DISSERTATIONES BIOLOGICAE UNIVERSITATIS TARTUENSIS
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REGULATION OF TRANSPOSITION OF TRANSPOSON TN*4652* IN *PSEUDOMONAS PUTIDA*

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PRESS

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CONTENTS

ORIGINAL PUBLICATIONS

This thesis is based on the following original publications:

I Hõrak, R. and Kivisaar, M. (1998) Expression of the transposase gene *tnpA* of Tn*4652* is positively affected by integration host factor. J. Bacteriol. **180**, 2822–2829.

II Hõrak, R. and Kivisaar, M. (1999) Regulation of the transposase of Tn*4652* by the transposon-encoded protein TnpC. J. Bacteriol. **181**, 6312–6318.

III Ilves, H., Hõrak, R. and Kivisaar, M. (2001) Involvement of σ^S in starvation-induced transposition of *Pseudomonas putida* transposon Tn*4652*. J. Bacteriol. **183**, 5445–5448.

IV Hõrak, R. and Kivisaar, M. Regulation of transposition of Tn*4652*: involvement of *Pseudomonas putida* integration host factor and transposon-encoded inhibitor TnpC. Manuscript.

ABBREVIATIONS

bp	base pair
DDE motif	conserved motif of two aspartic acid residues and a glutamic acid residue in the active site of transposase
Fis	factor for inversion stimulation
H-NS	histone-like nucleoid structuring protein
HU	heat-unstable nucleoid protein
IHF	integration host factor
IPTG	isopropylthio-β-D-galactoside
IS	insertion sequence element
ORF	open reading frame

INTRODUCTION

The genomes are not constant, rather they vary permanently. Multitude of genetic rearrangements occurs due to inversion, duplication, insertion, deletion, or translocation of DNA segments. In bacteria, two categories of recombination promote a variety of DNA rearrangements. In general homologous recombination, genetic material is exchanged between two homologous DNA loci. The other source for DNA recombination is transposition in which discrete DNA segments, called transposons, translocate to one of many nonhomologous target sites (see, for example, Hallet and Sherratt, 1997).

Transposons are widespread in nature, having been identified in the genomes of numerous organisms, from bacteria to humans. Transposition can alter the genome functionality. It is obvious that transposition of a mobile element into the particular gene inactivates it. However, insertion of a transposon can also activate the expression of neighbouring normally cryptic genes. Transposition can promote large DNA rearrangements including deletions, inversions and replicon fusions. Additionally, capability of transposons to transmit genetic information between cells makes transposons important tools in horizontal gene transfer. Thus, it is evident that mobile genetic elements have important roles in genome organisation and reorganisation and as a consequence — in the genome evolution.

Many transposons code only for factors that are needed for propagation of their DNA. By using functions of the host they can spread in the genome in a replicative mode, being able to overreplicate their host. Therefore, the transposons are often viewed as molecular parasites or as selfish DNA-s (Doolittle and Sapienza, 1980, Orgel *et al.*, 1980). However, the idea that mobile elements are primarily parasitic is one-sided. Transposons often code for genes, for example for antibiotic resistance confirming genes that could be useful for host under certain conditions. Really, the relationship between the transposable element and host genome may be highly variable ranging from parasitism to mutualism (Kidwell and Lisch, 2001).

Mobility of bacterial transposons is strictly regulated to low levels (10^{-3} to 10^{-8} per element per generation; Kleckner, 1990) in order to maintain the balance between their propagation and potential destructive mutagenic effects to their hosts. Actually, transposable elements stay mostly in the quiet state and translocate only in a narrow window of host cell cycle or solely in response to certain stimuli. Barbara McClintock, the discoverer of transposable elements, has suggested that transposition activity could be a response to challenges to the genome (McClintock, 1984). Indeed, it has been shown that different stresses such as carbon starvation, temperature effects and UV light can enhance transposition of bacterial mobile elements. Moreover, it has been hypothesised that

activation of transposition due to stress might serve as an adaptive response to overcome stress and to evolve the new traits (Wessler, 1996; Capy *et al.*, 2000).

So, there have been various interpretations about the nature of the transposons — from calling them parasites up to considering them as useful entities for the host genome. In order to understand the real interplay between the transposon and its host and their influence upon each other, it is important to find out the regulatory mechanisms that control the frequency of transposition. For most bacterial elements, the rate of transposition is primarily determined by the amount and activity of transposon-encoded specialised transposition recombinase called transposase. Up to now, a large variety of mechanisms limiting transposase gene expression or transposase protein activity have been described (reviewed in Kleckner, 1990). Furthermore, transposition reaction itself is mostly controlled by other transposon-encoded protein(s) and/or host factors. Involvement of host factors in the regulation of transposition indicates that these factors may be used for communication between the host and the transposon to signify whether the transposition is favoured or not. A popular idea is that transposition is modulated by cellular (and also probably by the extracellular) conditions being favoured when these conditions are poor (e.g. Kleckner, 1990; Shapiro, 1997; Capy *et al.*, 2000). However, there are only few well-understood examples of transposons, which switch their activity depending on different cellular signals (Lamrani *et al.*, 1999; Morillon *et al.*, 2000).

In the present thesis I will concentrate on the regulation of transposition of bacterial mobile elements with special attention to the relationship between the transposable element and its host. The experimental part of the thesis attempts to present an overview about the regulation of *Pseudomonas putida* transposon Tn*4652*, an interesting example among the bacterial transposons due to its ability to activate cryptic genes and to respond to the starvation-induced stress.

1. REVIEW OF LITERATURE

1.1. Overview of transposition

Transposons are discrete DNA segments that can move from one genetic location to another. The essential determinants of a transposon are terminal inverted repeat sequences that designate the transposon ends and the gene encoding for the transposase that performs the transposition reaction. The simplest transposable elements — IS elements (insertion sequence elements) — code only for these determinants (Fig. 1). Larger transposons can code other genes as well including, for example, genes for different antibiotic or metal resistance or genes for degradation of several cyclic organic compounds. Some transposons, called composite transposons, carry DNA with different genes between two IS elements. In this case both whole composite transposon as well as only one IS element are able to transpose.

