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Proteome analysis of *Salmonella enterica* serovar Typhimurium *fis* mutant

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Abstract

Salmonella enterica serovar Typhimurium is an enteric pathogen and a principal cause of gastroenteritis in humans. The factor-for-inversion stimulation protein (Fis) is known to play a pivotal role in the expression of *Salmonella* pathogenicity island (SPI)-1 genes in addition to various cellular processes such as recombination, replication, and transcription. In order to understand Fis function in pathogenicity of *Salmonella*, we performed two-dimensional gel electrophoresis and identified proteins whose expression pattern is affected by Fis using mass spectrometry. The results revealed various proteins that can be grouped according to their respective cellular functions. These groups include the genes involved in the metabolism of sugar, flagella synthesis, translation, and SPI expression. Changes in SPI expression suggest the possibility that regulation of genes in SPI-2 as well as SPI-1 is affected by Fis.

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Keywords: *Salmonella*; *fis*; Two-dimensional gel electrophoresis

1. Introduction

Salmonella enterica serovar Typhimurium is a Gram-negative bacterium that causes a self-limiting gastroenteritis in healthy humans while it leads to a typhoid-like disease in the mouse model [1]. Pathogenic *Salmonella* species taken through contaminated food contact with Peyer's patches of the small intestine and invade the specialized epithelial M cells of the follicle-associated epithelium of Peyer's patch tissue [2,3]. Bacterial entry into epithelial cells is mediated by the expression of genes encoding structural components of type III secretion system (TTSS) that exports effector proteins inducing actin rearrangement and ruffling of the host membrane, which results in engulfment of *Salmonella* [4]. Many genes related to invasion are clustered within a 40-kb region at 63 min of the chromosome, termed *Salmonella* pathogenicity island (SPI) 1 [5]. After invasion, these bacteria can survive and replicate within the *Salmonella*-containing vacuole in intestinal epithelial cells at early stages of disease [3] or tissue macrophages during systemic stages of disease [6]. Proteins encoded in SPI-2, a 40-kb pathogenicity island located at 31 min of the chromosome, are required for intracellular bacterial proliferation in vivo [7]. SPI-2$^-$ mutants inoculated by the intraperitoneal route reach the liver and spleen but do not multiply in these organs [8].

Fis is a histone-like DNA binding protein that was first identified as a factor from *Escherichia coli* that stimulates site-specific DNA inversion reactions mediated by *S. typhimurium* His [9] and by phage-derived Gin and Cin [10,11]. Fis was also shown to participate in other processes such as stimulation of phage λ excision [12], growth modulation of phage Mu [13], replication at oriC [14] and RNA synthesis [15]. It has been reported that Fis regulates the expression of HilA and InvF, two activators of SPI-1 [16]. A

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Abbreviations: *fis*, factor-for-inversion stimulation protein; SPI, *Salmonella* pathogenicity island; 2-DE, two-dimensional gel electrophoresis; TTSS, type III secretion system; LB, Luria–Bertani medium; EF-Tu, elongation factor Tu

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mutation in the *S. typhimurium fis* gene reduced the expression of *hilA* and *invF* and showed a severe virulence defect in a mouse model when administered orally. It is expected that more genes related to *Salmonella* pathogenesis are under Fis control considering the pleiotropic nature of Fis function. In this study, we utilized proteomic analysis to compare changes in gene expression patterns of *Salmonella* caused by mutation in *fis*. We tried to identify genes under Fis control involved in pathogenicity of *Salmonella* in order to understand the role of Fis in *Salmonella* virulence.

2. Materials and methods

2.1. Strains and culture condition

S. typhimurium UK1 was used as a wild-type and the *fis* mutant strain (SR1001) was constructed by P22 transduction using *S. typhimurium* RJ1827 provided by R.C. Johnson. To evaluate *fis* function on SPI expression in low-oxygen growth condition, 0.1% inoculum was used to inoculate Luria–Bertani (LB) medium and cells were grown non-agitated under mineral oil at 37°C to exponential (3 h after inoculation) or stationary (12 h after inoculation) phase.

