The transcriptional regulator IscR integrates host-derived nitrosative stress and iron starvation in activation of the vvhBA operon in Vibrio vulnificus

Garam Choi, Kyung Ku Jang, Jong Gyu Lim, Zee-Won Lee, Hanhyeok Im, and Sang Ho Choi

From the National Research Laboratory of Molecular Microbiology and Toxicology, Department of Agricultural Biotechnology, and Center for Food Safety and Toxicology, Seoul National University, Seoul 08826, South Korea

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For successful infection of their hosts, pathogenic bacteria recognize host-derived signals that induce the expression of virulence factors in a spatiotemporal manner. The fulminating food-borne pathogen Vibrio vulnificus produces a cytolysin/he-molysin protein encoded by the vvhBA operon, which is a virulence factor preferentially expressed upon exposure to murine blood and macrophages. The Fe-S cluster containing transcriptional regulator IscR activates the vvhBA operon in response to nitrosative stress and iron starvation, during which the cellular IscR protein level increases. Here, electrophoretic mobility shift and DNase I protection assays revealed that IscR directly binds downstream of the vvhBA promoter P_vvhBA, which is unusual for a positive regulator. We found that in addition to IscR, the transcriptional regulator HlyU activates vvhBA transcription by directly binding upstream of P_vvhBA whereas the histone-like nucleoid-structuring protein (H-NS) represses vvhBA by extensively binding to both downstream and upstream regions of its promoter. Of note, the binding sites of IscR and HlyU overlapped with those of H-NS. We further substantiated that IscR and HlyU outcompete H-NS for binding to the P_vvhBA regulatory region, resulting in the release of H-NS repression and vvhBA induction. We conclude that concurrent antirepression by IscR and HlyU at regions both downstream and upstream of P_vvhBA provides V. vulnificus with the means of integrating host-derived signal(s) such as nitrosative stress and iron starvation for precise regulation of vvhBA transcription, thereby enabling successful host infection.

Upon entering the host, enteropathogenic bacteria inevitably encounter drastic environmental changes and cope with harsh conditions raised by the host immune defense system. The ability to recognize environmental changes and produce appropriate virulence factors is essential for the pathogens to survive and develop diseases in the host (1, 2). Accordingly, pathogens have evolved sophisticated mechanisms to regulate the expression of specific virulence genes in response to various host-derived signals (1, 2). In an effort to understand the virulence gene regulation, numerous transcriptional regulators have been characterized (3). Notably, a number of transcriptional regulators use an iron-sulfur (Fe-S) cluster as a cofactor to sense environmental signals. Because the Fe-S cluster is easily disrupted under host-like conditions such as oxidative and nitrosative stress and iron starvation, it allows pathogens to promptly recognize and adapt to the host environment (4, 5).

The [2Fe-2S] containing transcriptional regulator IscR functions as a sensor of the cellular Fe-S cluster status and regulates Fe-S cluster biogenesis (6, 7). When the cellular Fe-S cluster level is sufficient to occupy IscR, [2Fe-2S]-IscR (holo-IscR) represses the isc operon (iscRSL-IscC) encoding IscR along with the proteins required for Fe-S cluster biogenesis. The [2Fe-2S] occupancy of IscR decreases under oxidative stress and iron starvation, resulting in apo-IscR lacking the clusters that relieves repression of the isc operon (7–11). As a result, the cellular apo-IscR protein level increases, and Fe-S cluster biogenesis is promoted (6, 7). IscR binds to two distinct Type 1 and Type 2 DNA motifs, depending upon the [2Fe-2S] occupancy of the protein (12, 13). Although holo-IscR binds to both Type 1 and Type 2 DNA motifs, apo-IscR exclusively binds to the Type 2 DNA motif (7, 11–14).

The opportunistic human pathogen Vibrio vulnificus is a causative agent of food-borne diseases ranging from gastroenteritis to life-threatening septicemia (15, 16). It has been reported that V. vulnificus exploits various transcriptional regulators such as the cAMP receptor protein (CRP), NanR, SmcR, and HlyU for the well-coordinated expression of its virulence factors (17–20). CRP and NanR recognize depletion of specific nutrients (18, 21), whereas SmcR senses increased cell density in the host to regulate virulence genes (19, 22). In addition, IscR and HlyU are preferentially induced by host cells or in septicemic patients, respectively, and also involved in activa-

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Figure 1. Expression of vvhBA upon exposure to murine blood and RAW 264.7 cells. A, the genes induced in V. vulnificus upon exposure to murine blood were identified by RNA-seq analysis. vvhBA was selected as the most highly induced extracellular toxin-encoding gene, and its induction was confirmed by qRT-PCR. Each column represents the vvhBA transcript level in V. vulnificus exposed to murine blood relative to M9G (negative control). Error bars represent the S.E. calculated using DeSeq2 for RNA-seq and the S.D. for qRT-PCR. B, V. vulnificus was exposed to DMEM (negative control) or RAW 264.7 cells in the presence or absence of L-NMMA. The vvhBA transcript levels were determined by qRT-PCR, and the vvhBA transcript level in the cells exposed to DMEM without L-NMMA was set to 1. Error bars represent the S.D. *, p < 0.05; ***, p < 0.0005; ns, not significant.

Expression of virulence genes in V. vulnificus (20, 23, 24). Particularly, IsrR also regulates numerous virulence genes in other pathogens such as Pseudomonas aeruginosa, Yersinia pseudotuberculosis, and Erwinia chrysanthemi, highlighting the importance of IsrR for bacterial pathogenesis (25–27).

