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Mutation of the *invF* Gene Encoding a *Salmonella* Pathogenicity Island 1 (SPI1) Activator Increases Expression of the SPI2 Gene, *sseA*

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In *Salmonella enterica*, many genes encoded within *Salmonella* pathogenicity islands (SPI) 1 and 2 are required to cause a range of diseases in a variety of hosts. The SPI1-encoded regulator HilD activates both the SPI1 and 2 genes at different times during growth in Luria-Bertani (LB) media. In this study, the expression levels of *hilD* during growth in LB were investigated. The data suggest that *hilD* expression is induced in the early stationary phase and decreases in the late stationary phase, when *sseA*, an SPI2 gene, is maximally expressed. However, HilD could act as an activator of *sseA* expression in the late stationary phase despite being present at low levels. *SseA* expression was investigated in SPI1 regulator mutant strains, *hilA*, *hilD* and *invF* mutants. As expected, *hilD* mutation decreased *sseA* expression. However, we found that *invF* mutation caused a 1.5-fold increase in *sseA* expression in not only LB but also M9 minimal media, which is thought to resemble an intracellular environment. InvF overexpression restored *sseA* expression to wild-type levels in an *invF* mutant but did not cause an additional reduction in *sseA* expression. These results suggest that SPI1 controls SPI2 expression either positively or negatively.

Keywords: *Salmonella* Typhimurium, *Salmonella* pathogenicity island (SPI), *invF*

Salmonella serovars are Gram-negative intracellular pathogens that cause a range of diseases in a variety of hosts [16]. Two major clinical manifestations characterize *Salmonella* infections: enteritis (salmonellosis), a self-limiting local intestinal inflammatory response, and enteric fever (typhoid), a systemic infection characterized by dissemination of the bacteria throughout the reticuloendothelial system via infected macrophages to the lymph nodes, liver, and spleen [12, 16, 17]. *Salmonella* has evolved through the acquisition of several pathogenicity islands, two of which, *Salmonella* pathogenicity island (SPI) 1 and 2, composed of 39 and 44 genes, respectively, play a key role in *Salmonella* pathogenesis [12]. Each pathogenicity island encodes type III secretion systems that are essential for virulence [11, 12]. SPI1 genes are necessary for invasion of epithelial host cells and thus intestinal colonization, leading to enteritis, whereas SPI2 genes are required for bacterial replication/survival inside macrophages and thus for systemic disease [11, 16].

The expression of SPI1 genes is governed mainly by transcriptional regulators encoded within SPI1, such as HilA, HilD, and InvF [9]. Bustamante *et al.* [3] reported that the SPI1-encoded regulator HilD can activate both SPI1 and SPI2 genes but at different times during the stationary phase of growth in Luria-Bertani (LB) medium: SPI2 genes are activated after bacteria have reached the late stationary phase and SPI1 expression has ceased. However, they monitored only the transcriptional level of *invF* and translational level of *hilA*, and not the level of *hilD* [3].

To determine whether the expression level of *hilD* is maintained during growth, we first constructed a *hilD*::*lacZY* chromosomal fusion strain, SR4221 (Table 1), using the one-step gene-inactivation method (i.e., λ red and FLP-mediated site-specific recombination system) [5, 8]. Briefly, a kanamycin cassette from pKD13 was amplified using the *hilD*-RF and -RR primers listed in Table 2, and the resultant PCR product (FRT-kan-FRT) was introduced into *hilD* in the chromosome by homologous recombination.

