the vvpE regulatory region |
| VVPE031          | TTAATTCTGACTGGACTGTCCAACG        | –236 to –214 | ChIP assay, electrophoretic mobility shift assay, |
| VVPE032          | AGCCACATCTACACACAAAAAT           | –36 to –56  | DNasel footprinting      |
| VVPE033          | AATTCTGCTAAGACTGTCC             | –235 to –212 | Amplification of DNA S and F3 |
| VVPE034          | ACTAGTAATTCATGTCCAAG            | –158 to –177 | DNA S                   |
| VVPE035          | CTTACAGGCGGCTAAAAAC             | –75 to –57  | DNA R                   |
| VVPE036          | GACACTGAGCTACTACA               | +7 to –11   | DNA R and F             |
| Hybridized to the vvpE coding region |
| VVPE-RTF         | GCGGTTGTGTCTCTGAATTTT           | VV2_0974   | Real time PCR           |
| VVPE-RTF         | GCGGTTGTGTCTCTGAATTTT           | VV2_0974   | Real time PCR           |
| VVPE0905         | GACGTTGTGTCTCTGAATTTT           | VV2_0974   | Primer extension        |
| Hybridized to the ihfA coding region |
| ihfA01           | GATACCTGCTCTCTGCTGCTGTAATAG     | VV1_2375   | Cloning of ihfA         |
| ihfA01-1         | GATACCTGCTCTCTGCTGCTGTAATAG     | VV1_2375   | Construction of the ihfA mutant |
| ihfA02           | GTGAAGACACCCCAGCTCTGCAGAATG     | VV1_2375   | Cloning of ihfA         |
| ihfA02-1         | GTGAAGACACCCCAGCTCTGCAGAATG     | VV1_2375   | Construction of the ihfA mutant |
| ihfA05           | GATACCTGCTCTCTGCTGCTGTAATAG     | VV1_2375   | Complementation of the ihfA mutant |
| ihfA06           | GTGAAGACACCCCAGCTCTGCAGAATG     | VV1_2375   | Complementation of the ihfA mutant |
| ihfA07           | GATACCTGCTCTCTGCTGCTGTAATAG     | VV1_2375   | Purification of IhfA    |
| Hybridized to the ihfB coding region |
| ihfB01           | ATACCTGACATTGCTGCTGCTGTAATAG   | VV1_2980   | Purification of IhfB    |
| ihfB01-1         | ATACCTGACATTGCTGCTGCTGTAATAG   | VV1_2980   | Purification of IhfB    |

* The oligonucleotides were designed using the genomic sequence of V. vulnificus CMCP6 (GenBank™ accession numbers AE016795 and AE016796).
* Regions of oligonucleotides not complementary to corresponding genes are underlined.
* Shown are the oligonucleotide positions, where +1 is the transcription start site of PS. Locus tag numbers are based on the database of the V. vulnificus CMCP genome.
* The amplified vvpE upstream DNA fragments are shown in Fig. 9B.

**TABLE 3**

Oligonucleotides used in PCR-mediated mutation of the vvpE promoter

| Oligonucleotide* | Oligonucleotide sequence (5'–3')b | Locationc | Use                   |
|------------------|----------------------------------|-----------|-----------------------|
| VVPE001          | GATACCTGACATTGCTGCTGCTGTAATAG   | –335 to –314 | Construction of all pHS-lux reporters4 |
| VVPE006          | GATACCTGACATTGCTGCTGCTGTAATAG   | 392 to 413 |                       |
| A266/197         | TTTGAGATCTTCTGCTGCTGTAATAG      | –196 to –180 | pHS206                |
| B266/197         | AGAGCAGACACTGAGTTGAGGGT         | –207 to –223 | pHS206                |
| A176/167         | AATTGAGATCTTCTGCTGCTGTAATAG     | –166 to –150 | pHS176                |
| A176/167         | AATTGAGATCTTCTGCTGCTGTAATAG     | –177 to –163 | pHS176                |
| A136/127         | AATAGTGAGATCTTCTGCTGCTGTAATAG  | –126 to –110 | pHS136                |
| A106/97          | ATAGTGAGATCTTCTGCTGCTGTAATAG   | –137 to –153 | pHS136                |
| B106/97          | ATAGTGAGATCTTCTGCTGCTGTAATAG   | –96 to –81  | pHS106                |
| A66/47           | TATAGTGAGATCTTCTGCTGCTGTAATAG | –107 to –122 | pHS106                |
| B66/47           | TATAGTGAGATCTTCTGCTGCTGTAATAG | –46 to –30  | pHS556                |
| A66/47           | TATAGTGAGATCTTCTGCTGCTGTAATAG | –57 to –73  | pHS556                |
| A66/47           | TATAGTGAGATCTTCTGCTGCTGTAATAG | –96 to –70  | pHS221 and pHS222     |
| A73/87           | TATAGTGAGATCTTCTGCTGCTGTAATAG | –82 to –116 (Δ –101 to –97) | pHS221 and pHS222 |
| B206/197         | TATAGTGAGATCTTCTGCTGCTGTAATAG | –82 to –127 (Δ –106 to –97) | pHS221 and pHS222 |

