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Low Cell Density Regulator AphA Upregulates the Expression of *Vibrio vulnificus iscR* Gene Encoding the Fe-S Cluster Regulator IscR

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IscR is a global transcriptional regulator that contributes to the pathogenesis of *Vibrio vulnificus*, a food-borne pathogen. In the present study, the regulatory mechanism for the *iscR* expression of *V. vulnificus* was evaluated. The expression of *iscR* was found to be upregulated by a transcriptional regulator AphA, a homologue of the low cell density regulator AphA of the *Vibrio* species, in the exponential phase of growth. The promoter activity of *iscR* appeared to be activated and repressed by AphA and IscR, respectively. EMSA and DNase I protection assay showed that both AphA and IscR bind to the *iscR* regulatory region and the binding site for AphA overlapped with part of the binding site for IscR. Further mutational analysis suggested that AphA upregulates the *iscR* expression only in the presence of functional IscR. An examination of the roles of AphA and the binding sites revealed that the binding of AphA would hinder the IscR-mediated repression of the *iscR* transcription. The combined results show that *V. vulnificus* AphA upregulates *iscR* expression by antagonizing its negative autoregulation.

Keywords: *V. vulnificus* AphA, *V. vulnificus iscR*

Introduction

The opportunistic Gram-negative pathogen *Vibrio vulnificus* has the ability to cause life-threatening septicemia contracted through the consumption of contaminated seafood as well as wound infections acquired through contact with the pathogen in shellfish or seawater (Strom and Paranjpye, 2000; Horseman and Surani, 2011). Numerous virulence factors account for the fulminating and destructive nature of *V. vulnificus* infections and contribute to survival and multiplication on or within a host as well as to disease development (for a recent review, Jones and Oliver, 2009). Most of the virulence factors of pathogenic bacteria act cooperatively to obtain maximum effectiveness during pathogenesis while their expression is coordinately controlled by common global regulators (Cotter and DiRita, 2000; Matson *et al*., 2007). This complex regulatory network from sensing environmental signals to expression of virulence factors is crucial for the overall success of pathogens during infection (Cotter and DiRita, 2000; Matson *et al*., 2007).

IscR is an iron-sulfur (Fe-S) cluster containing transcription factor and controls Fe-S cluster biogenesis by sensing the cellular Fe-S cluster status in *Escherichia coli* (Schwartz *et al*., 2001; Giel *et al*., 2013). When the cellular level of Fe-S cluster is adequate to occupy IscR, the resulting [2Fe-2S]-IscR (holo-IscR) represses the *isc* operon (*iscRSUA-hscBA-fdx*) encoding IscR and the proteins required for the biogenesis of the Fe-S cluster (for recent reviews, Johnson *et al*., 2005; Py and Barras, 2010). The repression is relieved by decreased [2Fe-2S] occupancy of IscR under the conditions of iron starvation or oxidative stress which are highly detrimental to maintaining the cluster, leading to an increase of the Fe-S cluster biogenesis (Zheng *et al*., 2001; Outten *et al*., 2004; Imlay, 2006; Yeo *et al*., 2006; Giel *et al*., 2013). IscR also controls 40 more genes including the genes involved in Fe-S cluster biogenesis and anaerobic respiration (Giel *et al*., 2006). Moreover, it has been reported that IscR proteins play essential roles in the virulence of pathogens such as *Erwinia chrysanthemi, Shigella flexneri*, and *Pseudomonas aeruginosa* (Rincon-Enriquez *et al*., 2008; Runyen-Janecky *et al*., 2008; Kim *et al*., 2009).

AphA is a PadR-family transcription factor that initiates the virulence cascade in *Vibrio cholerae* by activating the transcription of *tcpPH* with the LysR-type regulator AphB, resulting in the production of cholera toxin (CT) and toxin-coregulated pilus (TCP) (Kovacikova and Skorupski, 1999; Kovacikova *et al*., 2004). It is known that AphA is required for the virulence and biofilm formation of *V. cholerae* and *Vibrio parahaemolyticus* (Kovacikova and Skorupski, 2001; Yang *et al*., 2010; Wang *et al*., 2013). In addition, AphA is known as a master regulator of quorum sensing that operates at low cell density (LCD) in *Vibrio harveyi* and *V. cholerae*. At LCD, Qrr (quorum regulatory RNA) small noncoding RNAs activate the production of AphA, and AphA and Qrrs repress the production of LuxR (*V. harveyi*) or HapR (*V. cholerae*), the master quorum-sensing regulator that operates at high cell density (HCD) (Rutherford *et al*., 2011). Conversely, at HCD, LuxR or HapR represses the production of AphA. This reciprocal regulation enables AphA to be produced abundantly at LCD, in turn, effectively control the expression of its regulon (Kovacikova *et al*., 2004, 2005; Rutherford *et al*., 2011; van Kessel *et al*., 2013). Therefore, AphA could serve as a link between quorum sensing and

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Table 1. **Plasmids and bacterial strains used in this study**

