IHF is the limiting host factor in transposition of *Pseudomonas putida* transposon Tn4652 in stationary phase

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Summary

Transpositional activity of mobile elements is not constant. Conditional regulation of host factors involved in transposition may severely change the activity of mobile elements. We have demonstrated previously that transposition of Tn4652 in *Pseudomonas putida* is a stationary phase-specific event, which requires functional sigma S (Ilves et al., 2001, J Bacteriol 183: 5445–5448). We hypothesized that integration host factor (IHF), the concentration of which is increased in starving *P. putida*, might contribute to the transposition of Tn4652 as well. Here, we demonstrate that transposition of Tn4652 in stationary phase *P. putida* is essentially limited by the amount of IHF. No transposition of Tn4652 occurs in a *P. putida* ihfA-defective strain. Moreover, overexpression of IHF results in significant enhancement of transposition compared with the wild-type strain. This indicates that the amount of IHF is a bottleneck in Tn4652 transposition. Gel mobility shift and DNase I footprinting studies revealed that IHF is necessary for the binding of transposase to both transposon ends. *In vitro*, transposase can bind to inverted repeats of transposon only after the binding of IHF. The results obtained in this study indicate that, besides sigma S, IHF is another host factor that is implicated in the elevation of transposition in stationary phase.

Introduction

Transposons are discrete mobile DNA segments that can move throughout the genome(s). Transposable elements can be seen as intrinsic potential mutagenic agents, providing a broad range of genetic variation in natural populations of virtually all examined species. The mutagenic potential of transposons is usually precisely controlled by various host and element-encoded proteins. The involvement of host factors in transposition indicates that they may be used for communication between the transposon and the host bacterium. Differential regulation of host factors in response to changing environmental and physiological conditions may cause substantial alterations in frequency of transposition. Several recent studies have shown activation of transposons under different stress conditions (Lamrani et al., 1999; Chow and Tung, 2000; Ilves et al., 2001). Up to now, many regulatory mechanisms controlling the frequency of transposition have been described (Craig et al., 2002). However, some aspects in the regulation of transposition are not well understood. For example, it is not known how transpositional activity of mobile elements responds to different environmental signals. From the evolutionary point of view, it is worthwhile finding out whether the conditional activation of transposition is a regulated response or caused just by malfunction of control mechanisms.

Transpositional activity of mobile elements is usually modulated by various host factors. Many transposons use different histone-like proteins, such as IHF, HU, H-NS and Fis. These proteins are small and able to bind or wrap the DNA. Albeit relatively abundant in bacterial cells, concentration of these proteins depends on the growth phase and physiological condition of bacteria (Ali Azam et al., 1999). For example, the abundance of IHF has been shown to increase up to sevenfold in *Escherichia coli* and *Pseudomonas putida* during transition of cells from exponential growth to stationary phase (Ditto et al., 1994; Delic-Attree et al., 1996; Murtin et al., 1998; Teras et al., 2000; Valls et al., 2002). It is reasonable to suppose that such changes in IHF concentrations influence different processes regulated by IHF.

IHF, HU, H-NS and Fis are all involved in regulation of bacteriophage Mu. H-NS and Fis have negative influence upon Mu activity (Falconi et al., 1991; Betermier et al., 1993; Gomez-Gomez et al., 1997). Both HU and IHF are positive factors in Mu regulation (Craigie et al., 1985; Surette et al., 1989). IHF plays a dual role in transposition of phage Mu. First, it activates transposase gene expression from the Mu *Pₚ* promoter directly by activating *Pₚ* transcription and indirectly via alleviating the H-NS-medi-
ated repression (van Ulsen et al., 1996). Secondly, IHF binding to the Mu P₆ promoter region (which is part of the larger enhancer-like element) can facilitate the formation of MuA transposase complexes at the ends of the element in vitro (Surette et al., 1989; Allison and Chaconas, 1992). However, in vivo studies suggest that IHF is not essential for the Mu transposition reaction. Rather, it is important for the transcriptional regulation of Mu genes (Pato, 1989; Kano et al., 1993; Chaconas and Harsey, 2002).

Several mobile DNA elements carry IHF binding sites at one or both termini (Gamas et al., 1987; Wiater and Grindley, 1988; Huisman et al., 1989; Makris et al., 1990). For the transposon γ6 (Tn1000), it has been shown that IHF binds co-operatively with transposase to the ends of γ6 (Wiater and Grindley, 1988; Wiater and Grindley, 1990). However, γ6 transposon transposes equally well with or without IHF binding sites (Wiater and Grindley, 1990; May and Grindley, 1995), indicating that the effect of IHF in Tn1000 transposition seems to be only modulatory. Similar results were obtained with IS1: although IHF was shown to bind to both IS1 ends (Gamas et al., 1987), no clear effect of IHF on transposase expression or transposition of IS1 has been found. Instead, transposition of IS1 was shown to require another histone-like host factor, H-NS (Shiga et al., 2001).

Mostly, IHF has a positive effect upon transposition (Craigie et al., 1985; Morisato and Kleckner, 1987; Surette et al., 1989), although reports about a negative role of IHF can be found as well (Gama et al., 1992; Signon and Kleckner, 1995). For example, upon transposition and transposase expression of the composite transposon Tn10, IHF acts either positively or negatively depending on the location of the transposon – in the chromosome or in a multicopy plasmid respectively (Signon and Kleckner, 1995).

