Fis negatively affects binding of Tn4652 transposase by out-competing IHF from the left end of Tn4652

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Transposition activity in bacteria is generally maintained at a low level. The activity of mobile DNA elements can be controlled by bacterially encoded global regulators. Regulation of transposition of Tn4652 in *Pseudomonas putida* is one such example. Activation of transposition of Tn4652 in starving bacteria requires the stationary-phase sigma factor RpoS and integration host factor (IHF). IHF plays a dual role in Tn4652 translocation by activating transcription of the transposase gene *tnpA* of the transposon and facilitating TnpA binding to the inverted repeats of the transposon. Our previous results have indicated that besides IHF some other *P. putida*-encoded global regulator(s) might bind to the ends of Tn4652 and regulate transposition activity. In this study, employing a DNase I footprint assay we have identified a binding site of *P. putida* Fis (factor for inversion stimulation) centred 135 bp inside the left end of Tn4652. Our results of gel mobility shift and DNase I footprint studies revealed that Fis out-competes IHF from the left end of Tn4652, thereby abolishing the binding of TnpA. Thus, the results obtained in this study indicate that the transposition of Tn4652 is regulated by the cellular amount of *P. putida* global regulators Fis and IHF.

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INTRODUCTION

Transposition activity is generally maintained at a low level. The main reason for this is that high activity of random events and the accompanying mutagenic effect of genome rearrangements would be harmful to the organism (Doolittle *et al.*, 1984).

In bacteria transposition is controlled both by mechanisms intrinsic to the transposon and by external host-mediated control. Intrinsic control includes all regulatory mechanisms inherent to the transposable element itself. The second group of regulatory factors is encoded by the bacterial chromosome. However, the targets of regulation are the same as for intrinsic control: half-life or expression of the transposase or formation of the transpososome (Chandler, 2002).

The so called nucleoid-associated proteins, such as integration host factor (IHF), heat-unstable nucleoid protein (HU), histone-like nucleoid structuring protein (H-NS) and factor for inversion stimulation (Fis), form an important group of bacterial factors controlling transposi-

tion. These small proteins bind DNA and cause it to bend at an angle of 50–180° (Pan *et al.*, 1996; Spurio *et al.*, 1997; Swinger *et al.*, 2003). Depending on the location of binding sites for these proteins on the transposon DNA, transposition of mobile DNA elements can be influenced at different steps. In many cases the binding sites of nucleoid-associated proteins are located in or near to inverted repeats of mobile elements (Chandler, 2002). Therefore, these proteins may play roles in ensuring the correct architecture of the transpososome or maintaining the conformation necessary for productive transposition (Wiater & Grindley, 1988; Wardle *et al.*, 2005; Ward *et al.*, 2007).

Nucleoid-associated proteins can be a very important link between signals from the environment and the bacterial physiological state. At the same time, the amount of nucleoid-associated proteins depends on the growth phase of cells. For example, the amount of IHF is highest in early stationary phase but Fis is expressed only in exponentially growing cells and is almost absent in stationary-phase cells of *Escherichia coli* (Ali Azam *et al.*, 1999). The nucleoid-associated proteins are involved in regulation of transcription of large numbers of genes. This makes some of these proteins important transcriptional regulators in bacteria besides sigma factors (Goosen & van de Putte, 1995; Azam & Ishihama, 1999; Martinez-Antonio & Collado-Vides, 2003).

Abbreviations: Fis, factor for inversion stimulation; IHF, integration host factor.

Two supplementary tables, detailing the oligonucleotides used in this study and the PCR-amplified DNA fragments used in gel mobility shift and DNase I footprint assays, are available with the online version of this paper.

Fis is a small homodimeric nucleoid-associated protein that is found in the γ and β subdivisions of proteobacteria, which include the *Enterobacteriaceae* and *Pseudomonadaceae* (Beach & Osuna, 1998; Azam & Ishihama, 1999; Boswell *et al.*, 2004). Fis is quite well conserved among these families; for example *E. coli* and *Pseudomonas putida* Fis proteins share 81.7% identity at the deduced amino acid level as determined via <http://www.tigr.org>. Moreover, the most important amino acids from the DNA-binding domain are identical between *E. coli* and *P. putida* Fis (Boswell *et al.*, 2004). In most cases, information about Fis has been obtained from studies in *E. coli*, and only a few publications about the functioning of this protein in pseudomonads have been published (Kugelberg *et al.*, 2005; Yuste *et al.*, 2006). Due to binding to DNA *E. coli* Fis participates in several important processes for bacteria such as modulating DNA supercoiling (Schneider *et al.*, 1999, 2001; Travers *et al.*, 2001), which causes dramatic changes in the expression of a pool of genes (Amzallag, 2004; Travers & Muskhelishvili, 2005a, b), direct regulation of transcription of a large number of genes during different stages of growth (Xu & Johnson, 1995; Gonzalez-Gil *et al.*, 1996; Gutierrez-Rios *et al.*, 2007; Bradley *et al.*, 2007) and regulation of certain site-specific DNA recombination events (Johnson *et al.*, 1986; Finkel & Johnson, 1992; Dorgai *et al.*, 1993).