* The oligonucleotides were designed using the genomic sequence of V. vulnificus CMCP6 (GenBank™ accession numbers AE016795 and AE016796).
* Regions of oligonucleotides not complementary to corresponding genes are underlined.
* Shown are the oligonucleotide positions, where +1 is the transcription start site of PS.
* Constructs are depicted in Figs. 3A and 4A.

Briefly, VVPE001 that contained an XbaI restriction site followed by bases corresponding to the 5'-end of the vvpE promoter region was used in conjugation with one of the primers of B9 to amplify the 5' amplicons. The B9 antisense primers contained the mutant sequence (Table 3). Similarly, 3' amplicons were ampliﬁed using primers A9 and VVPE006 that carries a KpnI restriction site. The A5 sense primers contained a sequence complementary to the B5 primers. Similar experimental conditions were used for deletion of internal 5- and 10-bp sequences of the vvpE regulatory region, except that primers A6 and B6 were used in place of A3 and B3, respectively, as indicated in Table 3. Second stage PCR was performed using VVPE001 and VVPE006 as a pair of primers and the mixture of the two amplicons as the template to result in the vvpE regulatory region with the substitution or deletion mutant sequences. Construction of vvpE-luxAB Transcriptional Fusions and Measurement of Cellular Luminescence—The PCR products of the vvpE regulatory region with the mutant sequences were digested with XbaI and KpnI and inserted into pHK0011 that had been digested with the same enzymes. The latter plasmid carries promoterless luxAB luciferase genes (18). The resulting vvpE-luxAB fusion pH5 reporters (Table 1; see also Figs. 3A and 4A), as conﬁrmed by DNA sequencing, were then transferred into V. vulnificus ATCC29307 by conjugation. The cellular luminescence of the cultures was measured with a luminometer (Lumat model 9501, Berthold, Germany) and expressed in arbitrary relative light units (RLUs), as described previously (10).
library of *V. vulnificus* mutants generated by random transposon mutagenesis using a mini-Tn5 lacZ1 (24). A DNA segment flanking the transposon insertion was amplified by PCR as described previously (25). Because a data base search for homology to the amino acid sequence deduced from the resulting PCR product singled out the *V. vulnificus* IhfA, a subunit of IHF, a DNA fragment containing the whole *ihfA* open reading frame was amplified by PCR using the primers, *IhfA01* and *IhfA02* (Table 2). The amplified 1,520-bp DNA fragment was ligated into pGEM-T Easy (Promega, Madison, WI) to result in pHS301 (Table 1).

**Generation of the *ihfA* Deletion Mutant**—The *ihfA* gene on pHS301 was inactivated in *vitro* by deletion of about two-thirds (164 of 288 bp) of the *ihfA* open reading frame using the PCR-mediated linker-scanning mutation method as described above. Pairs of primers *IhfA01* and *IhfA01-1* (for amplification of the 5’ amplicon) or *IhfA02* and *IhfA02-1* (for amplification of the 3’ amplicon) were designed and used as listed in Table 2. The 164-bp deleted *ihfA* was amplified by PCR using the mixture of both amplicons as the template and *IhfA01* and *IhfA02* as primers. The resulting 1,356-bp DNA fragment containing Δ*ihfA* was ligated with SphI-Sall-digested pDM4 (43) forming pHS3021. To generate the Δ*ihfA* mutant by homologous recombination, *Escherichia coli* SM10 λ pir, tra (containing pHS3021) (26) was used as a conjugal donor to *V. vulnificus* ATCC29307. The conjugation and isolation of the transconjugants were conducted using the method previously described (18).