Among the virulence factors of V. vulnificus, a cytolytic hemolysin VhA is an extracellular pore-forming toxin essential for its hemolytic activity (28, 29). VhA is a product of the vvhA gene, which is cotranscribed with vhb encoding a chaperone-like protein required for the production of active VvHα (17, 30). In the present study, we discovered that the vvhBA expression is highly induced in V. vulnificus exposed to murine blood and murine macrophage RAW 264.7 cells. To elucidate the regulatory mechanisms by which V. vulnificus increases vvhBA expression in host-like conditions, the exact role of IsrR in vvhBA regulation was investigated. The vvhBA transcript and VvhA protein levels were compared in the WT and the isogenic iscr-deletion mutant (ΔiscR) under nitrosative stress and iron starvation. In addition, the combined effects of IsrR, HlyU, and the histone-like nucleoid-structuring protein (H-NS) on vvhBA regulation were analyzed at the molecular levels (31, 32). Consequently, this study demonstrated that IsrR is a sensor of host-derived nitrosative stress and iron starvation, and activates vvhBA transcription along with HlyU by relieving H-NS repression, contributing to the precise regulation of VvhA production during infection, which is essential for fitness and pathogenesis of V. vulnificus in the host.

Results

Expression of vvhBA is induced upon exposure to murine blood and macrophages

In an effort to identify virulence genes significantly induced in V. vulnificus upon invasion of the host bloodstream, transcriptomes of the bacteria exposed to murine blood or M9 minimal medium supplemented with 0.4% (w/v) glucose (M9G; negative control) were analyzed by RNA-seq. V. vulnificus exposed to murine blood differentially expressed 942 genes compared with that exposed to M9G; 491 genes were up-regulated and 451 genes were down-regulated (Dataset S1). Among the genes encoding extracellular toxins, expression of the vvhBA operon was the most elevated upon exposure to murine blood (about 26.0-fold; Fig. S1A), which was confirmed by quantitative RT-PCR (qRT-PCR) (about 81.1-fold; Fig. 1A). This result suggested that vvhBA is preferentially expressed in response to host-derived signals which exist in the murine bloodstream.

Because VvhA-dependent cytotoxicity of V. vulnificus toward murine peritoneal macrophages has been reported (33), we questioned whether the expression of vvhBA increases upon exposure to murine macrophage RAW 264.7 cells. As shown in Fig. 1B, V. vulnificus that was exposed to nitric oxide (NO)-producing RAW 264.7 cells increased vvhBA transcription compared with that exposed to Dulbecco’s modified Eagle’s medium (DMEM; negative control). Strikingly, the extent of increase in the vvhBA transcript level upon exposure to RAW 264.7 cells diminished significantly by addition of the NO synthase inhibitor L-Nω-monomethyl arginine citrate (L-NMMA) (Fig. 1B). This result indicated that NO is one of the murine macrophage-derived signals that V. vulnificus senses to induce vvhBA expression. Taken together, the combined results showed that vvhBA expression is induced under host-like conditions in response to certain host-derived signals.

IscR positively regulates vvhBA transcription

Our previous microarray analysis predicted that the expression of vvhBA is up-regulated by IsrR (24). Consistent with this, vvhBA transcript and VvhA protein levels were reduced in ΔiscR and restored by complementation (Fig. 2, A and B). This result confirmed that IsrR activates vvhBA expression mostly at the transcription level. Because IsrR exists in two forms, holo- and apo-IscR, whose regulatory characteristics are distinct from each other (12), we investigated whether apo-IscR activates vvhBA transcription. For this purpose, vvhBA transcript and VvhA protein levels were determined in the iscr3CA mutant of which the iscr coding region on the chromosome was replaced with iscr3CA encoding apo-locked IscR (34). Notably, vvhBA transcript and VvhA protein levels significantly increased in the iscr3CA mutant compared with the WT and ΔiscR (Fig. 2, C and D), demonstrating that apo-IscR is able to activate vvhBA transcription in vivo. Moreover, the iscr3CA protein level in the iscr3CA mutant was higher than the IscR protein level in the WT, possibly due to derepression of the isc
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Figure 2. The effect of iscR mutation on vvhBA expression. Total RNA and proteins were isolated from the V. vulnificus strains grown aerobically to an A_600 of 0.3. A and C, the vvhBA transcript levels were determined by qRT-PCR, and the vvhBA transcript levels in the WT were set to 1. Error bars represent the S.D. **, p < 0.005; ***, p < 0.0005; ns, not significant. B and D, the secreted VvhA and OmpU (internal control), and cellular IscR or IscR_{SCA} and DnaK (internal control) protein levels were determined by Western blot analysis. Molecular size markers (Bio-Rad) are shown in kDa. WT (pJH0311) and WT, a WT; ΔiscR (pJH0311) and ΔiscR, an iscR-deletion mutant; ΔiscR (pJH0311::iscR), an iscR-complemented strain with pKK1531; iscR_{SCA}, a strain expressing apo-locked IscR.

IscR activates vvhBA by sensing nitrosative stress and iron starvation

Because the Fe-S cluster is sensitive to nitrosative stress (4), we examined whether IscR mediates the induction of vvhBA expression in response to NO. The vvhBA transcript and VvhA protein levels were significantly elevated by an NO donor DEA NONOate, diethylammonium (Z)-1-(N,N-diethylamino)di-zen-1-ium-1,2-diolate, in the WT (Fig. 3, A and B), consistent with Fig. 1B. In contrast, vvhBA transcript and VvhA protein levels were not increased in response to DEA NONOate in ΔiscR (Fig. 3, A and B), suggesting that IscR responds to host-derived nitrosative stress and activates vvhBA transcription.

Meanwhile, our observation that many genes essential for V. vulnificus to survive under iron starvation were up-regulated in murine blood (Fig. 5B) led us to investigate the effect of iron starvation on vvhBA expression. As shown in Fig. 3, C and D, vvhBA transcript and VvhA protein levels significantly increased in the presence of an iron chelator, 2,2’-dipyridyl (DP) in the WT, which was not apparent in ΔiscR. This result revealed that IscR recognizes iron starvation as another environmental change in the host and induces vvhBA expression. Strikingly, the IscR level in the WT increased upon exposure to DEA NONOate and DP (Fig. 3, B and D), implying that induction of vvhBA expression is attributed to increased IscR levels. Combined with the previous observations (Fig. 2, C and D), we propose a model in which IscR senses nitrosative stress and iron starvation and shifts to the apo-form, which leads to release of repression of the isc operon, elevation of apo-IscR level, and subsequent activation of vvhBA transcription.