All *E. coli* nucleoid-associated proteins bind DNA non-specifically, but some, including Fis, show a preference for a particular DNA sequence. *E. coli* Fis preferentially binds to a 15 bp degenerate consensus core sequence, although it can contact up to 27 bp of DNA, depending on the flexibility of the flanking DNA (Pan *et al.*, 1996; Hengen *et al.*, 1997). The core consensus of the *E. coli* Fis binding site on DNA is GNTYAWWWWWTRANC (Y=pyrimidine, R=purine, W=A or T, and N=any nucleotide; Finkel & Johnson, 1992). The most important nucleotides in the binding sequence for Fis are G (position 1), A (position 5), T (position 11) and C (position 15) (Shao *et al.*, 2008). Additionally, the A or T nucleotides inside the core consensus (positions 6–10) and flanking the core consensus help Fis to bind to DNA (Shao *et al.*, 2008).

Although nucleoid-associated proteins are well studied, the involvement of these proteins in transposition has been studied only for a limited number of transposable elements, and moreover, only a few cases have been reported where Fis participates in the regulation of transposition: transposition of bacteriophage Mu (Betermier *et al.*, 1989), Tn5 (IS50) (Weinreich & Reznikoff, 1992) and IS2 (Lei *et al.*, 2007).

In this study we show that Fis might be involved in regulation of transposition of transposon Tn4652. Transposition of Tn4652, which is located in the chromosome of *P. putida*, is complexly regulated. This 17 kb transposon belongs to the Tn3 family (Tsuda & Iino, 1987) and is activated under stress conditions by sigma factor RpoS and IHF. Both proteins positively affect

transcription of the transposase gene *tnpA* (Hörak & Kivisaar, 1998; Ilves *et al.*, 2001). Additionally, IHF helps to form a correct transpososome. IHF binds to the inverted repeats of Tn4652 (Hörak & Kivisaar, 1998; Teras *et al.*, 2000) and facilitates transposase binding (Ilves *et al.*, 2004). Our previous studies have indicated that besides IHF some other *P. putida*-encoded factor could bind to the ends of Tn4652 and thereby regulate transposition (Teras *et al.*, 2000). The goal of this study was to investigate *in vitro* binding of *P. putida* Fis to the ends of Tn4652 and to study the possible effect of this binding on regulation of transposition of Tn4652. DNase I footprint and DNA mobility gel shift assays were used to examine the binding of Fis to the left and right ends of Tn4652, and competition of Fis with IHF and TnpA for the binding to the left end of the transposon.

METHODS

Bacterial strains, plasmids and media. Bacterial strains and plasmids used in this study are described in Table 1. Strains were grown on either complete Luria–Bertani (LB) medium (Miller, 1992) or minimal medium M9 (Adams, 1959) as indicated. Solid media contained 1.5% Difco agar. Antibiotics were added at the following concentrations: ampicillin, 100 $\mu\text{g ml}^{-1}$; and penicillin, 1500–3000 $\mu\text{g ml}^{-1}$. *E. coli* was incubated at 37 °C and *P. putida* at 30 °C. *E. coli* and *P. putida* were electrotransformed as described by Sharma & Schimke (1996). *E. coli* strain DH5 α (Invitrogen) was used for DNA cloning procedures; BL21(DE3) (Studier & Moffatt, 1986) was used for overexpression of proteins.

Overexpression and purification of proteins. For expression and purification of *P. putida* Fis protein the *fis* gene of *P. putida* was PCR amplified from chromosomal DNA of PaW85 by oligonucleotides *fishis*NcoI and *fishis*BamHI and cloned into *NcoI/BamHI*-opened pET19b (oligonucleotides used in PCR amplifications are listed in Supplementary Table S1, available with the online version of this paper). The resulting plasmid pET19-*hisfis* yielded an N-terminally His₆-tagged version of *P. putida* Fis.

P. putida IHF subunits were co-purified by a His₆ tag fused to the C terminus of IhfB. The purification of IHF was carried out as described previously (Ilves *et al.*, 2001).

