Hfq and ArcA Are Involved in the Stationary Phase-Dependent Activation of *Salmonella* Pathogenicity Island 1 (SPI1) Under Shaking Culture Conditions

Sangyong Lim†, Hyunjin Yoon†, Minjeong Kim†, Ahreum Han†, Jihae Choi†, Jeongjoon Choi†, and Sangryeol Ryu*?

1Research Division for Biotechnology, Korea Atomic Energy Research Institute, Jeongeup 580-185, Republic of Korea
2Department of Food Technology and Services, Eulji University, Seongnam 461-713, Republic of Korea
3Department of Agricultural Biotechnology, Center for Agricultural Biomaterials, and Research Institute for Agriculture and Life Sciences, Seoul National University, Seoul 151-921, Republic of Korea

Received: May 9, 2013
Revised: July 29, 2013
Accepted: September 8, 2013

First published online September 10, 2013
*Corresponding author
Phone: +82-2-880-4856;
Fax: +82-2-873-5095;
E-mail: sangryu@snu.ac.kr
†These authors contributed equally to this work.

In *Salmonella enterica* serovar Typhimurium, many genes encoded within *Salmonella* pathogenicity island 1 (SPI1) are required to induce intestinal/diarrheal disease. In this study, we compared the expression of four SPI1 genes (*hilA*, *invF*, *prgH*, and *sipC*) under shaking and standing culture conditions and found that the expression of these genes was highest during the transition from the exponential to stationary phase under shaking conditions. To identify regulators associated with the stationary phase-dependent activation of SPI1, the effects of selected regulatory genes, including *relA/spoT* (ppGpp), *luxS*, *ihfB*, *hfq*, and *arcA*, on the expression of *hilA* and *invF* were compared under shaking conditions. Mutations in the *hfq* and *arcA* genes caused a reduction in *hilA* and *invF* expression (more than 2-fold) in the early stationary phase only, whereas the lack of ppGpp and IHF decreased *hilA* and *invF* gene expression during the entire stationary phase. We also found that *hfq* and *arcA* mutations caused a reduction of *hilD* expression upon entry into the stationary phase under shaking culture conditions. Taken together, these results suggest that Hfq and ArcA regulate the *hilD* promoter, causing an accumulation of HilD, which can trigger a stationary phase-dependent activation of SPI1 genes under shaking culture conditions.

**Keywords:** *Salmonella* Typhimurium, *Salmonella* pathogenicity island 1 (SPI1), *hfq*, *arcA*

---

**Introduction**

*Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) is a gram-negative facultative intracellular anaerobe that can infect a wide range of hosts. The infection of mice with *S. Typhimurium* leads to a systemic disease similar to human typhoid that occurs following ingestion of *S. Typhi*. In humans, *S. Typhimurium* leads to a systemic disease similar to human typhoid that occurs following ingestion of *S. Typhi*. In humans, *S. Typhimurium* can cause both acute intestinal inflammation from gastroenteritis and a more debilitating disorder known as enterocolitis [10, 20]. *S. Typhimurium* invasion of host cells is a key step in the infectious cycle. Many of the genes required for intestinal penetration and invasion of host cells are encoded within the *Salmonella* pathogenicity island 1 (SPI1), located at centisome 63 of the *Salmonella* chromosome [26].

SPI1 gene expression requires the SPI1-specific transcriptional regulators HilA and InvF [16, 26]. HilA activates expression of the *prg/org* and *inv/spa* genes, which encode a functional type III secretion system (T3SS) apparatus [25, 26], whereas InvF is required to induce transcription of several effector genes encoded both within (*sic/sip* genes) and outside (*sigD* and *sopE*) SPI1 [12, 13]. Both regulators are governed by three other transcriptional regulators located inside (HilC and HilD) and outside (RtsA) SPI1. Of the three regulators, HilD appears to control the entire pathway [15, 16].

During its life cycle, *Salmonella* encounters various
environmental stresses in different niches. Therefore, SPI1 expression is also controlled in response to multiple environmental signals such as pH, oxygen tension, and medium osmolarity. Although it is known that the optimal expression of SPI1-specific regulators occurs under low-oxygen growth conditions, which can be created in an upright culture without agitation [2, 16, 22, 26], many studies have shown that genes encoded within SPI1 are also highly expressed in shaking cultures, especially during the transition from the exponential to stationary phase under shaking culture conditions [7, 31, 38, 41].

