Fis Is Essential for the Stability of Linear Plasmid pBSSB1 and Affects the Motility of *Salmonella enterica* Serovar Typhi

Haifang Zhang1, Bin Ni1, Xin Zhao1, Isaac Dadzie1, Hong Du1, Qiang Wang2, Huaxi Xu1, Xinxiang Huang1*

1 Department of Biochemistry and Molecular Biology, School of Medical Technology, Jiangsu University, Zhenjiang, Jiangsu, China, 2 State Key Laboratory of Pharmaceutical Biotechnology, Department of Biology, Nanjing University, Nanjing, Jiangsu, China

Abstract

pBSSB1 is a 27 kb non-bacteriophage-related linear plasmid first found in *Salmonella enterica* serovar Typhi (S. Typhi), but the mechanism underlying the replication of pBSSB1 is currently unknown. Previous reports showed that the factor for inversion stimulation (Fis) encoded by fis can affect the replication, transcription and other processes through binding DNA. Here, a fis deletion mutant of S. Typhi (∆fis) was prepared through the homologous recombination mediated by suicide plasmid and the loss of pBSSB1 in ∆fis was observed surprisingly by pulsed field gel electrophoresis (PFGE). Subsequently, the loss of pBSSB1 was verified by PCR and Southern blot. In addition, the motility of ∆fis was deficient and the flagellin of ∆fis could not be detected by 2-dimensional polyacrylamide gel electrophoresis. All these results show that Fis is essential for the stability of pBSSB1 and affects the motility of S. Typhi.

Introduction

The factor for inversion stimulation (Fis) encoded by fis is a small DNA-bending nucleotide-associated protein which plays a role in the transcriptional regulation of a number of genes in diverse bacterial species [1]. Fis was found initially as a co-factor of the site-specific recombination system. It was reported that Fis of *E. coli* is composed of two similar subunits and each subunit consists of 98 amino acids. In the structure of Fis, there is a typical 2-helix-turn-2-helix (helix-turn-helix, HTH) domain which could bind to the major groove of the DNA double helix [2]. Fis has wide regulatory roles, such as regulating the bacterial growth, virulence, and flagellum [3]. In addition, Fis can change the structure of bacterial nucleic acid and affect the replication, transcription and other processes through binding the DNA [4].

Plasmid is an extra chromosomal, self-replicating genetic element which in many cases is circular. In 1979, the first linear plasmid of prokaryote was found in *Streptomyces rochei* which could produce antibiotics [5]. So far linear plasmids have been found in about a dozen of *Streptomyces*, and the molecular size of these linear plasmids is between 12–640 kb [6]. Subsequently, another kind of linear plasmids were also found in *Borrelia* [7]. In 2007, a linear plasmid named pBSSB1 was reported to be present in *Salmonella enterica* serovar Typhi (S. Typhi) z66-positive strain by Baker et al [8]. pBSSB1, which is about 27kb-sized, is the first non-bacteriophage-related linear plasmid found in Enterobacteriaceae, and it mediates the unidirectional flagellar phase variation of S. Typhi z66-positive strain [8,9]. However, the mechanism underlying the replication of pBSSB1 is currently unknown.

The protein DnaA, which recognizes the origin of replication oriC, is essential for the DNA replication of Bacteria. With the help of DnaC protein, DnaB, a helicase can bind to oriC region to open the DNA double helix so as to initiate replication. Results of *in vitro* research show that Fis competes with DnaA protein for the origin of replication, oriC to affect DNA replication in *E. coli* [10,11]. Besides, Fis can bind to the transcription start site of dnaA operon to suppress its expression [12]. dnaA operon is composed of dnaA, dnaN and recF, coding for the DnaA protein which can recognize the origin of replication oriC, β subunit of DNA polymerase III which is responsible for the extension of the newly-replicated DNA chains and the RecF protein which is involved in the recombination and the repair of DNA, respectively [12,13]. In light of these functions of Fis, we hypothesized that Fis may play a very important role in the replication of pBSSB1.

In this study, we prepared a fis deletion mutant of S. Typhi (∆fis) through the homologous recombination mediated by suicide plasmid, and performed pulsed field gel electrophoresis (PFGE) to investigate the genome structure of ∆fis. It was surprisingly found that the 27 kb linear plasmid has disappeared in ∆fis. The loss of linear plasmid in ∆fis was verified by PCR and Southern blot. Moreover, only the complementary strain ∆fis[pBAD] could host this linear plasmid while the ∆fis and ∆fis[pBAD] cannot. These results suggest that fis is essential for the stability of plasmid pBSSB1. Since it was previously reported that the gene βfis' is located on pBSSB1 encodes the flagellum and is responsible for the motility of S. Typhi z66-positive strain [8], the motility and flagellin of ∆fis was examined by semi-solid agar plates and two-dimensional polyacrylamide gel electrophoresis respectively. The
results show that the motility of $\Delta fis$ was deficient and the flagellin of $\Delta fis$ could not be detected.