Tn4652 transposase TnpA was overexpressed as a fusion protein containing an N-terminal His₁₀ tag and *P. putida* Fis as a fusion protein containing an N-terminal His₆ tag. The purification procedure was the same for both of the proteins. *E. coli* BL21(DE3) harbouring plasmid pET19-*tnpA* or pET19-*hisfis* was grown in LB medium at 37 °C to an OD₅₈₀ of 0.5. The growth temperature was subsequently shifted to 18 °C and bacteria were adapted under these conditions for 30 min before induction of His₁₀-TnpA or His₆-Fis expression with 0.5 mM IPTG. After 4 h of induction, cells were pelleted and sonicated in buffer A (100 mM Tris/HCl, pH 7.5, 1 M NaCl, 30 mM imidazole, 0.1% NP-40, 1 mM PMSF, 10% glycerol). The cell lysate was centrifuged at 12 000 g for 30 min at 4 °C. The cleared supernatant was supplied with the Ni-NTA agarose matrix (Qiagen), and protein binding to the matrix allowed to occur by slowly rotating the test tube overnight at 4 °C. The Ni-NTA matrix was washed twice with 50 volumes of buffer A, and purified TnpA or Fis was eluted with buffer B (100 mM Tris/HCl, pH 7.5, 300 mM NaCl, 300 mM imidazole, 0.1% NP-40, 0.1 mM PMSF and 50% glycerol). Purified proteins were stored at –20 °C.

Table 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or description	Source/reference
<i>E. coli</i>		
DH5 α	<i>supE44 ΔlacU169 (ϕ80 <i>lacZ</i>ΔM15) <i>recA1 endA1 hsdR17 thi-1 gyrA96 relA1</i></i>	Invitrogen
BL21(DE3)	<i>hsdS gal (λclts857 <i>ind1 Sam7 nin5 lacUV5-T7 gene 1</i>)</i>	Studier & Moffatt (1986)
<i>P. putida</i>		
PaW85	Tn4652	Bayley <i>et al.</i> (1977)
Plasmids		
pBluescript KS	<i>E. coli</i> cloning vector (Ap ^r)	Stratagene
pET19b	Protein expression vector (Ap ^r)	Stratagene
pET19-tnpA	<i>tnpA</i> fused with histidine tag in pET19b (Ap ^r)	Hörak & Kivisaar (1999)
pET19b-hisfis	His ₆ -head-tagged <i>P. putida</i> <i>fis</i> cloned into pET19b opened with <i>NcoI/BamHI</i> (Ap ^r)	this study
pET11-ihfAihfB	pET11c containing <i>ihfA</i> and His ₆ -tail-tagged <i>ihfB</i> genes (Ap ^r)	Ilves <i>et al.</i> (2004)
pLA1-12	pEST1332 containing 334 nt of the left end of Tn4652 upstream of <i>pheBA</i> genes (Ap ^r)	Teras <i>et al.</i> (2000)
pRA1-12	pEST1332 containing 278 nt of the right end of Tn4652 upstream of <i>pheBA</i> genes (Ap ^r)	Teras <i>et al.</i> (2000)

Prediction of Fis-binding sites on the ends of Tn4652. Potential Fis-binding sequences at the ends of Tn4652 were predicted by using the *E. coli* Fis-binding sites matrix (Salgado *et al.*, 2004) and the matrix-scan program available at the Regulatory System Analysis Tools homepage (<http://embnet.ccg.unam.mx/rsa-tools/>). The whole sequence of Tn4652 was used for the prediction of potential Fis-binding sites whereas the 500 bp terminal sequences were more carefully investigated.

Gel mobility shift assay. PCR-amplified fragments used for the gel mobility shift assay are listed in Supplementary Table S2. Plasmids pLA1-12 and pRA1-12 were used for the amplification of the left- and right-end DNA of Tn4652, respectively. Labelled DNA probes were purified by 5% PAGE, eluted and resuspended in water. Binding reactions with different amounts of purified *P. putida* His-tagged IHF, His-tagged TnpA and His-tagged Fis were carried out with 1000 c.p.m. of labelled DNA fragment in 1 $\times$ footprint buffer (see below) in a final volume of 20 μ l. The mixtures were preincubated for 20 min at room temperature. After incubation, reaction mixtures were applied to a 5% non-denaturing polyacrylamide gel buffered with 0.5 $\times$ TBE (50 mM Tris, 60 mM boric acid, 5 mM EDTA; pH 7.5). Electrophoresis was carried out at 4 $^{\circ}$ C at 10 V cm⁻¹ for 3 h. Gels were dried under vacuum and exposed to a Typhoon Trio screen (GE Healthcare). Protein concentrations were measured by the Lowry method.