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

Strain or plasmid	Description	Reference or source
Strains		
SL1344	wild type serovar Typhimurium *xyl rpsL hisG*	Lab stock
SR4221	SL1344 *hilD*::*lacZY*, Kan^R	This study
SR3584	SL1344 *hilD*::HA	This study
BJ2398	SL1344 *hilA*::Tn5*lacZY*, *hilD*::*cam*, Tet^R, Cam^R	2
SR3432	SL1344 *hilD*::*cam*, Cam^R	This study
HH129	SL1344 *hilA*::Tn10, Tet^R	1
SVM579	SL1344 Δ*invF* (in-frame deletion of 465 bp)	4
SR3266	SL1344 *sseA*::*lacZY*, Kan^R	13
SY1051	SR3432 *sseA*::*lacZY*, Kan^R, Cam^R	This study
SY1052	HH129 *sseA*::*lacZY*, Kan^R, Tet^R	This study
SY1053	SVM579 *sseA*::*lacZY*, Kan^R	This study
Plasmids		
pKD46	*bla* P_{BAD} *gam beta exo* pSC101 oriTS	5
pKD13	*bla* FRT *ahp* FRT PS1 PS4 oriR6K	5
pCP20	*bla cat cI*857 λP_R *flp* pSC101 oriTS	5
pCE70	*ahp* FRT *lacZY*$^+$ t_{his} oriR6K	15
pJB5	*hilD-lacZY* reporter vector, Amp^R	2
pYD40	*invF-sicA* expression, Amp^R	6

This antibiotic resistance cassette was removed using the temperature-sensitive plasmid pCP20 carrying the FLP recombinase gene. FLP-mediated excision of the *kan* cassette left a single FRT site that served as an integration point for fusion. Transcriptional *lac* fusions were generated from the above constructs using pCE70 [15]. A stationary-phase culture that had been grown overnight with shaking was used as the stock culture. The stock culture was inoculated into fresh LB broth at a 1:100 dilution, and cells were cultivated at 37°C with shaking. The activity of chromosomal *hilD*::*lacZ* transcriptional fusions was analyzed by determining the β-galactosidase activity according to a standard method [14]. *HilD* expression increased rapidly, peaking at 4 h post-inoculation (p.i.), and decreased gradually thereafter (Fig. 1A).

HilD is capable of inducing its own expression [7]. Therefore, it is likely that the activity of chromosomal *hilD*::*lacZY* fusion does not mirror a real transcription because the *lacZY* fusion on the *hilD* gene causes mutation of *hilD*. To investigate only the *hilD* promoter activity, we transformed a single-copy *hilD-lacZY* reporter vector (pJB5; Table 1) into the wild-type strain. Although peak expression was delayed by 2 h, *hilD* activated 4 h after inoculation. However, *hilD-lacZY* expression from the plasmid pJB5 did not decrease as much as the chromosomal *hilD*::*lacZY* expression at 8 h p.i. (Fig. 1A and B). This suggests that HilD seems to play a more important role in its own regulation in the late stationary phase rather than in the early one. It is known that *hilD* is primarily regulated at the post-transcriptional level [9]. To check if the HilD protein is also produced differentially depending on the growth phase, we performed Western blot analysis of whole-cell lysates of strain SR3584, a derivative of wild-type *Salmonella* expressing HA-tagged HilD. This strain was also constructed by the one-step gene-inactivation method [5] using *hilD*-HAF/*hilD*-HAR primer sets (Table 2), as previously described in SR4221 construction. Insertion of the HA epitope did not affect the expression of SPI1 genes (data now shown). *Salmonella* strains encoding the HilD-HA protein were harvested by centrifugation at 4 and 12 h p.i., and lysates were prepared by lysis of cells using B-PER solution (Pierce). Lysates (20 μg protein) were separated by 12% SDS-PAGE, transferred to nitrocellulose membranes, and the HilD and DnaK proteins were 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). The HilD protein was more abundant in the early (4 h) stationary phase than in the late (12 h) one