Strain or plasmid	Relevant characteristics [a]	Reference or source
Bacterial strains		
V. vulnificus		
M06-24/O	Clinical isolate; virulent	Laboratory collection
JK093	M06-24/O with Δ*iscR*	Lim and Choi (2013)
JK131	M06-24/O with Δ*aphA*	This study
JK132	M06-24/O with Δ*aphA*, Δ*iscR*	This study
E. coli		
DH5α	λ^- ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17* (r_K^- m_K^-) *supE44 thi-1 gyrA relA1*; plasmid replication	Laboratory collection
S17-1λ*pir*	λ-*pir* lysogen; *thi pro hsdR hsdM*$^+$ *recA* RP4-2 Tc::Mu-Km::Tn7;Tp^r Sm^r; host for π-requiring plasmids; conjugal donor	Simon *et al.* (1983)
BL21(DE3)	F^- *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm* (DE3)	Laboratory collection
Plasmids		
pGEM-T Easy	PCR product cloning vector; Ap^r	Promega
pJK1201	pGEM-T Easy with 321-bp fragment of *iscR* upstream region; Ap^r	This study
pDM4	R6K γ *ori sacB*; suicide vector; *oriT* of RP4; Cm^r	Milton *et al.* (1996)
pJK1126	pDM4 with Δ*aphA*; Cm^r	This study
pET28a(+)	His_6 tag fusion expression vector; Km^r	Novagen
pJK0903	pET28a(+) with *aphA*; Km^r	This study
pJK0928	pET22b(+) with *iscR*; Ap^r	Lim and Choi (2013)
pBAD24	ColE1 *ori*; *ara*BAD promoter; Ap^r	Guzman *et al.* (1995)
pJK1011	pBAD24 with *aphA*; Ap^r	This study
pBBR-*lux*	Broad host range vector with promoterless *luxCDABE*; Cm^r	Lenz *et al.* (2004)
pJK1307	pBBR-*lux* with 456-bp fragment of *iscR* upstream region; Cm^r	This study
pJK1312	pBBR-*lux* with 164-bp fragment of *iscR* upstream region; Cm^r	This study
pJK1313	pBBR-*lux* with 148-bp fragment of *iscR* upstream region; Cm^r	This study

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

virulence gene expression in *Vibrio* species.

Recently, we have reported that *V. vulnificus* IscR is a global regulator required for pathogenesis and represses the *isc* operon including *iscR* itself (Lim and Choi, 2013). In an effort to further examine the regulatory characteristics of *iscR*, we observed that *V. vulnificus* AphA upregulates the expression of *iscR* during exponential growth. Then, we demonstrated that the *iscR* transcription is directly activated by AphA and repressed by IscR, respectively. Mutational analysis revealed that AphA upregulates the *iscR* transcription only in the presence of functional IscR and the result was confirmed by deletion analysis of the *iscR* regulatory region. It appears that AphA upregulates *iscR* expression by antagonizing its negative autoregulation.

Materials and Methods

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 LB medium supplemented with 2.0% (wt/vol) NaCl (LBS) at 30°C.

Generation of *aphA* and *aphA iscR* mutants

The *aphA* gene was inactivated *in vitro* by deletion of the *aphA* ORF (373-bp of 540-bp) using a PCR-mediated linker-scanning mutation method as described previously (Kim *et al.*, 2011). The primer pairs APHA001F and APHA001R (for amplification of the 5′ amplicon) and APHA002F and APHA002R (for amplification of the 3′ amplicon) were designed (Table 2). The 373-bp deleted *aphA* was amplified by PCR using a mixture of both amplicons as the template and APHA001F and APHA002R as primers. The resulting 1,730-bp DNA fragment containing the deleted *aphA* was ligated with SpeI-SphI-digested pDM4 (Milton *et al.*, 1996) to generate pJK1126 (Table 1). The *E. coli* S17-1 λ *pir*, *tra* strain (containing pJK1126) (Simon *et al.*, 1983) was used as a conjugal donor to *V. vulnificus* MO6-24/O and JK093 to generate the *aphA* mutant JK131 and *aphA iscR* double mutant JK132, respectively, as indicated in Table 1. The conjugation and isolation of the transconjugants were conducted as previously described (Kim *et al.*, 2011).

RNA purification and transcript analyses

Total cellular RNAs were isolated from the *V. vulnificus* strains grown in LBS using an RNeasy Mini Kit (Qiagen, USA) (Kim *et al.*, 2012). For quantitative real-time PCR (qRT-PCR), cDNA was synthesized using an iScript™ cDNA Synthesis Kit (Bio-Rad, USA), and real-time PCR amplification of the cDNA was performed by using a Chromo 4 real-time PCR detection system (Bio-Rad) with the specific primer pairs for each gene listed in Table 2. Relative expression levels of the specific transcripts were calculated by using the 16S rRNA expression level as the internal reference for normalization as described previously (Kim *et al.*, 2012).