DNase I footprinting. DNase I footprint assays were performed for the identification of *P. putida* Fis-binding sequence on the ends of Tn4652 and for competition experiments between IHF and Fis. PCR-amplified fragments were used for DNase I footprint assay and were generated as described above. To prepare one-strand-labelled DNA probes, one oligonucleotide used in PCR was labelled with [γ -³²P]ATP. PCR-amplified DNA fragments of the left and right end of Tn4652 used in footprint experiments are listed in Supplementary Table S2.

Labelled DNA probes were purified by 5% PAGE, eluted and resuspended in water. The binding reaction with different amounts of purified *P. putida* His-tagged IHF, TnpA and Fis was carried out with 30 000 c.p.m. of labelled DNA fragment in 1 $\times$ footprint buffer (24 mM Tris/HCl pH 7.5, 50 mM KCl, 10 mM MgCl₂, 1 mM CaCl₂, 0.1 mM EDTA, 5% glycerol, 0.1 μ g BSA μ l⁻¹ and 0.05 μ g salmon sperm DNA μ l⁻¹) in a final volume of 50 μ l. Mixtures were preincubated for 20 min at room temperature before the start of digestion by DNase I (1 U; Promega) for 0.5–3 min. Reactions were stopped by addition of 50 μ l stop solution (20 mM Tris/HCl pH 8.0, 20 mM EDTA, 0.8% SDS, 0.1 μ g salmon sperm DNA μ l⁻¹). The

footprinting reaction mixtures were subsequently extracted once with phenol/chloroform (1:1, v/v) and once with chloroform. Finally, the DNA was ethanol-precipitated. Dried reactions were resuspended in 5 μ l loading buffer (80% formamide, 0.3% bromophenol blue, 0.3% xylene cyanol, 10% glycerol, 10 mM EDTA pH 8.0) for electrophoretic fractionation on a 6% polyacrylamide, 8 M urea gel in 0.5 $\times$ TBE (pH 8.3) buffer. DNA sequencing reactions were performed with a Sequenase kit (version 2.0, US Biochemicals) and were loaded onto the gel as size markers. Electrophoresis was carried out at room temperature at 100 V cm⁻¹. After the run, gels were dried and exposed to a Typhoon Trio screen (GE Healthcare).

RESULTS

In silico analyses reveal six potential binding sites for Fis at the ends of Tn4652

Fis served as a good candidate for a host-encoded protein that could modulate transposition of Tn4652. Fis is well-studied in *E. coli* as a global regulator participating in many biological processes, including regulation of transposition (Weinreich & Reznikoff, 1992; Betermier *et al.*, 1993; Lei *et al.*, 2007). The DNA-binding domain of Fis is well conserved among γ -proteobacteria (Boswell *et al.*, 2004). For instance, the *E. coli* and *P. putida* Fis proteins share 81.7% identity at the deduced amino acid level as determined via <http://www.tigr.org>. Additionally, the most important amino acids from the DNA-binding domain are identical between *E. coli* and *P. putida* Fis (Boswell *et al.*, 2004). Since no DNA-binding sequences for *P. putida* Fis have been described, we used the Fis-binding consensus of *E. coli* for the prediction of Fis-binding sites on the ends of Tn4652.

We used the binding matrix of *E. coli* Fis (Salgado *et al.*, 2004) to predict the binding sites of Fis on both ends of Tn4652. Three putative Fis-binding sites on the left end and two binding sites on the right end of Tn4652 were predicted *in silico* (Table 2, Fig. 1a). The binding sites of Fis on the left end of Tn4652 were named LF and those on right end RF. The potential binding sites LF3a and LF3b

Table 2. Fis-binding sequences on the ends of Tn4652

The bold nucleotides in the consensus sequence of *E. coli* Fis ensure highly specific binding of *E. coli* Fis (Shao *et al.*, 2008). Nucleotides from the potential Fis-binding sites at the ends of Tn4652 that match with important nucleotides from *E. coli* Fis-binding consensus sequence are indicated in bold. The potential binding sites LF1 and RF1 were found by visual comparing of the matrix and the transposon sequence.

	Sequence (5'–3')	Position (bp) from ends of Tn4652	Weight score	P-value
Consensus of <i>E. coli</i> Fis*	GnTYA WWWW TRAnC			
LF1	atGTCCTAATTT TT GCctt	34–19		
LF2	ttGAGGAAGGTT T GATCta	125–140	4.4	1.6×10^{-3}
LF3a	atGCTCGATTAT T ACCTga	292–307	7.6	4.7×10^{-5}
LF3b	tcGATTATTACCTGAGCct	296–311	5.0	9.2×10^{-4}
RF1	atGTCCTAATTT TT GCctt	34–19		
RF2	atGCTGATGCTTT T GCctc	116–131	4.6	1.3×10^{-3}

*Finkel & Johnson (1992); Shao *et al.* (2008).

overlapped and were handled in experiments as one region, LF3.