**Materials and Methods**

**Bacterial Strains and Plasmids**

*S. Typhi* GIFU10007, a z66-positive wild-type strain was used in this study. Mutants and plasmids used in this work are listed in Table 1.

**Construction of the fis Deletion Mutant of S. Typhi**

Primers used in this study are listed in Table 2. To generate the $\Delta fis$, primer pairs F1A/B and F2A/B were used to amplify the fragments F1 (499-bp) and F2 (314-bp) located upstream and downstream of the gene fis, respectively. A *BamH*I site was added to the 5’-termini of primers F1A and F2B, and a *Sal*I site was added to the 5’-termini of primers F1B and F2A. Two fragments F1 and F2 were amplified from *S. Typhi* GIFU10007 and digested with *Sal*I and ligated with DNA Ligation Kit Ver.2 (TaKaRa) to form the homologous fragment, in which 159-bp of the gene fis was absent. The fragment was then inserted into the *BamHI* site of the suicide plasmid pGMB151, which carries a sucrose-sensitivity gene sacB. The suicide plasmid carrying the deletion of fis gene was transferred into wild-type strain by electroporation as previously described [14,15]. The mutant strain was selected by PCR with primers F1A and F2B. Finally, the selected candidate of the fis deletion mutant was confirmed by sequencing analysis and designated as $\Delta fis$.

**Pulsed-field Gel Electrophoresis (PFGE)**

A single colony of *S. Typhi* wild strain and mutant strain $\Delta fis$ was inoculated into 4 ml LB, and cultured overnight with shaking (250 r/min) at 37°C. Bacteria were collected by centrifugation (4000 r/min, 10 min, 4°C) and washed three times with buffer PIV (10 mmol/L Tris, 1 mol/L NaCl, pH7.6). The pelleted bacteria were resuspended in 1 ml buffer PIV and 5 times with buffer PIV (10 mmol/L Tris, 1 mol/L NaCl, pH7.6). Then mixed with 2% low melting agarose gel to make the cell plugs for PFGE. The cell plugs were digested with the fresh lysis buffer (6 mmol/L Tris, 0.1 mol/L EDTA, 1 mol/L NaCl, 0.5% Brij-50, 0.2% sodium deoxycholate, 0.5% SDS, RNaseA 20 µg/ml, lysozyme enzyme 1 mg/ml) overnight at 37°C. After being washed with buffer ES (0.5 mol/L EDTA, 1% SDS), the cell plugs were digested with buffer ESP (including protease K 100 µg/ml of the ES) overnight at 50°C to digest bacterial protein, and finally washed with TE buffer. The DNA of bacteria in the cell plugs was separated on 1.0% agarose gels by electrophoresis with a CHEF Mapper system (Bio-Rad, USA) in the 0.5 x TBE buffer. The electrophoresis was performed at 6 V/cm and 14°C. The pulse time increased from 1 to 20 s during 18 h run. DNA-PFGE marker (Bio-Rad, USA) was used as the size marker.

**Verification of the Absence of Linear Plasmid pBSSB1 in $\Delta fis$ by PCR and Southern-blot**

To verify the absence of linear plasmid pBSSB1 in $\Delta fis$, a pair of primers Pa(23175) and Pb(23750) (Table 2), which was designed according to the sequence of pBSSB1 reported previously [8], was used to amplify the corresponding fragments in order to investigate whether the linear plasmid pBSSB1 was absent in the mutant strain $\Delta fis$ of *S. Typhi*. In addition, the separated DNA fragments from PFGE was transferred onto the nitrocellulose membrane and subjected to Southern-blot with the biotin-labeled DNA fragments as the probe.