IscR binds downstream of P_{vvhBA} to activate vvhBA

To examine whether IscR directly binds to the vvhBA promoter P_{vvhBA}; electrophoretic mobility shift assays (EMSAs) were performed. Because IscR was purified and used under aerobic conditions, most purified IscR would be in the apo-form (11, 34). Addition of IscR to the radiolabeled DNA probe encompassing the P_{vvhBA} regulatory region (−353 to +148 from the transcription start site of vvhBA (17)) resulted in two retarded bands in a concentration-dependent manner (Fig. 4A). This result indicated that at least two binding sites of IscR with different DNA-binding affinities are present in the P_{vvhBA} regulatory region. The same unlabeled DNA fragment competed for IscR binding in a dose-dependent manner (Fig. 4A), confirming the specific binding of IscR to the P_{vvhBA} regulatory region.

To determine the precise location of the IscR-binding sites in the P_{vvhBA} regulatory region, DNase I protection assays were performed using the same DNA probe labeled with 6-carboxyfluorescein (6-FAM). IscR protected two regions extending from +48 to +75 (ISCRB2, centered at +61.5) and +89 to +118 (ISCRB3, centered at +103.5), respectively, from DNase I digestion (Fig. 4B). Increasing IscR levels revealed an additional protected region extending from −5 to +24 (ISCRB1, centered at +10) (Fig. 4B). The sequence of ISCRB1, ISCRB2, and ISCRB3 showed about 83, 93, and 93% identity, respectively, to the consensus IscR-binding sequence of Type 2 DNA motif, which both holo- and apo-IscR can bind (Fig. 4C) (12), supporting apo-IscR-mediated vvhBA activation (Fig. 2, C and D). Combined with the EMSA data (Fig. 4A), this result indicated that IscR binds directly to ISCRB2 and ISCRB3 with similar binding affinities, but binds relatively weakly to ISCRB1. It is noteworthy that all three binding sites of IscR are located downstream of P_{vvhBA} (Fig. 4C), which is unusual for a positive regulator.

operon (Fig. 2D) (6, 7). This result suggested that the elevated IscR_{SCA} level contributes to the increased vvhBA expression, because both holo- and apo-IscR bind to the Type 2 DNA motif with similar affinities in Escherichia coli (12).
**Figure 3. The effect of nitrosative stress and iron starvation on vvhBA and IscR expression.** Total RNA and proteins were isolated from the *V. vulnificus* strains grown aerobically to an *A*₆₀₀ of 0.3 and then exposed to 25 μM DEA NONOate for 20 min (A and B) or 50 μM DP for 10 min (C and D). A and C, the vvhBA transcript levels were determined by qRT-PCR, and the vvhBA transcript levels in the WT unexposed to DEA NONOate (A) or DP (C) were set to 1. Error bars represent the S.D. *p* < 0.05; **,** *p* < 0.0005; ns, not significant. B and D, the secreted VvhA and OmpU (internal control), and cellular IscR and DnaK (internal control) protein levels were determined by Western blot analysis. Molecular size markers (Bio-Rad) are shown in kDa. WT, a WT; ΔiscR, an iscR-deletion mutant.

**Figure 4. Specific binding of IscR to P\_vvhBA and sequences of the P\_vvhBA regulatory region.** A, a 501-bp DNA of the P\_vvhBA regulatory region (5 nM) was radiolabeled and then incubated with increasing amounts of IscR as indicated. For competition analysis, various amounts of the unlabeled DNA fragment were used as a self-competitor and added to a reaction mixture containing 5 nM radiolabeled DNA and 30 nM IscR. B1, a DNA-IscR complex; F, free DNA. B, the same DNA of the P\_vvhBA regulatory region (32.3 nM) was labeled with 6-FAM, incubated with increasing amounts of IscR as indicated, and then digested with DNase I. The regions protected by IscR are indicated by black boxes (ISCRB1, ISCRB2, ISCRB3). Nucleotide numbers shown are relative to the transcription start site of vvhBA, which was determined previously (17). C, sequence analysis of the P\_vvhBA regulatory region. The transcription start site of vvhBA and the putative translational initiation codon of VvhB are indicated by solid and dashed bent arrows, respectively. The putative −10 and −35 regions are underlined and the putative ribosome-binding site (AGGA) is boldface. The binding sequences of IscR are shown with the black boxes as described above. The binding sequences of HlyU (HLYUB; a white box) and H-NS (HNSB1, HNSB2, HNSB3, HNSB4, HNSB5, HNSB6; gray boxes) were determined later in this study (Fig. 6, C and D). The consensus sequences of the IscR-binding Type 2 DNA motif are indicated above the *V. vulnificus* DNA sequences.
HlyU and H-NS regulate vvhBA by directly binding to the P\textsubscript{vvhBA} regulatory region

To further understand how IscR activates vvhBA transcription despite binding downstream of P\textsubscript{vvhBA}, we investigated whether IscR interacts with other transcriptional regulator(s) in the P\textsubscript{vvhBA} regulatory region. Among the previously proposed transcriptional regulators of vvhBA, the exact regulatory mechanisms of HlyU and H-NS have not been elucidated yet (23, 32). Hence, the role of these two transcriptional regulators in vvhBA regulation was further examined. The vvhBA transcript and VvhA protein levels were reduced in the hlyU-deletion mutant (ΔhlyU), but elevated in the hns-deletion mutant (Δhns) (Fig. 5, A and B). The varied levels of vvhBA transcript and VvhA protein in the deletion mutants were restored by complementation (Fig. 5, C and D). This result confirmed that HlyU is a positive regulator, whereas H-NS is a negative regulator of vvhBA transcription.

