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Original Article

Transcriptional Analysis of the *iagB* within *Salmonella* Pathogenicity Island 1 (SPI1)

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HilA is a central regulator of *Salmonella* pathogenicity island 1 (SPI1), which is necessary for host invasion by *Salmonella* and induction of gastroenteritis. The *iagB* lies downstream of *hilA* and is thought to be co-transcribed with *hilA*, but *iagB* expression has not yet been analyzed directly. In this study, *iagB* expression in various mutant strains was measured to determine whether the expression pattern was similar to that of *hilA*. A β-galactosidase assay revealed that *iagB* expression was greater under shaking than standing culture condition. *iagB* expression was decreased in *relA*/*spoT* and *ihfB* mutants but not in *luxS* mutant, in line with previous reports on *hilA* expression. The *hilA* and *iagB* mRNA levels decreased by approximately 2-fold in *arcA* mutant grown aerobically and increased by approximately 10-fold in *fnr* mutant grown anaerobically. Although the fold changes in *hilA* and *iagB* mRNA level differed in *hfq* mutant strain, the patterns of time- and Hfq-dependent regulation were similar for both genes. Thus, *iagB* and *hilA* exhibited similar expression patterns in various mutational backgrounds and under different growth condition.

Key Words: *Salmonella* Typhimurium, *Salmonella* pathogenicity island 1 (SPI1), HilA, IagB

INTRODUCTION

Salmonellae are enteric pathogens that cause gastrointestinal and systemic diseases in a broad range of hosts and are believed to have diverged from the genus *Escherichia* approximately 150 million years ago (1, 2). Sequencing of the *Salmonella* and *Escherichia* genomes has shown that both species share extensive regions of homology but also that virulence genes cluster in localized regions of the *Salmonella* chromosome termed *Salmonella* pathogenicity islands (SPIs) (3, 4). Of the five major SPIs, SPI1, which is required for host invasion by *Salmonella* and induction of gastroenteritis, is well studied in terms of regulatory mechanisms (5~7).

To date, five Hil (hyperinvasion locus) proteins located inside (HilA to HilD) and outside (HilE) SPI1 have been characterized; these are associated with regulation of SPI1. HilD is the prime controller of such regulation, and HilD expression is, in turn, controlled at the post-transcriptional level by various regulatory factors such as FliZ (a flagellar protein) and HilE (6, 7). HilE negatively regulates SPI1 by inhibiting HilD activity via protein-protein interaction (8). HilA, which is the first-identified Hil protein, is a central regulator of all SPI1 genes (5, 9); *hilA* expression is regulated principally by HilD (10, 11). When HilC (also known as

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Table 1. The bacterial strains

Strains	Description	Reference or source
SL1344	wild type serovar Typhimurium *xyl rpsL hisG*	Lab stock
CS669	14028s *iagB*::*lacZY*, Kan^R	21
SR3306	SL1344 Δ*luxS*	25
JE3999	TR6538 *ihfB*::*cat*, Cam^R	35
SHJ2037	14028s *relA*::*kan*, *spoT*::*cat*, Kan^R, Cam^R	26
NC983	14028s *fnr*::Tn10, Tet^R	29
SY1001	SL1344 *iagB*::*lacZY*, Kan^R	This study
SY1002	SL1344 Δ*luxS*, *iagB*::*lacZY*, Kan^R	This study
SY1004	SL1344 *relA*::*kan*, *spoT*::*cat*, *iagB*::*lacZY*, Kan^R, Cam^R	This study
SY1005	SL1344 *ihfB*::*cat*, *iagB*::*lacZY*, Kan^R, Cam^R	This study
SR3560	SL1344 Δ*arcA*	22
SY1006	SL1344 *fnr*::Tn10, Tet^R	22
SY1101	SL1344 Δ*hfq*	22

SprA or SirC) is overproduced, the protein enhances SPI1 expression by binding upstream of *hilA* and *hilD* (11, 12~15), but a *hilC* null mutation has little effect on SPI1 function (11, 13, 16, 17).

HilA can act as an autorepressor under certain conditions, such as low osmolality and aeration (18). Therefore, it is likely that the β-galactosidase activity expressed by a *hilA*::*lacZY* chromosomal fusion, which is commonly used to monitor *hilA* expression levels, may not mirror the real expression of *hilA* under certain conditions because the *lacZY* fusion mutates. *hilB*, better known as *iagB* (invasion-associated gene), lies downstream of *hilA* and is thought to be co-transcribed with *hilA* (19). Mutation of *iagB* does not affect SPI1 function (20), and an *iagB*::*lacZY* fusion has thus been used as a reporter of *hilA* expression (19, 21). However, *iagB* expression has not yet been analyzed directly.