Table 2. Oligonucleotides used in this study

Oligonucleotide	Sequence (5′→3′)[a]	Use
APHA001F	CAACGAGAACTTGATGCAAAGCG	Deletion of *aphA*
APHA001R	TAAGGATCCATAGAAGCGGAGAATTCTTT	
APHA002F	TATGGATCCTTACGCCGTAATTTGATG	Deletion of *aphA*
APHA002R	CCCACACTTAACCTGTATTGATGCC	
APHA003F	CCATGGTTGCATCAACAAGAATGTAAA	Amplification of *aphA* ORF, purification of AphA
APHA003R	GTCGACGCCGAGGATATCCAGTT	
APHA004F	CCATGGTGTCATTACCACACGTAAT	Amplification of *aphA* ORF, *E. coli* dual plasmid system assay
APHA004R	CTGCAGCCACATTGTCTAGCGTA	
ISCR005F	AATAAAATGCGTCGATTGTTCAG	Amplification of *iscR* regulatory region (-194 ~+127)[b], Primer extension, EMSA, DNase I protection assay
ISCR005R	CTCGGAAATATCAGCCAGAGG	
ISCR006F	GAGCTCCAACAAAGCCTATATCCA	Amplification of *iscR* regulatory region (-329 ~+89)[b], *E. coli* dual plasmid system assay
ISCR006R	ACTAGTAATGCAGTGCCACATCCA	
ISCR007F	GAGCTCATAGGGATGAATACCTGACTAT	Amplification of *iscR* regulatory region (-75 ~+89)[b], *E. coli* dual plasmid system assay
ISCR008F	GAGCTCGACTATTTTAGTCAAATAAATACTTG	Amplification of *iscR* regulatory region (-59 ~+89)[b], *E. coli* dual plasmid system assay
ISCR_qRTF	GATATGCGGTAACGGCAATGCT	qRT-PCR
ISCR_qRTR	TAAGAGAGCGAAATCCCCTGACG	
APHA_qRTF	TTGGAACCGCAAGAAGGCAA	qRT-PCR
APHA_qRTR	AGTTGGGTGGGCTGTCGGTT	

[a] Regions of oligonucleotides not complementary to corresponding genes are underlined.
[b] Shown are the oligonucleotide positions, where +1 is the transcription start site of *iscR*.

To determine the transcription start site (TSS) of the *iscR* gene, a primer extension analysis was conducted as descried previously (Park *et al*., 2012). An end-labeled 21-base primer ISCR005R (Table 2) complementary to the coding region of *iscR* was added to the RNA and then extended with Super-Script II RNase H^- reverse transcriptase (Invitrogen, USA). The cDNA products were purified and resolved on a sequencing gel alongside sequencing ladders generated from pJK1201 (Table 1) with the same primer used for the primer extension. The primer extension products were visualized using a phosphorimage analyzer (BAS1500, Fuji Photo Film Co. Ltd., Japan).

Overexpression and purification of *V. vulnificus* AphA and IscR

The coding region of the *aphA* gene was amplified by a PCR using *V. vulnificus* MO6-24/O chromosomal DNA and primers APHA003F and APHA003R (Table 2). The 621-bp PCR product was subcloned into a His_6 tag expression vector, pET28a(+) (Novagen, USA), to result in pJK0903 (Table 1). His-tagged AphA protein was then expressed in *E. coli* BL21 (DE3) and purified by affinity chromatography according to the manufacturer's procedure (Qiagen). Similarly, the expression and purification of the His-tagged IscR were carried out using pJK0928 (Table 1), as described previously (Lim and Choi, 2013).

Electrophoretic mobility shift assay (EMSA) and DNase I protection assay

For EMSA, the 321-bp *iscR* regulatory region, extending from residues -194 to +127 from the TSS of *iscR*, was amplified by a PCR using ^{32}P-labeled ISCR005F and unlabeled ISCR005R as the primers (Table 2). The labeled 321-bp DNA (5 nM) fragment was incubated with varying concentrations of purified His-tagged IscR for 30 min at 30°C in a 20-μl reaction mixture containing 1× IscR-binding buffer (Giel *et al*., 2006) and 0.1 μg of poly(dI-dC) (Sigma). The protein-DNA binding reactions with AphA were the same as those with IscR, except that the 1× AphA-binding buffer was used (Kovacikova and Skorupski, 2001). Electrophoretic analyses of the DNA-protein complexes were performed as described previously (Kim *et al*., 2011).

The same 321-bp regulatory region was labeled by PCR amplification using a combination of ^{32}P-labeled and unlabeled primers, ISCR005F and ISCR005R, and used for DNase I protection assays. The binding of IscR or AphA to the labeled DNA was performed as described above for the EMSA, and DNase I digestion of the DNA-protein complexes followed the procedures previously described (Kim *et al*., 2011). After precipitation with ethanol, the digested DNA products were resolved on a sequencing gel alongside of sequencing ladders of pJK1201 generated using either ISCR005F (for the coding strand) or ISCR005R (for the non-coding strand) as the primer. The gels were visualized as described above for the primer extension analysis.

E. coli dual plasmid system

The coding region of the *aphA* gene was amplified by a PCR using a set of primers APHA004F and APHA004R (Table 2). The PCR product was subcloned into the pBAD24 vector and under an arabinose-inducible promoter (Guzman *et al*., 1995), to result in pJK1011 (pBAD-*aphA*) (Table 1). A set of *iscR-luxCDABE* transcriptional fusion reporters was created by subcloning a series of DNA fragments that overlapped the *iscR* regulatory region into pBBR-*lux* carrying promoterless *luxCDABE* (Lenz *et al*., 2004). Primer sets ISCR006F and ISCR006R (for pJK1307), ISCR007F and ISCR006R (for

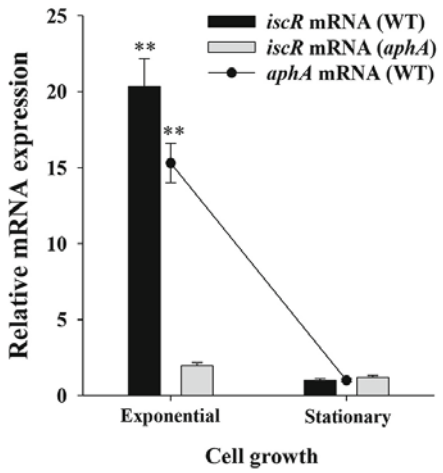

Fig. 1. Growth phase-dependent expression of *iscR* and *aphA*. Cultures of the wild type and the *aphA* mutant grown with LBS were harvested at A_{600} of 0.5 (for exponential phase) or 1.5 (for stationary phase) and used to isolate total cellular RNA. The relative mRNA level of *iscR* or *aphA* was determined by qRT-PCR analyses and normalized to the 16S rRNA expression level. The *iscR* or *aphA* mRNA level of the wild type grown to stationary phase was presented as 1. Error bars represent the SEM. **, $P<0.005$ relative to the wild type grown to stationary phase. WT, wild type; *aphA*, *aphA* mutant JK131.