**Complementary Expression of fis in $\Delta fis$**

Primers *P-fis*-A and *P-fis*-B (Table 2), specific to upstream and downstream regions of the gene fis were used to amplify a promoterless fis gene with *pfu* DNA polymerase (Fermentas). An *Neo*I site and a *Sal*I site were added to the 5’-termini of primers *P-fis*-A and *P-fis*-B, respectively. An approximately 297 bp ampiclon was inserted into the *Neo*I and *Sal*I sites of the expression vector pBAD/gIII (Invitrogen) to form the recombinant plasmid (pBAD/fis). The positive plasmid pBAD/fis was verified by digestion with *Neo*I and *Sal*I and sequence

---

**Table 1.** Strains and plasmids used in the present study.

| Strain or plasmid | Relevant characteristics | Reference or source |
|-------------------|--------------------------|---------------------|
| **Strains**       |                          |                     |
| S. Typhi GIFU10007 | wild-type strain; z66\(^+\) | 14                  |
| SY372:pfd    | *E. coli* host strain of suicide plasmid | Laboratory collection |
| $\Delta fis$    |                          | This work           |
| $\Delta fis$ (pBADI) | $\Delta fis$ containing pBAD empty vector | This work           |
| $\Delta fis$ (pBADfis) | $\Delta fis$ containing pBADfis recombinant plasmid | This work           |
| S. Typhi GIFU10007-1 | GIFU10007 containing pBSSB2; Kan\(^{a}\) | This work           |
| $\Delta fis$(pBADfis)(pBSSB2) | $\Delta fis$(pBADfis) containing pBSSB2; Kan\(^{a}\) | This work           |
| **Plasmids**     |                          |                     |
| pGMB151         | suicide plasmid; sacB; Amp\(^{a}\) | 14                  |
| pGMBfis         | pGMB151 containing fis   | This work           |
| pBAD/gIII       | Expression vector; Amp\(^{a}\) | 15                  |
| pBADfis         | pBAD containing fis      | This work           |
| pET-28a(c+)     | Kan\(^{a}\)              | Laboratory collection |
| pKD46           | Red helper plasmid; Amp\(^{a}\) | Laboratory collection |
| pBSSB2          | pBSSB1 containing a kanamycin resistance gene | This work           |

---

doi:10.1371/journal.pone.0037462.t001
Table 2. Primers used in this study.

| Primers      | Sequence(5'-3')        | Purpose                                            |
|--------------|------------------------|----------------------------------------------------|
| F1A(BamHI)   | AGGAGTCCGGCAGTTAAGGCAGAAAGT | fis mutant construction                           |
| F1B(SalI)    | CTTCGTGAGGATACCTGACCTGAGAGTT |                                                     |
| F2A(SalI)    | TATGCTGACTGCTGATGATG |                                                     |
| F2B(BamHI)   | TCAGATCCACACTACGCTGCAAT |                                                     |
| P-fis-A(NcoI)| TACCATGGATACCTGATGAGGACG | Complementary expression of fis in Δfis          |
| P-fis-B(SalI)| CTTCGTGAGGATACCTGACCTGAGAGTT |                                                     |
| Pa(23173)    | TAAACGATAGCCACACAGT | Confirmation of the presence or absence of pBSSB1 by PCR |
| Pb(23750)    | TCAAGGAAAGCTGAGAGTTGT |                                                     |
| P-Kana-A     | AATTGATAAAGGAAAGGTTTCCGATATAAAGTGCTGAGCTGACGTCAGTGA | Insertion of kanamycin cassette within pBSSB1 |
| P-Kana-B     | TAGTGGCTCAAACAGTATAGAATTGACAGAAGAAAGAGCAGAAGTTTCCGATATAAAGTGCTGAGCTGACGTCAGTGA |                                                     |
| ORF1-A       | GAGAAGATGGCCCGTAA | Confirmation of the insertion of kanamycin cassette within pBSSB1 by PCR |
| Kana-B       | ATGCTGATCATAACACCC |                                                     |

doi:10.1371/journal.pone.0037462.t002

analysis. The Δfis was transformed with pBADfis and designated as Δfis(pBADfis). As a control, the Δfis was also transformed with the empty vector pBAD/gIII and designated as Δfis(pBAD). Expression of fis in Δfis(pBADfis) was induced by L-arabinose (0.2% wt/vol).

Insertion of Kanamycin Cassette within pBSSB1

A kanamycin resistance gene was inserted within pBSSB1 between ORF001 and ORF002 using the lambda Red recombinase (one-step method) as described by Datsenko and Wanner [16]. First, the Red helper plasmid pKD46 was isolated and transformed into wild-type S. Typhi GIFU10007 by electroporation (2.5 kV, 600 ohms, 25 μF; Bio-Rad Gene Pulser). Then, the kanamycin resistance gene was amplified with the primers P-Kana-A and P-Kana-B (Table 2) using plasmid pET-28a-c(+) DNA as a template. PCR-amplified DNA was precipitated and resuspended in 10 μl of nuclease-free water. Re-suspended DNA was transformed by electroporation into S. Typhi GIFU10007 cells containing pKD46 plasmid as described above. Transformed cells were recovered for 1 h at 37°C in 100 μl of SOC, and then plated onto LB medium supplemented with 25 μg/ml kanamycin.