In this study, we explored *iagB* expression in certain mutant strain in which *hilA* expression has been reported to be altered, and found that the expression patterns of *hilA* and *iagB* were similar in the mutants tested.

MATERIALS AND METHODS

Growth conditions

Bacteria were routinely cultivated at 37℃ in LB broth containing 1% tryptone, 0.5% yeast extract, and 1% NaCl. A stationary-phase culture that had been grown overnight (~ 14 h) with shaking was used as the stock culture. The stock culture was used to inoculate fresh LB broth at 1:100 dilution and grown under shaking or standing conditions as previously described (22). The following antibiotics were used when necessary: 50 μg/ml kanamycin, 25 μg/ml chloramphenicol, and 15 μg/ml tetracycline.

Strain construction

The *S. enterica* serovar Typhimurium strains used in this study are described in Table 1. *Salmonella* Typhimurium SL1344 was used as a wild-type strain and isogenic derivatives of strain SL1344 were constructed by P22HT-mediated transduction (23). The *iagB*::*lacZY* fusion was also introduced into mutant strains via phage P22HT-mediated transduction, and the recipient strains were spread onto agar plates containing X-Gal (a β-galactosidase substrate yielding an insoluble blue

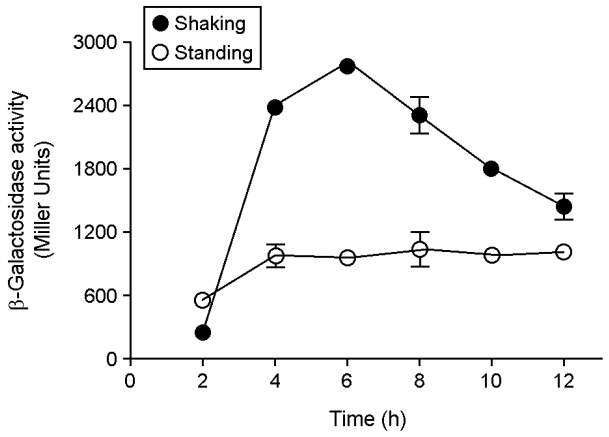

Figure 1. Transcriptional analysis of *iagB* under different growth conditions. The *S.* Typhimurium strain harboring a chromosomal *iagB*::*lacZY* fusion was grown in LB medium under shaking (●) or standing conditions (○). The expression level was determined by measuring the β-galactosidase activity (Miller units) at the indicated times. Data are presented as the mean ± standard error of three independent experiments performed in duplicate.

reaction product) for screening. The *lacZ* gene insertion was confirmed by PCR and DNA sequencing.

β-Galactosidase assay

Salmonella Typhimurium strains containing the chromosomal *lacZ* transcriptional fusion were assayed for β-galactosidase activity according to the standard method (23). Cells were centrifuged, resuspended in Z buffer (100 mM KPO_4, 10 mM KCl, 1 mM $MgSO_4$), and then permeabilized with sodium dodecyl sulfate (SDS) and chloroform. Assays were performed in Z buffer containing 30 mM β-mercaptoethanol by a kinetic method with a plate reader. Activities are normalized to the actual cell density (OD_{600}) and were always compared with activities of appropriate controls assayed at the same time.

Quantitative real-time PCR (qRT-PCR) analysis

Cultures grown under indicated conditions were mixed with 10% volume (v/v) of ice-cold phenol-ethanol mixture (5% phenol, 95% ethanol). Total RNA was prepared from bacterial cultures with RiboEX reagent (GeneAll; Seoul, Korea), treated with DNase, and purified using the RNeasy Mini Kit (Qiagen; Hilden, Germany) according to the manufacturer's instructions. For qRT-PCR analysis, cDNA was synthesized from 2 μg of total RNA using the Maxime RT Premix Kit (Intron; Seoul, Korea) according to the manufacturer's instructions. The SYBR green real-time PCR assay was performed in a reaction volume of 25 μl containing 12.5 μl SYBR Premix Ex Taq (Takara Bio; Osaka, Japan), 0.5 μM of each specific primer set, and 2 μl cDNA. The 7300 Real-Time PCR System (Applied Biosystems, Carlsbad, CA) was programmed for 40 cycles at 95℃ for 15 s and 52℃ for 30 s. The mRNA expression level of the target gene was normalized to the 16S rRNA expression level. The primer sequences were as follows: hilA-RT1 (5'-ccc tgc tac gct cag aaa a-3') and hilA-RT2 (5'-tcg agc agg atg acc aga a-3'); iagB-RT1 (5'-gac ccc tgc att tct gtc at-3') and iagB-RT2 (5'-ttt gcc gcg ata gaa agt ct-3'); and 16S-RT1 (5'-cga tcc cta gct ggt ctg ag-3') and 16S-RT2 (5'-cgc ttt acg ccc agt aat tc-3').