Tn4652 is a 17-kb-long derivative of the toluene degradation transposon Tn4651 that belongs to the Tn3 family of transposons (Tsuda and Iino, 1987). Previous study has established that expression of the Tn4652 transposase is positively affected by two P.putida host factors – stationary phase-specific RNA polymerase sigma factor σ⁵ (RpoS) and IHF (Hörak and Kivisaar, 1998; Ilves et al., 2001). Although IHF has only a modulatory effect on transcription from the transposase promoter, the level of transcription from this promoter is strongly repressed in an RpoS-minus background. Actually, both ends of the transposon can bind IHF (Hörak and Kivisaar, 1998; Teras et al., 2000). IHF binding sites locate just near the terminal inverted repeat sequences that are presumable binding sites for the transposase. This suggests that, besides activation of the tnpA promoter, IHF may participate in Tn4652 transposition directly, either by modulating the binding of transposase to the ends of the transposon or by influencing the formation of nucleoprotein complexes needed in subsequent transposition reactions.

The aim of this study was to elucidate the role of IHF in the regulation of Tn4652 transposition. For that, we monitored transposition of native 17-kb-long Tn4652 in IHF-defective and in IHF-overexpressing P.putida strains. The results indicate that IHF is necessary for the translocation of Tn4652 and that it regulates transposition in a concentration-dependent manner. Gel mobility shift and DNase I footprinting experiments revealed that IHF binding is a prerequisite for the binding of transposase TnpA to the ends of the transposon. Our results suggest that IHF might be used for conditional activation of Tn4652 in stationary phase bacteria when the concentration of IHF in bacteria increases. Thus, IHF could be another protein factor (besides σ⁵) involved in growth phase-dependent regulation of transpositional activity of Tn4652.

**Results**

**Transposition of Tn4652 is prevented in a P.putida ihfA-defective strain**

We have demonstrated previously that IHF moderately (approximately fourfold) enhances transcription from the transposase tnpA promoter of Tn4652 (Hörak and Kivisaar, 1998). Additionally, both ends of Tn4652 have been shown to bind IHF of P.putida (Teras et al., 2000). To explain the role of IHF in the regulation of Tn4652 transposition, we examined the movement of Tn4652 in the P.putida wild-type strain PaW85 and in its ihfA-defective derivative. Transposition of native chromosomally located Tn4652 was tested in an assay that detects transposition of Tn4652 into plasmid pEST1332 in front of promoterless phenol degradation genes pheBA (Ilves et al., 2001). Transposition of Tn4652 can activate the phe genes as a result of generation of a fusion promoter (Nurk et al., 1993), resulting in accumulation of phenol-using Phe⁺ mutants on phenol minimal plates. The beauty of this assay is its dynamic nature, i.e. it allows monitoring of transposition for 1 week or more during starvation of bacteria. As presented in Fig. 1A, the accumulation of Phe⁺ mutants in the ihfA-minus strain shows a drastic decrease compared with that in the wild-type P.putida. The appearance of Phe⁺ mutants in the ihfA-defective strain was reduced about two orders of magnitude compared with the wild-type P.putida. Polymerase chain reaction (PCR) analysis of the Phe⁺ mutants revealed that, in the wild-type strain, more than 95% of the mutants emerged as a result of transposition of Tn4652. At the same time, no transposition was detected in the ihfA-minus strain.

The fusion promoters generated by insertion of Tn4652 are positively affected by IHF, being about threefold more active in the wild type than in an IHF-defective background.
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In order to test whether these promoters are still strong enough to ensure the growth of the Phe\(^+\) mutants lacking IHF, we purified plasmid DNA from the Phe\(^+\) mutants carrying different Tn4652 insertions in pEST1332 and transformed an IHF-minus strain with these plasmids. The transformants could grow on phenol minimal plates. Thus, the level of transcription of the pheA gene under the control of different fusion promoters in an IHF-minus background is high enough to allow bacteria to grow on phenol. This convinced us that, if Tn4652 could transpose in an IHF-defective strain, we could detect this in our assay.

Despite the implication of IHF in many different regulatory processes, this protein is not essential for bacterial cell propagation under laboratory conditions (Friedman, 1988). However, there are hints in the literature that IHF might be important for survival of E. coli under starvation conditions. Starvation survival of an E. coli himA (ihfA) mutant was found to be severely impaired during 4 days of glucose starvation in liquid minimal medium (Nystrom, 1995). Our transposition assay also involves carbon starvation. Therefore, we estimated the viability of a P. putida ihfA-defective strain starving on phenol minimal plates. Cell viability measurement on phenol minimal plates was prepared as described previously (Ilves et al., 2001). No decrease was observed in the survival of IHF-defective P. putida during the 9 days of starvation (data not shown). Thus, this result suggests that drastically decreased accumulation of Phe\(^+\) mutants is not caused by decreased survival of the IHF-minus strain; rather, the transposition of Tn4652 is disabled.

Frequency of transposition of Tn4652 depends on the expression level of IHF

In order to find out whether complementation of the P. putida ihfA-defective strain with functional ihfA and ihfB genes could restore the transposition of Tn4652, the transposition assay was carried out in P. putida strain PaW85ihfA/tacIHF, which contains P. putida ihfA and ihfB genes under the control of the P\(_{tac}\) promoter and lac\(^R\) repressor in the chromosome, enabling artificial change in the level of IHF expression. According to Western blot analysis, induction with 0.01 mM and 0.5 mM IPTG resulted in a near wild-type expression level and overexpression of IHF respectively (data not shown). Therefore, to provide different expression levels of IHF in starving bacteria, the phenol minimal plates were supplied with either 0.01 mM or 0.5 mM IPTG or, alternatively, no IPTG was added. Data in Fig. 1B clearly demonstrate that complementation of the ihfA-minus strain with ihfA and ihfB genes restored the accumulation of Tn4652-linked Phe\(^+\) mutants on phenol plates. Furthermore, frequency of transposition depended on IHF concentration. A few Tn4652-linked Phe\(^+\) mutants emerged on phenol minimal plates when IHF was not induced. We suppose that this resulted from the leakiness of the P\(_{tac}\) promoter. Overexpression of IHF resulted in elevated frequency of transposition compared with that in the wild-type strain (Fig. 1B). These results not only indicate that IHF is involved in the transposition of Tn4652, but also demonstrate that the amount of IHF in the wild-type P. putida starving cells is a limiting factor in transposition of Tn4652.