In this study, we directly compared the expression patterns of SPI1 genes between shaking and standing culture conditions and found that the expression of SPI1 genes was highest during the transition from the exponential to stationary phase under shaking conditions. We also found that the RNA-binding protein Hfq and the transcriptional regulator ArcA were involved in this growth phase-specific activation of SPI1 genes.

Materials and Methods

Growth Conditions

Bacteria were routinely cultivated at 37°C 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 a 1:100 dilution and grown under shaking or standing conditions. Shaking cultures were grown on a platform shaker at 200 rpm in a 250 ml Erlenmeyer flask containing 50 ml of LB. The absorbance at 600 nm of the cultures was monitored using a Helios beta UV-Vis spectrophotometer (Thermo Scientific) at the times indicated after inoculation. Standing cultures were grown undisturbed in a 50 ml Falcon tube containing 50 ml of LB with the caps tightly sealed, standing upright. The following antibiotics were used when necessary: 50 μg/ml ampicillin, 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 through P22HT-mediated transduction [29]. To construct the arcA mutant, we used the one-step gene-inactivation method (i.e., λ red and FLP-mediated site-specific recombination system) [14]. Briefly, a chloramphenicol cassette from pKD13 was amplified using the arcA-RF and arcA-RR primers (Table 2), and the resultant PCR product (FRT-ahp-FRT) was introduced into arcA in the chromosome using a homologous recombination. This antibiotic resistance cassette was removed using the temperature-sensitive plasmid pCP20 carrying the FLP recombinase gene, and the resulting strain was designated SR3560. The hilD deletion mutant strain (SR4219) and the strain expressing 2HA-tagged HilD (SY1116) were constructed as described above using the hilD-RF/hilD-RR and hilD-2HAF/hilD-2HAR primer sets, respectively (Table 2). The insertion of the HA tag and deletion of the target genes were confirmed by PCR and nucleotide sequencing.

Table 1. The bacterial strains and plasmids used in this study.

