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NOTE

Temporal regulation of *Salmonella* pathogenicity Island 1 (SPI-1) *hilA* by Hfq in *Salmonella enterica* serovar typhimurium

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Abstract Hfq is a global regulatory RNA-binding protein abundant in many bacteria. In terms of virulence regulation in *Salmonella*, Hfq binds to and stabilizes the mRNA of *hilD*, a master regulator of *Salmonella* pathogenicity island 1 (SPI-1), and Hfq activates SPI-1 gene transcription during the late exponential-growth phase. In contrast to the positive roles of Hfq in SPI-1 regulation, we observed that the absence of Hfq increased the expression of SPI-1 regulators, including HilA and InvF, during the late stationary phase. This result suggests that Hfq has pleiotropic functions, controlling SPI-1 positively or negatively depending on the growth conditions.

Keywords *HilA* · *Hfq* · *Salmonella* · *Salmonella* pathogenicity islsland 1 (SPI-1)

Introduction

Salmonellae are enteric pathogens that cause gastroenteritis and enteric fever. They diverged from the genus *Escherichia* ~120–160 million years ago with the acquisition of a set of virulence genes, termed *Salmonella* pathogenicity island 1 (SPI-1) (Galan 1999). SPI-1, a 40-kb island located at centisome 63 of the genome, is composed of more than 39 genes encoding regulators, effectors, cognate chaperones, and components of a type III secretion system (SPI-1 T3SS) (Lostroh and Lee 2001). The SPI-1 T3SS plays diverse roles in multiple steps of *Salmonella* systemic infections, including invasion into host epithelial cells (Galan 1999), induction of proinflammatory responses (Hardt et al. 1998), stimulation of programmed cell death in macrophages (Hernandez et al. 2003), and proliferation within host cells (Que et al. 2013). Due to the multifaceted roles of SPI-1 in different locations and at different times, its expression is finely regulated by a coordinated regulatory network that integrates multiple environmental signals conclusively under the control of HilA, a direct activator of SPI-1 (Golubeva et al. 2012). HilD, another cognate activator encoded within SPI-1, is positioned at the hub of the regulatory network, linking multiple regulatory inputs to HilA; it is therefore regarded as a master regulator at the top of the SPI-1 regulatory circuit (Golubeva et al. 2012).

Hfq is one of the most abundant RNA-binding proteins in bacteria and has key roles in gene expression control (Vogel and Luisi 2011). In terms of SPI-1 regulation, Hfq was found to stabilize *hilD* mRNA and enhance HilD activity in late log/early stationary phase cultures at 4 h post-inoculation (p.i.) (Sittka et al. 2008; Lim et al. 2013). Accordingly, *hilA* promoter activity was reduced in Δ*hfq* strains (Sittka et al. 2007; Lim et al. 2013). However, we suggest that the influence of Hfq on SPI-1 varies depending on bacterial growth phases, based on the observation in this study that the expression of SPI-1 regulators, including HilA and InvF, in Δ*hfq* appeared to catch up with that in wild-type *Salmonella* in late stationary phase cultures.

Materials and methods

Bacterial strains and growth conditions

Wild-type *Salmonella enterica* serovar Typhimurium SL1344 was used as the parent strain in all of the strains

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constructed in this study. Wild-type *Salmonella* and its isogenic Δ*hfq* strain harboring the *hilA*::*lacZY* fusion, respectively, were constructed in a previous study (Lim et al. 2013). *Salmonella* strains expressing hemagglutinin (HA)-tagged HilA or HA-tagged InvF were constructed using the phage λ Red recombination system as described elsewhere (Datsenko and Wanner 2000; Lim et al. 2013). The primers used to amplify the kanamycin cassette from pKD13 (Datsenko and Wanner 2000) were hilA-HAF (5′-ttc aaa aga tgg aaa cag gat ccc cgc ttg att aaa tta cgg tat ccg tat gat gtt cct gat tat gct agc ctc tga tgt agg ctg gag ctg ctt cg-3′) and hilA-HAR (5′-att acg atg ata aaa aaa taa tgc ata tct cct ctc tca gat tat tcc ggg gat ccg tcg acc-3′) for HilA::HA expression, and invF-HAF (5′-tcg ccg cgg aaa tta tca aat att att caa ttg gca gac aaa tat ccg tat gat gtt cct gat tat gct agc ctc tga tgt agg ctg gag ctg ctt cg-3′) and invF-HAR (5′-cgc ggc aca tgc cag cac tct ggc caa aag aat atg tgt cta ttc cgg gga tcc gtc gac c-3′) for InvF::HA expression. Transfer of the resistance allele between strains was conducted by P22HT-mediated transduction (Maloy et al. 1996).