Overexpression of IHF does not increase transcription from the tnpA promoter

There are several ways in which IHF might be needed in the transposition of Tn4652. It has been shown earlier that
binding of IHF to the right end of Tn4652 can moderately enhance transcription from the transposase promoter (Hõrak and Kivisaar, 1998). Therefore, we controlled whether overexpression of IHF could increase transcription from the transposase promoter and thereby elevate transposition to the level seen in Fig. 1B. We measured the lacZ-fused transposase promoter (plasmid pKTlacZS/C) activities in P. putida wild-type, ihfA-defective and ihfA-defective strain carrying ihfAB genes under the control of the inducible P_tac promoter (PaWihfA/tacIHF). In the strain PaWihfA/tacIHF, the level of IHF was modulated by different concentrations of IPTG. Data presented in Fig. 2A show that the transcription activity from the transposase promoter depends on IHF, which is in good agreement with our previous results (Hõrak and Kivisaar, 1998). However, the level of transcription from the tnpA promoter under conditions of ihfAB overexpression (with 0.5 mM and 5 mM IPTG) did not exceed the transcription level observed in the wild-type strain, indicating that IHF overexpression cannot cause overproduction of transposase. To control whether IHF was really overexpressed, we prepared gel shift experiments with the same cell lysates that were used in the tnpA promoter expression studies. Data in Fig. 2B demonstrate that the amount of IHF–DNA complex increased with both Tn4652 left- and right-end DNA when IHF was overexpressed. In addition, we have compared the transposase expression originated from its own promoter in the wild-type and in the IHF-defective strain by quantitative Western blot analysis. We detected about two- to threefold lower amounts of TnpA in the IHF-minus strain compared with that in the wild-type strain. In accordance with experiments carried out with tnpA promoter fusions with lacZ (Fig. 2A), IHF overexpression did not result in the expression of transposase over the level seen in the wild-type strain (data not shown). Thus, all these data suggest that elevated transposition of Tn4652 in the case of IHF overexpression (Fig. 1B) is not caused by overproduction of transposase and points rather to the additional role of IHF in the regulation of Tn4652 transposition.

**IHF binding is a prerequisite for binding of transposase to both Tn4652 ends**

The first step in transposition involves recognition of transposon terminal inverted repeat sequences by the transposase. However, our attempts to demonstrate binding of Tn4652 transposase (TnpA) to transposon ends failed (data not shown). Therefore, using a gel shift assay, we tested whether IHF might be necessary for the TnpA binding to the transposon terminal inverted repeat sequences. When the transposon left- and right-end DNA were probed with either purified histidine-tagged IHF or TnpA, only IHF was able to retard DNA fragments (Fig. 3A and B, lane 2). No DNA probe shift was detected if TnpA was used in reactions (Fig. 3A and B, lane 5). However, when both proteins were mixed with labelled transposon end fragments, a super-shifted band was revealed in the gel indicating binding of TnpA (Fig. 3A and B, lanes 3 and 4). To test whether the preformed IHF–DNA complex (not only the presence of IHF in the reaction) is important for TnpA bind-

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**Fig. 2.** A. lacZ-fused transposase promoter (plasmid pKTlacZS/C) activities measured in P. putida wild-type strain PaW85 (wt), ihfA-defective PaWihfA (ihfA) and PaWihfAtacIHF, which is an ihfA-defective strain carrying ihfAB genes under the control of the inducible P_tac promoter (tacIHF). In strain PaWihfAtacIHF, the level of IHF was modulated by different concentrations of IPTG.

B. Specific binding of IHF to the right and left ends of Tn4652 in a gel mobility shift assay. The total cell lysates were prepared from bacteria used in a β-galactosidase assay and are designated as in (A). About 0.5 ng (1000 c.p.m.) of probe was incubated with 30 μg of crude cell lysate. The IHF–DNA complex is indicated by an arrow.
ing, we prepared binding reactions with mutated transposon left-end DNA. Nine mutations, supposed to disrupt both putative IHF binding sites but not the TnpA site, were introduced into the transposon left end (see Fig. 5A and Experimental procedures). Figure 3C shows that neither IHF nor TnpA can bind to the mutant left-end DNA fragment. Thus, these results suggest that, at least in vitro, binding of TnpA to transposon ends is strictly dependent on binding of IHF close to the transposase binding site.

To define further the interactions of IHF and TnpA with transposon ends, we analysed the protein–DNA complexes in DNase I reactions. Similar to the results of the gel mobility shift assay, the transposase alone could not bind either end of the transposon (Fig. 4A and B, lane 7). The IHF protein gave clear protection of DNA. The IHF-protected regions were between 35 and 76 bp in both the right and left ends (Figs 4 and 5). Thus, the DNase I footprints revealed identical positions of IHF binding sites in transposon right and left ends relative to the terminal inverted repeats. When IHF and TnpA were combined in the footprint reactions, the transposase alone could not bind either end of the transposon (Fig. 4A and B, lane 7). The IHF protein gave clear protection of DNA. The IHF-protected regions were between 35 and 76 bp in both the right and left ends (Figs 4 and 5). Thus, the DNase I footprints revealed identical positions of IHF binding sites in transposon right and left ends relative to the terminal inverted repeats. When IHF and TnpA were combined in the footprint reactions, the TnpA-specific protection of whole transposon end and 9–10 bp of flanking target DNA was observed (Figs 4 and 5). Interestingly, binding of TnpA extended the IHF-protected region inwards to the left and right terminus of transposase by 2–3 bp. This indicates that binding of TnpA alters the IHF interactions with DNA. Together, TnpA and IHF protected about 79 bp of the right and 56 bp of the left terminus of Tn4652 (Fig. 5). The binding of TnpA to both the left and right end resulted in the appearance of a hypersensitive site for DNase I in position 19 of either end when bottom strand was analysed. The hypersensitive sites resulted from DNase I cleavage as no cuts occurred when DNA was incubated with transposase and IHF in the absence of DNase I (data not shown). According to the results of gel shift and DNase I footprinting assays, we can conclude that IHF is essential for binding of TnpA to the transposon ends in vitro.