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>SL1344</td>
<td>Wild-type serovar Typhimurium xyl rpsL hisG Lab stock</td>
<td></td>
</tr>
<tr>
<td>EE658</td>
<td>SL1344 hisA::Tn5lacZY, Tet&lt;sup&gt;a&lt;/sup&gt; [4]</td>
<td></td>
</tr>
<tr>
<td>EE639</td>
<td>SL1344 invF::Tn5lacZY, Tet&lt;sup&gt;a&lt;/sup&gt; [4]</td>
<td></td>
</tr>
<tr>
<td>EE656</td>
<td>SL1344 proH::Tn5lacZY, Tet&lt;sup&gt;a&lt;/sup&gt; [4]</td>
<td></td>
</tr>
<tr>
<td>EE638</td>
<td>SL1344 sipC::Tn5lacZY, Tet&lt;sup&gt;a&lt;/sup&gt; [4]</td>
<td></td>
</tr>
<tr>
<td>HY4260</td>
<td>14028s hsp60, Kan&lt;sup&gt;a&lt;/sup&gt; [3]</td>
<td></td>
</tr>
<tr>
<td>SHJ2037</td>
<td>14028s relA::kan, spoT::cat, Kan&lt;sup&gt;a&lt;/sup&gt;, Cam&lt;sup&gt;a&lt;/sup&gt; [38]</td>
<td></td>
</tr>
<tr>
<td>EE699</td>
<td>TR6538 ihyB::cat, Cam&lt;sup&gt;a&lt;/sup&gt; [30]</td>
<td></td>
</tr>
<tr>
<td>SR3306</td>
<td>SL1344 ΔluxS [9]</td>
<td></td>
</tr>
<tr>
<td>SR3315</td>
<td>SL1344 ΔluxS, invF::Tn5lacZY, Tet&lt;sup&gt;a&lt;/sup&gt; [9]</td>
<td></td>
</tr>
<tr>
<td>SR3316</td>
<td>SL1344 ΔluxS, hilaA::Tn5lacZY, Tet&lt;sup&gt;a&lt;/sup&gt; [9]</td>
<td></td>
</tr>
<tr>
<td>SR3580</td>
<td>SL1344 ΔarcA This study</td>
<td></td>
</tr>
<tr>
<td>SR4219</td>
<td>SL1344 ΔhilD This study</td>
<td></td>
</tr>
<tr>
<td>SY1101</td>
<td>SL1344 Δhfq This study</td>
<td></td>
</tr>
<tr>
<td>SY1103</td>
<td>SL1344 Δhfq, invF::Tn5lacZY This study</td>
<td></td>
</tr>
<tr>
<td>SY1104</td>
<td>SL1344 Δhfq, hilD-:lacZ, Kan&lt;sup&gt;a&lt;/sup&gt; This study</td>
<td></td>
</tr>
<tr>
<td>SY1108</td>
<td>SL1344 Δhfq, ΔhilD This study</td>
<td></td>
</tr>
<tr>
<td>SY1116</td>
<td>SL1344 HilD-2HA, Kan&lt;sup&gt;a&lt;/sup&gt; This study</td>
<td></td>
</tr>
<tr>
<td>SY1117</td>
<td>SL1344 Δhfq, HilD-2HA, Kan&lt;sup&gt;a&lt;/sup&gt; This study</td>
<td></td>
</tr>
<tr>
<td>SY1121</td>
<td>SL1344 relA::kan, spoT::cat, hilaA::Tn5lacZY This study</td>
<td></td>
</tr>
<tr>
<td>SY1122</td>
<td>SL1344 relA::kan, spoT::cat, invF::Tn5lacZY This study</td>
<td></td>
</tr>
<tr>
<td>SY1123</td>
<td>SL1344 ihyB::cat, hilaA::Tn5lacZY This study</td>
<td></td>
</tr>
<tr>
<td>SY1124</td>
<td>SL1344 ihyB::cat, invF::Tn5lacZY This study</td>
<td></td>
</tr>
<tr>
<td>SY1125</td>
<td>SL1344 Δhfq, hilaA::Tn5lacZY This study</td>
<td></td>
</tr>
<tr>
<td>SY1126</td>
<td>SL1344 ΔarcA, hilaA::Tn5lacZY This study</td>
<td></td>
</tr>
<tr>
<td>SY1127</td>
<td>SL1344 ΔarcA, invF::Tn5lacZY This study</td>
<td></td>
</tr>
<tr>
<td>SY1128</td>
<td>SL1344 ΔarcA, ΔhilD This study</td>
<td></td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pKD46</td>
<td>bla&lt;sup&gt;b&lt;/sup&gt; P&lt;sub&gt;max&lt;/sub&gt; gam beta&lt;sup&gt;b&lt;/sup&gt; exo&lt;sup&gt;b&lt;/sup&gt; pSC101 oriTS [14]</td>
<td></td>
</tr>
<tr>
<td>pKD13</td>
<td>bla&lt;sup&gt;b&lt;/sup&gt; FRT&lt;sup&gt;b&lt;/sup&gt; ahp&lt;sup&gt;b&lt;/sup&gt; FRT&lt;sup&gt;b&lt;/sup&gt; P&lt;sub&gt;51&lt;/sub&gt; PS1 PS4 oriR6K [14]</td>
<td></td>
</tr>
<tr>
<td>pCP20</td>
<td>bla&lt;sup&gt;b&lt;/sup&gt; cat&lt;sup&gt;b&lt;/sup&gt; c857 λP&lt;sub&gt;x&lt;/sub&gt; fli&lt;sup&gt;b&lt;/sup&gt; pS&lt;sub&gt;101&lt;/sub&gt; oriT&lt;sub&gt;0&lt;/sub&gt;K [14]</td>
<td></td>
</tr>
<tr>
<td>pJB5</td>
<td>hilD-lacZY reporter vector, Amp&lt;sup&gt;b&lt;/sup&gt; [6]</td>
<td></td>
</tr>
</tbody>
</table>

**β-Galactosidase Assay**

*Salmonella* Typhimurium strains containing the chromosomal lacZY transcriptional fusion or carrying the lacZ reporter plasmids (pJB5) were assayed for β-galactosidase activity according to the standard method [29].