**RNA Purification and Transcript Analysis**—Total cellular RNAs from the *V. vulnificus* strains were isolated using an RNeasy minikit (Qiagen). For quantitative reverse transcription-PCR, cDNA was synthesized with an iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer’s procedure. Real time PCR amplification of the cDNA was performed with a PCR-mediated linker-scanning mutation method as described above. The 164-bp deleted *ihfA* was amplified by PCR using the mixture of both amplicons as the template and *IhfA01* and *IhfA02* as primers. The resulting 1,356-bp DNA fragment containing Δ*ihfA* was ligated with SphI-Sall-digested pDM4 (43) forming pHS3021. To generate the Δ*ihfA* mutant by homologous recombination, *Escherichia coli* SM10 λ pir, tra (containing pHS3021) (26) was used as a conjugal donor to *V. vulnificus* ATCC29307. The conjugation and isolation of the transconjugants were conducted using the method previously described (18).

**Measurement of Elastase Activity and Data Analysis**—Cultures of the *V. vulnificus* strains were grown at 30 °C under aeration, and the growth was monitored by measuring the A600 of the cultures. The cultures were harvested at an A600 value of 2.0, and the elastase activities in the stationary phase were determined according to the procedure previously described (32). Averages ± S.E. were calculated from at least three independent experiments.

**RESULTS**

SmcR Directly Binds to the *vvpE* Regulatory Region in Vivo—Because SmcR binding at −196.5 is unusually distant for the protein to interact directly with RNAP, we examined whether the SmcR binding to the *vvpE* regulatory region occurs indeed in *V. vulnificus*. For this purpose, the cross-linked chromatin from the wild type and *smcR* mutant HS03 cells was immunoprecipitated using the antibody against SmcR (Fig. 2). As positive controls, the input chromatin from both the wild type and HS03 appeared to carry the *vvpE* regulatory region when determined based on PCR using the primers VVPE031 and VVPE032 (Fig. 2). The primers were designed to specifically amplify the

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**FIGURE 2. SmcR binding to the *vvpE* regulatory region in vivo**. The cells were cross-linked, washed, and then sonicated to produce sheared chromatin as described under “Experimental Procedures.” The DNA was purified from the sheared chromatin before precipitation (input, positive control) and after precipitation in the presence (+) or absence (−) of the anti-SmcR antibody. The DNA was then amplified by PCR using primers VVPE031 and VVPE032 (Table 2). WT, wild type; smcR, *smcR* mutant.
vvpE regulatory region that contains the SmcR-binding site and is 201 bp in length (Table 2). After reversing the cross-links, the vvpE regulatory fragment was detected in the chromatin precipitate of the wild type, induced in the presence of the anti-SmcR antibody. The presence of the vvpE regulatory region in the precipitated chromatin was caused by the specific binding of the SmcR protein to the DNA, because no vvpE regulatory DNA was detected in the precipitate induced in the absence of the anti-SmcR antibody. Consistent with this, no detectable level of the vvpE regulatory fragment was present in the anti-SmcR immunoprecipitate of the smcR mutant (Fig. 2), verifying that the SmcR protein directly binds to the vvpE regulatory region in V. vulnificus as well as in vitro.

Mapping of cis-Acting Regulatory Elements in the vvpE Regulatory Region—To delineate the cis-DNA sequences required for activation of vvpE, transcriptional fusions of the mutant vvpE regulatory regions were made to the luxAB reporter gene (Fig. 3A). Culture luminescence was used to quantify the capacity of each vvpE upstream fragment to activate vvpE (Fig. 3B). For the V. vulnificus containing pHS201, a plasmid carrying an intact vvpE regulatory region, the luminescence activity was about 3.8 × 10⁶ RLU. The luminescence was reduced in the strains that carried either pHS206, pHS176, pHS136, or pHS56, indicating that the important cis-acting regulatory element(s) for the activation of vvpE was mutated in the vvpE regulatory region of the transcriptional fusions tested.

Because the sequence for SmcR binding extended from −207 to −186 (10, 17), the reduced luminescence from the cell containing pHS206 appeared to be due to the lack of part of the SmcR binding sequence on the transcriptional fusion. The sequences from −56 to −47 scored a reasonable homology to the UP element consensus sequences of the promoters recognized by the α-CTD of E. coli RNAP (Fig. 1) (33), implying that the vvpE regulatory region of pHS56 did not harbor UP element sufficient for the activation of vvpE by the V. vulnificus RNAP. The light produced by the cells carrying either pHS176 or pHS136 was significantly reduced, suggesting that the upstream region extending from −176 to −127 is also required for activation of vvpE by an as yet unknown mechanism. Interestingly, luminescence of pHS106 did not significantly decrease and was comparable with that of pHS201, indicating that the sequences from −106 to −97 did not harbor any cis-acting regulatory elements essential for the activation of vvpE.