**Discussion**

Transposable elements often generate insertions and cause other mutations in the genomic DNA. The mobility of transposons is usually strictly downregulated, probably to minimize the potential destructive mutagenic effects of transposition on their hosts. However, although transposition activity is generally maintained at a low level, a high frequency of transposition can occur under certain circumstances. For example, different stress conditions have been shown to induce activation of transposable elements (Wessler, 1996; Lamrani et al., 1999; Chow and Tung, 2000; Ilves et al., 2001). To understand the mutational impact of transposons in host genome evolution, it would be important to find out what kind of molecular mechanisms are responsible for conditional activation of mobile elements. Our present study throws light upon the role of IHF in the regulation of *P. putida* transposon Tn4652, and allows us to draw some conclusions about the contribution of IHF to stress-induced transposition of Tn4652.

IHF has been implicated in the regulation of several transposons as a modulating participant (see Introduction). IHF binds to inverted repeats of IS1 (Gamas et al., 1987) and close to the transposase binding site at both ends of γ16 transposon (Wiater and Grindley, 1988). However, there is no evidence that transposition of either IS1 or γ16 is altered in an IHF-defective *E. coli*. IS10 also carries an IHF binding site immediately adjacent to the transposase binding site at one end (Huisman et al., 1989). In vivo experiments show that, although IHF is not an essential factor in the regulation of Tn10/IS10, it modulates transposition either positively or negatively by activating chromosomal events or inhibiting transposition out of a multicopy plasmid (Signon and Kleckner, 1995). Only in the case of bacteriophage Mu has it been shown that IHF is essential, and Mu cannot proliferate in an IHF mutant (Pato, 1989). Yet, this defect can be overcome if Mu transposition proteins (MuA and MuB) are additionally supplied from a plasmid (Pato, 1989). Also, Mu can transpose in an IHF and H-NS double mutant (Kano et al., 2004).
IHF has been shown to counteract H-NS-mediated transcription repression of Mu phage transposition genes (van Ulsen et al., 1996). Thus, taken together, this suggests that IHF is not necessary in the transposition reaction of Mu but is essential in the transcriptional regulation of Mu proteins.

In this context, our finding that transposition of Tn4652 is impeded in an IHF-defective P. putida is quite exceptional and intriguing. IHF seems to be an essential host protein in the transposition of Tn4652, because we could not detect transposition in an IHF-minus strain (Fig. 1A). However, we must point out that, owing to the limits of our transposition assay, we can detect only up to 100-fold reduction in transposition. Therefore, we cannot totally exclude the possibility that some transpositional activity of Tn4652 might exist in

Fig. 4. Protection against DNase I cleavage by binding of IHF and transposase at the left (A) and right (B) ends of Tn4652. Only bottom strand protections are presented. Lanes 1–7 present DNase I reactions carried out in the absence of proteins (lane 1) or with IHF (lanes 2 and 3), transposase (lane 7), IHF and TnpA in combination (lanes 4–6). Lines to the right of the pictures designate protection regions against DNase I cleavage as follows: continuous lines for protection by IHF and dotted lines for protection of IHF and TnpA together. Positive base numbers indicate Tn4652 sequence, and negative numbers indicate flanking DNA sequence. The asterisks signify base 19 on each end that becomes hypersensitive to DNase I cleavage upon interaction with TnpA.
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an IHF mutant strain, although we are not able to detect that.

Involvement of IHF in the regulation of Tn4652 was first proposed because of its ability to enhance transcription from the transposase promoter (Hõrak and Kivisaar, 1998). The results obtained in this study confirm the role of IHF in the regulation of the transposase promoter (see Fig. 2) but also reveal another possible function for IHF in the regulation of Tn4652. Namely, IHF is needed for the stable binding of Tn4652 transposase to its cognate binding sites at both termini of the transposon in vitro (Figs 3 and 4). Therefore, we suppose that IHF might act as an accessory protein facilitating binding of the transposase also in vivo. Indeed, a strong impact of IHF on transposition frequency in vivo cannot be explained solely by a modest (up to fourfold) effect of IHF on transposase regulation. Here, it is appropriate to discuss comparative data concerning another host factor involved in the regulation of Tn4652 – stationary growth phase specific sigma factor σ5. Compared with IHF, σ5 has about 10 times stronger effect on the transposase promoter. Nevertheless, the transposition frequency of Tn4652 is at least 10 times higher in a σ5-defective P. putida (Ivics et al., 2001) than in an IHF-defective strain (this study, Fig. 1). Therefore, it is reasonable to suppose that, besides regulating the tnpA promoter, IHF also facilitates the contact of the transposase with transposon ends. However, we cannot rule out the possibility that, as a result of pleiotropic effects of IHF in bacteria (Friedman, 1988), there might be additional checkpoints in transposition, which are regulated by IHF.

The organization of TnpA and IHF binding sites in Tn4652 ends is very similar to that in E. coli transposon γ6 (Wiater and Grindley, 1988). IHF protects the region between 35 and 75 bp at both ends of γ6 and Tn4652. Transposase binding regions are also similar between γ6 and Tn4652, covering the whole transposon ends and extending by about 10–15 bp into the flanking target DNA. This is in contrast to the behaviour of Tn3 transposase, a relative of the transposases of γ6 and Tn4652 – in vitro transposase of Tn3 binds only to the inner region of the terminal inverted repeats, leaving the distal ends of the transposon and junction DNA free for DNase I cleavage (Ichikawa et al., 1987).