Bacteria grown in Luria–Bertani (LB) broth were added to fresh LB broth at a 1:100 ratio and cultivated at 37 °C with shaking. Kanamycin was added when required at 50 μg/ml.

β-galactosidase assay

Wild-type *Salmonella* and an isogenic Δ*hfq* strain harboring the chromosomal *hilA*::*lacZY* fusion were cultivated in LB broth and the activity of *hilA*::*lacZY* transcriptional fusions was analyzed by determining the β-galactosidase activity according to a standard method (Maloy et al. 1996).

Western blot analysis

Salmonella strains producing HA-tagged proteins were harvested by centrifugation at 4 and 12 h after inoculation. The cell lysates were prepared by lysis of cells using B-PER solution (Pierce, Rockford, IL) and lysates corresponding to 20 μg of protein were separated by 12 % SDS-PAGE. The proteins on the gel were transferred to nitrocellulose membranes and the membranes were incubated with anti-HA (Sigma, St. Louis, MO, USA) or anti-DnaK (Stressgen Biotechnologies Corp., Victoria, BC, Canada) antibodies and anti-mouse IgG horseradish peroxidase-linked antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in succession. The blots were developed using the WEST-ZOL detection system (Intron Biotechnology, Seongnam, Korea).

Results and discussion

In contrast to the known positive role of Hfq in SPI-1 regulation, we have observed that the *hilA* expression seemed to be increased by the absence of Hfq at 12 h p.i. in our previous study (Lim et al. 2013). To gain better insight into this issue, we first monitored *hilA* expression in a Δ*hfq* strain using *hilA*::*lacZY* chromosomal fusion across various growth phases (Fig. 1a). Wild-type *Salmonella enterica* serovar Typhimurium SL1344 and an isogenic Δ*hfq* strain harboring the *hilA*::*lacZY* fusion were cultivated in Luria–Bertani (LB) broth, and the activity of chromosomal *hilA*::*lacZY* transcriptional fusions was analyzed by determining the β-galactosidase activity every 2 h. As expected, *hilA* expression was reduced by approximately two-fold at 4 h p.i. in the Δ*hfq* strain when compared with wild type. However, a time-course observation demonstrated comparable *hilA* expression levels between the two strains at 6 h p.i. and higher *hilA* expression in the Δ*hfq* strain at later time points (the reduced expression of *hilA* after 6 h in wild type, while the increased expression of *hilA* in the Δ*hfq* strain was maintained for the remainder of the experiment).

The time-dependent differential effects of Hfq on *hilA* expression were further verified by comparing HilA protein levels between the wild-type and Δ*hfq* strains (Fig. 1b).