2.2. Protein preparation and two-dimensional gel electrophoresis (2-DE)

Cells of *S. typhimurium* UK1 and SR1001 were harvested and washed with low-salt buffer containing 3 mM KCl, 1.5 mM KH_2PO_4, 68 mM NaCl, and 9.0 mM NaH_2PO_4. The washed pellet was resuspended in solubilization buffer (9 M urea, 2% β-mercaptoethanol, 2% phamalyte (pH 3–10), and 8 mM phenylmethylsulfonyl fluoride) and sonicated with a Branson 250 sonifier [17]. To degrade the bacterial DNA and RNA, the lysate was mixed with 0.1 vol. of the solution containing 1 mg ml^{-1} DNase I, 0.25 mg ml^{-1} RNase A, and 50 mM $MgCl_2$ and incubated on ice for 30 min. Following ultracentrifugation at $32\,000 \times g$ for 20 min at 4°C to remove insoluble proteins and large macromolecular complexes, the supernatant was precipitated with acetone containing 10% trichloroacetic acid for 2 h at −20°C. The protein pellet obtained after centrifugation was resuspended in solubilization buffer and equal amounts of protein from *S. typhimurium* UK1 and *S. typhimurium* SR1001 were separated by isoelectric focusing in a pH gradient ranging from 4 to 7. In the second dimension, the proteins were separated according to their molecular mass by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and visualized by colloidal Coomassie blue staining. The same amount of 2D marker (Sigma) was mixed and applied together with the sample proteins to normalize the intensity of the protein spots from different gels.

2.3. Protein visualization and image analysis

The quantitative data were obtained from multiple gel runs and from PDQuest (Bio-Rad) analysis of the gel images. An estimate of the relative quantitative changes was made based on the average changes in protein intensity from three independent experiments and protein spots showing more than two-fold differences in the intensity between UK1 and SR1001 were considered to be regulated by Fis and digested with trypsin for mass spectrometry analysis.

2.4. Identification of proteins by mass spectrometry

Protein spots showing different levels depending on Fis were excised from stained gels and followed by in-gel digestion according to Mann's method [18]. Gel slices were washed twice with water and acetone–water (1:1) solution. To shrink gel pieces, acetonitrile was added and washed with 0.1 M NH_4HCO_3. After removal of all liquid by Speedvac concentrator, gel pieces were reduced and alkylated with 10 mM dithiothreitol and 55 mM iodoacetamide respectively. The gel pieces were dried again and the protein was digested by trypsin at 37°C overnight in digestion buffer (50 mM NH_4HCO_3, 5 mM NaCl, 12.5 mg ml^{-1} trypsin). The volume of the supernatant containing the generated peptides was reduced by Speedvac concentrator. Peptide mass fingerprinting was done with Voyager-DETM STR Biospectrometry Workstation (PerSeptive Biosystems, Framingham, MA, USA) and proteins were identified by peptide mass fingerprinting with the search programs MS-FIT (http://prospector.usuf.edu/ucsfhtml4.0/msfit.htm) and MASCOT (http://www.matrixscience.com) supplemented with the option for *Salmonella* in the NCBI database.

3. Results

3.1. Comparison of protein profiles between S. typhimurium *UK1 and SR1001*

Many of the proteins involved in virulence are expressed at particular conditions resembling the host environment. For example, the growth condition to induce *hilA* expression is known to include low oxygen, high osmolarity, high pH, and early exponential phase growth [19,20]. We compared protein profiles between UK1 and its isogenic *fis* mutant SR1001 grown in low-oxygen condition to exponential and stationary phases. Whole cell proteins from wild-type and *fis* mutant strain were analyzed by 2-DE (Fig. 1). Even though the expression level of Fis is highly regulated depending on the growth stage [21], the overall expression patterns of UK1 and SR1001 were similar between exponential and stationary phases of growth. These are exemplified by the comparison of FljB, FliC, SipA,