The binding of Fis to the left end of Tn4652 is specific

To verify the *in silico*-predicted binding sequences on the ends of Tn4652, gel mobility shift assays and DNase I footprint analyses were carried out with purified His-tagged Fis of *P. putida*. Since Fis is able to bind DNA non-specifically as well, although with lower affinity (Betermier *et al.*, 1994), we determined the concentration of *P. putida* Fis sufficient for non-specific binding of DNA in our assay conditions. For that purpose we used a 111 nt DNA segment of the *P. putida colR* (PP0901) gene. This DNA was chosen for the following reasons: (a) the *in silico* prediction did not reveal any Fis-binding sites in this DNA sequence and (b) since the consensus Fis-binding sequence is A/T rich, the G/C richness (68 %) of the fragment reduces the possibility that it contains a specific binding site for *P. putida* Fis. The formation of Fis–*colR* DNA complex was detected at concentrations of 1.7 μ M Fis or higher (Fig. 1f).

The binding affinity of Fis to the ends of the transposon Tn4652 was greatest when the labelled DNA fragment contained the potential binding site LF2. In this case, the concentration of Fis that was necessary for the formation of a Fis–DNA complex was 0.07 μ M (Fig. 1c). Fis was able to bind to LF3 only at a concentration of 0.34 μ M or higher (Fig. 1d), and to LF1 at a concentration of 1.7 μ M (Fig. 1b), which was considered as non-specific binding of Fis (Fig. 1f). No significant differences in the minimum concentrations of Fis for the formation of a complex with DNA containing LF2 were observed in the presence or absence of LF3 and LF1 (data not shown), which also demonstrated that LF2 is bound by Fis first. Fis bound to the RF1 site at a concentration of 1.7 μ M (Fig. 1e) and no specific binding to RF2 could be detected (data not shown). Thus, the results of the gel shift assay demonstrated that Fis binds

specifically to the left end of Tn4652 but not to the right end of this element.

To further study binding of Fis to the *in silico*-predicted binding sequences at the ends of Tn4652, DNase I footprint analysis was carried out with purified Fis of *P. putida*. Protection of DNA against DNase I digestion by Fis was clearly detected only in cases where the labelled DNA contained the putative Fis site LF2 (Fig. 2a, b). According to the results of DNase I footprint analysis, the Fis-protected area spans nucleotides 121–146 on the top strand of the left end of Tn4652 and nucleotides 118–142 on the bottom strand (Fig. 2c). As a result of the binding of Fis a DNase I hypersensitive nucleotide G appeared at position 130 on the top strand. The predicted sites LF1, LF3, RF1 and RF2 did not reveal any specific DNA protection by Fis against DNase I cleavage (data not shown). These data confirm the results of the gel mobility shift assay, demonstrating that Fis binds preferentially to LF2 and less efficiently to the other sites predicted *in silico*.

Fis and IHF compete for binding to the left end of Tn4652

Since both IHF and Fis had binding sites at the left end of Tn4652, gel mobility shift assays were carried out to perform competition experiments. The results of these experiments revealed that IHF and Fis could bind to the left end of Tn4652 both separately (Fig. 3a, b) and simultaneously (Fig. 3c, d). Although LF2 is located approximately 70 bp away from the IHF-binding site (Fig. 1a), our results indicated that binding of Fis may negatively affect binding of IHF to the left end of Tn4652. Possibly, after binding to the LF2 site, Fis may bind with lower affinity to the LF1 and LF3 sites as well, resulting in a DNA conformation which is not accessible for binding by IHF. Considering the binding specificity of IHF and Fis (Fig. 3a, b), we suppose that two kinds of DNA complexes are formed where Fis is always present: Fis–DNA (lower

Fig. 1. (a) Diagram of the left and right ends of Tn4652. The Tn4652 DNA is shown by bold lines and the flanking DNA by thin lines. An arrow shows the direction of transcription of the *tnpA* gene. The potential binding sites of Fis are indicated by dark grey boxes; the binding sites of IHF are shown by light grey boxes. A binding site of TnpA is indicated by a striped box on the left end of the transposon. The binding of TnpA to the right end of the transposon is shown in the article by Ilves *et al.* (2004). Positive base numbers indicate Tn4652 sequence; negative numbers indicate flanking DNA sequence. (b–d) Binding of Fis to the left end of Tn4652. The following PCR products containing the left end of Tn4652 were used for gel mobility assays: (b) a 146 bp PCR product containing the putative Fis-binding site LF1, (c) a 164 bp PCR product containing the site LF2; (d) a 184 bp PCR product containing the site LF3. (e) Binding of Fis to the right end of Tn4652, using a 201 bp PCR product containing the putative Fis-binding site RF1 from the right end of Tn4652 for gel mobility assays. (f) A 111 bp PCR product containing *P. putida colR* (PP0901) DNA was used as negative control.

bands) or Fis–IHF–DNA (upper bands) complexes (Fig. 3d), whereas IHF–DNA complexes were not detectable in the presence of Fis. Moreover, higher concentrations of Fis diminished the proportion of IHF–Fis–DNA complex in favour of the Fis–DNA complex (Fig. 3d). These data indicate that Fis out-competes IHF from the left end of Tn4652 at higher concentrations (0.1 μ M or higher).