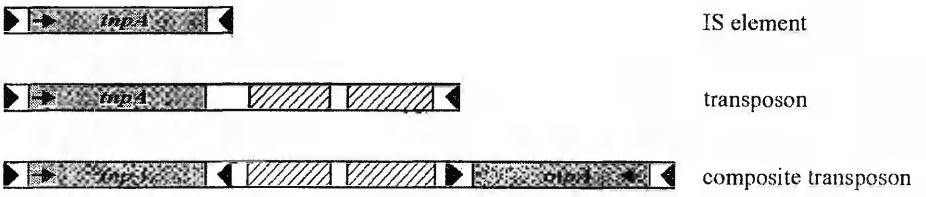

Figure 1. Organization of different types of bacterial transposable elements. Transposase genes (tnpA) are designated by grey boxes. The terminal inverted repeats are indicated with black triangles. Hatched boxes picture the different transposon-carried genes.

In the first step of transposition the transposase specifically interacts with the sequences at both ends of the mobile element. These terminal inverted repeats sequences are unique and characteristic to each type of transposable element. After specifically binding to inverted repeats the transposase catalyses the DNA cleavage and rejoining to a new target site. The transposon ends are joined to the target DNA in a staggered fashion, and the resulting gaps are filled in by using host replication functions (reviewed in Mizuuchi, 1992; Craig, 1996). This generates the target site duplications on either side of inserted transposon. The length of these direct duplications is characteristic for each transposon and can vary from 2 to 14 bp (reviewed in Mahillon and Chandler, 1998).

In the thesis I will concentrate on the transposition of bacterial mobile DNA elements. However, hereby I want to point out that translocation of a transposon and integration of a virus into the chromosome of the host are mechanistically

similar reactions. Actually, many aspects in transpositional recombination have been resolved in the study of bacteriophage Mu regulation.

1.2. Types of transposition mechanisms

Transposition can be described as a three-step process. In the first two steps, specific DNA cleavage at the transposon ends and subsequent strand transfer into target DNA occurs. In the third step, the transpositional intermediate is processed by host DNA repair or replication machinery. The basic biochemical mechanism underlying the transpositional recombination is remarkably similar between diverse mobile elements. However, the outcomes of the transposition process may differ essentially while some important differences exist between different types of transposition mechanisms (reviewed in Mizuuchi, 1992).

The transposition mechanisms of bacterial transposons can be divided into two major types of reactions: non-replicative or cut-and-paste transposition and replicative transposition. The fundamental differences between the cut-and-paste and replicative transposition mechanisms lie in the DNA cleavage type at the transposon ends and as a consequence in the different outcomes of the transposition reaction.

1.2.1. Replicative transposition

In replicative transposition, entire mobile element is directly copied by DNA replication during translocation process. If the replicative transposition occurs into the same DNA molecule it may lead to deletion or inversion of DNA region between target and original location of transposon. Replicative transposition from one replicon to another results in generation of a structure called cointegrate in which the donor and target replicon are joined by directly repeated copies of the transposon at each junction (Fig. 2).

The critical steps in transposition are the DNA breakage reactions at each transposon end promoted by transposase. The mode how transposase cuts the DNA largely determines the type of transposition mechanism. In replicative transposition, process begins with the cleavage of only one DNA strand at each end of transposon, liberating the 3' ends of the element (Fig. 3). After transfer of free transposon ends to the target DNA, the two replicons (donor and target) will be linked while no cleavage at the 5' ends of transposon has been occurred (Craigie and Mizuuchi, 1985; Craigie and Mizuuchi, 1987). This intermediate structure is often called the Shapiro intermediate from the name of the scientist who was one of the first to suggest the model for cointegrate formation and resolution (Shapiro, 1979; Arthur and Sherratt, 1979). Because the staggered

target site cut, the Shapiro intermediate has gaps next to the ends of the transposon. Free 3' ends of the target DNA are used as primers for DNA replication through the gaps and entire transposon to generate the cointegrate. The cointegrate can be subsequently resolved by recombination between two copies of the transposon yielding to a restored donor molecule and a target replicon now containing a copy of the transposon (reviewed in Hallet and Sherratt, 1997; Craig, 1996). Many transposons, for example Tn*3* and its relatives, encode for separate site-specific recombination system that can resolve the cointegrate (Grindley *et al.*, 1982, Stark *et al.*, 1989). Recombination occurs between the *res* sites of the two copies of transposon and is catalysed by element-encoded recombinase resolvase (Shapiro, 1979; Arthur and Sherratt, 1979). Replicatively moving transposons that do not code for resolution function use host-encoded homologous recombination system to resolve the cointegrate.

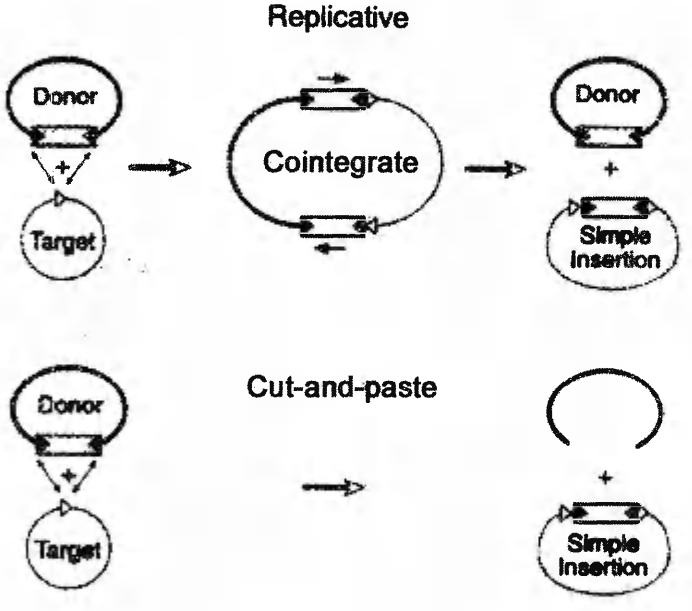

Figure 2. Schematic presentation of replicative and cut-and-paste transpositions. The product of replicative transposition is cointegrate in which the donor and target replikon are fused by two copies of the transposon (rectangle). The cointegrate will be resolved by site-specific recombintion between the two transposon copies. Both modes of transposition result in duplication of the target site (open triangles) (from Hallet and Sherratt, 1997).