pJK1312), and ISCR008F and ISCR006R (for pJK1313) were used in the PCR reactions (Table 1 and 2). The resulting *iscR-luxCDABE* fusion pJK reporters (Table 1; see also Fig. 6) were confirmed by DNA sequencing. *E. coli* DH5α strains were cotransformed with pBAD24 (control) or pJK1011 and one of the pJK reporters. The cells were grown at 37°C in LB media containing 100 μg/ml (wt/vol) ampicillin, 20 μg/ml (wt/vol) chloramphenicol, and 0.2% (wt/vol) arabinose (Sigma). The luminescence in the exponential-phase cells was measured with an Infinite™ M200 microplate reader (Tecan, Switzerland). The relative luminescence unit (RLU) was calculated by dividing the luminescence by the A_{600}, as described previously (Hwang *et al.*, 2013).

Data analyses and statistics

Averages and standard errors of the means (SEM) were calculated from at least three independent experiments. All data were analyzed by Student's *t* tests with the SAS program (SAS software; SAS Institute Inc.). Significance of differences between experimental groups was accepted at a *P* value of <0.005.

Results

Effects of the cell growth and *aphA* mutation on the *iscR* expression

In order to examine the effect of the *V. vulnificus* growth on the expression of *iscR*, the levels of *iscR* mRNA in the wild type were determined in exponential or stationary growth

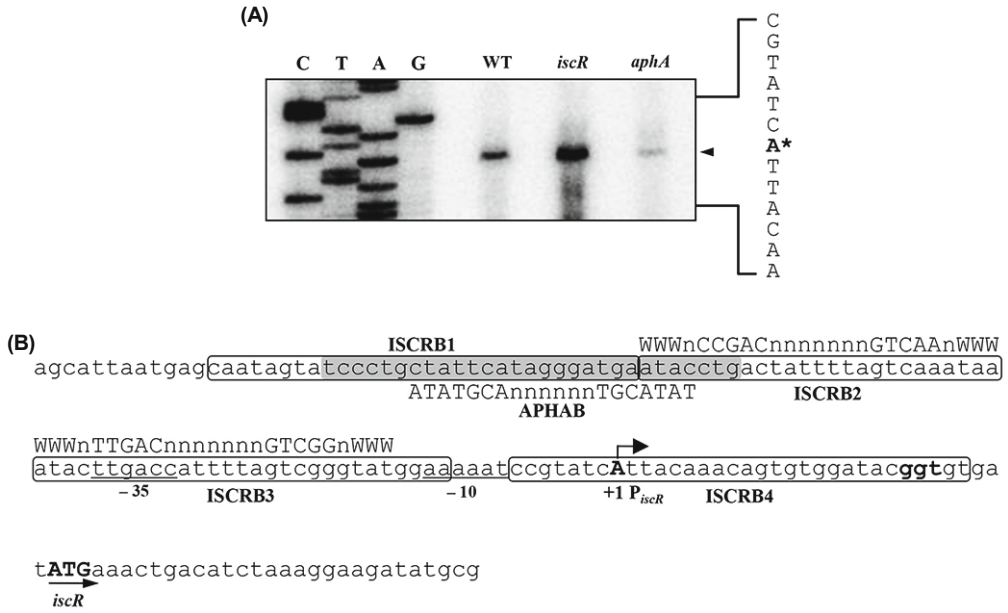

Fig. 2. Activities of P_{iscR} in *V. vulnificus* with different genetic backgrounds and sequence analysis of the *iscR* regulatory region. (A) The P_{iscR} activities were determined separately by primer extension of the RNA derived from the wild type and isogenic mutants as indicated. Each total RNA was prepared from the exponential-phase cells (A_{600} 0.5). Lanes *G, A, T,* and *C* represent the nucleotide sequencing ladders of pJK1201. An *asterisk* indicates the transcription start site (TSS) of P_{iscR}. WT, wild type; *iscR*, *iscR* mutant JK093; *aphA*, *aphA* mutant JK131. (B) TSS of P_{iscR} is indicated by *bent arrow*, and the positions of putative -10 and -35 regions are *underlined*. The IscR- and AphA-binding sequences (ISCRB1, 2, 3, and 4 and APHAB) determined later in this study (from the results of both coding and noncoding strands; Fig. 4) are represented as *open boxes* and a *gray box*, respectively. The ATG translation initiation codon and the putative ribosome-binding site (ggt) are indicated in *boldface*. The consensus sequences for the binding of IscR from *E. coli* (Rajagopalan *et al.*, 2013) and AphA from *V. parahaemolyticus* (Sun *et al.*, 2012) are shown above and below the *V. vulnificus* DNA sequence, respectively. *W*, A or T; *Y*, C or T; *R*, A or G; *n*, any base.