Fis affects binding of TnpA to the left transposon end

As a next step we investigated whether the binding of Fis to the left end of the transposon would affect the transposition of Tn4652. We were not able to investigate transposition *in vivo* due to the impossibility of deleting

the *fis* gene in *P. putida*, presumably because this gene is essential and the Fis-minus phenotype is lethal for *P. putida*. Additionally, the overexpression of Fis caused growth arrest of *P. putida* on minimal medium (data not shown), needed for the transposition assay developed by Kasak *et al.* (1997). Therefore we focused on *in vitro* experiments. We have previously shown that the transposase TnpA of Tn4652 binds to the ends of the transposon *in vitro* only in the presence of IHF (Ilves *et al.*, 2004). The results of the current study demonstrated that Fis negatively affects binding of TnpA to the left end of Tn4652 and confirmed the necessity of IHF for TnpA binding to the left end of Tn4652 (Fig. 4). New DNA–protein complexes were identified by gel mobility shift

Fig. 2. Protection of the left end of Tn4652 against DNase I cleavage by binding of Fis to the predicted LF2 site on (a) the bottom and (b) the top strand. A 166 bp PCR product from the left end of Tn4652 that contained the predicted binding site LF2 was used for the DNase I footprint analysis. Lines at the right of the panels indicate the regions protected by Fis from DNase I cleavage. The numbers indicate the positions of nucleotides on the Tn4652 sequence. The arrow marks base 128 on the left end of Tn4652, which becomes hypersensitive to DNase I cleavage upon interaction with Fis. (c) DNA sequence of the left end of Tn4652 protected by Fis. The LF2 predicted Fis-binding site is boxed. The protection against DNase I cleavage by binding of Fis to the top and bottom strand is shown by brackets. The nucleotide G that becomes hypersensitive to DNase I cleavage upon interaction with Fis is in bold.

assay in the presence of three proteins (Fig. 4a, dotted arrows). We presume that the slowest-migrating complex contains all three proteins, IHF, TnpA and Fis. This protein–DNA complex was seen in the presence of 0.2 μM Fis. Increasing the concentration of Fis to 0.5 or 0.9 μM in the binding reactions resulted in reduced amounts of this complex in the gel shift assays (Fig. 4a).

The identification of specific protein–DNA complexes in gel mobility shift assays is complicated. Since binding each of the studied proteins Fis, IHF and TnpA had character-

istic patterns of protection against DNase I cleavage, we decided to perform DNase I footprint analysis in the presence of different proteins in the reaction mixture. In the first reaction, IHF and TnpA were incubated with DNA 5 min before addition of Fis (Fig. 5a), and in the second reaction Fis was incubated with DNA before the addition of IHF and TnpA (Fig. 5b). When IHF was incubated first with the left end of Tn4652, the efficiency of DNA protection by IHF depended on the concentration of Fis (Fig. 5a). IHF was out-competed from the left end DNA of Tn4652 by Fis when the concentration of Fis was four times

Shao *et al.* (2008) high affinity of *E. coli* Fis to artificially designed 28 bp stretches of DNA is observed *in vitro* when at least three out of four conserved nucleotides (at positions 1, 5, 11 and 15; see Table 2) are present in the binding site. All five predicted binding sites at the ends of *Tn4652* contained at least three of these conserved nucleotides. In spite of this *P. putida* Fis had different

binding affinity to these sites as determined by the gel shift assay. Therefore, it is possible that the flanking DNA sequence is more important for *P. putida* Fis binding than for the *E. coli* protein.