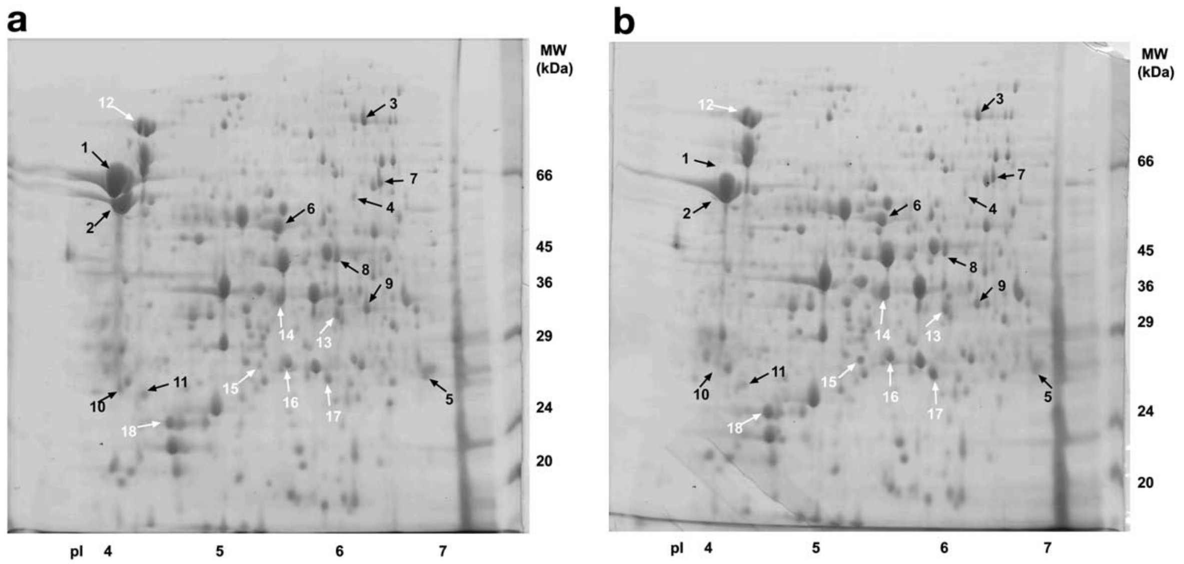

Fig. 1. 2-DE protein profiles of *fis*$^+$ strain *S. typhimurium* UK1 (a) and *fis*$^-$ strain *S. typhimurium* SR1001 (b). Cells were grown in low-oxygen condition in LB broth to stationary phase. Isoelectric focusing was performed with IPG strips ranging from pH 4 to pH 7 and second dimensional separation was accomplished on 12% polyacrylamide gels. Proteins of which expression is upregulated more than two-fold by Fis are indicated with black arrows and proteins of which expression is downregulated more than two-fold by Fis are marked with white arrows. Identification of proteins corresponding to numbered arrows is shown in Table 1.

SseC, and SopE2 expression in wild-type and *fis* mutant strains at exponential and stationary phase of growth, in which we could not find much difference in their expression patterns (Fig. 2): proteins positively regulated by Fis in stationary phase showed a similar positive regulation in exponential phase.

In three separate experiments, the mean values of spot intensities were compared between UK1 and SR1001 and we observed 11 spots showing higher levels in the wild-type strain than in the *fis* mutant, suggesting those proteins are probably upregulated by Fis (Fig. 1). Additionally, seven spots with higher levels in *S. typhimurium* SR1001 than *S. typhimurium* UK1 were detected, indicating the possibility that the synthesis of these proteins is

Table 1
Identification of possible Fis-induced proteins and Fis-repressed proteins by MALDI-TOF