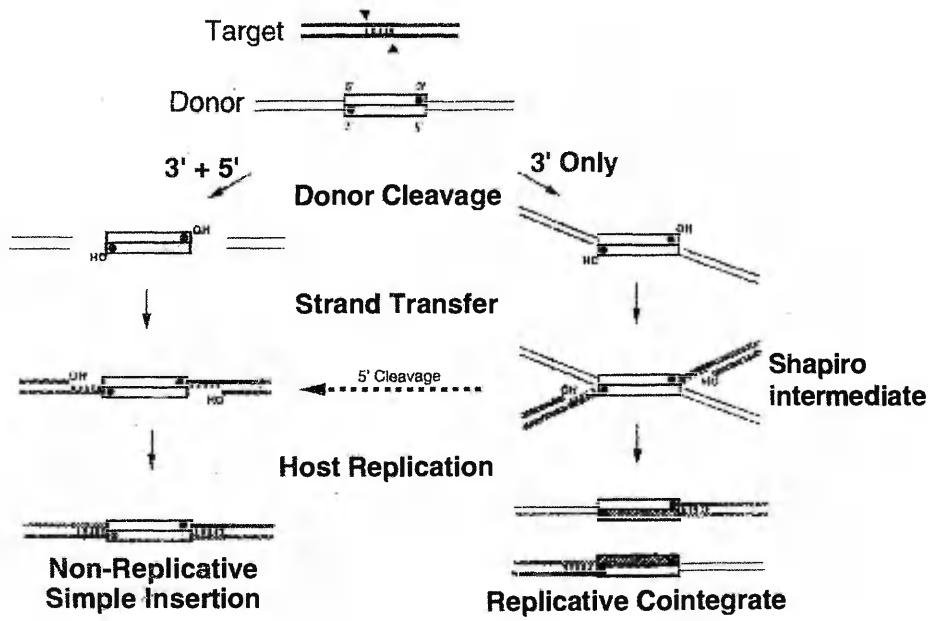

Figure 3. Chemical steps in replicative and cut-and-paste transposition. The transposon (solid box) donor DNA is shown by thin line. Target DNA is pictured by thick line. Note, that the Shapiro intermediate may result both in cointegrate formation or simple insertion, as is the case of bacteriophage Mu transposition in the lytic or lysogenic cycle, respectively (see the text) (from Craig, 1996).

The best-studied examples of transposons that transpose via the replicative transposition pathway are ampicillin resistance-encoding transposon Tn*3* and its relatives. They all move through a cointegrate as transposition intermediate and they code for recombination functions for cointegrate resolution (reviewed in Sherratt, 1989). An interesting example is bacteriophage Mu that uses replicative transposition mechanism to propagate its genome during the lytic growth cycle. Multiple rounds of replicative transposition can generate about 100 progeny phage particles per cell in less than one hour (Pato, 1989).

1.2.2. Cut-and-paste transposition

In the non-replicative or cut-and-paste mechanism, the transposon is cut out of the donor site by double-strand breaks (Fig. 2). The process is carried out by a transposase and the excised transposon can be seen as a transposition intermediate which will be transferred to the target site. Similarly to the replicative transposition, the transposon transfer occurs through the joining of the 3' ends of the

element to staggered positions of the target DNA (Fig. 3). In this process small gaps (several nucleotides), flanking the inserted transposon, are generated. These gaps are repaired by host functions creating direct repeats at both ends of transposon that is characteristic of transpositional recombination.

Elements known to move through the cut-and-paste transposition are Tn*5* (Reznikoff *et al.*, 1999), Tn*7* (Bainton *et al.*, 1991; Gary *et al.*, 1996), and Tn*10* (Benjamin and Kleckner, 1989; Bolland and Kleckner, 1995). Many IS-elements translocate in this fashion as well (reviewed in Mahillon and Chandler, 1998). Bacteriophage Mu that uses replicative transposition in the lytic growth cycle, can also transpose via the non-replicative mechanism. In the lysogenic cycle of life, Mu is integrated into the host chromosome without replicating the viral genome (Pato, 1989). However, the non-replicative transposition of bacteriophage Mu does not involve the double-strand breaks at the Mu ends but only free 3' ends are produced (Fig. 3). These 3' ends are then joined to the target DNA yielding a branched DNA intermediate (Shapiro intermediate), which can be resolved, by nucleolytic cleavage and gap repair to generate a simple insert (Craigie and Mizuuchi, 1985; Craigie and Mizuuchi, 1987).

Thus, not all non-replicative transposition reactions involve an excised transposon. On the whole, it is not easy to determine by inspection of the transposition products whether the element translocates via a non-replicative or replicative pathway. Transposition often appears to be replicative: mostly the transposon copy at the original donor site does not get lost even in the case of non-replicative transposition. Indeed, during the cut-and-past reaction the broken donor molecule is rarely resealed and might be lost. However, bacterial replicons (even the chromosome) are usually present in multiple copies in the same cell. Therefore, the transposon donor locus of pre-transposition state can be restored by recombinational repair (Craig, 1996). In this case the non-replicative transposition gives the same outcome as replicative transposition.

1.3. Molecular view of transposition

Although in detail, there are important variations in the transposition reactions from one mobile element to another, the basic biochemical reactions underlying the different transposition pathways of bacteria and eukaryotes are extremely similar. For example, the eukaryotic mobile elements, such as retroviruses (e.g. HIV-1) and retrotransposons, insert themselves into target DNA mechanistically similar to bacterial transposons. The proteins performing the reaction in these cases are called integrases (Polard and Chandler, 1995; Haren *et al.*, 1999).

Central to all transposition reactions is the cutting of DNA that precisely exposes the free 3' ends of the mobile element and subsequent joining of these ends to the target DNA. Transposition reactions occur within elaborate protein-

nucleic acid complexes called synaptic complexes. These complexes contain DNA substrates (transposon ends and sometimes also target DNA) that are juxtapositioned by oligomerised transposase molecules. The proper assembly of synaptic complexes prior to activation of transposase catalytic activity is likely to be a key regulatory step in transposition.