Finally, the insertion of kanamycin cassette into pBSSB1 was confirmed by PCR with the primers ORF1-A and Kana-B (Table 2). The pBSSB1 plasmid with the kanamycin resistance gene insert was designated as pBSSB2, and S. Typhi GIFU10007 harboring pBSSB2 was designated as S. Typhi GIFU10007-1.

Transformation of Δfis, Δfis(pBAD) and Δfis(pBADfis) with pBSSB2

The pBSSB2 plasmid was isolated from S. Typhi GIFU10007-1 using an alkaline lysis method originally described by Kado and Liu [17]. The quality and quantity of the extracted pBSSB2 DNA were tested by electrophoresis and an ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, USA) respectively. Then, Δfis(pBADfis), Δfis(pBAD) and Δfis was transformed with the pBSSB2 DNA by electroporation (2.5 kV, 600 ohms, 25 μF; Bio-Rad Gene Pulser). Transformed cells were screened on the LB medium supplemented with 25 μg/ml kanamycin. Transformation of Δfis or Δfis(pBAD) or Δfis(pBADfis) with pBSSB2 was finally confirmed by PCR with primers Pa(23173) and Pb(23750) (Table 2).

Motility Assay

Bacteria were cultured overnight at 37°C in LB broth. Each 4 μl of culture was inoculated into the centre of a 0.3% semisolid LB agar plate containing L-arabinose (0.2% wt/vol). The plates were incubated at 37°C for 10 hours and motility was assessed qualitatively by examining the diameter of circular swimming which was formed by the growing motile bacterial cells.

Two-dimensional Polyacrylamide Gel Electrophoresis and Mass Spectrometry Analysis

Bacterial proteins were extracted from wild-type and fis mutant strain strains. The proteins were firstly separated by isoelectrofocusing electrophoresis and then subjected to SDS-PAGE electrophoresis (Bio-rad). After staining with Coomassie Brilliant Blue G-250, differential expression of bacterial proteins between the wild-type and fis mutant strains were detected and analyzed by Mass spectrometry.

Results

The Loss of Linear Plasmid pBSSB1 in Δfis of S. Typhi

The DNA of bacteria in the agarose, which was not digested by restriction enzymes, was separated by PFGE. As shown in Figure 1(A), two DNA bands were stained and the upper band may be the chromosomal DNA while the lower band is the linear plasmid pBSSB1 DNA. This result indicates that the linear plasmid pBSSB1 was lost in the Δfis of S. Typhi. To better verify the loss of the linear pBSSB1 plasmid, we used biotin labeled DNA fragment which was amplified by a pair of specific primers designed according to the sequence of pBSSB1 plasmid as probes to hybridize the DNA bands from PFGE by Southern blot (figure 1(B)). In addition, a specific DNA fragment of pBSSB1 cannot be detected by PCR with primers designed according to the pBSSB1 DNA sequence (data not shown). Moreover, the pBSSB2
plasmid DNA can only be transformed into the complementary strain Δfis(pBADfis) while the Δfis and Δfis(pBAD) cannot host the plasmid pBSSB2 DNA. These results show that Fis is essential for the stability of this linear plasmid in S. Typhi.

Discussion

Although linear plasmids are relatively common in bacterial species such as *Streptomyces* and *Borrelia*, pBSSB1 is the first non-bacteriophage-related linear plasmid to be described in the Enterobacteriaceae that contains no detectable homology sequence of bacteriophage [8]. However, little is known about the replication on plasmids and bacterial chromosomes, previously used to predict the internal origin of bi-directional linear replication [22,23]. It was reported that the change in GC skew ((G-C)/(G+C)) is present in the middle of pBSSB1 and this region may be the origin of the replication [8]. Therefore, it is speculated that the replication of pBSSB1 is initiated from the middle and prolonged bi-directionally and Fis may be essential for the initiation of replication of this plasmid. In addition, it has been suggested that pBSSB1 may possess terminal protein (Tp) covalently bound to the 5' end of the DNA, which is very similar to linear plasmids from *Streptomyces* [8]. Many linear plasmids are replicated bi-directionally from an internal origin, which leaves single-stranded gaps of 250-300 nt at the 3' ends, and these gaps are proposed to be patched by Tp-primed DNA synthesis [24,25]. Therefore, Fis may also affect the replication of pBSSB1 through the regulation on the Tp of this plasmid. All these hypotheses need further experiments to clarify.