**Western Blot Analysis**

*Salmonella* Typhimurium cells encoding the HilD-2HA protein were grown under shaking culture conditions and harvested by centrifugation at 4 and 12 h after inoculation. The cell lysates were prepared by lysis of cells using B-PER solution (Pierce). Lysates (20 µg protein) were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes, and the HilD and DnaK proteins detected using anti-HA (Sigma) and anti-DnaK (Assay Designs) antibodies, respectively. The blots were developed using anti-mouse IgG horseradish peroxidase-linked antibody (Santa Cruz Biotechnology) with the WEST-ZOL detection system (Intron).

**Real-Time PCR Analysis**

A 20 ml culture was added to a tube containing 2.5 ml of ice-cold ethanol/phenol stop solution (5% water-saturated phenol in ethanol). After centrifugation, total RNA was isolated from the cell pellets using the hot phenol method (http://bugarrays.stanford.edu/protocols/rna/Total_RNA_from_Ecoli.pdf), followed by purification with the RNEasy Mini Kit (Qagen) and RNase-free DNase (Qiagen). For real-time PCR analysis, cDNA was synthesized from 1 µg of DNase-treated total RNA using the cDNA synthesis kit (PhileKorea Technology, Inc.) as per the manufacturer’s protocol. The SYBR green real-time PCR assay was performed in a reaction volume of 25 µl containing 12.5 µl of SYBR Premix Ex Taq (Takara), 0.5 µM of each specific primer set, and 2 µl of cDNA. The Eco Real-Time PCR System (Illumina) was programmed for 40 cycles at 95°C for 10 sec, 60°C for 20 sec, and 72°C for 15 sec. The mRNA expression level of hilD was normalized to the 16S rRNA expression level.

**Results**

**Maximal Induction of SPI1 Genes Under Shaking Culture Conditions**

The effects of growth conditions on the expression of the SPI1 genes *hilA*, *invF*, *prgH*, and *sipC* were analyzed using chromosomal lacZY fusions under both shaking and standing conditions (Fig. 1). The expression levels were monitored up to 12 h post-inoculation (p.i.). Gene expression was higher under shaking culture conditions compared with standing culture conditions. This result clearly shows that SPI1 is most active under shaking culture conditions, especially when cells are grown to the early stationary growth phase.

**Hfq and ArcA Down-Regulate SPI1 Only in the Early Stationary Phase Under Shaking Culture Conditions**

To identify the regulators relevant to the stationary phase-dependent activation of SPI1, we selected candidate genes from previous studies and measured the *hilA* and *invF* expressions in mutants at the early (4 h p.i.) and late (12 h p.i.) stationary phases. It is known that stationary-phase induction of SPI1 genes depends on the signal molecule ppGpp, which is synthesized by the RelA and SpoT proteins [38, 41]. As expected, *hilA* and *invF* showed a very
low expression in the ppGpp null mutant background (ΔrelAΔspoT strain) under shaking culture conditions (Fig. 2). The luxS gene, which encodes a synthase that produces a quorum-sensing signal molecule termed autoinducer (AI-2), is necessary for the growth-dependent induction of a subset of SPI1 genes, including invF and InvF-dependent genes, but not hila [9]. However, as those experiments were performed only under standing culture conditions, we investigated hila and invF expression in a strain lacking luxS under shaking culture conditions and found that luxS deletion affected only invF expression (Fig. 2). It has been recently reported that mutants lacking IHF (integration host factor) did not induce hila upon entering the stationary phase [32], and we confirmed the role of IHF in SPI1 gene activation under shaking culture conditions (Fig. 2). hild mRNA is bound by Hfq, which is one of the most