Next, EMSAs revealed that each transcriptional regulator binds specifically to the P\textsubscript{vvhBA} regulatory region (Fig. 6, A and B). Based on this result, the precise binding sites of HlyU and H-NS in the P\textsubscript{vvhBA} regulatory region were determined by DNase I protection assays. HlyU clearly protected the region extending from −128 to −114 (HLYUB, centered at −121) from DNase I digestion (Fig. 6C). In addition, the regions protected by H-NS extended from −119 to −109 (HNSB1, centered at −114), −98 to −76 (HNSB2, centered at −87), +16 to +26 (HNSB3, centered at +21), +60 to +74 (HNSB4, centered at +67), +87 to +99 (HNSB5, centered at +93), and +105 to +129 (HNSB6, centered at +117) (Fig. 6D). Notably, the binding sites of both IscR and HlyU overlapped with those of H-NS, which were located throughout the P\textsubscript{vvhBA} regulatory region (Fig. 4C). In addition, IscR and HlyU bound to the P\textsubscript{vvhBA} regulatory region at 30 nm, whereas H-NS showed binding at a much higher concentration of 200 nm (Figs. 4A, and 6, A and B). The combined results suggested that IscR and HlyU compete with H-NS for binding to the P\textsubscript{vvhBA} regulatory region and exhibit higher DNA-binding affinities.

IscR and HlyU outcompete H-NS by simultaneously binding to the P\textsubscript{vvhBA} Regulatory region

To determine whether IscR and HlyU compete with H-NS for binding, EMSAs were performed using reaction mixtures containing a fixed concentration of H-NS with various amounts of IscR. As the concentration of IscR increased, the DNA–H-NS complex (B3) was completely replaced by DNA–IscR complexes (B1) (Fig. 7A). This result indicated that IscR outcompetes H-NS that binds to the P\textsubscript{vvhBA} Regulatory region. Similarly, EMSAs with a fixed concentration of H-NS and increasing amounts of HlyU revealed that HlyU also relieves H-NS binding to the P\textsubscript{vvhBA} regulatory region (Fig. 7B). Then, the effect of IscR and HlyU on the binding of each other to the P\textsubscript{vvhBA} regulatory region was examined. When IscR and HlyU were incubated together in the reaction mixture, the DNA–IscR–HlyU complexes (B4), as well as DNA–IscR (B1) and DNA–HlyU complexes (B2), were observed (Fig. 7C). This result showed that IscR and HlyU can bind simultaneously to the P\textsubscript{vvhBA} Regulatory region, and they do not compete for binding sites. Taken together, the combined results suggested that IscR and HlyU bind simultaneously with strong DNA-binding affinities to alleviate H-NS binding from the P\textsubscript{vvhBA} regulatory region. Because these three transcriptional regulators did not regulate the expression of one another (Fig. S2), we hypothesized that IscR, together with HlyU, relieves H-NS repression of vvhBA transcription by binding downstream of P\textsubscript{vvhBA}.

IscR and HlyU alleviate H-NS repression to activate vvhBA additively in vivo

To ascertain whether IscR and HlyU activate vvhBA by relieving H-NS repression \textit{in vivo}, vvhBA transcript and VvhA protein levels were compared in the WT and various deletion mutants. Interestingly, vvhBA transcript and VvhA protein levels were comparable in Δhns, the iscR hns double-deletion mutant (ΔiscRΔhns), and the h-ns-deleted iscR\textsubscript{ACA} mutant (iscR\textsubscript{ACA}Δhns) (Fig. 8, A and B). This observation that IscR did not affect vvhBA transcription in the absence of H-NS sug-
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Figure 6. Specific binding of HlyU and H-NS to P\(_{vvhBA}\). A and B, a 501-bp DNA of the P\(_{vvhBA}\) regulatory region (5 nM) was radiolabeled and then incubated with increasing amounts of HlyU (A) or H-NS (B) as indicated. For competition analysis, various amounts of the unlabeled DNA fragment were used as a self-competitor and added to a reaction mixture containing 5 nM radiolabeled DNA and 30 nM HlyU (A) or 300 nM H-NS (B). B2, a DNA-H-NS complex; B3, a DNA-H-NS complex; F, free DNA. C and D, the same DNA of the P\(_{vvhBA}\) regulatory region (32.3 nM) was labeled with 6-FAM, incubated with increasing amounts of HlyU (C) or H-NS (D) as indicated, and then digested with DNase I. The regions protected by HlyU and H-NS are indicated by a white box (HLYUB) and gray boxes (HNSB1, HNSB2, HNSB3, HNSB4, HNSB5, HNSB6), respectively. Nucleotide numbers shown are relative to the transcription start site of vvhBA.

Discussion

The \textit{V. vulnificus} vvhBA operon encodes a well-studied extracellular pore-forming toxin VvhA, which exhibits powerful hemolytic and cytolytic activities \cite{28, 33}. VvhA is actually expressed during infection and contributes to the severe intestinal tissue damage, subsequent invasion of \textit{V. vulnificus} into the bloodstream, and dissemination of the pathogen to other organs in a murine infection model \cite{29, 33}. In this study, RNA-seq analysis identified vvhBA as the most highly induced extracellular toxin-encoding gene in \textit{V. vulnificus} upon exposure to murine blood (Fig. 1A and Fig. S1A). In addition, vvhBA expression increased upon exposure to NO-producing murine macrophage RAW 264.7 cells (Fig. 1B). These observations indicate that \textit{V. vulnificus} recognizes certain host-derived signal(s) to induce vvhBA expression during infection.

Our next concern was with the host-derived signal(s) that \textit{V. vulnificus} senses to activate vvhBA. Interestingly, the Fe-S cluster containing transcriptional regulator IscR activated vvhBA transcription in response to nitrosative stress and iron starvation (Figs. 2 and 3). This IscR-mediated activation of VvhA could be beneficial for \textit{V. vulnificus} because the pathogen inevitably encounters host-derived nitrosative stress and iron starvation during infection \cite{35, 36}. In the host, NO is produced by immune cells, particularly by macrophages, as a primary antimicrobial agent \cite{35}. Simultaneously, free iron is depleted by the host iron-sequestering system to prevent outgrowth of IscR merely relieves H-NS repression. Furthermore, this result showed that IscR does not act as a roadblock for RNA polymerases \textit{in vivo} despite its unusual binding sites on the P\(_{vvhBA}\) regulatory region. Similarly, vvhBA transcript and VvhA protein levels in the \textit{hly} hns double-deletion mutant (\(\Delta hly U\Delta hns\)) were significantly lower than those in \(\Delta hns\) (Fig. S3), indicating that HlyU also does not positively affect vvhBA transcription in the absence of H-NS. These results supported our hypothesis that IscR and HlyU relieve repression of vvhBA transcription by outcompeting H-NS for binding to the P\(_{vvhBA}\) regulatory region.