phase with qRT-PCR. The level of *iscR* mRNA in exponential-phase cells was more than 20-fold higher when compared to that in the stationary-phase cells (Fig. 1), suggesting that *V. vulnificus iscR* is primarily expressed during exponential growth. Because it has been reported that AphA is a master quorum sensing regulator operating at low cell density (LCD) in *Vibrio* species (Rutherford *et al*., 2011), we tested whether *V. vulnificus* AphA, a homologue of the LCD regulator AphA, controls the expression of *iscR*. The level of *aphA* mRNA in the wild type, determined by qRT-PCR, was almost 15-fold higher in the exponential-phase cells than in the stationary-phase cells (Fig. 1), implying that the high level of *iscR* mRNA in the exponential-phase cells correlates with the increased cellular level of AphA. This result suggests that AphA may act as a LCD regulator, as reported in other *Vibrio* species (Rutherford *et al*., 2011; Sun *et al*., 2012), and play a role in the growth phase-dependent variation of *iscR* expression. Consistent with this, the level of *iscR* mRNA in the *aphA* mutant was almost 10-fold less than that in the wild type during exponential growth while no significant difference was observed between them during stationary growth (Fig. 1). Therefore, the combined results suggest that *V. vulnificus* AphA upregulates the expression of *iscR* in a growth phase-dependent manner.

Effects of *iscR* or *aphA* mutation on the activity of the *iscR* promoter

We have reported that the expression of *V. vulnificus iscR* is negatively autoregulated (Lim and Choi, 2013). To confirm the effects of IscR or AphA on the transcription of *iscR*, the activities of the *iscR* promoter were compared for the wild type, the *iscR*, and *aphA* mutants grown to exponential phase by primer extension analyses. A single reverse transcript was identified from the RNAs isolated from the bacterial cells (Fig. 2A). The 5′-end of the *iscR* transcript, located 28-bp upstream of the translational initiation codon of the *iscR* gene, was subsequently designated +1 and the putative promoter constituting this transcription start site (TSS) was named P_{iscR} (Fig. 2B). Based on the intensity of the reverse transcripts, the P_{iscR} activity was significantly increased in the *iscR* mutant and decreased in the *aphA* mutant (Fig. 2A). These results led us to conclude that the activity of P_{iscR} in *V. vulnificus* is under the negative control of IscR and the positive control of AphA. The sequences for the -10 and -35 regions of P_{iscR} were assigned based on similarity to the consensus sequences of the *E. coli* σ^{70} promoter (Fig. 2B).

IscR and AphA bind specifically to the *iscR* regulatory region

The 312-bp DNA fragment encompassing the *iscR* regulatory region was incubated with increasing amounts of IscR (Fig. 3A) or AphA (Fig. 3B) and then subjected to electrophoresis. As shown in Fig. 3A, the addition of IscR resulted in a concentration-dependent ladder of four retarded bands, indicating that at least two binding sites with different affinities for IscR are present within the *iscR* regulatory region. The binding of IscR was also specific because assays were performed in the presence of 100 ng poly(dI-dC), a nonspecific competitor. In a second EMSA, the same but unlabeled 321-bp DNA fragment was used as a self-competitor to confirm the specific binding of IscR. The unlabeled 321-bp DNA competed for the binding of IscR in a dose-dependent manner (Fig. 3A, lanes 6–9), confirming that IscR binds specifically to the DNA. In similar DNA-binding assays, AphA also showed specific binding to the *iscR* regulatory region (Fig. 3B). The addition of AphA resulted in a single retarded band, suggesting that a single binding site for AphA is present in the *iscR* regulatory region (Fig. 3B).

Identification of binding sites for IscR and AphA using DNase I protection analysis

To determine the precise location of the IscR- and AphA-binding site in the *iscR* regulatory region, a DNase I protection analysis was performed with the same 321-bp DNA fragment used for the EMSA. When the sequences were mapped with 25 nM IscR, two IscR footprints extended from -95 to -66 and from -7 to +25 relative to the TSS of P_{iscR} (Figs. 4A and 2B). When increasing the IscR, two additional regions extending from -65 to -41 and from -40 to -12 were protected from DNase I digestion (Figs. 4A and 2B). This sequential protection with increasing IscR was consistent with the previous observation that at least two binding sites

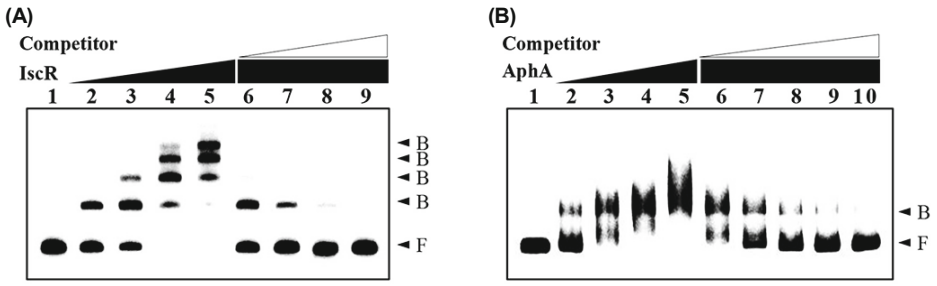

Fig. 3. EMSA for binding of IscR and AphA to the *iscR* regulatory region. A 321-bp DNA fragment of the *iscR* regulatory region was radioactively labeled and then used as a DNA probe. The radiolabeled fragments (5 nM) were mixed with increasing amounts of IscR (A) or AphA (B) and then resolved on a 5% polyacrylamide gel. For *A*, 0, 5, 10, 20, and 30 nM of IscR in the lanes 1-5, respectively. For *B*, 0, 20, 40, 60, and 80 nM of AphA in the lanes 1-5, respectively. For competition analysis, the same but unlabeled 321-bp DNA fragment was used as a self-competitor DNA. Various amounts of the self-competitor DNA were added to the reaction mixture containing the labeled DNA (5 nM) prior to the addition of 30 nM IscR (A) or 80 nM AphA (B). For *A*, 25, 75, 150, and 300 nM of competitor DNA in the lanes 6-9, respectively. For *B*, 10, 20, 30, 50, and 100 nM of competitor DNA in the lanes 6-10, respectively. B, bound DNA; F, free DNA.