In contrast to the transposase of Tn4652, γ6 transposase can bind in vitro to terminal inverted repeats also without the IHF, which is in good agreement with the dispensability of IHF in the transposition of γ6 in vivo (Wiater and Grindley, 1990; May and Grindley, 1995). Nevertheless, it seems paradoxical that, despite co-oper-
ative binding between IHF and ψ6 transposase in vitro, IHF does not play any essential role in transposition other than stimulation of transpositional immunity of target DNA (Wiater and Grindley, 1990; May and Grindley, 1995). However, it has been shown that E. coli HU and IHF can substitute for each other in many processes including, for example, IS10 and phage Mu transposition in vitro (Morisato and Kleckner, 1987; Surette and Chaconas, 1989). Therefore, it might be that HU can replace IHF in the transposition of ψ6 as well. To our knowledge, this possibility has not been tested. Secondly, IHF can affect ψ6 transposition only in certain conditions. We measured Tn4652 transposition during carbon starvation conditions in which IHF-caused effects may somehow be enhanced, for example as a result of the ability of IHF to change the level of DNA supercoiling (Surette and Chaconas, 1989). In starving bacteria, negative supercoiling of DNA is essentially decreased (Balke and Gralla, 1987). However, DNA negative supercoiling is known to be an important requirement for transposition (Mizuuchi, 1992). In vitro experiments with Mu and Tn10 have shown that IHF is able to serve as a supercoiling relief factor (Surette and Chaconas, 1989; Chalmers et al., 1998). This suggests that the requirement for IHF in transposition might be more prominent in situations in which the supercoiling level is decreased.

Why do certain transposons carry IHF binding sites at their ends and what is their true role and evolutionary significance? In most cases, IHF seems to act positively on transposition frequency. Considering a general opinion that transposons are selfish elements, this question can be approached from the position of either the host or the transposon. For the transposon, it is beneficial to use host factors for the activation of transposition. For the host, it does not seem to be reasonable to contribute to transpositional mutagenic processes. Nevertheless, it has been shown that the activity of several transposable elements increases in response to stress. It has been proposed that elevated transposition during stress may reflect a survival strategy because promoted mutagenesis processes may potentially contribute to survival in unfavourable conditions (Chao et al., 1983; Wessler, 1996; Kidwell and Lisch, 1997; 2001; 2002; Shapiro, 1997; 1999; Capy et al., 2000). Transposon Tn4652 is an interesting example among the bacterial transposons because of its ability to respond clearly to starvation-induced stress (Ilves et al., 2001). Our data suggest that activation of Tn4652 under starvation conditions is not an occasional malfunction of transposition control mechanisms; it is rather an induced process provoked by the host. We suggest that at least two factors can be responsible for starvation-induced transposition of Tn4652. First, in starving bacteria, transposition of Tn4652 is elevated on account of direct control of tnpA promoter by the stationary phase sigma factor σ5, which is induced in order to improve the survival of cells under stressful conditions (Ilves et al., 2001). Secondly, the results obtained in this study imply that IHF may also be involved in the enhancement of transposition of Tn4652 in stationary phase P. putida. The amount of IHF is known to increase by nearly one order of magnitude in E. coli and P. putida when bacteria go from exponential to stationary phase of growth (Ditto et al., 1994; Delic-Attree et al., 1996; Murtin et al., 1998; Teras et al., 2000; Valls et al., 2002). Here, we show that transposition of Tn4652 depends essentially on IHF concentration. Artificial overexpression of IHF resulted in elevated transposition of Tn4652 compared with that in the wild-type strain (Fig. 1B). This indicates that the concentration of IHF in wild-type cells is limiting and cannot saturate the potential of transpositional machinery of Tn4652. Here, we want to point out that no transposition of Tn4652 can be detected in exponentially growing bacteria, even in the case of overexpression of IHF (unpublished results). Results of our previous study (Ilves et al., 2001) suggest that, in exponentially growing cells, transposition of Tn4652 is limited by σ5. Consequently, regulation of transposition by IHF can occur only in stationary phase cells in which transposase expression is already activated by σ5. Summing up, the data in this study indicate that IHF might be one of the host factors that links transposition with the physiological state of the host. It would be interesting to know whether it could also be true for other transposons containing IHF binding site(s).

It has been demonstrated that mutation rates increase in bacteria under stress situations (see, e.g. Bjedov et al., 2003). However, intense debate continues as to whether stress-induced mutagenesis is a consequence of a genetically programmed evolutionary strategy or a result of accidental malfunction of mutagenesis control mechanisms (Lenski and Sniegowski, 1995; Metzgar and Wills, 2000; Radman et al., 2000; Rosenberg, 2001; Kivisaar, 2003; Roth et al., 2003). Our study suggests that activation of Tn4652 transposition in starving bacteria is not an accident but is a host-induced process. In the light of these results, it may not be far-fetched to conclude that the host ‘uses’ the potential of the transposon transiently to promote mutagenesis processes under conditions in which it could be potentially useful, for example under conditions of starvation.

**Experimental procedures**

**Bacterial strains, plasmids and media**

The bacterial strains and plasmids used in this study are described in Table 1. Bacteria were grown on Luria–Bertani (LB) medium (Miller, 1992) or on M9 minimal medium (Adams, 1959) containing 0.2% glucose. Phenol minimal plates with
Table 1. Bacterial strains and plasmids.