Fig. 1. Transcriptional analysis of SPI1 gene expression. Various strains harboring the transcriptional lacZ fusions with SPI1 genes were grown in LB medium under shaking (●) or standing conditions (○). The expression level of each gene was determined by measuring the β-galactosidase activity (Miller units) at the indicated times. Cell optical densities at 600 nm of wild-type culture grown under shaking and standing conditions were monitored at the same time points as when β-galactosidase activities were measured. The mutant culture densities were not significantly different from those of the wild type (data not shown). Data are presented as the mean ± standard deviation of three independent experiments containing duplicate samples.
abundant RNA-binding proteins in bacteria [3, 36]. When
grown to the early stationary phase under shaking culture
conditions, the \( \Delta hfq \) strain fails to activate the SPI1
transcription factor cascade [37]. The mutation of \( hfq \)
caus ed a reduction in \( hilA \) and \( invF \) expression (more than
2-fold) in the early stationary phase (4 h p.i.), but the
expression at 12 h p.i. remained at wild-type levels (Fig. 2).

The Hfq-dependent stationary-phase expression of SPI1
genes was also clearly observed in an InvF-regulated gene,
\( sipC \): a \( sipC::lacZ \) chromosomal fusion expressed 3,761 ± 292
and 1,120 ± 12 Miller units of \( \beta \)-galactosidase at 4 and 12 h
p.i., respectively, in wild-type cells, whereas the \( sipC::lacZ \)
fusion in \( \Delta hfq \) cells expressed 1,486 ± 192 and 830 ± 67
Miller units of \( \beta \)-galactosidase at the same time points (data
not shown).

ArcA (aerobic respiratory control) is a response regulator of
a two-component system and has generally been implicated
in activating and repressing many genes in response to
changes in redox states [34]. In this study, we tested the
effect of \( arcA \) mutation on SPI1 genes because \( arcA \)
transcription markedly decreased when the cultures reached
a stationary phase under aerobic conditions [35]. Interestingly,
a disruption in \( arcA \) reduced the level of \( hilA \) and \( invF \)
expression at 4 h p.i., but the expression was almost
restored to the wild-type level at 12 h p.i. (Fig. 2). Taken
together, these results demonstrate that the role of Hfq and
ArcA in SPI1 induction is specific to the transition from the
exponential to the stationary phase, whereas ppGpp, LuxS,
and IHF are related to the overall activation of SPI1 under
shaking culture conditions.

**Hfq and ArcA Can Affect \( hilD \) Promoter Activity**

The defect of SPI1 activation in the \( \Delta hfq \) strain was well
pronounced when grown to the early stationary phase under shaking conditions [36, 37], but the effect of \( hfq \)
mutation on SPI1 gene expression was not examined in the
late stationary phase. To confirm the growth phase-specific
role of Hfq, we compared the HiiD protein levels between
wild-type and \( \Delta hfq \) cells under shaking culture conditions
through western blotting for an HA tag fused to HiiD. The
insertion of the HA epitope did not affect the expression of
SPI1 genes (data not shown). The \( hfq \) mutation decreased
HiiD production compared with the wild-type at 4 h p.i.,
and 3B). As expected, $P_{\text{hilD}}$ exhibited reduced transcription in $\Delta$hilD and $\Delta$hfq strains compared with the wild type. The stationary phase-dependent activation of $P_{\text{hilD}}$ was attenuated in $\Delta$hilD and completely disappeared in $\Delta$hfq; $P_{\text{hilD}}$ activity increased gradually over time in the $\Delta$hfq strain (Fig. 3B). Interestingly, however, expression from $P_{\text{hilD}}$ was barely detected in $\Delta$hilD$\Delta$hfq (Fig. 3B). This finding suggests that Hfq can affect expression from $P_{\text{hilD}}$ even in the absence of hilD. The hilD-independent effect of Hfq on $P_{\text{hilD}}$ was verified using a hilD::lacZY chromosomal fusion strain lacking functional hilD (data not shown).

