[SII-3]

Analysis of *Salmonella* Pathogenicity Island (SPI) Gene Expression

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Many pathogenic bacteria harbor large clusters of virulence genes that act together in a complex virulence function and it has been known that the genes are acquired by horizontal transfer (Groisman and Ochman, 1997). These virulence gene clusters are referred to as pathogenicity islands and five pathogenicity islands are known in *Salmonella* (SPI, *Salmonella* pathogenecity islands). SPI-1 and SPI-2 encode distinct type III secretion system. Type III secretion systems are used by many bacterial pathogens to deliver virulence factors to the host cell and interfere with or subvert normal host cell signaling pathways. SPI-2 at 30cs on the chromosome is required for bacterial growth in macrophages and is involved in the late, systemic stages of infection (reviewed by Ohl and Miller, 2001). Hensel et al (1997) showed that 13 genes in *ssaK/U* operon encoding components of type III secretion apparatus were transcriptionally coupled and *ssaJ* and *ssaK* are transcribed separately. Since analysis of the predicted protein sequences of the 3 genes from *ssaH* to *ssaJ* showed that they are all transcribed in the same orientation, RT-PCR analysis was performed to determine whether these genes are cotranscribed. As can be seen from figure 1, it was evident that *ssaH, ssaI,* and *ssaJ* were transcribed as a single polycistronic mRNA from the promoter upstream of *ssaH*. To determine the transcription start site of the *ssaH* promoter and to characterize the regulatory proteins involved in *ssaH* transcription, the *ssaH* transcription was analyzed with primer extension analysis using *Salmonella* at various growth stages. The *ssaH* expression was induced dramatically in stationary phase (Fig. 2). Several pleiotropic regulators of *Salmonella* such as *rpoS*, *phoP*, and *ompR* are known to play an important role in pathogenicity of *Salmonella* because mutation of any one of these genes are known to render *Salmonella* avirulent (Dorman et al., 1989; Coynault et al., 1996; Groisman, 2001). Effects of mutations in *rpoS*, *phoP*, and *ompR* on *ssaH* transcription were tested. In either *phoP*⁻ or *rpoS*⁻ strain, the expression of *ssaH* was induced in stationary phase as was in wild type strain. In contrast, *ssaH* expression was reduced strikingly in *ompR* mutant in stationary growth phase (Fig. 2A). HilA and InvF are major regulatory protein of SPI1 and *ssrAB* encodes a sensor and kinase of two-component system in SPI2. Effects of these genes on the transcription regulation of *ssaH* were also investigated. As expected, SPI-1 regulator HilA did not affect *ssaH* expression but *ssaH* expression was reduced in the absence of SsrB. However, *ssaH* expression was reduced dramatically in the absence of another SPI-1 regulator InvF (Fig. 2B). Mutations in either *ihf* or *fis* gene caused the very low level of *ssaH* transcription whereas the *ssaH* transcription could be induced normally in *hns* mutant (Fig. 2C). Although it has been known that Fis levels drop precipitously in late exponential and stationary phases (Ball et al., 1992),

Fis seems to act as an activator in stationary phase. Fis is one of the most abundant nucleoid-associated proteins in exponentially growing cells. In order to examine whether Fis influenced *ssaH* expression directly or not, DNase I footprinting experiment was carried out using DNA fragment containing *ssaH* regulatory region. As shown in figure 3, it was observed that there were four Fis binding sites on the *ssaH* promoter region and Fis binding affinity was different for each site. Site I and II were located at positions –141 to –112 and –104 to –75 with its core sequence centered at -125 and –86, respectively. Site III and IV overlapping with core region of *ssaH* promoter were centered at –40 and +2, respectively. To study the effects of Fis on intracellular replication of *Salmonella*, RAW 264.7 cells were infected with UK1 grown to stationary phase and gentamicin protection assay was performed. The intracellular replication rate was reduced in *fis* mutant compared with wild type (Fig. 4). The reduced replication may be the result of the reduced transcription of *ssaH* in the absence of Fis (Fig. 2C). Our results indicated that Fis might also play a role in the regulation of SPI-2 gene expression.