The Correct Phasing of SmcR Binding Is Required for the Activation of vvpE—One possible mechanism for the activation of vvpE by SmcR bound at −196.5 is that a transcription factor(s) bring SmcR to RNAP for their direct interaction. To examine this possibility, the transcriptional fusions of the vvpE regulatory region with either 5- or 10-bp deletions within the sequences from −106 to −97 were constructed to the luxAB reporter genes, and their luminescence was compared with that of pHS201 (Fig. 4). Luminescence of pHS221 carrying the 5-bp deletion from −101 to −97 significantly decreased. Because the sequences from −106 to −97 were not essential for the vvpE expression and the 5-bp deletion results in a half-integral turn of DNA, the decreased luminescence of pHS221 presumably resulted from the altered phasing of SmcR on the transcriptional fusion. In contrast, luminescence of pHS222 carrying the 10-bp deletion from −106 to −97 was greater than that of pHS201, indicating that reducing the distance between SmcR and RNAP increased the vvpE expression only when a specific phasing of SmcR was maintained. These results suggested that SmcR contacts directly with RNAP for the activation of vvpE. However, because SmcR did not appear to bend the vvpE upstream DNA (data not shown), the results suggested that the
direct contact between SmcR and RNAP was presumably mediated by yet unidentified protein(s).

Effect of ihfA Mutation on Production of Elastase—To examine the role of IHF, the V. vulnificus ihfA isogenic mutant was constructed by allelic exchanges. Double cross-overs, in which the wild type ihfA on the V. vulnificus chromosome was replaced with the ΔihfA allele, were confirmed using PCR as previously described (data not shown) (10). The ihfA mutant chosen for further analysis was named HS05 (Table 1). For the wild type, elastase was produced and reached a maximum at 29 units during stationary growth (Fig. 5A). When compared with parental wild type, HS05 produced much less elastase that was almost 4-fold lower than that of the wild type, indicating that the elastase expression is positively regulated by IHF. Real time PCR was used to confirm positive regulation of the elastase expression by IHF and revealed that the vvpE transcript level also significantly decreased as a result of ihfA inactivation. These results demonstrated that IHF activates vvpE at the transcription level.

We examined whether the introduction of pHS303 carrying a recombinant ihfA could complement the decrease of the vvpE expression in HS05. For this purpose, pHS303 was constructed by subcloning ihfA amplified by PCR using the primers IhfA05 and IhfA06 into the broad host range vector pRK415 (Table 1) (34). The elastase activity and vvpE transcript level of HS05 (pHS303) was restored to the wild type levels (Fig. 5B). Therefore, the decreased vvpE expression in HS05 was confirmed to result from the inactivation of functional ihfA rather than any polar effects on genes downstream of ihfA. Because SmcR has been previously demonstrated as a key activator for the vvpE expression (10), it is perhaps not surprising that the residual levels of elastase and vvpE transcript in HS05 were still significantly higher than those observed in the smcR mutant HS03 (Fig. 5).
The addition of IHF at a concentration of 200 nM resulted in a shift of the 201-bp DNA fragment to a single band with a slower mobility. The binding of IHF was also specific, because assays were performed in the presence of 1 μg of poly(dI-dC) as a nonspecific competitor. In a second gel mobility shift assay, the same, but unlabeled, 201-bp DNA fragment was used as a self-competitor DNA. Various amounts of the self-competitor DNA were added to the reaction mixture containing 7 nM of the labeled DNA prior to the addition of 400 nM of IHF. The DNase I footprinting performed with IHF revealed two clear protection patterns in the upstream region of PS extending from −183 to −165 (centered at −174, IHFB1) and from −137 to −125 (centered at −131, IHFB2), respectively (Figs. 1 and 8A). Several nucleotides also showed enhanced cleavages, which have been frequently observed in DNase I protection analyses of the binding sites of transcriptional regulatory proteins with DNA bending activities, such as cAMP receptor protein (10). Both sequences were almost equally protected by the same level of IHF, indicating that IHF bound to the two sites with a comparable affinity. The pattern of protection was consistent with the result of gel mobility shift assays where only a single DNA-IHF complex was produced (Fig. 7). These IHF-binding sites were further supported by the finding that the sequences spanning from −176 to −167 and −136 and −127 mapped by mutational analysis of the vvpE upstream region are essential cis-regulatory elements for the vvpE expression (Fig. 3).