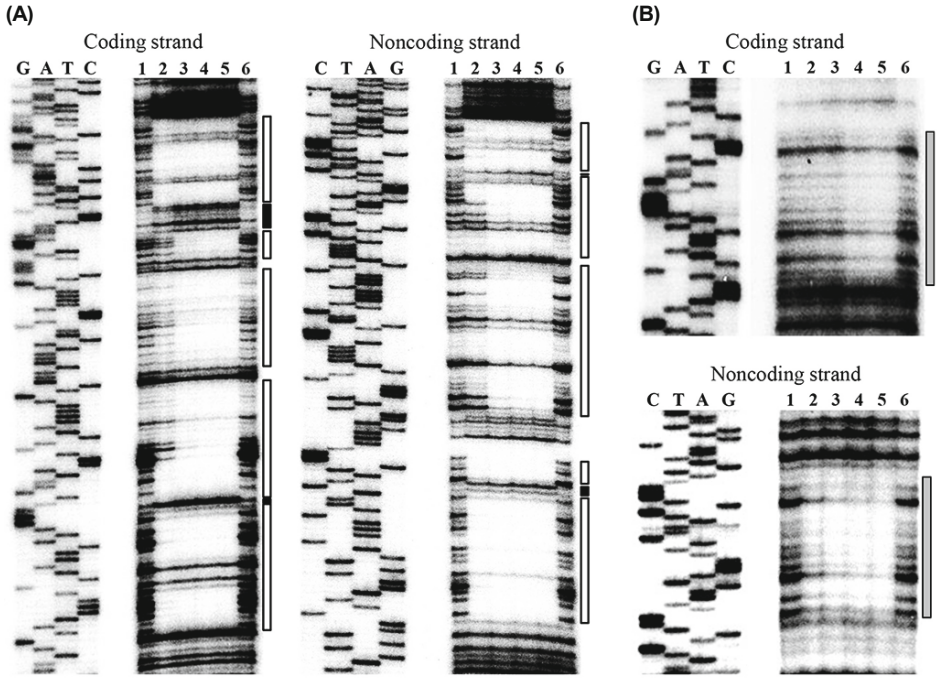

Fig. 4. Identification of binding sites for IscR and AphA. DNase I protection analysis of IscR (A) and AphA (B) binding to the *iscR* regulatory region. The ^{32}P-labeled 321-bp DNA fragments were incubated with increasing amounts of IscR or AphA and then digested with DNase I. (A) Lanes 1 and 6, no IscR added; 2–5, IscR at 25, 50, 100, and 200 nM, respectively. (B) Lanes 1 and 6, no AphA added; 2–5, AphA at 200, 400, 600, and 800 nM, respectively. Lanes *G, A, T,* and *C* represent nucleotide sequencing ladders of pJK1201. The regions protected by IscR are indicated by the *open boxes* and the nucleotides showing enhanced cleavage are indicated by *black boxes* (A). The regions protected by AphA are indicated by *gray boxes* (B).

with different affinities for IscR are present in the regulatory region (Fig. 3A). The regions extending from -95 to -66, from -65 to -41, from -40 to -12, and from -7 to +25 were named as ISCRB1, ISCRB2, ISCRB3, and ISCRB4 to represent the IscR-binding sites 1, 2, 3, and 4, respectively (Fig. 2B). Because sequences of ISCRB3 overlap with the sequences of -10 and -35 regions of P_{iscR}, and ISCRB4 is located downstream of the -10 region comprising +1 of P_{iscR} (Fig. 2B), bound IscR would be expected to prevent RNA polymerase (RNAP) binding and movement. This idea supported our earlier observation that IscR negatively regulates the activity of P_{iscR} (Fig. 2A).

A similar DNase I protection analysis was performed with AphA, and the DNase I footprinting revealed a clear protection pattern in the *iscR* regulatory region extending from -87 to -59 relative to the TSS of P_{iscR} (Figs. 4B and 2B). This position for AphA binding in the *iscR* regulatory region (APHAB) overlapped with a part of ISCRB1 and ISCRB2 (Fig. 2B), leading us to hypothesize that AphA upregulates the *iscR* expression by hindering the IscR binding on the *iscR* regulatory region. Taken together, these observations confirm that IscR and AphA control the activity of P_{iscR} directly by binding to the *iscR* regulatory region.