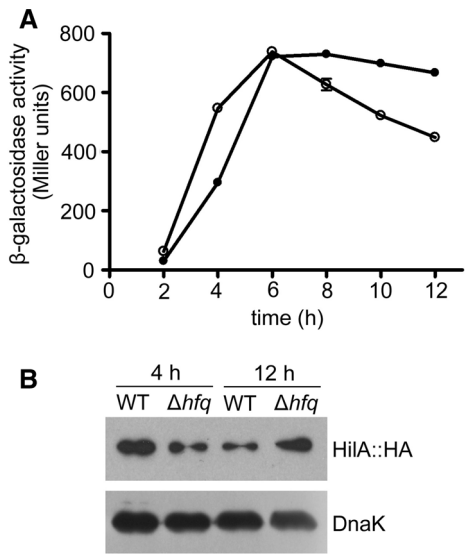

Fig. 1 Growth phase-dependent pleiotropic effects of Hfq on *hilA* expression. **a** Wild-type *Salmonella* Typhimurium SL1344 and isogenic Δ*hfq* strains harboring *hilA*::*lacZY* fusion were cultivated in LB broth at 37 °C, and the activity of *hilA*::*lacZY* was determined using a β-galactosidase assay every 2 h and represents the average result from three independent biological cultures. **b** Wild-type and Δ*hfq* *Salmonella* strains producing HA-tagged HilA were grown in LB broth for 4 and 12 h and the levels of HilA::HA were compared by Western blot analysis using anti-HA antibodies. The amount of protein loaded in each lane was normalized to the level of intracellular DnaK

Salmonella strains expressing HA-tagged HilA were cultivated in LB medium and subjected to Western blot analysis as described in Materials and Methods. In accordance with the β-galactosidase assay results, the absence of Hfq decreased HilA levels during the late log phase (4 h p.i.), whereas it increased HilA levels during the late stationary phase (12 h p.i.).

HilA can activate the expression of *inv*/*spa* and *prg*/*org* operons, which encode components of the SPI1-T3SS machinery, by binding to the *invF* and *prgH* promoters (Lostroh and Lee 2001). InvF, which is transcriptionally activated by HilA and HilD, was also differentially regulated by Hfq between the late log and late stationary phases; decreased InvF expression at 4 h p.i. and increased InvF expression at 12 h p.i. were observed in the Δ*hfq* strain compared with wild type (Fig. 2). Taken together, these results suggest that Hfq activates the transcription of SPI-1 genes by stabilizing *hilD* mRNA during the late log phase but decreases their expression during the late stationary phase; however, the role of Hfq in the negative regulation at the late stationary phase remains undetermined.

Hfq binds 727 *Salmonella* mRNAs to control their translational efficiency in combination with small noncoding RNAs (sRNAs) (Sittka et al. 2008). To gain further insights into the role of Hfq in the late stationary phase-dependent negative regulation of SPI-1 genes, we searched for regulators in the SPI-1 regulatory circuit, the expression of which might be affected by Hfq under late stationary phase conditions. CsrA, a global regulatory RNA-binding protein, binds to a region within the *hilD* mRNA transcript that overlaps with the Shine-Dalgarno sequence and prevents its translation, leading to accelerated turnover (Martinez et al. 2011). CsrA transcription is in turn activated by RpoS, an alternative sigma factor, responding to stressful conditions such as the starvation-induced stationary phase of growth (Mukherjee et al. 1998; Dong and Schellhorn 2010; Knudsen et al. 2013). RpoS mRNA is a representative target of Hfq, whose translation is released by the coordinated binding of Hfq and sRNAs (Lease and Woodson 2004). The translation of *rpoS* mRNA is self-repressed in the absence of Hfq by a stem loop within its 5′ leader; thus, *Salmonella* Δ*hfq* strains suffer from the lack of RpoS under starvation-induced stationary phase growth (Brown and Elliott 1996). The depletion of RpoS caused by the absence of Hfq downregulates CsrA expression, which may relieve the repression of SPI-1 genes under late

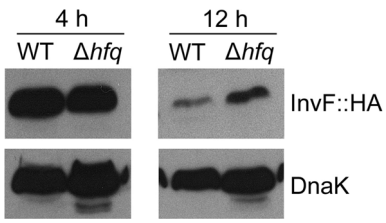

Fig. 2 Differential regulation of InvF by Hfq between the late log and late stationary phases of growth. The expression level of InvF::HA was compared between wild-type and Δ*hfq* *Salmonella* strains at 4 and 12 h post-inoculation using Western blot analysis. Equal amounts of cell lysate (20 μg protein) were loaded in each lane, and the level of InvF protein produced in each strain was normalized to the intracellular DnaK level