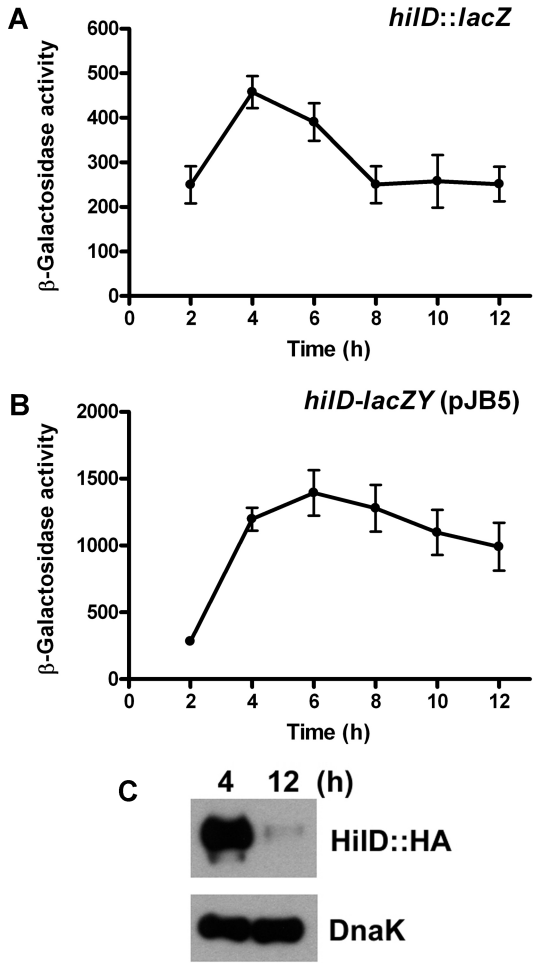

Fig. 1. Analysis of *hilD* expression in LB. The expression of chromosomal *hilD*::*lacZ* transcriptional fusion (A) and a single-copy *hilD-lacZY* reporter vector (pJB5) (B) were analyzed by measuring β-galactosidase activity under shaking culture conditions. Data represent the mean and standard deviation of three independent experiments with duplicate samples. (C) Western blots of whole-cell lysates of a *Salmonella* Typhimurium SL1344 strain carrying a chromosomal HA-tagged HilD were performed using a monoclonal anti-HA antibody. Equivalent amounts of protein were analyzed for each time point. As a loading control, DnaK was detected using a monoclonal antibody.

(Fig. 1C). Taken together, these results indicate that *hilD* also shows growth phase-dependent expression similar to other SPI1 genes such as *hilA* and *invF*.

Considering that SPI2 genes are expressed during the late stationary phase during growth in LB medium [3], it seems unusual that the expression of HilD, an activator of SPI2, is lower at that time (Fig. 1). To determine whether HilD activates SPI2 genes regardless of its abundance, we first monitored the expression of *sseA*, the first gene of the SPI2 operons (*sseA*-*G*) to determine the point in time for maximal induction. *sseA* expression was activated following entry into the stationary phase, but peaked later (12 h) than the *hilD* expression (Figs. 1B and 2A). At this point in time, we investigated the possibility that, in addition to *hilD*, other SPI1 regulators such as *hilA* and *invF* might be involved in controlling the expression of SPI2 genes. An isogenic *hilD* mutant strain, SR3432, was obtained through P22HT-mediated transduction to the wild-type of a mutant allele (*hilD*::*cam*) from a *hilA*/*hilD* mutant strain, BJ2398. The SY1051, SY1052, and SY1053 mutant strains containing the chromosomal *sseA*::*lacZ* fusion were obtained through P22HT-mediated transduction of the *lacZ* fusion from strain SR3266 into strains SR3432 (*hilD*$^-$), HH129 (*hilA*$^-$), and SVM579 (*invF*$^-$), respectively [14]. The expression of *sseA* was significantly reduced in the *hilD* mutant but unaffected in the *hilA* mutant (Fig. 2B), which is in agreement with previous results [3]. HilD activates the expression of *hilA* and *ssrAB*, the genes encoding the central regulators of SPI1 and SPI2, by binding directly to their promoter regions [3, 18]. HilD shows a higher binding affinity to the *hilA* promoter region than that of *ssrAB* [3]. Considering the low level of HilD during the late stationary phase (Fig. 1C), it seems that additional factors might be required to assist in binding to the *ssrAB* regulatory region. However, we cannot rule out the possibility that even low levels of HilD may activate SPI2 expression without aiding of other factors.

Interestingly, *invF* mutation caused a 1.5-fold increase in *sseA* expression (Fig. 2B). To complement the *invF* defect,

Table 2. Oligonucleotides used in this study.