IHF Induces a Protein-Protein Interaction between SmcR and RNAP—Although IHF associated with the activation of the vvpE expression was identified, its mechanism of the activation had yet to be determined. Accordingly, the cross-linked chromatin from the wild type, smcR mutant HS03, and ihfA mutant HS05 cells were immunoprecipitated using the antibody against SmcR (Fig. 9A). As positive controls, the input chromatin from the wild type, HS03, and HS05 appeared to carry the SmcR-dependent promoter PS rather than PL.

Identification of the IHF-binding Sites—As shown in Fig. 8A, the DNase I footprinting performed with IHF revealed two clear protection patterns in the upstream region of PS extending from −183 to −165 (centered at −174, IHFB1) and from −137 to −125 (centered at −131, IHFB2), respectively (Figs. 1 and 8A). Several nucleotides also showed enhanced cleavages, which have been frequently observed in DNase I protection analyses of the binding sites of transcriptional regulatory proteins with DNA bending activities, such as cAMP receptor protein (10). Both sequences were almost equally protected by the same level of IHF, indicating that IHF bound to the two sites with a comparable affinity. The pattern of protection was consistent with the result of gel mobility shift assays where only a single DNA-IHF complex was produced (Fig. 7). These IHF-binding sites were further supported by the finding that the sequences spanning from −176 to −167 and −136 and −127 mapped by mutational analysis of the vvpE upstream region are essential cis-regulatory elements for the vvpE expression (Fig. 3).

IHF Binds Specifically to the vvpE Regulatory Region—The 201-bp DNA fragment encompassing the vvpE regulatory region was incubated with increasing amounts of IHF (IhfA and IhfB) and then subjected to electrophoresis. As seen in Fig. 7, the addition of IHF at a concentration of 200 nM resulted in a shift of the 201-bp DNA fragment to a single band with a slower mobility. The binding of IHF was also specific, because assays were performed in the presence of 1 μg of poly(dI-dC) as a nonspecific competitor. In a second gel mobility shift assay, the same, but unlabeled, 201-bp DNA fragment was used as a self-competitor to confirm the specific binding of IHF to the vvpE regulatory region (Fig. 7). The unlabeled 201-bp DNA competed for the binding of IHF in a dose-dependent manner (Fig. 7), confirming that IHF binds specifically to the DNA. These results suggested that IHF activates PS by directly binding to the vvpE regulatory region.

FIGURE 6. Activities of PL and PS in V. vulnificus with different genetic backgrounds. The vvpE promoter activities were determined separately by primer extension of the RNA derived from the wild type, ihfA mutant, and complemented strain, as indicated. Total RNA was prepared from the log phase (L; A_{600} = 0.6) and stationary phase (S; A_{600} = 2.0) of each culture. Lanes G, A, T, and C represent the nucleotide sequencing ladders of pKC980. The asterisks indicate the sites of the transcription starts for PS and PL, respectively. WT, wild type; ihfA, ihfA mutant.

Effect of IHF on vvpE Expression Is Mediated through PS—The presence of two promoters for vvpE expression raises the question of whether the activation by IHF is through the stationary phase-induced promoter (PS) or the constitutive promoter (PL). To answer this question, the activities of PL and PS in the wild type and ihfA mutant were determined by primer extension analyses (Fig. 6). As such, RNAs were prepared from cultures grown to the log phase or stationary phase. PL activities were observed in the cells grown to both the log phase and the stationary phase. Plus, when determined based on the intensity of the bands of the reverse transcripts, the PL activities were not significantly changed by the inactivation of ihfA. These results were consistent with previous observations by the current authors in which the activity of PL was found to be decreased PS activity was restored to the wild type level. Accordingly, these results indicate that IHF is involved in the regulation of vvpE transcription by activating the SmcR-dependent promoter PS rather than PL.

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![Flowchart](https://via.placeholder.com/150)
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**FIGURE 8.** IHF-binding sites in the *vvpE* regulatory region. 

**A**, DNase I protection analysis of IHF binding to the *vvpE* regulatory region. Lane 1, no IHF added; lanes 2–4, IHF at 200, 400, and 600 nM, respectively. Lanes G, A, T, and C represent the nucleotide sequencing ladders of pKC980. The nucleotides showing an enhanced cleavage in the presence of IHF are indicated by the black boxes, whereas the regions protected by IHF are indicated by the shaded boxes. **B**, schematic representation of the *vvpE* regulatory region with the proposed binding sites for SmcR (SB), IHF (IHF1 and IHF2), and RNAP, where +1 is the transcription start site of PS. The arrow represents the transcriptional direction and coding region of *vvpE*, and the solid line represents the upstream region of *vvpE*, respectively. The sequences for binding of SmcR, IHF, and RNAP were determined previously (10, 18). The sequences for the binding of IHF, proposed by the DNase I protection assay, are represented by shaded boxes. The consensus sequences for the binding of IHF from *E. coli* (37) were shown below the *V. vulnificus* DNA sequence. W, A or T; R, A or G; N, any base.