AphA upregulates the *iscR* expression only in the presence of functional IscR

We further investigated the role of AphA in *iscR* regulation by comparing the levels of *iscR* mRNA in the exponential-phase cells of the wild type, the *aphA*, *iscR*, and *aphA iscR* double mutants with qRT-PCR analyses (Fig. 5). To measure the level of *iscR* mRNA in the cells in which the *iscR* gene is disrupted, a set of primers, ISCR_qRTF and ISCR_qRTR, was designed to hybridize to the nucleotides of the *iscR* coding region upstream from the deletion site of the *iscR* mutant (Table 2). The levels of *iscR* mRNA in the *iscR* and *aphA iscR* double mutants did not show any significant difference (Fig. 5), suggesting that the inactivation of *aphA* did not affect the *iscR* expression in the absence of functional IscR. At the same time, the difference in the *iscR* mRNA level between the *aphA* and *aphA iscR* double mutants was approximately 6-fold higher than that between the wild type and the *iscR* mutant, revealing that IscR more repressed its own expression in the absence of functional AphA than in

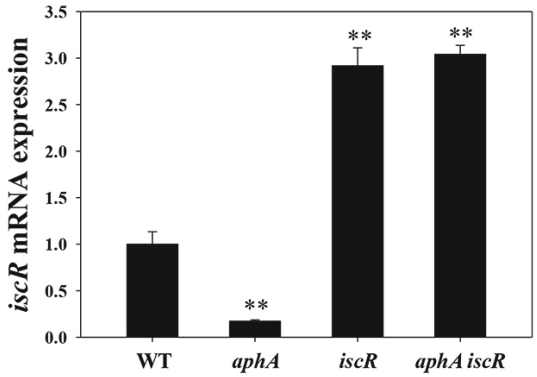

Fig. 5. Expression of *iscR* in *V. vulnificus* with different genetic backgrounds. Cultures of the wild type, the *aphA*, *iscR*, and *aphA iscR* double mutants grown with LBS were harvested at A_{600} of 0.5 and used to isolate total cellular RNA. The *iscR* mRNA level was determined by qRT-PCR analyses and normalized to the 16 S rRNA expression. The *iscR* mRNA level of the wild type was presented as 1. Error bars represent the SEM. **, $P<0.005$ relative to the wild type. WT, wild type; *aphA*, *aphA* mutant JK131; *iscR*, *iscR* mutant JK093, *aphA iscR*, *aphA iscR* double mutant JK132.

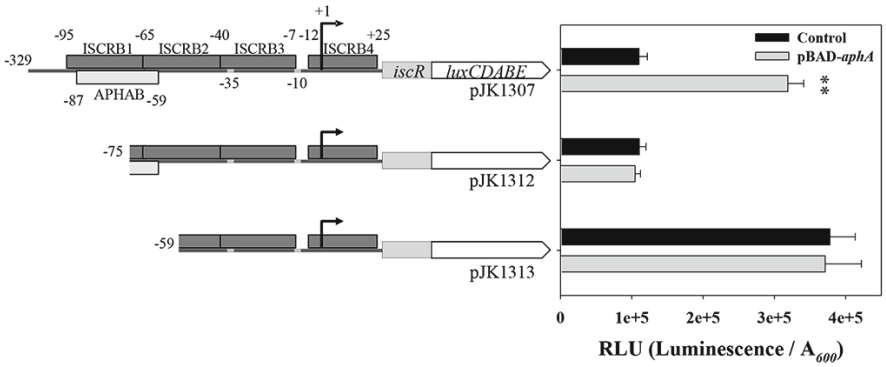

Fig. 6. Deletion analysis of the *iscR* regulatory region. A series of PCR fragments carrying the intact- or deleted-regulatory region of *iscR* was subcloned into pBBR-*lux* to create pJK1307, pJK1312, or pJK1313 (pJK reporters) as shown on the *left*. The proposed -10 and -35 regions and the binding sites for IscR (ISCRB1, 2, 3 and 4) and AphA (APHAB) were indicated as shown in Fig. 2B. Activities of each promoter were determined by using an *E. coli* dual plasmid system assay, as described in 'Materials and Methods'. The relative luminescence unit (RLU) was measured in exponential-phase cells and calculated by dividing the luminescence by the A_{600} of each strain. Error bars represent the SEM. **, $P<0.005$ relative to the each control vector-containing strain.

the presence of AphA (Fig. 5). The combined results were consistent with our hypothesis that AphA positively regulates *iscR* transcription by hindering IscR-repression on the *iscR* regulatory region (Figs. 2B and 4).

Examination of the roles of AphA and binding sites in the control of P_{iscR} activity

A series of *iscR-luxCDABE* transcriptional fusions carrying an intact regulatory region of *iscR* (pJK1307) or the deleted-regulatory regions of *iscR* (pJK1312 and pJK1313) was constructed as shown in Fig. 6. The activities of P_{iscR} were evaluated by measuring the cellular luminescence of *E. coli* DH5α strains which were cotransformed with combinations of one of the pJK reporters and AphA-expressing plasmid pJK1011 (pBAD-*aphA*) or pBAD24 (control) (Fig. 6). For pJK1307, the cellular luminescence of the AphA-expressing strain was about 3-fold higher when compared to the control strain (Fig. 6, upper panel), indicating that the activity of P_{iscR} was positively controlled by AphA *in vivo*, consistent with the previous transcript analyses (Figs. 1 and 2A). When the chromosomal *iscR* gene of *E. coli* was deleted (Yeo *et al.*, 2006), the activity of P_{iscR} highly increased and the induction of AphA no longer affected the activity of P_{iscR} (data not shown), suggesting that *E. coli* IscR could repress the activity of P_{iscR} in *V. vulnificus* and the overexpressed-*V. vulnificus* AphA hindered the binding of *E. coli* IscR on P_{iscR}.