Primer name	Sequence (5'→3')*	Purpose
hilD-RF	aca tct gaa aac ggc gtt ctc ctg tac gaa gga tac aag *gtg tag gct gga gct gct tc*	λ Red deletion of *hilD*; *lacZY* insertion
hilD-RR	ttt caa tac aac ttt tgt tgt tac agt tac tgc aac ctt t*at tcc ggg gat ccg tcg acc*	
hilD-HAF	aaa act acg cca tcg aca ttc ata aaa atg gcg aac cat tat ccg tat gat gtt cct gat tat gct agc ctc taa *gtg tag gct gga gct gct tc*	λ Red deletion of *hilD*; HA epitope insertion
hilD-HAR	ctg ata gag cgt gtt aat gcg cag tct gaa ttt taa att *att ccg ggg atc cgt cga cc*	

*P1, P2, and P4 priming sites in pKD3 and pKD13 are shown in italics.

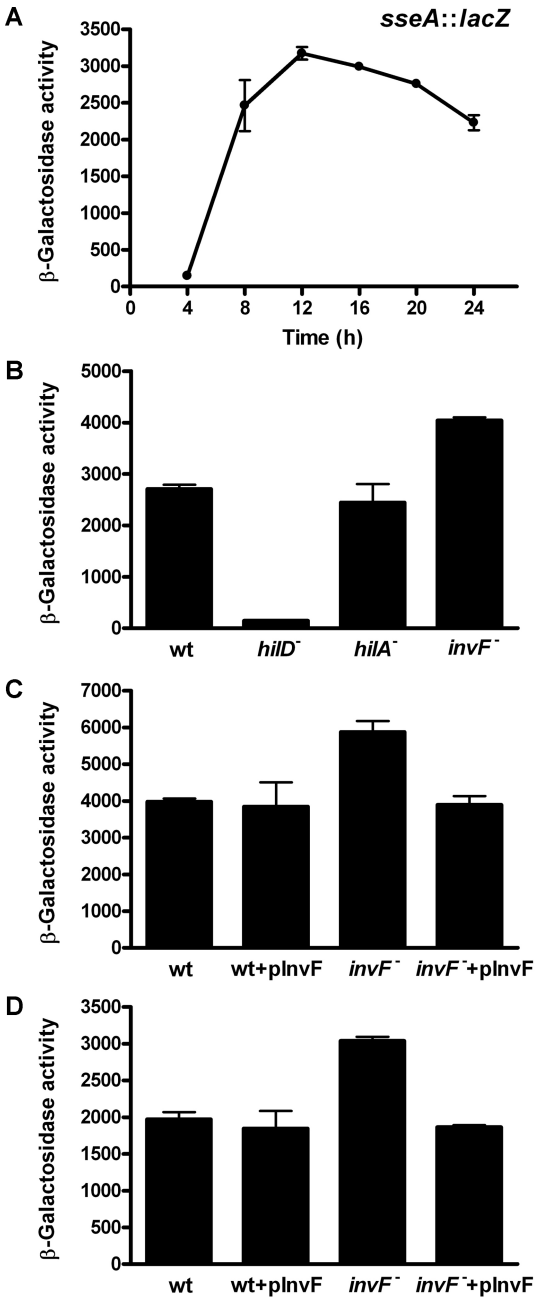

Fig. 2. Effect of *invF* mutation on *sseA* expression. (A) *SseA* expression was examined using a chromosomal *lacZ* fusion strain by measuring β-galactosidase activity under shaking culture conditions. (B) Expression of the *sseA*::*lacZ* fusion was determined in cultures of wild-type *Salmonella* Typhimurium and its *hilD*⁻, *hilA*⁻, and *invF*⁻ derivatives grown for 12 h in LB medium. The level of *sseA* expression was also determined in the wild-type and *invF* mutant strains with or without the *invF* complementation plasmid pInvF (pYD40) grown for 12 h in (C) LB and (D) M9 minimal media. Data represent the mean and standard deviation of three independent experiments with duplicate samples.