**FIGURE 9.** IHF induces direct interaction between SmcR and RNAP. 

A, direct interaction between SmcR and RNAP on the *vvpE* regulatory region was analyzed using a ChIP assay. The cells were cross-linked, washed, and then sonicated to produce sheared chromatin as described under “Experimental Procedures.” DNase I was added to the sheared chromatin to reduce their average sizes. The DNA was purified from the DNase I-treated chromatin precipitate induced in the presence of IHF or the anti-SmcR antibody. The presence of functional IHF, because no R fragment was detected in the chromatin precipitate induced in the *ihfA* mutant. It was noteworthy that the direct interaction of SmcR with RNAP was dependent on the presence of functional IHF, because no R fragment was detected in the chromatin precipitate induced in the *ihfA* mutant. This dependence of SmcR on IHF for direct interaction with RNAP, along with the two IHF-binding sites observed between SmcR- and RNAP-binding sites, suggests a possible model whereby IHF introduces a bend to the *vvpE* regulatory region and thus brings SmcR to RNAP by looping the *vvpE* regulatory DNA was proposed and confirmed by ChIP assays.

**DISCUSSION**

Among global regulators controlling numerous genes contributing to pathogenesis as well as survival of the pathogenic *Vibrio* spp., *Vibrio cholerae* HapR, *Vibrio parahemolyticus* OpaR, *Vibrio anguillarum* VanT, and *V. vulnificus* SmcR have been demonstrated as LuxR homologues (4–7, 10, 15–17). Although a number of the promoters regulated by LuxR homologues have been reported (3, 11, 12, 17, 35, 36), the molecular mechanism by which the proteins modulate the expression of the promoters has yet to be extensively studied (10–12, 35). Therefore, the question of whether the LuxR homologues collaborate with any other regulator proteins and whether (and how) the LuxR homologues interact directly with RNAP for regulation of the target promoters has not yet been addressed.

IHF is a member of the nucleoid-associated proteins (for a recent review, see Ref. 21) and a heterodimeric protein composed of the α and β subunits, encoded by the *ihfA* (*himA*) and *ihfB* (*himD*) genes, respectively. Among the nucleoid-associated proteins, IHF has long been an exception because it recognizes and binds to a specific asymmetric binding site on DNA. The consensus IHF-binding sequence from *E. coli* is WATCAANNNTTR, where W stands for A or T and R stands for A or G (37). The *V. vulnificus* IHF-binding

After reversing the cross-links, both the S and R fragments were detected in the chromatin precipitate of the wild type, induced in the presence of the anti-SmcR antibody. The presence of the DNA fragment for RNAP binding (R) in the chromatin precipitate indicated that the SmcR protein directly contacts RNAP to enhance the activity of PS. Consistent with this, the R fragment was not detected in the anti-SmcR immunoprecipitate of the *smcR* mutant. It was noteworthy that the direct interaction of SmcR with RNAP was dependent on the presence of functional IHF, because no R fragment was detected in the chromatin precipitate induced in the *ihfA* mutant. This dependence of SmcR on IHF for direct interaction with RNAP, along with the two IHF-binding sites observed between SmcR- and RNAP-binding sites, suggests a possible model whereby IHF introduces a bend to the *vvpE* regulatory region and thus brings SmcR to RNAP by looping the *vvpE* regulatory DNA was proposed and confirmed by ChIP assays.

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sequences in the vvpE regulatory region, IHFB1 and IHFB2, scored 85 and 69% homologies to the consensus sequence, respectively (Fig. 8B). IHF has been known as a global regulator to control the transcriptions of over 100 genes of various functions in E. coli (38). The amino acid sequences of the V. vulnificus IhfA and IhfB were 87 and 81% identical to those of IhfA and IhfB from E. coli, respectively, and their identity appeared evenly throughout the whole proteins (data not shown). It is not yet clear whether IHF also acts as a global regulator in V. vulnificus. Nonetheless, the similarity in the binding sequences and the high level of identity in the amino acid sequences of IHF proteins from V. vulnificus and E. coli indicated that they might perform a similar function in their global gene regulation.