To compare the hilD expression between the wild-type and arcA mutant strains, we performed a real-time PCR assay under shaking conditions. When the RNA was harvested from $\Delta$arcA during the early stationary phase (4 h p.i.), the hilD mRNA level was reduced by approximately 3-fold compared with the wild type (Fig. 4A). The hilD mRNA level was not significantly changed in the late stationary phase (12 h p.i.), irrespective of arcA mutation (Fig. 4A). The mutation in arcA decreased the expression of $P_{\text{arc-lacZ}}$ only during the early stationary phase (Fig. 4B). Although $P_{\text{arc-lacZ}}$ expression was reduced in the $\Delta$arcA$\Delta$hilD double mutant in the late stationary phase (Fig. 4B), this decrease seems to have been caused by hilD mutation, not arcA mutation, because there was no further reduction in $P_{\text{hilD}}$ expression in $\Delta$arcA$\Delta$hilD compared with the $\Delta$hilD mutant (Fig. 4B). Taken together, these data show that the effect of Hfq and ArcA on $P_{\text{hilD}}$ expression is specific to the transition from the exponential to the stationary phase under shaking conditions, in which case Hfq can affect $P_{\text{hilD}}$ through both hilD-dependent and hilD-independent mechanisms.

**Discussion**

We monitored the transcriptional levels of four SPI1 genes using chromosomal lacZ fusions under both shaking and standing culture conditions, and found that SPI1 is most active at the onset of the stationary phase under shaking culture conditions (Fig. 1). Growth phase-dependent activation of SPI1 genes during the stationary phase under shaking conditions has been previously reported [7, 21, 24, 32, 38]. The enhanced activation of SPI1 under shaking culture conditions is consistent with previous observations that *Salmonella* cells from aerobic cultures in the late logarithmic phase are maximally invasive [23, 39] and fully induce macrophage death [28]. Many previous studies have shown that SPI1 gene expression decreased significantly under shaking culture conditions when SPI1 expression levels whereas both strains showed similar levels of HilD at 12 h p.i. (Fig. 3A), which is consistent with the expression patterns of hilA and invF in hfq mutants (Fig. 2).

Although production of HilD is largely controlled at the post-transcriptional level [16, 19], transcriptional regulation is a basic step in bacterial gene regulation. Since HilD can induce its own expression [15, 16], the decrease in HilD in $\Delta$hfq likely decreases the hilD promoter ($P_{\text{hilD}}$) activity. To investigate the effect of hfq mutation on hilD transcription, we monitored the hilD promoter ($P_{\text{hilD}}$) activity using a single-copy hilD-lacZ reporter vector, pJB5. Under shaking culture conditions, $P_{\text{hilD}}$ also showed a stationary phase-dependent activation, similar to other SPI1 genes (Figs. 1 and 3B). As expected, $P_{\text{hilD}}$ exhibited reduced transcription in $\Delta$hilD and $\Delta$hfq strains compared with the wild type. The stationary phase-dependent activation of $P_{\text{hilD}}$ was attenuated in $\Delta$hilD and completely disappeared in $\Delta$hfq; $P_{\text{hilD}}$ activity increased gradually over time in the $\Delta$hfq strain (Fig. 3B). Interestingly, however, expression from $P_{\text{hilD}}$ was barely detected in $\Delta$hilD$\Delta$hfq (Fig. 3B). This finding suggests that Hfq can affect expression from $P_{\text{hilD}}$ even in the absence of hilD. The hilD-independent effect of Hfq on $P_{\text{hilD}}$ was verified using a hilD::lacZ chromosomal fusion strain lacking functional hilD (data not shown).

To compare the hilD expression between the wild-type and arcA mutant strains, we performed a real-time PCR assay under shaking conditions. When the RNA was harvested from $\Delta$arcA during the early stationary phase (4 h p.i.), the hilD mRNA level was reduced by approximately 3-fold compared with the wild type (Fig. 4A). The hilD mRNA level was not significantly changed in the late stationary phase (12 h p.i.), irrespective of arcA mutation (Fig. 4A). The mutation in arcA decreased the expression of $P_{\text{arc-lacZ}}$ only during the early stationary phase (Fig. 4B). Although $P_{\text{arc-lacZ}}$ expression was reduced in the $\Delta$arcA$\Delta$hilD double mutant in the late stationary phase (Fig. 4B), this decrease seems to have been caused by hilD mutation, not arcA mutation, because there was no further reduction in $P_{\text{hilD}}$ expression in $\Delta$arcA$\Delta$hilD compared with the $\Delta$hilD mutant (Fig. 4B). Taken together, these data show that the effect of Hfq and ArcA on $P_{\text{hilD}}$ expression is specific to the transition from the exponential to the stationary phase under shaking conditions, in which case Hfq can affect $P_{\text{hilD}}$ through both hilD-dependent and hilD-independent mechanisms.