1.3.1. Binding of the transposase to the ends of transposon

Mobile elements carry at least two determinants that are essential in transposition: two transposon ends and transposase gene. Inverted repeat sequences at both transposon ends are sites onto which specifically binds the transposase. Transposases are multidomainal proteins: they contain a specific DNA-binding domain (for binding to transposon ends), a catalytic core domain (for DNA cleavage and strand transfer) and a non-specific DNA-binding domain (for binding to target DNA). Additionally, in order to form the synaptic complex essential for initiation of transposition, transposase molecules oligomerise bringing the two transposon ends together. Actually, only after oligomerization and synaptic complex formation the transposase converts from a catalytically inactive molecule to an active one (reviewed in Mizuuchi, 1992).

Why is assembly of synaptic complex a prerequisite step for the subsequent chemical activities? Detailed studies of synaptic complexes of bacteriophage Mu and Tn*5* transposases with relevant transposon ends have enlightened several aspects of transposition machinery (Savilahti and Mizuuchi, 1996; Davies *et al.*, 2000). Resolution of the three-dimensional structure of Tn*5* transposase complexed with Tn*5* transposon end DNA revealed that the architectural organisation of synaptic complex explains the transposase activation in this complex. Namely, in the synaptic complex, the catalytic centre of transposase subunit bound to one DNA end is precisely positioned at the other end of transposon (Davies *et al.*, 2000). It means that the subunit bound to one DNA end cleaves and joins the other end — the transposase performs so-called *trans*-catalysis (Fig. 4). Biochemical studies of the transposase-DNA complex of bacteriophage Mu indicate a similar architecture and *trans*-catalysis by Mu transposase (Savilahti and Mizuuchi, 1996). It is assumed that also other members of transposase and integrase family employ similar organisational structure for co-ordinate cutting and religating the DNA (Davies *et al.*, 2000; Williams and Baker, 2000).

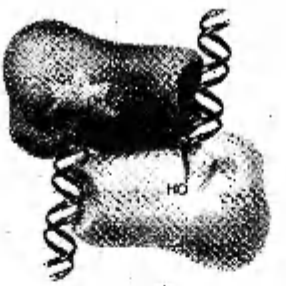

Figure 4. Organization of the transposase-DNA complex of Tn*5*. Transposase molecule bound to the one transposon end catalyses the nicking of the other end (from Williams and Baker, 2000).

Catalytic domains of transposases and integrases are characterised by a common catalytic triad of acidic residues, two aspartic acid residues and a glutamic acid residue known as the DDE motif (reviewed in Polard and Chandler, 1995; Haren *et al.*, 1999). These three conserved residues are well separated in the primary sequence with a spacer of about 50–70 residues between the two aspartic acid and about 35 residues between the second aspartic acid and the glutamic acid residue (Baker and Luo, 1994). Studies of several transposases have shown that mutating any one of these acidic residues abolishes the catalytic activity of protein (Baker and Luo, 1994; Bolland and Kleckner, 1996; Sarnovsky *et al.*, 1996; Naumann and Reznikoff, 2000). It was proposed that DDE motif constitutes a catalytic pocket which binds and co-ordinates divalent metal ions known to be essential in transposition reaction (Baker and Luo, 1994). This suggestion has been confirmed recently by resolution of the three-dimensional structure of Tn*5* transposase-DNA complex (Davies *et al.*, 2000).

1.3.2. DNA breakage and joining

All transposition reactions analysed so far appear to utilise the same basic chemical strategy for joining transposon ends to target DNA: 3' termini of transposon are created by hydrolytic cleavage and subsequently used in direct nucleophilic attacks on target DNA to perform the strand transfer. *In vitro* studies of chemical steps of transposition have revealed that these two reactions are performed by one catalytic centre of transposase or integrase molecule (Baker and Luo, 1994). Both the DNA cleavage and joining steps seem to occur by a one-step transesterification mechanism and there is no evidence for covalent protein-DNA intermediates. In the first reaction, an activated water molecule performs a nucleophilic attack, hydrolysing one strand at the each end of transposon to expose a 3'OH group (Fig. 5). Next, activated 3'OH groups carry out nucleophilic attacks on target DNA (see for example Mizuuchi, 1992). Ca-

talysis of both these transesterification steps requires divalent cations such as Mg^{2+} or Mn^{2+} (Junop and Haniford, 1996; Sarnovsky *et al.*, 1996).

Nonreplicative DNA transposons, e.g. Tn*10*, Tn*5*, Tn7 in *E. coli* and Tc*3* in *C. elegans*, move by a double-strand cleavage mechanism (Bainton *et al.*, 1991; Reznikoff, 1993; van Luenen *et al.*, 1994; Bolland and Kleckner, 1996). The 5' end strand cleavage has been shown for Tn*5* and Tn*10* transposons to occur via a two-step process whereby the 3'OH group generated from the initial strand cleavage step attacks the complementary strand to form a hairpin structure (Fig. 5). Next, hydrolysis of the hairpin intermediate results in blunt-ended DNA at the transposon end (Kennedy *et al.*, 1998; Bhasin *et al.*, 1999). Thus, as demonstrated with Tn*5* and Tn*10*, transposase can catalyse four subsequent chemical reactions: fist-strand nicking, hairpin formation, hairpin resolution and strand transfer.

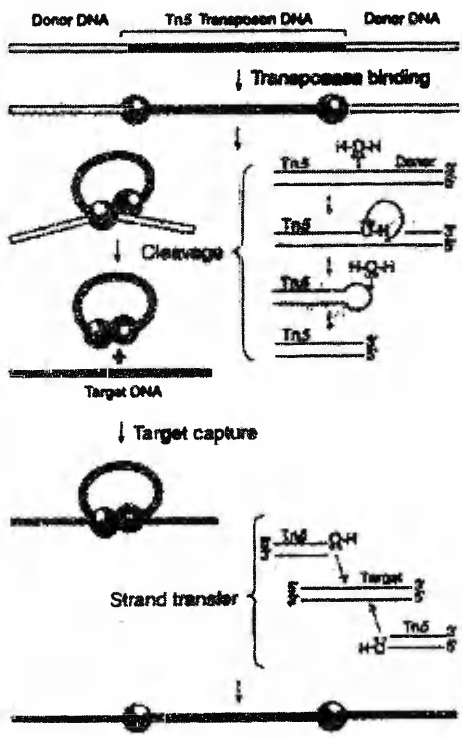

Figure 5. Schematic diagram of the Tn*5* cut-and-paste transposition mechanism (from Davies *et al.*, 2000).