Although IHF can control bacterial transcription by different mechanisms, its ability to bend DNA as much as 180° and thus facilitate a protein–protein interaction between an upstream activator and RNAP represents a key feature as a transcriptional regulator (39). In addition to the SmcR-binding site within the vvpE regulatory region mentioned above, many of the known binding sites of SmcR and LuxR are also unusually distant from the promoter (17, 35). For example, LuxR binding at region A (centered at −250.5) and at region B (centered at −117) for activation of luxCDABEGH operon in V. harveyi is also exceptionally distant (35). It is apparent from this study that the ability of SmcR to function as a transcriptional activator depends on the presence of IHF that brings the protein at −165.5 to RNAP for direct contacts (Figs. 5, 6, and 9). This finding, along with the previously observed LuxR-binding sites well removed from the target promoter, suggests that the involvement of other transcriptional factors that promote direct contacts between the LuxR homologues and RNAP may be a common feature inherited in the regulation of genes by quorum sensing.

Additional levels of control for the precise coordinate expression of the virulence factors may be obtained through the participation of multiple global regulators. Elastolytic protease is a putative virulence factor that has been proposed to account for the destructive nature of V. vulnificus infections. Elastase has been hypothesized as an important virulence factor for V. vulnificus by several studies (for a recent review, see Refs. 40). Nonetheless, it is essential to understand the mechanism whereby the expression pattern and level of elastase are modified during infection to further understand the role of elastase in pathogenesis. We previously demonstrated that vvpE expression is activated by the RpoS-dependent promoter, PS, in the stationary phase, which is under the control of SmcR (10, 18). RpoS (σ^53 or σ^26) is a stationary phase-specific sigma factor and controls the expression of numerous genes responsible for increased resistance of bacteria to a range of environmental stresses (41, 42). What seems likely is that RpoS would make expression of vvpE temporally coordinated along with expression of the stress resistance genes when V. vulnificus cells encounter increased stresses imposed by the host immune defense system during infection. LuxR homologues, including SmcR, of Vibrio spp. are proposed to sense the place where their cell densities reach higher than critical levels (1, 2). It is still difficult to define the additional signal(s) integrated by IHF into the regulation of vvpE; however, it is likely that IHF allows more precise tuning of the elastase expression by optimizing SmcR activation of the RpoS-dependent promoter PS. Whereas the collaboration between RpoS, SmcR, and IHF and its implications in pathogenesis of V. vulnificus has yet to be explored further, the overall success of the organism during pathogenesis would be enhanced through this fine tuning of the temporally and spatially coordinated expression of elastase.