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<th>Genotype or construction</th>
<th>Source or reference</th>
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<td>E. coli TG1</td>
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<tr>
<td>CC118 λ pir</td>
<td>Δ[ara-leu] araD ΔlacX74 galE galK phoA20 thi-1</td>
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<td>S17-1 λ pir</td>
<td>Tp' Sm' recA thi pro (r m') RP4::2-Tc::Mu::Km Tn7 λpir</td>
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</tr>
<tr>
<td>P. putida PaW85ihfAtacIHF</td>
<td>P. putida ihfA and ihfB under the control of the P_tac promoter and the lacI promoter</td>
<td>This study</td>
</tr>
</tbody>
</table>

Plasmidsa

<table>
<thead>
<tr>
<th>Plasmidsa</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBluescript KS</td>
<td>E. coli cloning vector (Ap')</td>
</tr>
<tr>
<td>pEST1332</td>
<td>Plasmid containing promoterless pheBA genes (Ap')</td>
</tr>
<tr>
<td>pKTlacZS/C</td>
<td>Plasmid containing trpA promoter fused with reporter gene lacZ (Ap')</td>
</tr>
<tr>
<td>pET19b</td>
<td>Protein expression vector (Ap')</td>
</tr>
<tr>
<td>pET19-trpA</td>
<td>trpA is fused with histidine tag in pET19b</td>
</tr>
<tr>
<td>pET11c</td>
<td>Protein expression vector (Ap')</td>
</tr>
<tr>
<td>pET11c-ihfAbhis</td>
<td>pET11c containing ihfA and his6-tail-tagged ihfB genes</td>
</tr>
<tr>
<td>pUTetPF</td>
<td>pUTmini-Tn5luxAB carrying P. putida ihfA and ihfB under control of P_tac promoter and lacI promoter</td>
</tr>
<tr>
<td>KS-ihfA</td>
<td>625 bp P. putida ihfA gene containing PCR fragment</td>
</tr>
<tr>
<td>KS-ihfAKm</td>
<td>cloned into Sad--XbaI-opened pBluescript KS</td>
</tr>
<tr>
<td>pGP704L</td>
<td>Delivery plasmid for homologous recombination (Ap')</td>
</tr>
<tr>
<td>pGP704L-ihfAKm</td>
<td>pGP704L containing Sad--EcoRI fragment of ihfA::Km from KS-ihfAKm</td>
</tr>
<tr>
<td>pRK2013</td>
<td>Helper plasmid for conjugal transfer of pGP704L (Km')</td>
</tr>
<tr>
<td>KSLHihf+</td>
<td>183 bp DNA fragment containing mutated IHF binding site of left end (132 bp of left end + 51 bp of neighbouring DNA) cloned into XbaI--HindIII-opened pBluescript KS</td>
</tr>
</tbody>
</table>

a. Oligonucleotides used for construction of the plasmids are presented in Table 2.

1.5% Difco agar contained 2.5 mM phenol as the carbon source. Antibiotics were added at the indicated final concentrations: ampicillin, 100 μg ml⁻¹, kanamycin, 50 μg ml⁻¹ and tetracycline, 10 μg ml⁻¹ for E. coli; carbenicillin, 1500 μg ml⁻¹, kanamycin, 50 μg ml⁻¹ and tetracycline, 40 μg ml⁻¹ for P. putida. P. putida was incubated at 30°C and E. coli at 37°C. E. coli was transformed with plasmid DNA as described by Hanahan (1983). P. putida was electrotransformed according to the protocol of Sharma and Schimke (1996).

Construction of plasmids and strains

Oligonucleotides used in PCR amplifications are listed in Table 2. For construction of the P. putida PaW85 ihfA-defective strain, the ihfA gene was amplified by PCR from the genomic DNA of P. putida PaW85. The oligonucleotides IhfASacN and IhfAXbaC were used for amplification (Table 2). The amplified DNA fragment was cloned into pBluescript KS cleaved with SacI and XbaI to obtain KS-ihfA. For interruption of the ihfA coding region, the Eco47III fragment containing the Kmr gene from pUTmini-Tn5 Km2 was inserted into the NruI-cleaved ihfA gene in KS-ihfA. The resulting ihfA-Km sequence was cloned from KS-ihfAKm as a SacI--EcoRI fragment into delivery plasmid pGP704L (Pavel et al., 1994). The interrupted ihfA gene was inserted into the chromosome of P. putida PaW85 by homologous recombination. Plasmid pGP704L-ihfAKm, which does not replicate in P. putida, was conjugatively transferred from E. coli CC118 λ pir (Herrero et al., 1990) into P. putida PaW85 using helper plasmid pRK2013 (Figurski and Helinski, 1979). The selected PaW85ihfA knock-out was controlled by PCR analysis. The lack of functional IHF protein in the knock-out was also verified by testing the cell lysates prepared from P. putida PaW85ihfA by immunoblot and gel mobility shift analyses.

For complementation of the IHF-defective strain with functional IHF, the ihfA and ihfB genes under the control of the P_tac promoter and lacI promoter were introduced into P. putida PaW85ihfA. Mating between E. coli S17-1 λ pir (Miller and Mekalanos, 1988) carrying pUTetPF (Teras et al., 2000; Table 1) and P. putida PaW85ihfA and subsequent selection of tetracycline resistance transconjugants enabled us to obtain the IHF-overexpressing strain PaW85ihfAtacIHF. The selected PaW85ihfA knock-out was controlled by PCR analysis. The lack of functional IHF protein in the knock-out was also verified by testing the cell lysates prepared from P. putida PaW85ihfA by immunoblot and gel mobility shift analyses.

For clotting the ihfA and ihfB genes into the protein expression vector pET11c, the ihfA–ihfB-containing fragment was amplified from plasmid pUTtetPF (Teras et al., 2000) using oligonucleotides IhfANde and IhfBhisBam. Use of these oligonucleotides enabled us to design suitable cloning sites...
and his6-tag into the C-terminus of IhfB. The PCR-amplified DNA fragment was cloned into Ndel–BamHI-opened pET11c. The resulting plasmid pET11c-ihfBhis encodes both subunits of IHF – the IhfA and the IhfB with a C-terminal histidine tag.