1.4. Regulation of transposition

1.4.1. Frequency of transposition

Successful maintenance of transposable elements requires that the transposon can overreplicate its host. However, this overreplication cannot exceed the delicate balance between the necessity to propagate itself and the potentially deleterious impact to the host. Therefore, the transposition occurs rarely and the frequency of transposition is mostly tightly downregulated (reviewed in Kleckner, 1990). For instance, transposition of Tn*3* family transposons normally occurs at frequencies of 10^{-5} to 10^{-7} per cell generation (Sherratt, 1989). Still, there are some transposons that can transpose at much higher frequency. Transposon Tn*7* transposes at high frequency (up to 10^{-1}) to a single specific site in *E. coli* chromosome. On the other hand, Tn*7* transposition into other sites is much less efficient (Waddell and Craig, 1988). Bacteriophage Mu is the most active element — during replicative transposition more than 100 new copies of the viral genome in less than an hour can be generated (Pato, 1989). The name of bacteriophage Mu is derived from its ability to mutate genes.

The mechanisms of transposition regulation vary among transposons studied. Although, some aspects are common to all. For example, the rule is that the transposase is never expressed at a high amount. Also, transposase catalytic activity is controlled both by transposon-encoded and by host factors. Generally, all these regulatory mechanisms ensure low-frequency transposition (reviewed in Kleckner, 1990).

1.4.2. Regulation of transposase expression and activity

The frequency of transposition is limited primarily by the amount of active transposase (Kleckner, 1990). For now, many different mechanisms are described that maintain a low level of transposase and/or control the transposase activity.

1.4.2.1. Regulation of transposase transcription

Several transposase genes are characterised by weak promoters. For example, the promoter for transposase of IS*10* (pIN) is essentially weak and transcription from this promoter is even more inhibited by transcription from the opposite lying promoter pOUT (Simons *et al.*, 1983). Transposase promoters often overlap with inverted repeat sequences at the transposon end permitting autoregulation by the transposase itself (Mahillon and Chandler, 1998).

Transposons often code for transcriptional repressors that inhibit transcription from the transposase promoter. For example, expression of transposase

protein of bacteriophage Mu is under the negative control of Mu-encoded repressor protein c (Krause and Higgins, 1986). Transposon Tn*3* and other elements of the same subfamily are subjected to negative regulation by the element-encoded resolvase. In these elements, transposase and resolvase are transcribed divergently from the promoters in a *res* region and binding of resolvase to the *res* region inhibits transcription of both the transposase (*tnpA*) and the resolvase (*tnpR*) genes (Sherratt, 1989). The *insA* gene product of IS*1* (which is N-terminal part of IS*1* transposase) inhibits IS*1* transposition by two ways. Binding of *insA* to the terminal inverted repeats both represses expression of transposase gene and prevents binding of transposase to the transposon ends (Machida and Machida, 1989; Zerbib *et al.*, 1990).

Besides of transposase regulation by mobile element-encoded proteins, the transposase expression can be modulated by various host factors as will be described in more detail below (see section 1.4.5.). For instance, transposase promoters of several transposons are regulated by DNA adenine methylation (*dam*). IS*50*, IS*10* and Tn*903* have GATC methylation sites located in the –10 regions of the transposase gene promoters and transcription from these promoters is elevated up to 10-fold in *dam*-minus strain (Roberts *et al.*, 1985; Yin *et al.*, 1988). In the IS*10* transposition, *dam* methylation seems to play the dual role. One GATC site lies in the transposase promoter region. Methylation of this site decreases transcription from the promoter. The other GATC site occurs within the transposase-binding site, which is involved in the transposition reaction. Mutation in *dam* gene increases IS*10* transposition about 100-fold (Roberts *et al.*, 1985). The important biological consequence of *dam* regulation is that transposition should occur only during a limited period of the cell cycle, shortly after replication.

1.4.2.2. Regulation of transposase translation

For many transposable elements, e.g. Tn*3*, IS*10*, IS*5*, IS*1* and Mu, the transposase expression is largely restricted by the inefficient translation of transposase mRNA (reviewed in Kleckner, 1990). Translation of the Tn*3* transposase (*tnpA*) transcript is very inefficient because of a poor ribosome-binding site (RBS). Mutations creating a strong Shine-Dalgarno (SD) sequence in RBS increase expression of the *tnpA* of Tn*3* approximately 30-fold (Casadaban *et al.*, 1982).

Translation of transposase mRNAs of IS*10* and IS*30* is inhibited by antisense RNAs (Simons and Kleckner, 1983; Arini *et al.*, 1997). Pairing of transposase mRNA with IS*10*-encoded antisense RNA sequesters the Shine-Dalgarno sequence and AUG start codon of transposase transcript preventing ribosomes from efficient initiation of translation (Ma and Simons, 1990). Additionally, pairing of antisense RNA with *tnpA* mRNA destabilises the transposase transcript because of the cleavage of the duplexed molecule by ribonuclease III (Case *et al.*, 1990). IS*30* encodes for a 150-bp-long antisense RNA

which can form a RNA-RNA duplex with the transposase mRNA impeding the migration of the ribosomes in the central part of the transposase gene (Arini *et al.*, 1997).

One of the mechanisms involved in transposase regulation is programmed translational frameshifting. Several IS elements encode for two consecutive overlapping ORFs placed in different reading phases. Slippage of the elongating ribosome between the ORFs can lead to synthesis of an intact transposase (Chandler and Fayet, 1993). Typically a —1 frameshift occurs at the so-called "slippery" codons. Slippage of the ribosome can be stimulated by stem-loop structures located downstream of the "slippery" codons. The frequency of frameshifting is low — only about 1% of elongating ribosomes may slip and synthesise the fusion protein (Escoubas *et al.*, 1991). Elements known to use programmed translational frameshifting in transposase synthesis are, for example, the members of IS*1* and IS*3* families (Sekine and Ohtsubo, 1989; Polard *et al.*, 1991; Sekine *et al.*, 1994; Hu *et al.*, 1996).