There was only one potential Fis-binding site, LF2, which had high affinity for *P. putida* Fis protein according to the

Fig. 5. Protection against DNase I cleavage by binding of IHF and TnpA on the left end of the Tn4652 top strand. A 479 bp PCR product from the left end of Tn4652 was used for DNase I footprint analysis. It contained all three predicted Fis-binding sites: LF1, LF2 and LF3a/b. Positive base numbers indicate Tn4652 sequence; negative numbers indicate flanking DNA sequence. The arrow marks base -12 on the target sequence, which becomes hypersensitive to DNase I cleavage upon interaction with TnpA. Lines to the right of the panels designate the regions protected against DNase I cleavage as follows: continuous lines for protection by IHF and dotted lines for protection of IHF and TnpA together. Two kinds of reaction mixtures were used for DNase I footprint analysis: (a) after 5 min incubation of labelled DNA, Fis was added to the mixture of IHF and TnpA, and thereafter reactions were incubated for 15 min; (b) after 5 min incubation of labelled DNA and Fis, IHF and TnpA were added to the reaction mixture and thereafter reactions were incubated for 15 min. (c) A fragment of the left-end sequence of Tn4652 and flanking DNA. The LF1 predicted binding site of Fis is boxed. Protection against DNase I cleavage by binding of IHF on the top chain is shown by a continuous bracket and protection against DNase I cleavage by binding of IHF and TnpA is shown by a dotted bracket. The nucleotide A that becomes hypersensitive to DNase I cleavage upon interaction with IHF and TnpA is marked in bold.

gel mobility shift assay and verified also by DNase I footprint analysis. Binding of Fis induced a DNase I hypersensitive site in LF2. Notably, the Fis-induced DNase I hypersensitive nucleotide G is located in one of the possible hypersensitive positions of the Fis-binding core consensus of *E. coli* (Hengen *et al.*, 1997; Shao *et al.*, 2008). While the protection of Fis was not seen on the LF1 and LF3 sites, it is possible that Fis has only one strong binding site, LF2, on the left end of Tn4652, because the other binding sites, LF1 and LF3, had lower affinity to *P. putida* Fis, and Fis did not properly protect these sequences in the DNase I footprint assay.

Since the IHF-binding site is located between LF2 and LF1, the next question was whether Fis affects the binding of IHF to the left end of Tn4652. Based on the results of the gel mobility shift assay (Fig. 4) we concluded that the increasing of the concentration of Fis negatively affects IHF binding. The results of DNase I footprint analyses supported this opinion. When the left-end DNA of Tn4652 was first exposed to Fis, and IHF was added to the reaction mixture 5 min later, IHF was unable to bind DNA (Fig. 5b). However, when IHF was bound to the left end prior to Fis, the repressive effect of Fis to IHF binding was remarkably weaker (Fig. 5a). Since the preferred binding site for Fis, LF2, and the binding site for IHF are located 70 bp from each other, the simultaneous binding of these two proteins should not be obstructed, as is seen in the gel mobility shift analyses (Fig. 3d). Therefore, it is possible that Fis first binds to the LF2 site, which stimulates the binding of Fis to the weaker sites. The binding of Fis may result in a DNA conformation that hinders binding of IHF. The negative effect of Fis is clearly seen in DNase I footprint analyses where the left end DNA was first exposed to Fis (Fig. 5b), which means that the formation of a DNA conformation where the IHF-binding site is hidden takes longer than binding of Fis to a single binding site, for example LF2.

Since we detected the Fis–DNA complex at a Fis concentration of 80 nM, one may ask whether repression of IHF binding to the left end DNA of Tn4652 would occur due to DNA coating by Fis. For example, it is known that specific complexes between *E. coli* Fis and a 100 bp DNA fragment from phage λ DNA appear at approximately

10 nM concentrations of Fis, and the DNA was coated by Fis already at a concentration of 20 nM. Moreover, higher concentrations of Fis may cause coating of longer DNA fragments with protein and thereafter stabilize DNA loops by Fis–Fis interactions. It is speculated that a similar situation may occur *in vivo*, causing repression of transcription or DNA nucleation (Skoko *et al.*, 2006). However, although both ends of Tn4652 contain IHF-binding sites between two predicted Fis sites at similar distance (Fig. 1a), no repressive effect of Fis on the binding of IHF to the right end of Tn4652 was seen (Fig. 6), which indicates that Fis may form a repressive complex with DNA at higher concentrations only when it has the possibility to bind to high-affinity sites first.

Interestingly, the results of the competition experiments carried out with Fis and IHF proteins in gel mobility shift assays revealed that in the presence of Fis, IHF–DNA complex was not detectable. However, the formation of Fis–IHF–DNA or Fis–DNA complexes was always seen (Fig. 3d). These data indicate that, differently from IHF, Fis could bind equally well to Tn4652 left-end DNA both alone and when IHF was already bound to the DNA.