For mutation of the IHF binding site in the left-end DNA of Tn4652, two mutant oligonucleotides, Hv1 and Hv2 (Table 2), were used in PCR amplification. Two mutant PCR fragments were generated: one with the aid of Hv1 and TnL and the other using Hv2 and LHnear (Table 2). The Hv1–TnL fragment cleaved with SspI and the Hv2–LHnear fragment cleaved with DraI were ligated at cleavage sites to obtain a TnL–LHnear fragment. This joined DNA fragment, containing nine mutations in two potential IHF binding sites in the transposon left end (Fig. 5), was cleaved with XbaI and HinDIII and cloned into XbaI–HindIII-opened pBluescript KS. The resulting plasmid KSLHihf* was used as a PCR template for the generation of a mutant left-end DNA fragment for gel shift mobility assay.

**Enzyme assays**

For β-galactosidase (β-Gal) measurement, the P. putida cells were grown overnight in LB medium. The assay was performed in cell suspension by modification of the standard protocol of Miller (1992) as described previously (Tover et al., 2001).

**Transposition assay**

Transposition of native Tn4652 was examined in an assay that involves starvation of bacteria on phenol minimal plates (Kasak et al., 1997; Ilves et al., 2001). The assay enables the detection of insertions of chromosomal Tn4652 into the target plasmid pEST1332, which contains promoterless phenol degradation genes pheBA. Transposition of Tn4652 results in activation of phe genes resulting from the generation of a functional promoter (Kasak et al., 1997). In consequence, the Phe+ mutants accumulate on phenol minimal plates. More than 95% of the Phe+ mutants emerge as a result of transposition of Tn4652 into pEST1332 (Ilves et al., 2001). The transposition assay was carried out with P. putida PaW85 wild-type strain, with its ihfA-defective derivative PaW85ihfA, and with IHF-overexpressing strain PaW85ihfAtacIHF. Bacteria were grown overnight in LB medium at 30°C and washed with M9 solution. Approximately 10³ cells of five independent overnight cultures of each of the three P. putida strains compared were spread onto phenol minimal plates, and the accumulation of mutant Phe+ colonies was monitored upon incubation of the plates at 30°C during 7 days. To obtain different expression levels of IHF in the cells of P. putida PaW85ihfAtacIHF, the phenol minimal plates were supplied with either 0.01 mM or 0.5 mM IPTG or no IPTG was added. For proving the transposition of Tn4652 into pEST1332, a random portion of Phe+ mutants was analysed by PCR with oligonucleotides pheA, TnR0 and TnL (Table 2).

**Overexpression and purification of proteins**

Tn4652 transposase TnpA was overexpressed as a fusion protein containing an N-terminal decahistidine (his₁₀) tag. The protein was purified as described previously (Hõrak and Kivisaar, 1999). The purified TnpA was stored in storage.

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**Table 2. Oligonucleotides.**

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Description (complementary positions)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>TnR0</td>
<td>5′-TGCCATTGAAACATCCCTCCCAA (positions 136–157 inside Tn4652 right end)</td>
</tr>
<tr>
<td>RHnear</td>
<td>5′-GGAGGAAAAAGGGTAGGGCAGA (positions 30–49 in the DNA flanking the right end of Tn4652 in the chromosome)</td>
</tr>
<tr>
<td>TnL</td>
<td>5′-CGTAAAGCTTCCCTAATGGATGGCTGAAG (positions 111–132 inside Tn4652 left end)</td>
</tr>
<tr>
<td>XbaI</td>
<td>5′-GAGATATCTAGAGAAAAAGGCTC (positions 35–56 in the DNA flanking the left end of Tn4652 in the chromosome)</td>
</tr>
<tr>
<td>For cloning of the P. putida ihfA gene:</td>
<td></td>
</tr>
<tr>
<td>IhtASacN</td>
<td>5′-CAAGAAGCCTCTGTAGATAGAAAAAGCCTTGC (positions 122–143 upstream of the ATG start codon of ihfA)</td>
</tr>
<tr>
<td>Sacl</td>
<td>5′-TCATCTAGATCAGCTAGACAGCCTGC (positions 155–176 downstream of the TAA stop codon of ihfA)</td>
</tr>
<tr>
<td>For cloning of the P. putida ihfB genes:</td>
<td></td>
</tr>
<tr>
<td>IhfANde</td>
<td>5′-AAGGCAATGAGGTGCTTGACGAAAGC (positions 1–20 in the ihfA gene)</td>
</tr>
<tr>
<td>Ndel</td>
<td>5′-CTTGTTAAATTTAGCCAGAAAAGGCTC (positions 27–62 inside the Tn4652 left end)</td>
</tr>
<tr>
<td>IhfBhisBam</td>
<td>5′-ATCAGGATCCCTAATATGATGATGATGATGATGGTGGTGCGCCTCCCTC (positions 282–303 in the ihfB gene)</td>
</tr>
<tr>
<td>BamHI</td>
<td>5′-GATCCTAAGATTATCATTACCGCGCT (complementary to the Ptac promoter)</td>
</tr>
<tr>
<td>For mutagenesis of IHF binding site in Tn4652 left end</td>
<td></td>
</tr>
<tr>
<td>Hv1</td>
<td>5′-CAAGAATGAGGTCGAAACGCTC (positions 58–84 inside the Tn4652 left end)</td>
</tr>
<tr>
<td>SspI</td>
<td>5′-CACTTTAATAATTTGGAGAAAAAGGCTC (positions 27–62 inside the Tn4652 left end)</td>
</tr>
<tr>
<td>Hv2</td>
<td>5′-CACTTTAATAATTTGGAGAAAAAGGCTC (positions 27–62 inside the Tn4652 left end)</td>
</tr>
<tr>
<td>For control PCR</td>
<td></td>
</tr>
<tr>
<td>Prtac</td>
<td>5′-AAGGCAATGAGGTGCTTGACGAAAGC (complementary to the Ptac promoter)</td>
</tr>
<tr>
<td>PheA</td>
<td>5′-TGCTCAAGATTATCATTACCGCT (positions 11–32 in the pheA coding region)</td>
</tr>
</tbody>
</table>