RESULTS

High-level expression of *iagB* under shaking culture conditions

SPI1 genes, including *hilA*, exhibit characteristic expression patterns under various culture conditions. In shaking culture, *hilA* expression increases rapidly, peaks at 6 h post-inoculation (p.i.), and decreases gradually thereafter. In standing culture, *hilA* expression increases slowly and then reaches a plateau (22). The expression pattern of *iagB* (monitored using the chromosomal *iagB*::*lacZY* transcriptional fusion) was similar. Under shaking culture conditions, *iagB* expression peaked at 6 h p.i. (Fig. 1). Under standing culture condition, *iagB* expression increased until 4 h p.i. and was not noticeably changed thereafter (Fig. 1). Thus, *iagB* exhibited the same expression pattern as did other SPI1 genes under both shaking and standing culture conditions.

Reduced *iagB* expression in ppGpp-deficient and *ihfB* mutants

We monitored *iagB* expression levels in certain mutant strains in which SPI1 gene expression is altered. Assessments were made in both the early (4 h p.i.) and late (12 h

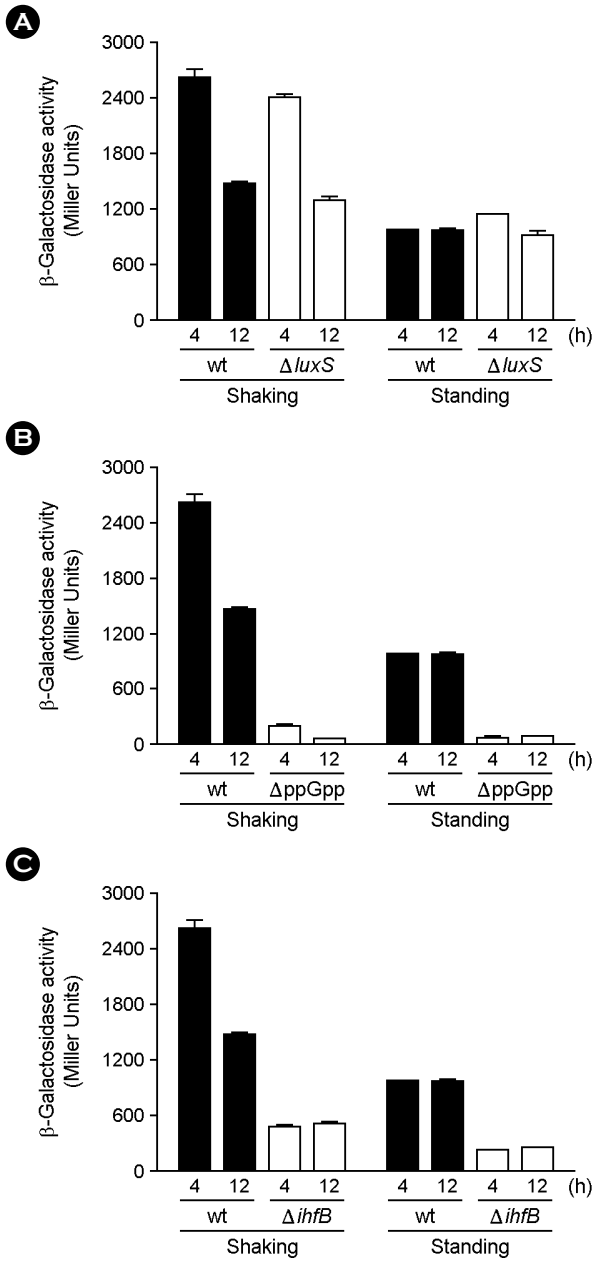

Figure 2. Transcriptional analysis of *iagB* in different mutant strains. The β-galactosidase activities (Miller units) of chromosomal *iagB*::*lacZY* were measured in Δ*luxS* **(A)**, ΔppGpp (Δ*relA*/Δ*spoT*) **(B)**, and Δ*ihfB* **(C)** strains at 4 h p.i. (early stationary phase) and 12 h p.i. (late stationary phase) under shaking and standing culture conditions, respectively. Data are presented as the mean ± standard error of three independent experiments performed in duplicate.