Group	Spot	Protein	Gene	MW (kDa)	p*I*	Fold change[a]
Fis-induced proteins						
Flagella	1	phase-2 flagellin	*fljB*	52.54	4.75	306.0
	2	phase-1 flagellin	*fliC*	51.34	4.76	2.2
SPI	3	SipA, SPI-1 effector protein	*sipA*	74.18	6.20	2.1
	4	SseC, SPI-2 translocon	*sseC*	53.31	6.32	2.3
	5	SopE2, SPI-1 effector protein	*sopE2*	26.60	9.37	2.2
Translation	6	EF-Tu	*tufB*	43.25	5.30	3.0
Sugar metabolism	7	lipoamide dehydrogenase	*lpdA*	50.60	5.87	3.5
	8	mannose 1-phosphate guanylyl transferase	*manC*	54.03	6.40	2.1
	9	mannose-specific enzyme II AB	*manX*	34.99	5.82	2.9
	10	putative sugar binding protein	STY2817	31.10	5.37	168.9
	11	2-deoxyribose-5-phosphate aldolase	*deoC*	28.56	5.66	2.5
Fis-repressed proteins						
Chaperone	12	DnaK	*dnaK*	69.23	4.83	0.5
Catabolism	13	putative aldose 1-epimerase	*yeaD*	32.83	5.71	0.43
	14	putative carbamate kinase	STM4466	33.35	5.45	0.47
	15	propanediol utilization: polyhedral bodies	*pudB*	24.10	5.43	0.36
	16	adenylate kinase	*adk*	23.53	5.53	0.48
	17	class B acid phosphatase	*aphA*	26.17	6.9	0.34
	18	6-*N*-aminoglycoside acetyltransferase	*aacC4*	19.17	4.50	0.47

Mass finger prints of numbered protein obtained by MALDI-TOF were analyzed with MS-Fit or MASCOT. Proteins were grouped depending on their respective functions in the cells. MW and p*I* mean theoretical values depicted in NCBI database.

[a] Ratio of protein spot intensity of *fis* mutant/wild-type.