The relationship between IscR and HlyU in vvhBA regulation was also investigated \textit{in vivo}. The vvhBA transcript and VvhA protein levels in the \textit{iscr} hlyU double-deletion mutant (\(\Delta iscR\Delta hly U\)) were significantly lower than those in either \(\Delta iscR\) or \(\Delta hly U\) (Fig. 8, C and D). This result revealed that these two transcriptional regulators have an additive effect in activating vvhBA transcription \textit{in vivo}. The elevated IscR\(_{3CA}\) level did not increase vvhBA transcript and VvhA protein levels in the hlyU-deleted iscR\(_{3CA}\) mutant (iscR\(_{3CA}\Delta hly U\)) compared with those in either \(\Delta iscR\) or \(\Delta hly U\) (Fig. 8, C and D), indicating that IscR alone cannot induce vvhBA expression to the WT level in the absence of HlyU. Taken together, the combined results demonstrated that both IscR and HlyU are required for complete release of H-NS repression, leading to full activation of vvhBA transcription \textit{in vivo}. 

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In general, transcriptional regulators that bind downstream of target promoters prevent the binding and progress of RNA polymerases as negative regulators (18). Nevertheless, a few positive regulatory systems such as MetR, PhoP, DnaA, and Rns still bind downstream of their target promoters (42–46). Likewise, IscR binds downstream of the target promoters to positively regulate prx3 and gbpA in V. vulnificus, the suf operon and ydiU in E. coli, and the yscW-lcrF operon in Y. pseudotuberculosis (27, 34, 39, 41). However, unlike these conserved and typical regulatory roles of IscR according to its binding sites, our results showed that V. vulnificus IscR atypically binds downstream of P_{vvhBA} as an antirepressor.

In a similar way, H-NS represses rtxA in V. vulnificus, but HlyU can disrupt the whole H-NS nucleoprotein complex by binding to a single site located far upstream of the rtxA promoter, resulting in antirepression of rtxA (38). However, our study revealed that IscR and HlyU bind simultaneously to the P_{vvhBA} regulatory region (Fig. 7C) and additively induce vvhBA transcription in vivo (Fig. 8, C and D). These results indicate that both antirepressors are required to bind upstream as well as downstream of P_{vvhBA} for complete disruption of a possible H-NS nucleoprotein complex and antirepression of vvhBA. To our knowledge, this is the first report describing the additive mode of antirepression for gene activation.

Because the Fe-S cluster is one of the most abundant enzymatic cofactors in fundamental cellular processes, the isc operon, iscRSUA-hiscBA-fdx, is highly conserved in many bacteria (10). IscR binds downstream of the iscR promoter in V. vulnificus, and iscR, yadR, and yhgl promoters in E. coli to act as a negative regulator (39, 40). In contrast, IscR binds upstream of the target promoters to positively regulate prx3 and gbpA in V. vulnificus, the suf operon and ydiU in E. coli, and the yscW-lcrF operon in Y. pseudotuberculosis (27, 34, 39, 41). However, unlike these conserved and typical regulatory roles of IscR according to its binding sites, our results showed that V. vulnificus IscR atypically binds downstream of P_{vvhBA} to activate vvhBA as an antirepressor.

Figures

**Figure 7. Interactions between IscR, HlyU, and H-NS in binding to P_{vvhBA}**

A and B. A 501-bp DNA of the P_{vvhBA} regulatory region (5 nM) was radiolabeled and then incubated with a mixture of 200 nM H-NS and increasing amounts of either IscR (A) or HlyU (B) as indicated. C, the same radiolabeled DNA (5 nM) was incubated with a mixture of 30 nM HlyU and increasing amounts of IscR as indicated. B1, a DNA-IscR complex; B2, a DNA-HlyU complex; B3, a DNA-H-NS complex; B4, a DNA-IscR-HlyU complex; F, free DNA.

invading pathogens (36). Under these hostile conditions, the IscR-mediated induction of VvhA could disrupt the macrophages and components of the iron-sequestering system (33, 37), thereby contributing to survival of V. vulnificus in the host.

Then, we tried to figure out the molecular mechanism by which IscR activates vvhBA transcription. Intriguingly, IscR directly bound downstream of P_{vvhBA} (Fig. 4), which is unusual for a positive regulator. In addition to IscR, HlyU and H-NS acted as positive and negative regulators for vvhBA transcription, respectively, by directly binding to the P_{vvhBA} regulatory region (Figs. 5 and 6). We verified that IscR and HlyU effectively relieve the H-NS binding with higher DNA-binding affinities (Fig. 7, A and B) and do not further affect vvhBA transcription in the absence of H-NS (Fig. 8, A and B, and Fig. S3). These results demonstrate that IscR and HlyU function as antirepressors against H-NS rather than direct activators for vvhBA transcription.

H-NS binds to multiple AT-rich regions throughout the promoter, which possibly results in the formation of nucleoprotein filaments and subsequent RNA polymerase trapping (31, 38). In
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Upon entering the host, V. vulnificus induces vvhBA expression in response to drastic environmental changes. IscR, along with HlyU, which is preferentially produced in the host (23), activates vvhBA by relieving H-NS repression by sensing nitrosative stress and iron starvation. Additionally, CRP activates vvhBA expression via Class I activation under certain nutrient-depleted conditions (17, 21). Meanwhile, a repressive interaction of H-NS and Fur at P\textsubscript{vvhBA} would be relieved in response to the increase in temperature and iron starvation in the host, respectively (32, 48). Taken together, the transcriptional regulators integrate diverse host-derived signals to collaboratively regulate vvhBA transcription during infection. Solid lines indicate activation of vvhBA by positive regulators, whereas dashed lines show relieved repression of vvhBA by negative regulators in the host. The transcription start site of vvhBA and the putative translational initiation codon of VvhB are indicated by solid bent arrows. The putative −10 and −35 regions, and ribosome-binding site (RBS) are underlined. ISCRB, an IscR-binding site; HLYUB, a HlyU-binding site; HNSB1, an H-NS-binding site; CRPB, a CRP-binding site; FURB, a Fur-binding site.

the legend to Fig. 9. IscR, together with HlyU, relieves H-NS repression of vvhBA by sensing nitrosative stress and iron starvation. Although the exact environmental signal affecting the regulatory activity of HlyU is still unknown, it is verified that HlyU is preferentially expressed in the host (23). CRP activates vvhBA possibly by sensing the depletion of specific nutrients (17). At the same time, H-NS repression is weakened at elevated temperatures in the host (32), and the iron-Fur complex repression is also alleviated under iron starvation (48), leading to increased vvhBA expression during infection. These transcriptional regulators take advantage of the multiple binding sites encompassing both downstream and upstream of P\textsubscript{vvhBA} for tight regulation of vvhBA in response to various host-derived signals.