Our results indicate that Fis modulates TnpA binding by changing the binding of IHF. It has been shown previously that TnpA binds *in vitro* to the ends of Tn4652 only in the presence of IHF (Ilves *et al.*, 2004). Since Fis binds the left end of Tn4652 specifically at the LF2 site and influences IHF binding, the modulation of TnpA binding to the left end was investigated. We found that *P. putida* Fis does not directly influence the binding of TnpA to the left end of Tn4652 (Figs 5b, 6a, lane 14), but an increase of Fis concentration in gel mobility shift assays retarded the binding of TnpA due to modulation of binding of IHF (Fig. 4). Moreover, interesting results were obtained if the order of addition of the proteins into the reaction mixtures was changed in DNase I footprint assays. Preincubation of DNA with Fis weakened the TnpA protection against DNase I cleavage, as seen by the weakening of a hypersensitive site (Fig. 5b). This result is interesting, because IHF was not able to protect DNA against the DNase I cleavage under these reaction conditions, but protection by TnpA still occurred. At the same time, IHF was needed for this protection, as the protection by TnpA

Fig. 6. (a) Protection against DNase I cleavage by binding of IHF to the right end of Tn4652 top strand. A 202 bp PCR product from the right end of Tn4652 that contained putative Fis-binding sites RF1 and RF2 was used for DNase I footprint analysis. Numbers indicate the right-end sequence of Tn4652. The line on the right designates the regions protected against DNase I cleavage upon interaction with IHF. Two kinds of reaction mixtures were used for DNase I footprint analysis: after 5 min incubation of labelled DNA and IHF, Fis was added to the reaction mixture and thereafter reactions were incubated for 15 min (lanes 1–7), or after 5 min incubation of labelled DNA and Fis, IHF was added to the reaction mixture and thereafter reactions were incubated for 15 min (lanes 8–15). (b) DNA sequence of the right end of Tn4652. The RF1 predicted Fis-binding site is boxed. The protection against DNase I cleavage by binding of IHF on the top strand is marked by a bracket.

did not occur in the absence of IHF in the binding reaction (Fig. 5b, lane 14). These results therefore clearly show that in the presence of Fis IHF is needed for TnpA binding to the left end of Tn4652. It is possible that in the presence of Fis some DNA, however, stays bound by IHF, which in turn is sufficient to support weak TnpA binding. The exposure of the left end DNA to Fis after preincubation with IHF and TnpA protein mixture changed the binding specificity of TnpA. At higher concentrations of Fis, TnpA protects DNA against DNase I cleavage more efficiently near the terminus of the left end of the transposon and the target sequence (Fig. 5a, lanes 13 and 14). One may speculate that this has an effect on the precise location of transposase and may inhibit transposition in general.

Our previous studies have indicated that *P. putida*-encoded proteins IHF and RpoS activate transposition of Tn4652 in stationary phase (Hörak & Kivisaar, 1998; Ilves *et al.*, 2001, 2004). The results presented in the current study indicate that Fis could be involved in downregulation of the translocation of Tn4652 in *P. putida*. It is known that in bacteria these proteins are expressed during different physiological states. The expression of two of these, RpoS and IHF, is upregulated in stationary-phase cells. Compared to exponentially growing cells, the cellular amount of RpoS increases three- to fivefold when the growth rate of *P. putida* starts to decelerate (Bertani *et al.*, 2003). The amount of IHF increases approximately fivefold in stationary-phase cells, and accretion of IHF occurs with a sudden burst in early stationary phase (Valls *et al.*, 2002). In contrast, from experiments in *E. coli* it is known that the amount of Fis, as well as *fis* mRNA, increases sharply when bacteria are suddenly shifted into fresh, rich growth medium but the amount of Fis quickly decreases (up to 500-fold) in the late-exponential growth phase (Ali Azam *et al.*, 1999). The changes in the amount of Fis have not been measured in *P. putida*. However, it has been demonstrated by RT-PCR that levels of *fis* mRNA in *P. putida* decrease only two- to threefold upon entry into stationary phase (Yuste *et al.*, 2006). Considering the expression pattern of global regulators affecting the transposition of Tn4652, the movement of this element might be tightly controlled by the physiological state of *P. putida*. Under conditions of nutrient abundance, transposition of Tn4652 can be avoided by a high amount of Fis. Since the cellular amount of IHF as well as TnpA is low in exponentially growing *P. putida*, inhibition of transposition by Fis is efficient. When the growth rate of the bacteria decelerates and they enter stationary phase, increased amounts of IHF and RpoS induce transcription of *tnpA*. Moreover, the amount of Fis starts to decrease, allowing IHF to bind to the ends of Tn4652, which is crucial for TnpA binding and initiation of transposition. Thus transposition of Tn4652 is precisely controlled by *P. putida* itself and is induced only in stressed bacteria. Increasing frequency of transposition may accelerate the evolution of bacteria under non-optimal growth conditions by increasing the probability of occurrence of beneficial mutations.

However, it is extremely important for any organism to avoid accumulation of mutations under optimal growth conditions when the organism might risk loss of the advantage for fast consumption of nutrients.

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