Previous studies showed that *S. Typhi* z66 positive strain is a biphasic *Salmonella* serovar, harbouring the fis gene in the chromosome and fliC gene in the pBSSB1 plasmid [8,14]. FljAz66, which is encoded by fisA66 gene located downstream of fliC gene, can inhibit the expression of fisC similar to most biphasic *Salmonella* [9,15]. In this study, the loss of plasmid pBSSB1 in Δfis may relieve the repression of FljAz66 on the expression of fisC. Previous study showed that the expression of the agellar biosynthesis was strongly repressed in the fis mutant [3]. In the present study, we also found that many genes contributing to agellar biosynthesis and motility were strongly down regulated in

**Figure 1. Identification of linear plasmid pBSSB1 by PFGE (A) and Southern-blot (B).**
doi:10.1371/journal.pone.0037462.g001

**Figure 2. Effect of Fis on the motility of *S. Typhi* GIFU10007.**
doi:10.1371/journal.pone.0037462.g002
\( \Delta_{fis} \) by microarray analysis of the differential expression between the wild-type and \( \Delta_{fis} \) (data not shown). Therefore, Fis might affect the bacterial motility due to the loss of pBSSB1 plasmid and the decreased expression of \( fliC \) in \( \Delta_{fis} \) of S. Typhi.

In summary, this study is the first to demonstrate that Fis is essential for the stability of pBSSB1 and affects the motility of S. Typhi.

References

1. Browning DF, Grainger DC, Busby SJ (2010) Effects of nucleoid-associated proteins on bacterial chromosome structure and gene expression. Curr Opin Microbiol 13: 773–780.

2. Yuan HS, Finkel SE, Feng JA, Kaczor-Grzeskowiak M, Johnson RC, et al. (1991) The molecular structure of wild-type and a mutant Fis protein: Relationship between mutational changes and recombinational enhancer function or DNA binding. Proc Natl Acad Sci U S A 88: 9558–9562.

3. Kelly A, Goldberg MD, Carroll RK, Danino V, Hinton JC, et al. (2004) A global role for Fis in the transcriptional control of metabolism and type III secretion in Salmonella enterica serovar Typhimurium. Microbiology 150: 2037–2053.

4. Cameron AD, Stoebel DM, Dorman CJ (2011) DNA supercoiling is differentially regulated by environmental factors and FIS in Escherichia coli and Salmonella enterica. Mol Microbiol 80: 85–101.

5. Hayakawa T, Otake N, Yonehara H, Tanaka T, Sakaguchi K (1979) Isolation and characterization of plasmids from Streptomyces. J Antibiot 32: 1348–1350.

6. Zheng L, Cheng Q, Tian X, Zhao L, Qin Z (2010) Characterization of the replication, transfer, and plasmid/lytic phage cycle of the Streptomyces plasmid- phage pZL12. J Bacteriol 192: 3747–3754.

7. Plasterk RH, Simon MI, Barbour AG (1985) Transposition of structural genes to an expression sequence on a linear plasmid causes antigenic variation in the bacterium Borrelia hermsii. Nature 318: 257–263.

8. Baker S, Hardy J, Sanderson KE, Quail M, Goodhead I, et al. (2007) A novel linear plasmid mediates flagellar variation in Salmonella Typhi. PLoS Pathog 3: e59.

9. Baker S, Holt K, Whitehead S, Goodhead I, Perkins T, et al. (2007) A linear plasmid truncation induces unidirectional flagellar phase change in H: z66 positive Salmonella Typhi. Mol Microbiol 66: 1207–1218.

10. Ryan VT, Grimwade JE, Camera JE, Crooke E, Leonard AC (2004) Escherichia coli prereplication complex assembly is regulated by dynamic interplay among Fis, IHF and DnaA. Mol Microbiol 54: 1347–1359.

11. Gilé H, Egan JB, Roth A, Messer W (1991) The Fis protein binds and bends the origin of chromosomal DNA replication, oriC, of Escherichia coli. Nucleic Acids Res 19: 4167–4172.

12. Wold S, Crooke E, Skarstad K (1996) The Escherichia coli Fis protein prevents initiation of DNA replication from oriC in vivo. Nucleic Acids Res 24: 3527–3532.

Acknowledgments

We thank T. Ezaki (Gifu University) for bacterial strains and continuous support.

Author Contributions

Concepted and designed the experiments: XH HZ HX. Performed the experiments: HZ BN XZ HD. Analyzed the data: HZ QW. Contributed reagents/materials/analysis tools: HZ BN. Wrote the paper: HZ ID XH.