Figure 8. IscR relieves H-NS repression of vvhBA in cooperation with HlyU in vivo. Total RNA and proteins were isolated from the V. vulnificus strains grown aerobically to an A\textsubscript{600} of 0.3. A and C, the vvhBA transcript levels were determined by qRT-PCR, and the vvhBA transcript levels in the WT were set to 1. Error bars represent the S.D. *, p < 0.05; **, p < 0.005; ns, not significant. B and D, the secreted VvhA and OmpU (internal control), and cellular IscR or IscR\textsubscript{3CA}, H-NS, HlyU, and DnaK (internal control) protein levels were determined by Western blot analysis. Molecular size markers (Bio-Rad) are shown in kDa. WT, a WT; Δhns, an hns-deletion mutant; ΔiscRΔhns, an iscR hns double-deletion mutant; iscR\textsubscript{3CA}Δhns, an hns-deletion mutant expressing apo-locked IscR; ΔiscR, an iscR-deletion mutant; ΔhlyU, a hlyU-deletion mutant; ΔiscRΔhlyU, an iscR hlyU double-deletion mutant; iscR\textsubscript{3CA}ΔhlyU, a hlyU-deletion mutant expressing apo-locked IscR.

Figure 9. A proposed model for the regulation of vvhBA by multiple transcriptional regulators during host infection. Upon entering the host, V. vulnificus induces vvhBA expression in response to drastic environmental changes. IscR, along with HlyU, which is preferentially produced in the host (23), activates vvhBA by relieving H-NS repression by sensing nitrosative stress and iron starvation. Additionally, CRP activates vvhBA expression via Class I activation under certain nutrient-depleted conditions (17, 21). Meanwhile, a repressive interaction of H-NS and Fur at P\textsubscript{vvhBA} would be relieved in response to the increase in temperature and iron starvation in the host, respectively (32, 48). Taken together, the transcriptional regulators integrate diverse host-derived signals to collaboratively regulate vvhBA transcription during infection. Solid lines indicate activation of vvhBA by positive regulators, whereas dashed lines show relieved repression of vvhBA by negative regulators in the host. The transcription start site of vvhBA and the putative translational initiation codon of VvhB are indicated by solid bent arrows. The putative −10 and −35 regions, and ribosome-binding site (RBS) are underlined. ISCRB, an IscR-binding site; HLYUB, a HlyU-binding site; HNSB1, an H-NS-binding site; CRPB, a CRP-binding site; FURB, a Fur-binding site.
**IsrR-mediated vvhBA activation in host environments**

In summary, this study demonstrated that vvhBA, encoding a cytolysin/hemolysin of *V. vulnificus*, is preferentially expressed upon exposure to murine blood and macrophages. The positive regulator IsrR activates vvhBA transcription by sensing host-derived nitrosative stress and iron starvation. IsrR exerts its effect additively with another positive regulator HlyU by binding downstream of P_{vvhBA} to relieve binding of the negative regulator H-NS from the P_{vvhBA} regulatory region. The collaborative regulation by multiple global regulators allows more precise tuning of vvhBA expression through integrating various host-derived signals encountered during infection, and thereby enhances the overall success of *V. vulnificus* pathogenesis.

**Experimental procedures**

**Strains, plasmids, and culture conditions**

The strains and plasmids used in this study are listed under Table S1. Unless otherwise noted, the *V. vulnificus* strains were grown aerobically in Luria-Bertani (LB) medium supplemented with 2% (w/v) NaCl (LBS) at 30°C and their growth was monitored spectrophotometrically at 600 nm (A_{600}). When required, 100 μg/ml of ampicillin was added to the medium. The murine macrophage RAW 264.7 cells were grown in DMEM containing 10% fetal bovine serum (VWR, Radnor, PA) and appropriate antibiotics (100 units/ml of penicillin G and 100 μg/ml of streptomycin (Gibco-BRL, Gaithersburg, MD)) in air supplemented with 5% CO₂ at 37°C. To induce NO production, the RAW 264.7 cells were suspended in fresh DMEM containing 500 μg/ml of L-arginine (Sigma), which is a known NO synthase inhibitor (39, 40).

**RNA purification and RNA-seq analysis**

To analyze transcriptomes differentially expressed upon exposure to murine blood, about 8 × 10⁷ CFU of *V. vulnificus* were inoculated to 800 μl of murine blood or M9G (negative control). For two biological replicates, the *V. vulnificus* cells and murine blood were prepared from two independent colonies or mice, respectively, on the same day. The mixture was incubated at 37°C with rolling for 1 h and then centrifuged at 250 × g for 3 min to harvest supernatant containing bacterial cells. Total RNA from the *V. vulnificus* cells was isolated and quantified using an RNeasy® Mini Kit (Qiagen, Valencia, CA) and a NanoDrop One® Microvolume UV-visible Spectrophotometer (Thermo Scientific, Waltham, MA), respectively. Strand-specific cDNA libraries were constructed and sequenced using HiSeq 2500 (Illumina, San Diego, CA) by Lab-Genomics (Seongnam, Gyeonggi, South Korea) as described previously (20). The raw sequencing reads were mapped to the *V. vulnificus* MO6–24/O reference genome (GenBank™ accession numbers: CP002469 and CP002470), and the expression level of each gene was calculated as reads per kilobase of transcript per million mapped sequence reads (RPKM) value using EDGE-pro version 1.3.1 (Estimated Degree of Gene Expression in PROkaryots) (51). The RPKM values were normalized and analyzed statistically using DESeq2 version 1.26.0 to identify differentially expressed genes (greater than 2-fold change with p < 0.05) when exposed to murine blood (52). All manipulations for murine blood sampling were performed following the National Institutes of Health Guidelines for Humane Treatment and approved by the Animal Care and Use Committee of Seoul National University (SNU-170116-1).