Several transposons have mechanisms for the protection of transposase expression from the external promoters that may occur if the element is inserted into an actively transcribed gene. For example, transcripts that read through the end of Tn*10* or IS*50* do not express the transposase because of an mRNA secondary structure that sequesters the translation initiation signals (Davis *et al.*, 1985; Schulz and Reznikoff, 1991).

1.4.2.3. Inhibition of transposase activity

For the effective transposition reaction, formation of a stable and co-ordinated synaptic complex between transposase molecules bound to each transposon end is a prerequisite. Therefore, proteins which interact with transposase or which can compete with transposase for the DNA-binding sites can affect the transposase activity. For example, IS*50* encodes for an inhibitor (Inh) that is translated in the same reading frame as the transposase (Tnp) but lacks the N-terminal 55 amino acids, required for sequence-specific binding with the transposon ends (Isberg *et al.*, 1982; Johnson *et al.*, 1982; de la Cruz *et al.*, 1993). It has been shown that Inh protein inhibits transposition due to forming transpositionally inactive heterodimers with transposase (Braam *et al.*, 1999). Interestingly, these Tnp-Inh heterodimers present even better binding activity to DNA as compared to the transposase homodimers (de la Cruz *et al.*, 1993). Thus, Tnp-Inh multimers seem to act by dual mechanism: they titrate out the active transposase pool and by binding to the transposon ends they block the transposase binding sites for the active form of transposase (de la Cruz *et al.*, 1993).

The negative regulator of IS*1* is also translated from the same reading frame as the transposase but differently to the Inh protein of IS*50*, it contains the DNA-binding domain and lacks the catalytic domain (Machida and Machida, 1989). Specific binding of IS*1* inhibitor to the transposon ends inhibits transpo-

sition probably by competition with the transposase for their cognate site within the ends of IS*1* (Zerbib *et al.*, 1990). Similar inhibition of transposase action is proposed also for IS*3* (Sekine *et al.*, 1997).

1.4.2.4. Transposase *cis*-activity

Many transposases are characterised by preferential *cis*-action, i.e., they act effectively only at their site of synthesis (Kleckner, 1990). Transposase *cis*-activity can exceed its *trans*-activity of several orders of magnitude and this phenomenon has been observed for a variety of transposable elements including IS*1* (Machida *et al.*, 1982), IS*10* (Morisato *et al.*, 1983), IS*50* (Isberg *et al.*, 1982; Johnson *et al.*, 1982) and IS*903* (Derbyshire *et al.*, 1990). The preferential *cis* action probably reduces the rate of accumulation of transposon copies in the cell because the particular transposon can use only the transposase synthesised by itself. Thus, increased copy number of the element can result in only a linear increase in transposition frequency not in an exponential increase.

Some transposases have been observed to be unstable proteins, which obviously partially explains the preferential *cis*-action of these transposases. For example, transposase of IS*903* is sensitive to the *E. coli* Lon protease and it can function up to 1000-fold more efficiently if its gene is located close to its binding site (Derbyshire *et al.*, 1990). In addition, poor expression of transposase protein due to inefficient translation initiation is supposed to complement the *cis* preference of the IS*903* transposase (Derbyshire and Grindley, 1996). The inefficient translation together with low half-life of the transposase message seems to be the main reason for preferential *cis* action also for IS*10* transposase (Jain and Kleckner, 1993b).

The degree of *cis* action of a transposase may also be influenced by its oligomerization state. Oligomerization of transposase monomers bound to transposon ends is important to form the transpositional synaptic complex. However, premature oligomerization of transposase before binding to transposon DNA might lead to inactivation of the protein. It has been hypothesised that inhibition of transposase due to premature oligomerization could be reason for *cis*-preference of some transposases. For example, oligomerization seems to regulate transposase *cis*-activity of IS*5* (Wiegand and Reznikoff, 1992). As mentioned above, Tn*5* encodes for inhibitor protein Inh that inactivates the transposase Tnp through the oligomerization with it. However, Tnp itself can inhibit Tn*5* transposition as well, when encoded *in trans* (DeLong and Syvanen, 1991; Wiegand and Reznikoff, 1992). That has been suggested to occur by the formation of inactive Tnp multimers and it was hypothesised that premature dimerization may mask the DNA-binding domain of Tnp (Weinreich *et al.*, 1994).

Most transposases have the sequence-specific DNA binding domains in the N-terminal region of the protein. This arrangement may permit the binding

of a nascent transposase tethered to the translation machinery to the transposon end. The idea is supported by the observations that the presence of the C-terminal region of both the IS*50* and IS*10* transposases appears to mask the DNA binding domain and reduce binding activity (Jain and Kleckner, 1993a; Weinreich *et al.*, 1994). The fact that the incomplete transposase molecule may have a higher affinity to the transposon ends than the complete molecule, leads to *cis* but not *trans* activity.

1.4.3. Target site selection

Transposons can insert at various sites of the host genome. Target-choice specificity varies largely for different transposons: some elements exhibit considerable target site selectivity while others seem to insert into quite random targets. Nevertheless, transposition never occurs absolutely randomly and some degree of target preference has been observed in every case studied so far. Mostly, the direct interaction of transposase with target DNA determines the target site selection. For instance, the selection of an IS*10* target site is mediated by direct interaction of transposase with the target DNA (Bender and Kleckner, 1992a). Yet, target site can be selected through transposase interaction with accessory proteins, as is the case of Tn*7* (Craig, 1997).

Some transposons insert preferentially into a specific sequence. For example, IS*91* has been shown to insert specifically 5' to either one of the tetranucleotides 5'-GAAC or 5'-CAAG, and always in the same relative orientation in respect to the sequence of the target (Mendiola and de la Cruz, 1989). A lot of other transposons show preference for some more or less strict sequences as well: IS*10* often inserts into the symmetric NGCTNAGCN heptanucleotide (Halling and Kleckner, 1982), bacteriophage Mu prefers pentanucleotide C-Py-G/C-Pu-G (Haapa-Paananen *et al.*, 2001), IS*231*A chooses mostly the sequence $GGG(N)_5CCC$ (Hallet *et al.*, 1994), transposable elements Tc1 and Tc3 of the nematode *C. elegans* insert into the dinucleotide TA (Plasterk, 1996). However, the abovementioned sequences are not sufficient to confer target specificity as the base pairs flanking the target sequence also contribute significantly to target-site selection (Bender and Kleckner, 1992b; Haapa-Paananen *et al.*, 2001).