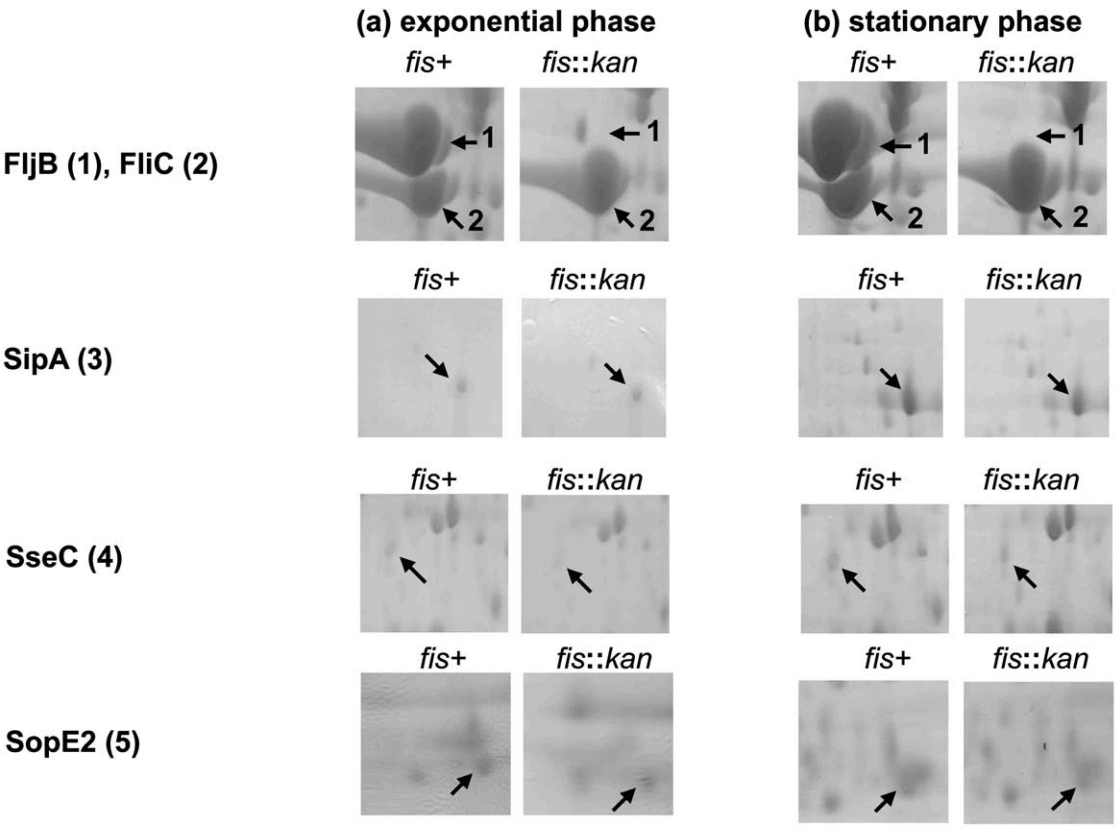

Fig. 2. Comparison of protein expression patterns in exponential phase (a) and stationary phase (b). To examine the effects of Fis amounts on gene regulation, total proteins were prepared from *S. typhimurium* UK1 (*fis*$^+$) and *S. typhimurium* SR1001 (*fis*::*kan*), which were grown to exponential phase and stationary phase. The possible Fis-induced or Fis-repressed proteins in stationary phase reproduced similar expression patterns in exponential phase. For example, flagellin proteins (FljB and FliC) and SPI-related proteins (SipA, SseC, and SopE2) are upregulated by Fis regardless of bacterial growth phase. Spot numbers used in Fig. 1 and Table 1 are indicated in parentheses.

repressed by Fis. Fis exhibited similar regulatory effects in three independent experiments. These Fis-regulated proteins were cut out from the gels and analyzed by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry after in-gel digestion. Protein identification was accomplished by MS-Fit and MASCOT and the results are demonstrated in Table 1. The potential Fis-induced proteins could be divided into several groups depending on their cellular functions. Those groups include the proteins involved in translation, flagellar synthesis, sugar metabolism, and the pathogenicity of *Salmonella*. In the case of Fis-repressed proteins, they were proteins mainly involved in catabolism.

3.2. Fis stimulates the expression of proteins involved in translation

The spot corresponding to elongation factor Tu (EF-Tu) showed higher expression levels in wild-type than in *fis* mutant. EF-Tu, together with elongation factor Ts, is required for the elongation step of translation following translation initiation complex formation. The reduced level of EF-Tu in the *fis* mutant strain probably indicates that Fis can affect the translation elongation step in addition to transcription of the tRNA operon and the rRNA operon reported in the case of *E. coli* [15,22].

3.3. Fis is involved in SPI expression

Positive regulation of SPI-1 genes by Fis was expected considering the fact that *hilA*, a transcriptional regulator of SPI-1, is upregulated by Fis [16]. 2-DE map comparison led to the detection of several SPI-related proteins induced in the presence of Fis. These included the SPI-1-related effector proteins SipA and SopE2 and the SPI-2 translocon protein SseC. SipA, a protein secreted through the needle complex encoded by the *inv* and *spa* genes of SPI-1, interacts with skeletal actin filaments in the host cell, leads to membrane ruffling and rearrangement, and enhances bacterial uptake [23]. SopE2, an effector protein secreted by SPI-1 TTSS, is encoded outside SPI and involved in invasion by stimulating membrane ruffling via guanidine nucleotide exchange on Rho GTPase CDC42 [24].

The level of SseC was reduced in the *fis* mutant strain compared with wild-type. SseB, SseC, and SseD are assembled into complexes on the bacterial cell surface and function as the translocon for the translocation of SPI2 effector proteins such as SspH1 and SspH2 into infected host cells [25].

3.4. Fis regulates flagellar synthesis

S. typhimurium undergoes phase variation to alternatively express two different types of flagellin subunit protein, FljB or FliC, and the flagella phase variation involves the inversion of approximately 1 kb of DNA containing the promoters of the *fljB* and *hin* genes encoding a recombinase [26,27]. Fis facilitates Hin-mediated DNA exchange to generate a site-specific inversion. We found *S. typhimurium* UK1 could produce flagellar protein FljB or FliC alternatively but *S. typhimurium* SR1001 could produce FliC only (Fig. 1), suggesting that Hin-mediated DNA inversion was not possible in the *fis* mutant strain. In addition to defective phase variation in the *fis* mutant, the expression level of FliC was reduced in *S. typhimurium* SR1001 (Fig. 2).