References

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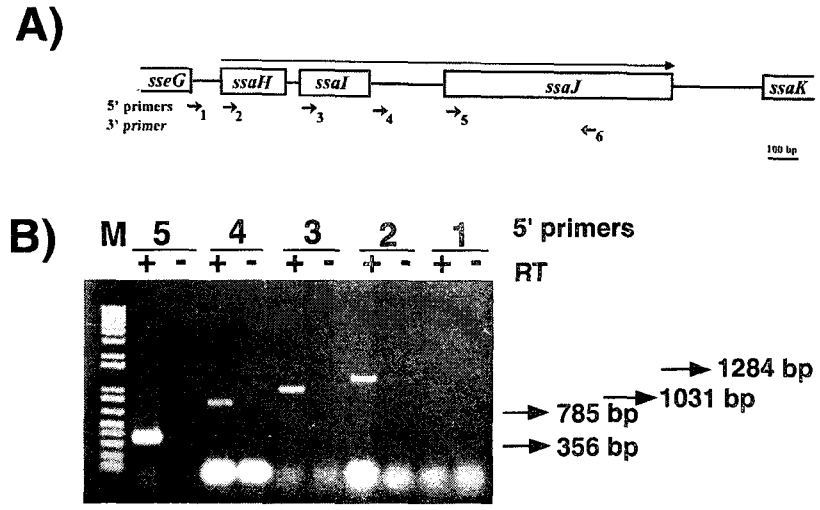

Figure 1. Transcriptional organization of the *ssaH* to *ssaJ* genes using RT-PCR analysis. (A) Schematic diagram of the genetic organization of the *ssaH*, *ssaI*, *ssaJ*, and boundary genes. The direction of transcription is indicated by solid arrow above the gene map. The approximate positions of 5' and 3' primers used in RT-PCR are indicated by numbered arrows below the map. (B) Results of the RT-PCR assays. 5' primers used in each reaction are listed on top of the panel. cDNA was amplified from *S. typhimurium* UK1 whole-cell RNA by using the 3' primer and PCR amplified with the same 3' primer and the indicated 5' primer. As a control, each RT-PCR was done without the addition of RT (- lanes). The size of each band is indicated.

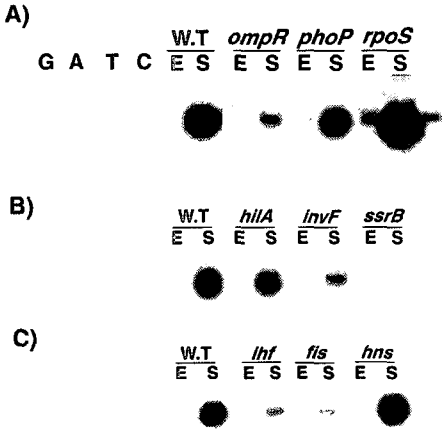

Figure 2. *In vivo* transcription analysis of *ssaH* promoter in wild type and various mutants of *S. typhimurium*. Total RNA was extracted from (A) SF530 (wild type), YK3092 (*ompR*), SR2001 (*phoP*), and JF2690 (*rpoS*); (B) SR2002 (*hilA*), SR2003 (*invF*), and SR2004 (*ssrB*); (C) SR2005 (*ihf*), SR1001 (*fis*), and YK3064 (*hns*) grown in LB at different stages of the growth cycle. E, early exponential phase (A_{600}=0.6); S, stationary phase (A_{600}=3.5). Fifty μ g of total RNA was used for primer extension analysis. Sequence ladder was generated using the same end-labeled primer used for primer extension analysis.