**qRT-PCR**

Relative vvhBA transcript levels in the total RNA isolated from *V. vulnificus* grown under various environmental conditions were determined by qRT-PCR. In detail, *V. vulnificus* was grown in LBS to an A_{600} of 0.5 and then exposed to RAW 264.7 cells at a multiplicity of infection of 10 or DMEM (negative control) for 1 h in the presence or absence of 500 μM ML-NMMA (Sigma), which is a known NO synthase inhibitor (50, 53). Moreover, *V. vulnificus* was exposed to 25 μM DEA NONOate (Cayman Chemical, Ann Arbor, MI) for 20 min or 50 μM DP (Sigma) for 10 min when necessary. cDNA was synthesized from 1 μg of the total RNA by using the iScript™ cDNA synthesis kit (Bio-Rad). Real-time PCR amplification of the cDNA was performed by using the Chromo 4 real-time PCR detection system (Bio-Rad) with pairs of specific primers (Table S2) as described previously (54). Relative expression levels of the vvhBA transcript were calculated by using the 16S rRNA expression level as an internal reference for normalization (54).

**Generation and complementation of ΔiscR, ΔhlyU, Δhns, Δfur, ΔiscRΔhlyU, ΔiscRΔhns, ΔiscRΔfur, ΔhlyUΔhns, ΔiscR3CAΔhlyU, and ΔiscR3CAΔhns**

ΔiscR JK093, the ΔiscR_{3CA} mutant JK128, and ΔhlyU ΔZJW141 were previously constructed and used in this study (Table S1) (24, 34, 54). For construction of Δhns, the hns gene was inactivated in *vvhBA* deletion by deletion of the ORF of hns (228 of 408 bp) using the PCR-mediated linker-scanning mutation method as described previously (41). Briefly, the primer pairs HNS01-F and HNS01-R, and HNS02-R were used for amplification of the 5' and 3' amplicon, respectively (Table S2). The 288-bp deleted hns was amplified by PCR using a mixture of both amplicons as the template, and HNS01-R and HNS02-R as primers. The resulting product was ligated into Spel-SphI-digested pDM4 to generate pEJ1502 (Table S1) (55). Similarly, pJK1121 carrying the 292-bp deleted fur on pDM4 was constructed using the primers FUR01-F, FUR01-R, FUR02-F, and FUR02-R (Table S2). *E. coli* S17-1 Δpir strain containing pEJ1502, pJK1121, or pZW1401 was used as a conjugational donor to *V. vulnificus* MO6–24/O, ΔiscR, ΔhlyU, the ΔiscR_{3CA} mutant to construct Δhns JK151, Δfur JK114, ΔiscRΔhlyU JK161, ΔiscRΔhns JK151, ΔiscRΔfur JK116, ΔhlyUΔhns JK152, ΔiscR_{3CA}ΔhlyU JK1611, or ΔiscR_{3CA}Δhns JK1620, respectively (Table S1) (54, 56). The conjugation and isolation of transconjugants were conducted using the method described previously (41).

To complement the mutations, pZW1510 carrying the hlyU gene on the broad host-range vector pJH0311 was used in this study (Table S1) (54, 57). Similarly, the ΔiscR and Δhns genes were amplified by PCR using pairs of specific primers listed in Table S2 and cloned into pJH0311 to create pKK1531 and pGR1713, respectively (Table S1). The plasmids were transferred into the appropriate mutants by conjugation as described above.
Protein purification and Western blot analysis

To overexpress IscR and HlyU, pK0928 carrying the iscR gene on pET-22b(+) (Novagen, Madison, WI) and pYU1317 carrying the hlyU gene on pET-28a(+) (Novagen) were constructed previously and used in this study (Table S1) (24, 54). Similarly, the hns and ompU genes were subcloned into pET-28a(+) and pHis-parallel1 using pairs of specific primers listed in Table S2 to create pKK1636 and pKK1615, respectively (Table S1)(58). Because injection of the VvhA protein is toxic to rabbit, the 5’-terminal region of vvhA (amino acids 1–261) was amplified by PCR using the primer pair VVHBA01-F and VVHBA01-R (Table S2), subcloned into pET-28a(+) to create pKK1643 (Table S1). The resulting His6-tagged IscR, HlyU, H-NS, OmpU, and truncated VvhA were expressed in E. coli BL21 (DE3) and purified by affinity chromatography according to the manufacturer’s procedure (Qiagen).

The purified IscR, HlyU, H-NS, OmpU, and truncated VvhA were used to raise rabbit polyclonal antibodies against the respective V. vulnificus proteins (AB Frontier, Seoul, South Korea). For Western blot analysis, V. vulnificus exposed to various environmental conditions were harvested and fractionated into cells and supernatants by centrifugation. The cells were lysed using B-PERTM Bacterial Protein Extraction Reagent with 100 µl of BSA (pore size 0.2 µm; GE Healthcare, Chicago, IL) and concentrated using Amicon Ultra-15 (cut-off 30 kDa; Millipore, Burlington, MA). The protein levels of IscR, HlyU, H-NS, and DnaK in the clear cell lysates or VvhA and OmpU in the supernatant concentrates were determined as described previously (20, 41).

EMS A and DNase I protection assay

For EMSAs, a 501-bp vvhBA promoter region (−353 to +148 from the transcription start site of vvhBA) was amplified by PCR using unlabeled VVHBA02-F and [γ-32P]ATP-labeled VVHBA02-R as primers (Table S2). The radiolabeled probe DNA was then incubated with purified IscR, HlyU, and H-NS for 30 min at 30°C in a 20-µl reaction mixture containing binding buffer (40 mM Tris-Cl (pH 7.9), 70 mM KCl, 1 mM DTT, and 100 µg of BSA) and 0.1 µg of poly(dI-dC) (Sigma) as a nonspecific competitor. For competition analysis, the same but unlabeled 501-bp DNA fragment was used as a self-competitor DNA. Electrophoretic analysis of the DNA-protein complexes was performed as described previously (20).