When the region of ISCRB1 and APHAB were deleted (pJK1312), there was no difference in the luminescence between the AphA-expressing and control strains (Fig. 6, middle panel). These results show that AphA and the APHAB region are essential for the upregulation of P_{iscR} activity. When the regulatory region was further deleted to ISCRB2 (pJK1313), the luminescences of both strains were increased to about 3.7×10^5 RLU (Fig. 6, lower panel) corresponding to the luminescence of the strain containing pJK1307 and pBAD-*aphA*. The combined results led us to conclude that bound AphA on the APHAB region would hinder IscR-binding to the ISCRB2 region which is essential for negative autoregulation, resulting in the AphA-mediated upregulation of P_{iscR} activity.

Discussion

The present study identified *V. vulnificus* AphA, a homologue of the transcriptional regulator AphA in *V. cholerae*, *V. harveyi*, and *V. parahaemolyticus* (De Silva *et al.*, 2005; Rutherford *et al.*, 2011; Sun *et al.*, 2012). The amino acid sequence deduced from the *V. vulnificus* AphA protein exhibits a high level of identity (87–92%) with those in other *Vibrio* species (data not shown). Furthermore, *V. vulnificus* AphA contains an N-terminal winged helix DNA binding domain and C-terminal antiparallel coiled coil dimerization domain at positions corresponding to those of *V. cholerae* AphA (De Silva *et al.*, 2005). These observations suggest that the properties of AphA as a transcriptional regulator may be highly conserved throughout the *Vibrio* species. AphA in *V. cholerae* initiates the virulence cascade by activating the transcription of *tcpPH* with another transcriptional regulator AphB (Kovacikova *et al.*, 2004). However, a search of the genome sequences for *V. vulnificus* CMCP6, YJ016, and MO6-24/O to find any *tcpPH* gene homologues with a substantial level of identity was not successful (data not shown), suggesting that both *V. vulnificus* AphA and AphB may have different roles in gene regulation that contribute to virulence. Consistently, our group has reported that *V. vulnificus* AphB regulates many genes involved in the acquisition and metabolism of nutrients and growth and adaptation in the host (Jeong and Choi, 2008).

The mechanism for the negative autoregulation of *iscR* transcription has already been studied extensively in *E. coli* (Schwartz *et al.*, 2001; Giel *et al.*, 2006, 2013). However, the nucleotide sequences of the *iscR* regulatory region in *V. vulnificus* were somewhat different from those in *E. coli* (Giel *et al.*, 2006, 2013). DNase I protection analysis revealed that there are four IscR-binding sites in the regulatory region of *V. vulnificus iscR* (ISCRB1~ISCRB4 in Fig. 2B). The ISCRB2 and ISCRB3 regions were completely conserved with the *E. coli* IscR-binding sites A and B, which are critical for negative autoregulation (Fig. 2B; Giel *et al.*, 2013). Consistently, *V. vulnificus* IscR also repressed its own expression by directly binding to the regulatory region of *iscR* (Figs. 2B and 4A). Meanwhile, the ISCRB1 and ISCRB4 regions have not been previously reported in *E. coli*, although the position of ISCRB4 partially overlapped the *E. coli* IscR-binding site C,

which has no effect on the P_{iscR} activity (Giel *et al.*, 2013). The roles of ISCRB1 and ISCRB4 regions in the expression of *iscR* require further investigation.

The assigned sequences for APHAB (CTATTCATAGGG ATGAATAC; *underline*, palindrome; Fig. 2B) scored 80% similarity to a consensus sequence for AphA binding in *V. parahaemolyticus* (the ATATGCAN$_6$TGCATAT, see Ref. Sun *et al.*, 2012). This observation confirmed that AphA binds to the *iscR* regulatory region directly (Figs. 3B and 5B). Our results show that AphA positively controls the P_{iscR} activity by hindering the negative autoregulation of *iscR* (Figs. 2B and 6). However, the APHAB region only overlapped with part of the ISCRB1 and ISCRB2 regions, not with the ISCRB3 and ISCRB4 regions. This implies that IscR could repress the P_{iscR} activity by only binding to the ISCRB3 or ISCRB4 region, even though AphA occupies the APHAB region. The mechanism may enable *V. vulnificus* to maintain the cellular Fe-S cluster homeostasis regardless of cell density.

It is noteworthy that the *iscR* expression is upregulated by AphA in the exponential-phase cells, namely at LCD (Fig. 1). We speculate that during the initial stage of infection in which the cell density is low, the LCD regulator AphA is highly expressed, leading to IscR production. The elevated IscR, in turn, facilitates the initiation of *V. vulnificus* pathogenesis by upregulating the genes involved in survival and virulence (Lim and Choi, 2013). In fact, *V. cholerae* AphA is known to play a role in connecting quorum sensing with virulence by sensing a low cell density and initiating the virulence cascade (Matson *et al.*, 2007). We further inspected whether *E. coli* or other *Vibrio* species (*V. harveyi*, *V. cholerae*, and *V. parahaemolyticus*) also possess the conserved AphA-binding site at a position corresponding to the *V. vulnificus* APHAB region, but we could not find any putative AphA-binding site in them (data not shown). Consistently, no one has reported the *iscR* gene as an AphA regulon in the previous transcriptome analyses of *V. cholerae* and *V. harveyi* (Kovacikova *et al.*, 2005; Rutherford *et al.*, 2011; van Kessel *et al.*, 2013). Therefore, AphA-mediated control of *iscR* expression is possibly an unusual feature of *V. vulnificus*. Overall, it is possible to suggest that *V. vulnificus* AphA may have been differentially evolved to control virulence gene expression according to the pathogenesis mechanism.

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