**Discussion**

We monitored the transcriptional levels of four SPI1 genes using chromosomal lacZ fusions under both shaking and standing culture conditions, and found that SPI1 is most active at the onset of the stationary phase under shaking culture conditions (Fig. 1). Growth phase-dependent activation of SPI1 genes during the stationary phase under shaking conditions has been previously reported [7, 21, 24, 32, 38]. The enhanced activation of SPI1 under shaking culture conditions is consistent with previous observations that *Salmonella* cells from aerobic cultures in the late logarithmic phase are maximally invasive [23, 39] and fully induce macrophage death [28]. Many previous studies have shown that SPI1 gene expression decreased significantly under shaking culture conditions when SPI1 expression levels whereas both strains showed similar levels of HilD at 12 h p.i. (Fig. 3A), which is consistent with the expression patterns of hilA and invF in hfq mutants (Fig. 2).

Although production of HilD is largely controlled at the post-transcriptional level [16, 19], transcriptional regulation is a basic step in bacterial gene regulation. Since HilD can induce its own expression [15, 16], the decrease in HilD in $\Delta$hfq likely decreases the hilD promoter ($P_{\text{hilD}}$) activity. To investigate the effect of hfq mutation on hilD transcription, we monitored the hilD promoter ($P_{\text{hilD}}$) activity using a single-copy hilD-lacZ reporter vector, pJB5. Under shaking culture conditions, $P_{\text{hilD}}$ also showed a stationary phase-dependent activation, similar to other SPI1 genes (Figs. 1 and 3B). As expected, $P_{\text{hilD}}$ exhibited reduced transcription in $\Delta$hilD and $\Delta$hfq strains compared with the wild type. The stationary phase-dependent activation of $P_{\text{hilD}}$ was attenuated in $\Delta$hilD and completely disappeared in $\Delta$hfq; $P_{\text{hilD}}$ activity increased gradually over time in the $\Delta$hfq strain (Fig. 3B). Interestingly, however, expression from $P_{\text{hilD}}$ was barely detected in $\Delta$hilD$\Delta$hfq (Fig. 3B). This finding suggests that Hfq can affect expression from $P_{\text{hilD}}$ even in the absence of hilD. The hilD-independent effect of Hfq on $P_{\text{hilD}}$ was verified using a hilD::lacZ chromosomal fusion strain lacking functional hilD (data not shown).

To compare the hilD expression between the wild-type and arcA mutant strains, we performed a real-time PCR assay under shaking conditions. When the RNA was harvested from $\Delta$arcA during the early stationary phase (4 h p.i.), the hilD mRNA level was reduced by approximately 3-fold compared with the wild type (Fig. 4A). The hilD mRNA level was not significantly changed in the late stationary phase (12 h p.i.), irrespective of arcA mutation (Fig. 4A). The mutation in arcA decreased the expression of $P_{\text{arc-lacZ}}$ only during the early stationary phase (Fig. 4B). Although $P_{\text{arc-lacZ}}$ expression was reduced in the $\Delta$arcA$\Delta$hilD double mutant in the late stationary phase (Fig. 4B), this decrease seems to have been caused by hilD mutation, not arcA mutation, because there was no further reduction in $P_{\text{hilD}}$ expression in $\Delta$arcA$\Delta$hilD compared with the $\Delta$hilD mutant (Fig. 4B). Taken together, these data show that the effect of Hfq and ArcA on $P_{\text{hilD}}$ expression is specific to the transition from the exponential to the stationary phase under shaking conditions, in which case Hfq can affect $P_{\text{hilD}}$ through both hilD-dependent and hilD-independent mechanisms.
were compared between bacterial cells incubated without agitation overnight, and bacterial cells incubated with shaking at equivalent optical densities (OD₆₀₀ 0.4–0.5) [4, 5, 15, 25, 27]. We also found that SPI1 gene expression increased by 2- to 6-fold under standing culture conditions when β-galactosidase activities were compared at similar culture densities (between the standing cultures grown for 12 h and the shaking cultures grown for 2 h, Fig. 1). In conclusion, SPI1 expression is differentially activated depending on the culture conditions; specifically, a temporary up-regulation coincides with entry into the stationary growth phase under shaking culture conditions, whereas steady expression is observed during growth under standing culture conditions.