Tn*7* is unique among transposons by its ability to transpose at high frequency into one major target site in the *E. coli* chromosome termed *att*Tn*7* (Craig, 1991). Four proteins encoded by Tn*7* — TnsA, TnsB, TnsC and TnsD — are required for Tn*7* insertion into *att*Tn*7*. TnsD binds specifically to *att*Tn*7* and directs the other Tns proteins together with the ends of the transposon to this site (Bainton *et al.*, 1993). It has been proposed that distortion of target DNA caused by TnsD serves as a signal to recruit the transposition complex (Kuduvalli *et al.*, 2001). Tn*7* can transpose also into other, non-*att*Tn*7* sites. However, it occurs at much lower frequency and TnsE, the fifth Tn*7* encoded protein is needed to choose the suitable non-*att*Tn*7* site (Waddell and Craig,

1988). Actually, the initiation of Tn*7* transposition is controlled by target selection, while no DNA breakage reactions happen before the assembly of transposition complex with target DNA (Bainton *et al.*, 1991; Bainton *et al.*, 1993). This contrasts, for example, with IS*10* which transposition reactions (breaks at the transposon ends) can be initiated in the absence of an appropriate target (Sakai and Kleckner, 1997).

Some transposons select the target probably by the DNA structure. For example, target choice can be influenced by DNA bending (Hallet *et al.*, 1994), the degree of DNA supercoiling (Lodge and Berg, 1990), the level of transcription of potential target (Bernardi and Bernardi, 1988; Casadesus and Roth, 1989; DeBoy and Craig, 2000) and replication (Bernardi and Bernardi, 1987; Wolkow *et al.*, 1996). Mostly, transcription of a target DNA has been observed to reduce the frequency of insertion of transposons (Casadesus and Roth, 1989; Wang and Higgins, 1994; DeBoy and Craig, 2000). It is supposed that such a strategy may serve to direct transposition away from the most essential genes, i.e. those being actively transcribed (Craig, 1997).

Transposition regulation of several bacterial mobile elements often facilitates horizontal transmission of the transposon (Craig, 1996). One strategy for horizontal transfer could be the insertion of the transposon into plasmids, which readily move into other cells. Indeed, some transposable elements prefer plasmids as insertion targets. For example, transposon Tn*3* preferentially transposes into plasmids than into the chromosome (Kretschmer and Cohen, 1977). Tn*7* possesses the similar preference of target choice: the preferred non-*att*Tn*7* targets for Tn*7* are conjugating plasmids (Wolkow *et al.*, 1996).

1.4.4. Transposon copy number control and transposition immunity

Any type of transposition can lead to an increase in the number of transposon copies within a cell. Therefore, the total frequency of transposition will increase with increasing transposon copy number if each of these copies acts independently. To avoid the exponential increase in transposon copy number, many elements have regulatory mechanisms that sense the copy number of the element and reduce the frequency of transposition per copy as the number of transposon copies per cell increases. Experiments with differentially marked Tn*5*s showed that the frequency of transposition of an individual Tn*5* decreased proportionally with the total number of copies of the element present in a cell (Johnson and Reznikoff, 1984a).

One mechanism that limits the rate of accumulation of transposon copies is that many transposases are preferentially *cis* acting, i.e. they are not freely diffusible to other transposon copies within the cell (see section 1.4.2.4.). Another important feature is the combination of a *cis*-acting transposase and a *trans*-acting negative regulator (reviewed in Kleckner, 1990). For example, IS*10* encodes a *trans*-acting negative regulator (antisense RNA) which effectiveness

increases with increasing concentration, i.e. with increasing transposon copy number (Simons and Kleckner, 1983). As a consequence, the transposition frequency per transposon copy decreases. Similarly to IS*10*, the negative regulators of IS*50* and IS*1* can also effectively function in *trans* and inhibit transposition (Yin and Reznikoff, 1988; Machida and Machida, 1989; Zerbib *et al.*, 1990).

Several transposable elements including members of the Tn*3* family (Lee *et al.*, 1983; Wiater and Grindley, 1990a; Wiater and Grindley, 1990b), bacteriophage Mu (Adzuma and Mizuuchi, 1988; Darzins *et al.*, 1988), and Tn*7* (Hauer and Shapiro, 1984; Arciszewska *et al.*, 1989) exhibit an interesting phenomenon known as transposition immunity, sometimes called target immunity. These elements transpose much less frequently into a plasmid replicon that already contains a copy of the transposon than into a replicon lacking the transposon. This kind of transposition inhibition is not global but it is *cis* specific since only the target already containing a copy of the transposon becomes "immune" to further transposition of the same transposon. Experiments with Tn*7* have established that target immunity can act over distances of at least 190 kb in the chromosome of *E. coli* (DeBoy and Craig, 1996). However, transposition of Tn*7* into a more distant site 1.9 Mb away in the same DNA is not inhibited (DeBoy and Craig, 1996).

The signal that confers immunity to a target DNA is provided by the ends of the transposon and by the transposase bound to the ends (Adzuma and Mizuuchi, 1988; Maekawa *et al.*, 1996). In the case of Mu and Tn*7* also transposon-encoded accessory proteins MuB and TnsC, respectively, are involved. MuB and TnsC select transposition target for relevant transposon. However, these proteins are actively removed from potential target DNAs containing Mu or Tn*7* ends and this is promoted by transposase bound to the transposon end DNA (Adzuma and Mizuuchi, 1988; Adzuma and Mizuuchi, 1989; Stellwagen and Craig, 1997).

The transposition immunity is important in limiting the copy number of transposon within cells. However, the immunity may likely also serve as a barrier to self-insertion. This might be especially important in the case of quite large transposable elements such as Mu (35 kb) (Adzuma and Mizuuchi, 1988; Darzins *et al.*, 1988) and Tn*7* (14 kb) (Stellwagen and Craig, 1997).