p.i.) stationary phases under shaking and standing culture conditions. InvF is a SPI1-specific transcriptional regulator and is activated in a HilA- or HilD-dependent manner (24). Deletion of the *luxS* gene, which encodes an enzyme producing a quorum-sensing signaling molecule (an autoinducer; AI-2), decreases *invF* expression but has no effect on *hilA* expression (25). Thus, we first measured *iagB* expression (using the *iagB*::*lacZY* transcriptional fusion) in an *luxS* mutant strain (Δ*luxS*). *iagB* expression in this strain was comparable to that in the wild-type strain under all culture conditions tested (Fig. 2A). The signal molecule guanosine tetraphosphate (ppGpp), synthesized by the RelA and SpoT proteins, is necessary for induction of *hilA* and *invF* expression (26). A *relA*/*spoT* double mutant strain deficient in ppGpp (ΔppGpp) exhibited severely reduced expression of *iagB* (Fig. 2B). Integration host factor (IHF), a bacterial nucleoid-associated protein, is also known to be required for *hilA* induction (27). As expected, *iagB* expression was induced less effectively in *ihf* mutants than in the wild-type strain (Fig. 2C). Taken together, and comparing the *iagB* expression pattern to the previously reported *hilA* expression pattern (22), we found that the expression profile of *iagB* was similar to that of *hilA* in the three mutant strains tested.

Increased *iagB* expression in *fnr* mutants

ArcA (aerobic respiratory control) and FNR (fumarate and nitrate reductase) are well-known oxygen-sensitive global regulators of gene expression (28, 29). SPI1 gene expression is not regulated significantly by ArcA under anaerobic conditions (28), but ArcA is necessary for *hilA* induction upon entry into the stationary phase under shaking culture conditions (22). However, any role for FNR in SPI1 regulation remains controversial; *hilA* has been reported to be either inhibited (29) or induced (7, 30) in *fnr* mutants (Δ*fnr*) under anaerobic conditions. Thus, we compared *hilA* and *iagB* expression at 4 h p.i. in cultures grown under shaking and standing conditions using qRT-PCR. As expected, disruption of *arcA* caused approximately 2-fold decreases in both *hilA* and *iagB* mRNA levels under shaking culture conditions and no significant change under standing culture conditions (Fig. 3). In Δ*fnr* strains, both the *hilA* and *iagB* expression levels increased approximately 10-fold (compared to wild type) under standing culture conditions (Fig. 3). These findings

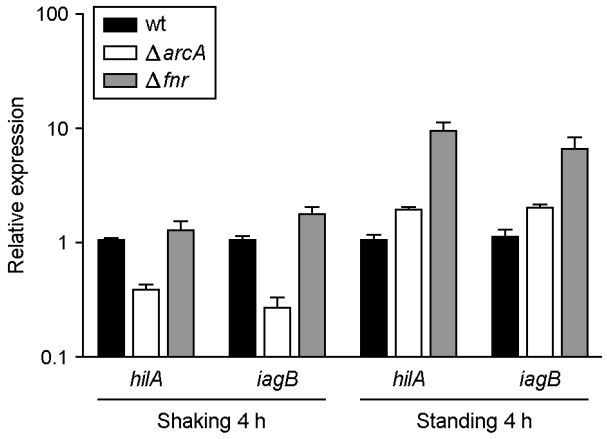

Figure 3. Transcriptional analysis of *hilA* and *iagB* in *arcA* and *fnr* mutants. The mRNA levels of *hilA* and *iagB* were determined by qRT-PCR. Total RNA was isolated from strains grown for 4 h under shaking and standing culture conditions. The values for the relative expression were determined by defining the mRNA levels from the wild-type strain as 1. The expression levels of the target genes were normalized to 16S rRNA gene. Data are presented as the mean ± standard error of three independent experiments performed in duplicate.

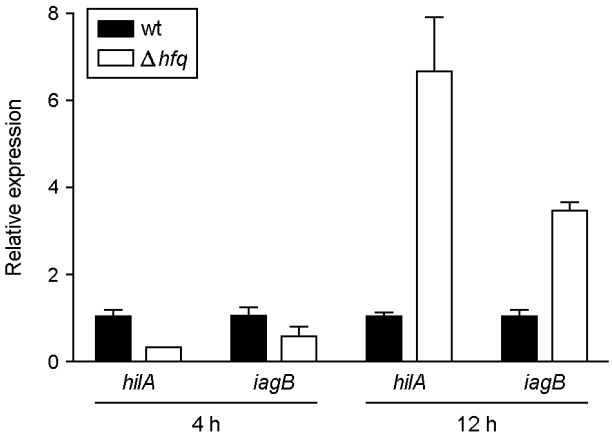

Figure 4. Transcriptional analysis of *hilA* and *iagB* in *hfq* mutants. The mRNA levels of *hilA* and *iagB* were determined by qRT-PCR. Total RNA was isolated from strains grown for 4 h and 12 h under shaking culture conditions. The values for the relative expression were determined by defining the mRNA levels from the wild-type strain as 1. The expression levels of the target genes were normalized to 16S rRNA gene. Data are presented as the mean ± standard error of three independent experiments performed in duplicate.