To identify the regulatory elements responsible for SPI1 activation under shaking culture conditions, we examined hilA and invF expression under various mutational backgrounds. Of the five mutant strains evaluated (which are involved in the growth phase-dependent activation of SPI1), ΔhilD and ΔarcA abrogated the stationary phase-dependent activation of hilA and invF (Fig. 2). Two global transcriptional regulators, Fnr and ArcA, have generally been implicated in activating a large number of anaerobic genes and repressing many aerobic genes in low-oxygen growth conditions [34]. There have been contradictory results of Fnr effects on SPI1 expression: the transcription of most SPI1 genes, including hilA, was reduced in the fnr mutant in one report [18], whereas other reports show increased hilA expression in fnr mutants under anaerobic conditions [19, 42]. It was previously reported that ArcA could not regulate SPI1 genes under anaerobic conditions [17]. Here, however, a disruption in arcA decreased the levels of hilA, invF, and hilD expression during the early stationary phase under shaking culture conditions (Figs. 2 and 4). Considering that several genes are under complex dual control of Fnr and ArcA [8, 43], and that Fnr is involved in the anaerobic activation of arcA transcription [11], a more comprehensive experiment that can define the roles of Fnr and ArcA is necessary to elucidate the regulation mechanism of SPI1.

Because hilD mRNA is a direct Hfq target [36], loss of Hfq association with hilD mRNA in ΔhigF strains in the early stationary phase can reduce HilD protein synthesis (Fig. 3A), thereby reducing SPI1 expression (Fig. 2). In addition, P₅₆₆ expression decreased in hfq mutants (Fig. 3B) because HilD is the positive regulator for hilD promoter activation [6, 15]. The fact that a lack of hfq dramatically decreased P₅₆₆ expression in the absence of hilD (Fig. 3B) suggests that Hfq can increase P₅₆₆ activity by stabilizing hilD mRNA and/or interacting with other RNAs that affect P₅₆₆ expression. On the other hand, ArcA seems to have a role in P₅₆₆ regulation independent of HilD in the early stationary phase (Fig. 4B). In the absence of hilD, a mutation of hfq has a greater effect on hilD expression when compared with the effect of arcA mutation (Figs. 3B and 4B). Since Hfq controls the expression of almost one fifth of all Salmonella genes [36], it is likely that Hfq and ArcA share a regulatory circuit of SPI1. There is a small regulatory RNA, ArcZ, which is dependent on Hfq and is controlled by ArcA [31]. Thus, we tested the effect of ArcZ on hilA expression, but the absence of ArcZ did not affect the expression (data not shown).

It was recently shown that SPI1 gene expression is activated by an increase in P₅₆₆ activity. When the activating signals are sufficiently strong, hilD is expressed, which activates SPI1 [33]. Based on our results, Hfq and ArcA seem to convey the signals activating P₅₆₆ when cells enter the stationary phase under shaking conditions. It was found that SPI1 is heterogeneously expressed in S. Typhimurium populations [21], and the proportion of cells expressing SPI1 increases during the late logarithmic phase of aerated batch cultures, in which the growth retardation is attributable to the SPI1 expression [40]. The subpopulation capable of forming the SPI1 T3SS invades the gut tissue and is killed by the intestinal innate immune defenses while preparing the ground for a successful infection [1]. Although the underlying mechanisms of self-destructive cooperation are not revealed, Hfq and/or ArcA may be associated in controlling the level of SPI1 expression in response to environmental signals such as oxygen and nutrient depletion and metabolite accumulation, which are common in the lumen of the host’s intestine and in the early stationary phase in LB batch culture.

**Acknowledgments**

This research was supported by the WCU (World Class University) program through the NRF funded by the Ministry of Education, Science and Technology (R32-2008-00-10183-0), and the Nuclear R&D program of the Ministry of Education, Science and Technology (MEST), Republic of Korea.

**References**

2. Altier C. 2005. Genetic and environmental control of