REFERENCES

1. Quin, C., and Greenberg, E. P. (2002) Nat. Rev. Mol. Cell Biol. 3, 685–695
2. Ng, W. L., and Bassler, B. L. (2009) Annu. Rev. Genet. 43, 197–222
3. Waters, C. M., and Bassler, B. L. (2006) Genes Dev. 20, 2754–2767
4. McDougal, D., Rice, S. A., and Kjelleberg, S. (2001) J. Bacteriol. 183, 758–762
5. Jolbling, M. G., and Holmes, R. K. (1997) Mol. Microbiol. 26, 1023–1034
6. McCarter, L. L. (1998) J. Bacteriol. 180, 3166–3173
7. Croxatto, A., Chalker, V. J., Lauritz, J., Jass, J., Hardman, A., Williams, P., Cámara, M., and Milton, D. L. (2002) J. Bacteriol. 184, 1617–1629
8. Zhu, J., Miller, M. B., Vance, R. E., Dziejman, M., Bassler, B. L., and Mekalanos, J. J. (2002) Proc. Natl. Acad. Sci. U.S.A. 99, 3129–3134
9. Beyhan, S., Bilecen, K., Salama, S. R., Casper-Lindley, C., and Yildiz, F. H. (2007) J. Bacteriol. 189, 388–402
10. Jeong, H. S., Lee, M. H., Lee, K. H., Park, S. J., and Choi, S. H. (2003) J. Biol. Chem. 278, 45072–45081
11. Kovacikova, G., and Skorupski, K. (2002) Mol. Microbiol. 46, 1135–1147
12. Lin, W., Kovacikova, G., and Skorupski, K. (2007) Mol. Microbiol. 64, 953–967
13. McDougal, D., Rice, S. A., and Kjelleberg, S. (2000) Gene 248, 213–221
14. Roh, J. B., Lee, M. A., Lee, H. J., Kim, S. M., Cho, Y., Kim, Y. J., Seok, Y. J., Park, S. J., and Lee, K. H. (2006) J. Biol. Chem. 281, 34775–34784
15. Shao, C. P., and Hor, L. I. (2001) J. Bacteriol. 183, 1369–1375
16. Lee, J. H., Rhee, J. E., Park, U., Ju, H. M., Lee, B. C., Kim, T. S., Jeong, H. S., and Choi, S. H. (2007) J. Microbiol. Biotechnol. 17, 325–334
17. Lee, D. H., Jeong, H. S., Jeong, H. G., Kim, K. M., Kim, H., and Choi, S. H. (2008) J. Biol. Chem. 283, 23610–23616
18. Jeong, H. S., Jeong, K. C., Choi, H. K., Park, K. J., Lee, K. H., Rhee, J. H., and Choi, S. H. (2001) J. Biol. Chem. 276, 13875–13880
19. Barnard, A., Wolfe, A., and Busby, S. (2004) Curr. Opin. Microbiol. 7, 102–108
20. Browning, D. F., and Busby, S. J. (2004) Nat. Rev. Microbiol. 2, 57–65
21. Dorman, C. J. (2009) Adv. Appl. Microbiol. 67, 47–64
22. Rhee, J. E., Kim, K. S., and Choi, S. H. (2005) J. Bacteriol. 187, 7870–7875
23. Lee, J. H., Kim, M. W., Kim, B. S., Kim, M. S., Lee, B. C., Kim, T. S., and Choi, S. H. (2007) J. Microbiol. 45, 146–152
24. de Lorenzo, V., Herrero, M., Jakubzik, U., and Timmis, K. N. (1990) J. Bacteriol. 172, 6668–6672
25. Kim, H. I., Lee, J. H., Rhee, J. E., Jeong, H. S., Choi, H. K., Chung, H. J., Ryu, S., and Choi, S. H. (2002) J. Microbiol. Biotechnol. 12, 318–326
26. Miller, V. L., and Mekalanos, J. J. (1988) J. Bacteriol. 170, 2575–2583
27. Lee, B. C., Lee, J. H., Kim, M. W., Kim, B. S., Oh, M. H., Kim, K. S., Kim, T. S., and Choi, S. H. (2008) Infect. Immun. 76, 1509–1517
28. Sambrook, J., and Russell, D. W. (2001) Molecular Cloning: A Laboratory Manual, 3rd Ed., pp. A1.7–A1.18, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
29. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
30. Lee, J. H., and Choi, S. H. (2006) Mol. Microbiol. 60, 513–524
31. Choi, H. K., Park, N. Y., Kim, D. I., Chung, H. J., Ryu, S., and Choi, S. H. (2002) J. Biol. Chem. 277, 47292–47299
32. Jeong, K. C., Jeong, H. S., Rhee, J. H., Lee, S. E., Chung, S. S., Starks, A. M., Escudero, G. M., Gulig, P. A., and Choi, S. H. (2000) Infect. Immun. 68, 9365

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JOURNAL OF BIOLOGICAL CHEMISTRY 9365
Regulation of V. vulnificus Elastase Gene

33. Estrem, S. T., Gaal, T., Ross, W., and Gourse, R. L. (1998) Proc. Natl. Acad. Sci. U.S.A. 95, 9761–9766
34. Keen, N. T., Tamaki, S., Kobayashi, D., and Trollinger, D. (1988) Gene 70, 191–197
35. Miyamoto, C. M., Smith, E. E., Swartzman, E., Cao, J. G., Graham, A. F., and Meighen, E. A. (1994) Mol. Microbiol. 14, 255–262
36. Pompeani, A. J., Irgon, J. J., Berger, M. F., Bulyk, M. L., Wingreen, N. S., and Bassler, B. L. (2008) Mol. Microbiol. 70, 76–88
37. Craig, N. L., and Nash, H. A. (1984) Cell 39, 707–716
38. Arfin, S. M., Long, A. D., Ito, E. T., Toller, L., Richle, M. M., Paegle, E. S., and Hatfield, G. W. (2000) J. Biol. Chem. 275, 29672–29684
39. Rice, P. A., Yang, S., Mizuuchi, K., and Nash, H. A. (1996) Cell 87, 1295–1306
40. Miyoshi, S., and Shinoda, S. (2000) Microbes Infect. 2, 91–98
41. Lange, R., and Hengge-Aronis, R. (1991) Mol. Microbiol. 5, 49–59
42. Tanaka, K., Takayanagi, Y., Fujita, N., Ishihama, A., and Takahashi, H. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 3511–3515
43. Milton, D. L., O’Toole, R., Horstedt, P., and Wolf-Watz, H. (1996) J. Bacteriol. 178, 1310–1319