are consistent with those of previous reports to the effect that FNR serves as a negative regulator of *hilA* expression under anaerobic conditions (7, 30). In addition, the *hilA* and *iagB* expression patterns were similar in Δ*arcA* and Δ*fnr* mutants (Fig. 3). The fact that *hilA* and *iagB* showed similar fold changes in mRNA levels suggests that the genes are co-transcribed into a polycistronic mRNA.

Temporal regulation of *iagB* by Hfq

Mutants lacking the global regulatory RNA-binding protein Hfq exhibited decreased and increased *hilA* expression in the early (4 h p.i.) and late (12 h p.i.) stationary phases, respectively, under shaking culture conditions (31). We compared the expression of *hilA* and *iagB* in *hfq* mutants (Δ*hfq*) under such culture conditions, using qRT-PCR. The *hilA* mRNA levels decreased 3.5-fold at 4 h p.i. and increased 6.6-fold at 12 h p.i. (Fig. 4). Interestingly, the fold changes in *iagB* mRNA levels measured in the Δ*hfq* strain were not synchronized precisely with those in *hilA*, which exhibited a 1.9-fold decrease at 4 h p.i. and a 3.4-fold increase at 12 h p.i. (Fig. 4). Although the effect of Hfq on *iagB* expression was mild compared to the Hfq effect on *hilA* expression, the time-dependent effect of Hfq on *hilA*, which has been previously reported (31), was also evident on *iagB*.

DISCUSSION

In SPI1, the 3' end of the stop codon of *hilA* is followed by 18 base pairs (bp) of an intergenic region that ends at the start codon of *iagB*. The genes nearest to the *hilA-iagB* region are *hilD* and *sicP*. *hilD* lies 1,090 bp upstream from the start codon of *hilA*. *sicP*, located 54 bp downstream from the stop codon of *iagB*, is transcribed divergently from *iagB*. These suggest that *hilA* and *iagB* might constitute an operon and thus be transcribed into a polycistronic mRNA. In this study, we explored whether *hilA* and *iagB* were co-transcribed in *luxS*, *relA*/*spoT*, *ihf*, *arcA*, *fnr* and *hfq* mutants under two culture conditions (shaking and standing) and by comparing *iagB* expression patterns to those of *hilA*. The *hilA* and *iagB* expression profiles were generally similar under all conditions tested, but *iagB* was less expressed than *hilA* by mutating *hfq* (Figs. 1 to 4).

HilD is a primary point of integration of regulatory signals to *hilA*; many regulatory systems and environmental signals control *hilA* expression via HilD (7). The RNA-chaperone protein Hfq binds to and stabilizes *hilD* mRNA (32). Therefore, *hfq* mutation reduces the *hilD* mRNA level and thus HilD protein synthesis (32), thereby reducing *hilA* promoter activity (33). However, the HilA protein level in a Δ*hfq* mutant was restored to only 60% of the wild-type level despite the overexpression of HilA (33). Recently, it has been found that Hfq binds to the 5'-untranslated region (UTR) of *hilA* mRNA (34). Taken together, these findings suggest that Hfq may regulate *hilA* and *iagB* expression not only transcriptionally, but also post-transcriptionally. Hfq may activate the promoter of the *hilA-iagB* operon in conjunction with HilD, and also modulate *hilA-iagB* mRNA stability by interacting with the 5'-UTR of the polycistronic mRNA.

In the *hfq* mutation background, both the fold decrease and increase in *iagB* mRNA levels were lower than those of *hilA*. As Hfq likely affects the stability of the *hilA-iagB* polycistronic mRNA (34), we infer that *iagB* may have an additional start site internal to the operon, causing a monocistronic *iagB* mRNA to be produced under both shaking and standing culture conditions, thereby reducing the relative influence of Hfq on *iagB* expression. For example, the *iagB*::*lacZY* fusion produced approximately 2,400 Miller units (MU) of β-galactosidase at 4 h p.i. under shaking culture conditions (Fig. 1), whereas the *hilA*::*lacZ* fusion produced approximately 900 MU under the same conditions (22). This supports the idea that a promoter within the operon may mediate *iagB* expression. Further experiments are needed to explore whether a monocistronic *iagB* mRNA is in fact produced, and if the transcript is expressed constitutively.

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