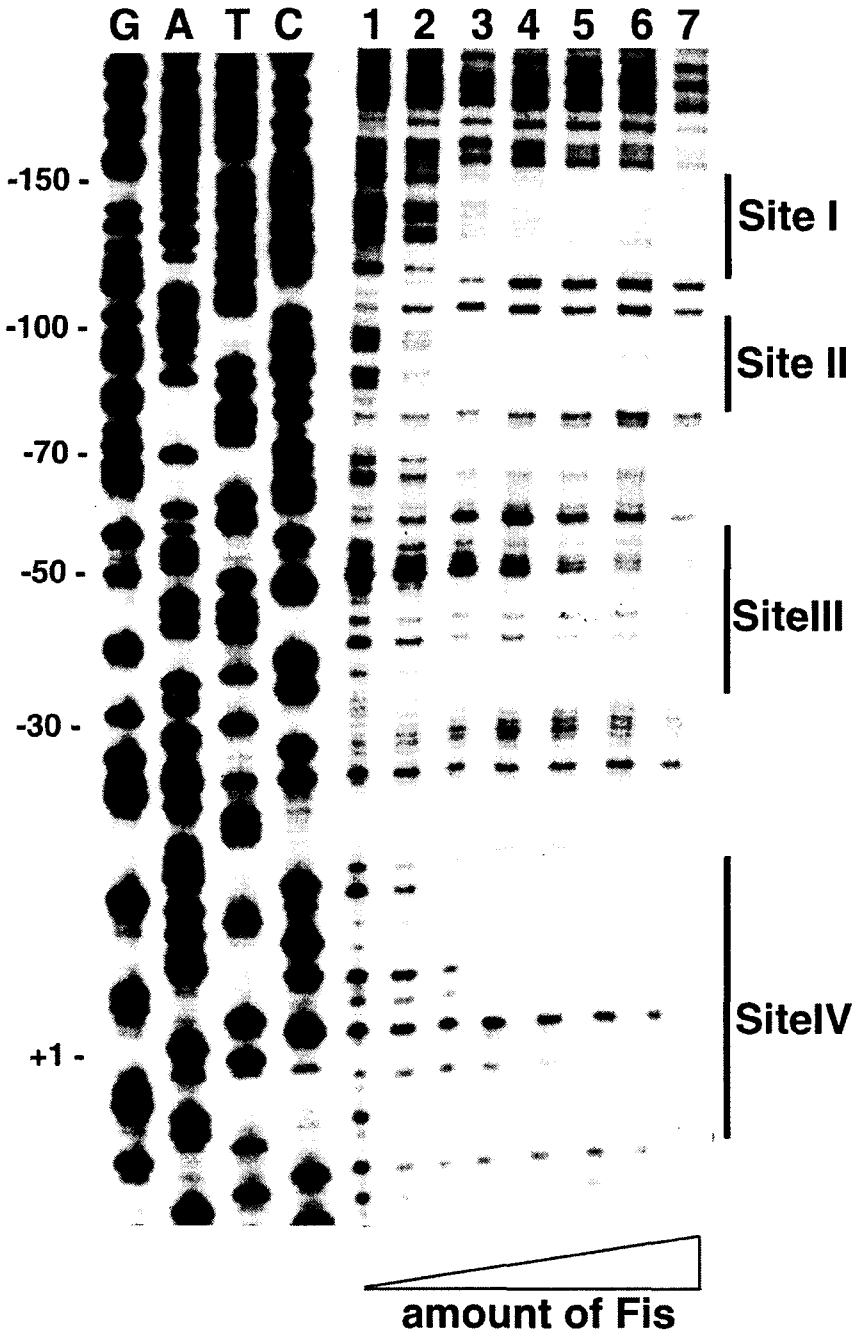

Figure 3. Footprint of Fis on the *ssaH* promoter DNA. The DNA fragment containing *ssaH* promoter region was obtained by PCR amplification using ^{32}P-end labeled reverse primer (non-coding strand). Protected regions for four Fis sites are indicated with lines and marked I through IV. G,A,T,C is sequencing ladder. Lanes 1 through 7, 0, 31.25, 62.5, 125, 250, 500, and 1000 nM of Fis, respectively. Numbering on the left is based on the transcription start point of the *ssaH* promoter as +1.

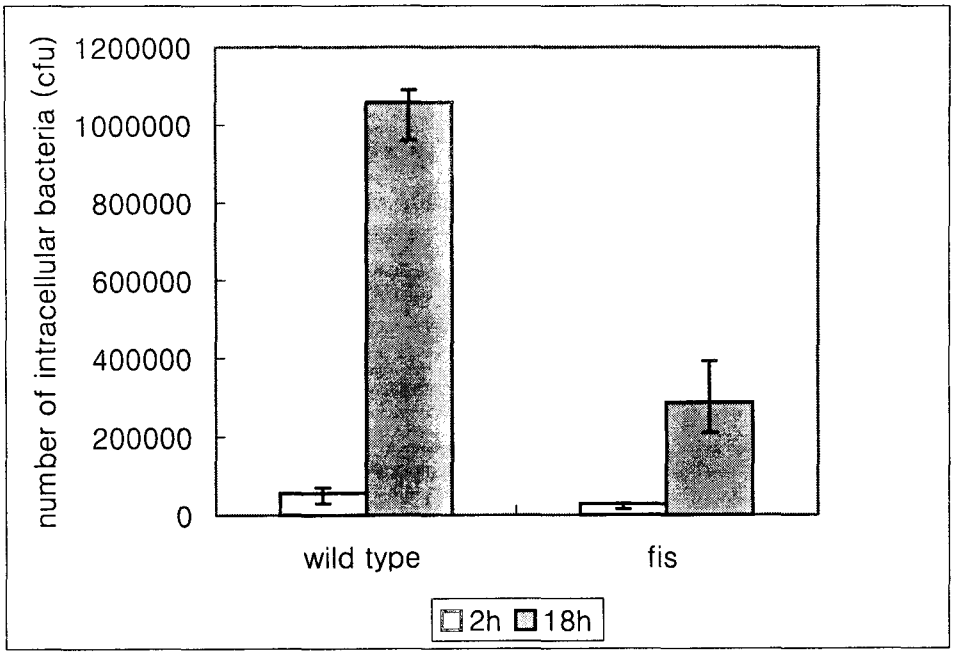

Figure 4. Intracellular survival and replication of *S. typhimurium* in macrophage-like cell line RAW 264.7. RAW 264.7 cells were infected with UK1 (wild type) and *fis* mutant strain grown at stationary phase in LB. The graph shows the number of cfu of intracellular bacteria at 2 h (white bars) and at 18 h (grey bars) after infection. These assays were performed at least three times for each strain and standard deviation is shown.