we transformed the pYD40 plasmid expressing InvF with a cofactor, SicA, under the control of the *lac* promoter (Table 1) into the wild-type and *invF* mutant strains, the respectively. IPTG (1 mM) was added to medium to induce the expression of *invF* and *sicA*. InvF overexpression did not affect *sseA* expression in the wild-type, but restored *sseA* expression to the wild-type level in the *invF* mutant strain (Fig. 2C). This suggests that InvF controls *sseA* indirectly because *sseA* expression was not reduced, irrespective of InvF overexpression. HilD is not required for expression of the SPI2 genes when *Salmonella* is grown in minimal media containing low concentrations of magnesium and phosphate [3]. Under these growth conditions, *ssrAB* expression depends instead on other regulators, such as OmpR, PhoP, and SlyA [10]. Thus, we next investigated whether InvF could affect *sseA* expression in M9 minimal medium. M9 medium comprised 13 μg/L $Na_2HPO_4 \cdot 7H_2O$, 3 g/L KH_2PO_4, 0.5 g/L NaCl, and 1 g/L NH_4Cl. It was supplemented with 2 mM $MgSO_4$, 10 mM glucose, and 0.004% L-histidine. In this medium, the expression of *sseA* increased (Fig. 2D), suggesting that InvF negatively regulates *sseA* expression during the stationary phase in LB and M9 media.

In this study, we demonstrated sequential activation of the SPI1 and SPI2 genes during the stationary phase by monitoring *hilD* and *sseA* expression. This study incorporates InvF that controls the expression of SPI1 in the SPI2 regulatory pathway. A previous study revealed that among the *hilD*, *hilA*, and *invF* mutants, the expression of an SPI2 gene (*ssaG*) was only changed in the *hilD* mutant [3]. This difference can be attributed to differences in the experimental model; in our study, *sseA* expression was examined using the chromosomal *sseA*::*lacZ* fusion, whereas the previous study used a plasmid carrying a transcriptional *ssaG-cat* fusion. It is likely that either cis- or trans-elements involved in *ssaG* gene expression might be disturbed on the plasmid. It is generally believed that the expressions of both the SPI1 and SPI2 genes are inversely regulated: under conditions that fully induce SPI1 expression, SPI2 expression is at minimal levels, and *vice versa* [11]. The increased expression of SPI2 in the absence of *invF* is a clue to the existence of a new type of regulatory network in SPI1 and SPI2 cross-talk.

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국문초록

Salmonella Pathogenicity Island 1(SPI1)의 발현조절 유전자 *invF*의 변이가 SPI2 유전자(*sseA*)의 발현에 미치는 영향

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살모넬라(*Salmonella*)의 염색체에 존재하는 병원성 유전자의 집합체인 *Salmonella* pathogenicity island(SPI)1 과 2는 살모넬라가 유발하는 다양한 질병에 중요한 역할을 한다. SPI1의 발현을 유도하는 HilD는 Luria-Bertani(LB) 배지 조건에서 SPI2의 발현 활성인자로 작용하는 것으로 알려져 있으나 LB 배지 내에서 *hilD* 유전자의 발현 양상은 아직까지 연구되지 않았다. 본 연구에서는 LB 배지에 살모넬라를 배양하면서 *hilD* 유전자의 발현과 단백질 양을 조사하였으며 SPI2 유전자인 *sseA*의 발현과 비교하였다. *hilD*의 발현은 대수 증식기 경과 후 정지기(stationary phase)로 전환되는 시기에 비약적으로 증가하였으나 *sseA*의 발현은 정지기 후반부에 최대로 증가하였다. 즉, 후반 정지기에서 HilD 단백질은 낮은 수준으로 존재함에도 불구하고 SPI2의 발현을 유도한다는 것을 알 수 있었다. SPI1의 다른 발현 조절인자인 *hilA*와 *invF*의 변이체에서 *sseA*의 발현을 살펴본 결과 *invF*의 변이는 *hilD*와는 다르게 배지 조건에 상관없이 오히려 *sseA*의 발현을 증가시켰다. 또한, InvF의 과발현은 *sseA* 발현을 정상 수준으로 복원시켰지만 추가적인 감소는 일으키지 않는다는 것을 알 수 있었다. SPI1은 HilD를 이용하여 SPI2의 발현을 유도하지만 반대로 InvF를 이용하여 발현을 억제하기도 하는 이중적인 조절 기전을 가지고 있는 것으로 판단된다.