Similarly, the same 501-bp vvhBA promoter region was amplified by PCR using unlabeled VVHBA02-F and 6-FAM-labeled VVHBA02-R as primers for DNase I protection assays (Table S2). The binding of IscR, HlyU, and H-NS to the labeled DNA was performed as described above, and DNase I digestion of DNA-protein complexes followed the procedures as described previously (54). The digested DNA products were precipitated with ethanol and eluted in sterilized H2O, and then analyzed using an ABI 3730xl DNA analyzer (Applied Biosystems, Foster City, CA) with Peak Scanner™ Software version 1.0 (Applied Biosystems) (59).

IscR-mediated vvhBA activation in host environments

Data analysis

Averages and S.D. were calculated from at least three independent experiments. Statistical analysis was performed by the Student’s t-test using GraphPad Prism 7.0 (GraphPad Software).

Data availability

All data presented in this paper are contained within the manuscript and supporting information. The raw data of RNA-seq analysis were deposited in NCBI Sequence Read Archive (SRA) database under accession numbers PRJNA560127.


Acknowledgments—We thank Eun Jung Na and Bityeoul Kim for kindly providing Δhns EJ151 and the HlyU-expressing plasmid pYU1317, respectively. We are also grateful to all members of the Choi laboratory for valuable discussions and technical support.

References

IscR-mediated vvhBA activation in host environments


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**IscR-mediated vvhBA activation in host environments**


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**IscR-mediated vvhBA activation in host environments**


The transcriptional regulator IscR integrates host-derived nitrosative stress and iron starvation in activation of the \textit{vvhBA} operon in \textit{Vibrio vulnificus}
Garam Choi, Kyung Ku Jang, Jong Gyu Lim, Zee-Won Lee, Hanhyeok Im and Sang Ho Choi

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SUPPORTING INFORMATION

IscR integrates host-derived nitrosative stress and iron starvation to activate the cytolysin/hemolysin vvhBA operon of Vibrio vulnificus

Garam Choi, Kyung Ku Jang, Jong Gyu Lim, Zee-Won Lee, Hanhyeok Im, and Sang Ho Choi

Table S1. Bacterial strains and plasmids used in this study.

Table S2. Oligonucleotides used in this study.

Figure S1. Extracellular toxin-encoding genes and iron uptake-related genes up-regulated upon exposure to murine blood.

Figure S2. Cellular protein levels of IscR, HlyU, and H-NS are unaffected by one another.

Figure S3. HlyU relieves H-NS repression of vvhBA in vivo.

Figure S4. The effect of fur mutation on vvhBA expression.

Dataset S1. List of differentially expressed genes in V. vulnificus exposed to murine blood. (Excel file)
Table S1. Bacterial strains and plasmids used in this study.

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
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*Tp<sup>r</sup>, trimethoprim-resistant; Sm<sup>r</sup>, streptomycin-resistant; Cm<sup>r</sup>, chloramphenicol-resistant; Ap<sup>r</sup>, ampicillin-resistant; Km<sup>r</sup>, kanamycin-resistant; MCS, multiple cloning site.
Table S2. Oligonucleotides used in this study.

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<td><strong>For EMSA and DNase I protection assay</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VVHBA02-F</td>
<td>CCACACCTTTTTTATGCTAATC</td>
<td>Amplification of P_{vvhB}</td>
</tr>
<tr>
<td>VVHBA02-R</td>
<td>GGCTGACATTTTGTTGATAC</td>
<td>regulatory region</td>
</tr>
</tbody>
</table>

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

*b Regions of oligonucleotides not complementary to the corresponding genes are underlined.*
Figure S1. Extracellular toxin-encoding genes and iron uptake-related genes up-regulated upon exposure to murine blood. Among the *V. vulnificus* genes identified to be induced upon exposure to murine blood by RNA-seq analysis, the extracellular toxin-encoding genes (A) or iron uptake-related genes (B) were presented. Each column represents the expression of each ORF in *V. vulnificus* exposed to murine blood relative to M9G (negative control). Error bars represent the S.E. calculated using DeSeq2. Locus tags are based on the *V. vulnificus* MO6-24/O genome sequence (GenBank™ accession numbers: CP002469 and CP002470), and the product of each gene is presented on the right.
Figure S2. Cellular protein levels of IscR, HlyU, and H-NS are unaffected by one another. Total proteins were isolated from the *V. vulnificus* strains grown aerobically to an *A*₆₀₀ of 0.3 (Log phase) and 2.5 (Stationary phase). The cellular IscR, HlyU, H-NS, and DnaK (internal control) protein levels were determined by Western blot analysis. Molecular size markers (Bio-Rad) are shown in kDa. WT, a WT; ΔiscR, an iscR-deletion mutant; ΔhlyU, a hlyU-deletion mutant; Δhns, an hns-deletion mutant.
Figure S3. HlyU relieves H-NS repression of vvhBA in vivo. Total RNA was isolated from the V. vulnificus strains grown aerobically to an A600 of 0.3. The vvhBA transcript levels were determined by qRT-PCR, and the vvhBA transcript level in the WT was set to 1. Error bars represent the S.D. **, p < 0.005; ns, not significant. WT, a WT; Δhns, an hns-deletion mutant; ΔhlyUΔhns, a hlyU hns double-deletion mutant.
Figure S4. The effect of fur mutation on vvhBA expression. Total RNA was isolated from the *V. vulnificus* strains grown aerobically to an $A_{600}$ of 0.3. The *vvhBA* transcript levels were determined by qRT-PCR, and the *vvhBA* transcript level in the WT was set to 1. Error bars represent the S.D. ***, $p < 0.0005$; ****, $p < 0.0001$. WT, a WT; Δfur, a fur-deletion mutant; ΔiscRΔfur, an iscR fur double-deletion mutant.