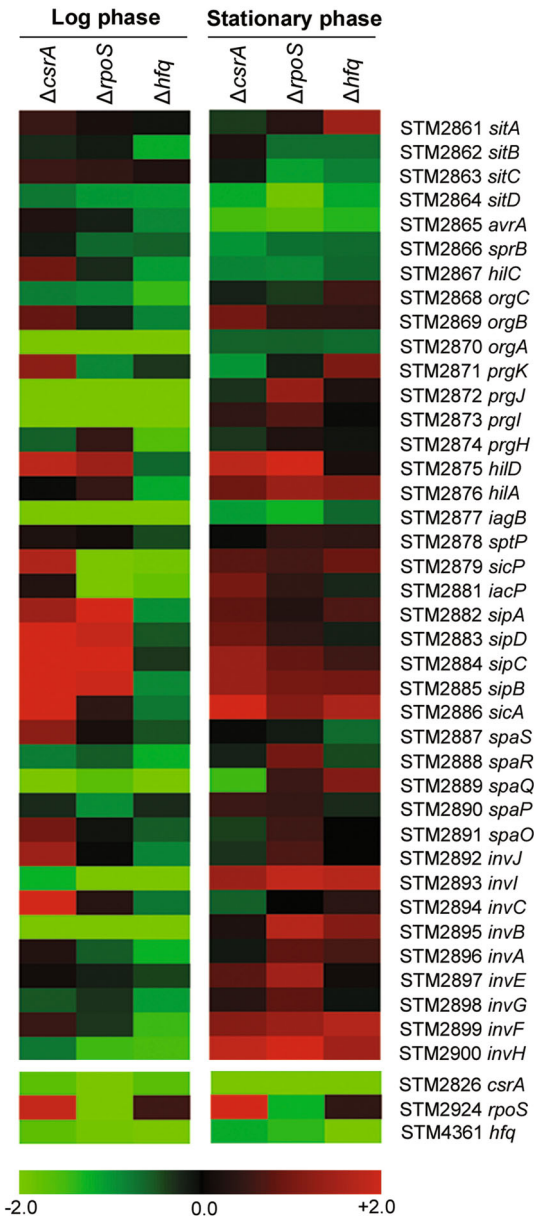

Fig. 3 Transcriptomic analysis of SPI-1 genes in Δ*csrA*, Δ*rpoS*, and Δ*hfq* strains. The expression ratio, as determined by the microarray results, of a given SPI-1 gene in a mutant strain relative to the wild-type strain was computed in the LB log and stationary phases. *Green* and *red* represent a logarithmic ($\log_2$) decrease and increase in the mutant, respectively. *Rows* indicate individual genes within SPI-1, while columns correspond to regulatory mutants under two growth conditions

stationary phase conditions. Knudsen et al. (2013) demonstrated that the downregulation of SPI-1 genes in *clpP* mutant strains was abolished by the deletion of *rpoS*, which most likely nullified the increase in CsrA caused by the deletion of *clpP*.

To investigate the possible indirect regulation of SPI-1 genes by Hfq via RpoS and CsrA, we compared the transcriptional profiles of SPI-1 genes in Δ*hfq*, Δ*rpoS*, and Δ*csrA* strains under log and stationary phase conditions (Fig. 3). Transcriptomic data for the Δ*hfq*, Δ*rpoS*, and Δ*csrA* strains were retrieved from a previous study (Yoon et al. 2009) and reanalyzed. In accordance with the results presented in this study, the effects of Hfq on the expression of SPI-1 genes differed between the log and stationary phases. The transcription of SPI-1 genes in the absence of Hfq was decreased in log phase LB cultures, whereas it tended to be elevated in stationary phase LB cultures. Interestingly, the SPI-1 expression patterns in the Δ*rpoS* and Δ*csrA* strains paralleled each other in the two growth phases, and SPI-1 gene transcription appeared increased in both strains during the stationary phase. Furthermore, the negative effects of RpoS and CsrA on SPI-1 expression were more distinct during the stationary phase, when the expression of *rpoS* is elevated in response to nutrient depletion. Given that the transcriptional responses of the SPI-1 region were similar among the Δ*hfq*, Δ*rpoS*, and Δ*csrA* strains during the stationary phase, we intend to explore the roles of RpoS and CsrA in the negative regulation of SPI-1 by Hfq during late stationary phase growth in a future study.

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