* a. The sites for restriction enzymes are underlined. Bold letters indicate the initiation and termination codons. The mutagenized nucleotides in Hv1 and Hv2 are double underlined.
buffer (30 mM Tris-HCl, pH 7.0, 0.13 mM EDTA, 1.3 mM β-mercaptoethanol, 130 mM NaCl, 300 mM imidazole, 50% glycerol) at −20°C.

_Pseudomonas putida_ IHF subunits were co-purified with the aid of a histidine tag (his₆) fused to the C-terminus of IhfB. For overexpression of IhfA and IhfB-his₆, the _E. coli_ BL21(DE3)-harbouring plasmid pET11c-ihfABhis was grown in LB medium at 37°C up to an optical density of 0.5 (OD₅₆₂). The culture was subsequently transferred to 18°C and adapted there for 30 min before induction of IhfA and IhfB-his₆ expression with 0.5 mM IPTG. After 4 h of induction, cells were pelleted and sonicated in buffer A [100 mM Tris-HCl, pH 7.0, 1 M NaCl, 50 mM imidazole, 0.1% NP-40, 1 mM phenylmethylsulphonyl fluoride (PMSF), 10% glycerol]. The cell lysate was centrifuged at 12 000 rpm for 30 min at 4°C. The cleared supernatant was supplied with the Ni-NTA agarose matrix (Qiagen), and protein binding to the agarose occurred by slowly rotating the test tube overnight at 4°C. The agarose matrix was washed twice with 50 volumes of buffer A, and the purified protein was stored at −20°C.

**DNase I footprinting**

The DNA fragments for DNase I footprinting were amplified by PCR using the purified chromosomal DNA of _P. putida_ PaW85 as a template. Oligonucleotides used in PCRs are listed in Table 2. One oligonucleotide was end-labelled by phosphorylation with [α-³²P]-ATP, allowing the specific labeling of one DNA strand. The following DNA fragments were used in footprinting experiments: (i) RH – a 206 bp DNA fragment containing the right end of transposon Tn4652 (157 bp from the right terminus of Tn4652 and 49 bp of flanking DNA), amplified using primers TrnR0 and RHnear; (ii) LH – a 188 bp DNA fragment containing the left end of the transposon (132 bp from the left terminus of Tn4652 and 56 bp of flanking DNA), amplified using primers TrnL and LHnear. The labelled DNA fragments were purified by native 5% polyacrylamide gel electrophoresis, eluted and resuspended in the water. For the binding reaction, different amounts of purified _P. putida_ his-tagged IHF and/or TnpA proteins were combined with 30 000 c.p.m. of labelled DNA fragment, 25 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 1 mM CaCl₂, 0.1 mM EDTA, 50 mM KCl, 5 μg of BSA, 1 μg of salmon sperm DNA and 5% glycerol in a final volume of 50 μl. When no protein was added, the TnpA storage buffer was added to the reaction. Mixtures were preincubated for 25 min at room temperature before the start of digestion by DNase I (1 U; Promega) for 4 min. Reactions were stopped by the addition of 50 μl of a solution containing 0.1 M EDTA, 0.1% sodium dodecyl sulphate, 1.6 M ammonium acetate and 20 mg of sonicated salmon sperm DNA per ml. The footprinting reaction mixtures were subsequently extracted once with phenol and chloroform (1:1 v/v) and once with chloroform and, finally, the DNA was precipitated with ethanol. The DNA fragments were resuspended in 5 μl of sequence loading buffer (deionized formamide containing 10 mM EDTA, 0.3% bromophenol blue and 0.3% xylene cyanol) and loaded on a 6.5% polyacrylamide gel that contained 8 M urea. DNA sequencing reactions were performed with a Sequenase version 2.0 kit (US Biochemicals) and were loaded on a sequencing gel as size markers. After the run, the gels were dried and exposed to a PhosphorImager screen (Molecular Dynamics).

To determine whether either protein preparation contained an intrinsic DNA ‘nicking’ activity, an additional control was performed by incubation of DNA with each protein at the highest concentration used in footprinting, but without the addition of DNase I.

**Gel mobility shift assay**

For gel mobility shift assay, the binding buffer and the DNA-binding conditions were the same as those used in the footprinting assay. The reaction volume was reduced to 25 μl. ³²P end-labelled fragments were also the same as in the footprinting experiment, except for an additional left-end DNA fragment containing mutations in the IHF binding site (Fig. 5). After incubation, the reaction mixture was applied to a 5% non-denaturing polyacrylamide gel buffered with 0.5x Tris-borate–EDTA (pH 7.5). Electrophoresis was carried out at 4°C at 10 V cm⁻¹ for 3 h. The gels were dried under vacuum and exposed to a PhosphorImager screen (Molecular Dynamics).

When cell lysates were used in the gel shift assay, the total protein concentration was measured by the Bradford method (Bradford, 1976).

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**References**


Kivisaar, M., Hörak, R., Kasak, L., Heinaru, A., and Habicht, J. (1990) Selection of independent plasmids determining phenol degradation in *Pseudomonas putida* and the...
cloning and expression of genes encoding phenol monooxygenase and catechol 1,2-dioxynogenase. Plasmid 24: 25–36.