3.5. Fis modulates expression of various proteins involved in metabolic processes

Proteins regulated by Fis in other cellular processes were primarily enzymes and transport proteins involved in the metabolism of sugars. Among them, we found that the mannose-specific enzyme IIAB showed a higher level in wild-type than in the *fis* mutant, indicating a role of Fis in the phosphoenol pyruvate:carbohydrate phosphotransferase system. Fis-repressed proteins were associated with diverse functions including catabolism of sugar and amino acid and heat shock protein synthesis.

4. Discussion

Fis is an 11.2-kDa protein that binds to specific DNA sites displaying a highly degenerate consensus sequence [28]. Binding to its sites causes a conformational change in DNA facilitating recombination. Mutational analysis showed that the carboxy-terminus is required for binding and bending of DNA and the amino-terminus is responsible for stimulation of the DNA inversion reaction [29,30]. The intracellular Fis concentration varies extensively in response to nutritional conditions. In (early) exponential phase, the level of Fis protein reaches over 40 000 dimers per cell but following maximal expression, the amount rapidly decreases to very low levels entering stationary phase [21]. In order to understand the role of Fis in virulence gene expression of *S. typhimurium*, we analyzed proteins that are differentially expressed in the presence and absence of *fis* and detected 11 possible proteins upregulated by Fis and seven proteins downregulated by Fis. These proteins are involved in diverse cellular functions such as translation, sugar metabolism, flagellar synthesis, and virulence. Protein spots expressed differentially depending on Fis in stationary phase also showed similar expression patterns in exponential phase (Fig. 2). These results suggest that Fis may affect gene expression in stationary phase even though Fis concentration is low in stationary phase.

We found that *S. typhimurium* cannot undergo phase variation in the absence of Fis. In addition to this defect in flagellin variation, the *fis* mutant appeared to produce a lower amount of FliC than the wild-type strain. Transcription of *fliC*, the gene encoding phase 1 flagellin, is positively regulated by FliA. FliA is an alternative sigma factor required for the expression of operons encoding flagellin, the chemotaxis machinery, and the flagellar motor [31]. It has been suggested that there exists an overlap between the regulatory mechanisms that control flagellar and invasion gene expression. Lucas et al. reported that FliZ activates expression of *hilA* [32] and Eichelberg and Galan [33] reported that FliA controls expression of genes associated with TTSS in *Salmonella* serovar Typhi. Iyoda et al. [34] showed that a mutation in *fliZ* diminished not only the expression of the flagellar operon, but also the transcription level of *hilA*, reducing the amounts of excreted SPI-1-encoded proteins. If the reduced FliC expression in the *fis* mutant was caused by the low *fliA* expression, Fis may be the key factor linking motility and virulence because *fliZ* is cotranscribed with *fliA* in *S. enterica* serovar Typhimurium [35]. The expression of three SPI-related proteins is likely to be upregulated by Fis as revealed by our proteome analysis of a *fis* mutant. Two of them are components of SPI-1 TTSS and the involvement of Fis in regulation of SPI-1 gene expression was reported previously [16]. Additionally, we detected one SPI-2 TTSS-related protein, SseC, that showed increased levels in the presence of Fis, suggesting the possibility of an association of Fis with SPI-2 expression. These results suggest that Fis may affect expression of genes in SPI-2 as well as SPI-1. Therefore, it is possible that Fis effects on virulence gene expression in *Salmonella* are wider than previously reported [16]. We are trying to elucidate more detailed function of Fis in regulation of expression of various genes identified in this study.

5. Concluding remarks

In an attempt to elucidate the role of Fis in virulence gene regulation in *Salmonella* we have studied proteome changes caused by *fis* mutation. We could find several virulence genes from both SPI-1 and -2 whose expressions are affected by Fis in addition to genes involved in various aspects of cellular physiology. Comprehensive protein profiles affected by Fis would help us to better understand the potential role of Fis in pathogenicity of *Salmonella*.

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