1.4.5. Host factors in transposition

As already mentioned above, transposition activity of mobile elements is frequently modulated by various host factors. The involvement of host factors in transposition indicates that these proteins may be used for communication between the transposon and its host bacterium. Differential regulation of host factors in response to changing physiological and/or environmental conditions may cause substantial alterations in the frequency of transposition. Host proteins

may participate in regulation of transposase expression or directly in the transposition reaction. Naturally, the DNA replication and repair, required to complete the transposition and performed by the host machinery, are subjected to host control (Craig, 1996).

Many transposons employ different histone-like proteins, such as HU, IHF, H-NS and Fis. These proteins are small and able to bend or wrap the DNA. Albeit relatively abundant in bacterial cell, the concentration of these proteins depends on the growth phase and the physiological conditions of the bacteria. For example, Fis levels vary dramatically during the course of cell growth and in response to changing environmental conditions. The intracellular level of Fis protein in exponential growth phase cells of *E. coli* was found to be more than 500-fold higher than in stationary phase cells (Ball *et al.*, 1992; Ali Azam *et al.*, 1999). The abundance of IHF, on the contrary, was shown to increase up to seven-fold during the transition of cells from exponential growth to the 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 changes in the amount of host factors involved in regulation of mobile element may affect frequency of transposition.

HU, IHF, H-NS, and Fis are all involved in the regulation of the bacteriophage Mu, either by controlling Mu transposase expression or participating directly in the transposition reaction (Surette *et al.*, 1989; Allison and Chaconas, 1992; Gama *et al.*, 1992; van Drunen *et al.*, 1993; van Ulsen *et al.*, 1996). Both HU and IHF stimulate Mu transposition (Craigie *et al.*, 1985; Surette *et al.*, 1989), H-NS and Fis influence negatively Mu activity (Falconi *et al.*, 1991; Betermier *et al.*, 1993; Gomez-Gomez *et al.*, 1997). IHF plays a dual role in the transposition of phage Mu. First, it activates the expression of transposase gene from the P_e promoter of Mu, indirectly via alleviating the H-NS-mediated repression and directly by activating P_e transcription (van Ulsen *et al.*, 1996). Second, IHF binding to the Mu P_e promoter region (that is part of a larger enhancer-like element) can also facilitate the formation of MuA transposase complexes at the ends of the element (Surette *et al.*, 1989; Allison and Chaconas, 1992).

IHF is involved in transposition of other transposable elements as well. Several mobile DNA elements carry IHF-binding sites at one or both termini (Makris *et al.*, 1990; Gamas *et al.*, 1987; Wiater and Grindley, 1988; Huisman *et al.*, 1989). For transposon γδ (Tn*1000*), it has been shown that IHF binds cooperatively with the transposase to the ends of γδ and stimulates transpositional immunity of the element (Wiater and Grindley, 1988; Wiater and Grindley, 1990a). However, while the wild-type γδ transposon transposed equally well with or without the IHF binding sites (Wiater and Grindley, 1990a; May and Grindley, 1995), the effect of IHF in transposition of Tn*1000* seems to be only modulatory. Similar results were obtained with IS*1*: although IHF was shown to bind to both IS*1* ends (Gamas *et al.*, 1987) no clear effect of IHF on transposase

expression or transposition of IS*1* has been found. Instead, transposition of IS*1* requires another histone-like host factor — H-NS (Shiga *et al.*, 2001).

Mostly, IHF affects positively upon the transposition (Craigie *et al.*, 1985; Morisato and Kleckner, 1987; Surette *et al.*, 1989), although reports can be found about the negative role of IHF (Signon and Kleckner, 1995; Gama *et al.*, 1992). For example, upon the transposition and transposase expression of the composite transposon Tn*10*, IHF acts either positively or negatively depending on where the transposon is located — in the chromosome or in the multicopy plasmid, respectively (Signon and Kleckner, 1995). Generally, IHF is supposed to play an architectural role in transposition since IHF binding with its cognate site induces sharp DNA bending that can facilitate the assembly of protein-DNA complexes (Surette *et al.*, 1989; Allison and Chaconas, 1992; Chalmers *et al.*, 1998).

Transposition of IS*10*, IS*50* and IS*903* is regulated by *E. coli* Dam methylase. Transposition of these elements is favoured just after replication when the DNA is hemimethylated (Roberts *et al.*, 1985; Yin *et al.*, 1988). Transposition regulation by DNA adenine methylation should be specifically advantageous to the elements that transpose by cut-and-paste mechanism while they leave behind a gap in the chromosome. Transposition just after replication could ensure that a second copy of the donor chromosome is intact and the broken copy of the chromosome will be repaired (Kleckner, 1990). Another bacterial replication protein, which has been implicated in Tn*5* transposition, is DnaA (Reznikoff, 1993). However, the exact molecular mechanism of DnaA action in Tn*5* transposition is unknown.

Most transposons require supercoiled DNA substrates for an efficient transposition reaction (Mizuuchi, 1992). DNA-binding histone-like proteins, discussed above, can regulate transposition by modulating the supercoiling status of DNA (Chalmers *et al.*, 1998). Additionally, gyrase and topoisomerase I, known to influence DNA supercoiling, have been shown to be important in the transposition of some transposons. For instance, transposition of Tn*5*, phage Mu and probably Tn*3* require DNA gyrase activity (Isberg and Syvanen, 1982; Pato and Banerjee, 1996; Maekawa *et al.*, 1996). Topoisomerase I is involved in the positive regulation of Tn*5* transposition (Sternglanz *et al.*, 1981; Yigit and Reznikoff, 1998). Interestingly, topoisomerase I seems to interact directly with Tn*5* transposase and it is supposed that this interaction could stimulate insertion of Tn*5* into supercoiled DNA (Yigit and Reznikoff, 1999).

1.4.6. Transposition and stress

Mostly the transpositional activity of mobile elements is greatly suppressed, yet there are several examples of transposons that are activated under the conditions in which fast genetic changes are needed, i.e. under different stresses (Kidwell and Lisch, 1997; Skaliter *et al.*, 1992; Lamrani *et al.*, 1999).

Different mutator mechanisms can be induced by stress. In bacteria, for example, exist a regulatory network, called SOS system, which is induced in response to DNA damage and which can generate genetic alterations in response to environmental stress (e.g. radiation, chemicals, starvation). Interestingly, there seems to be direct connection between transposition of some elements and SOS response. Transposition of IS*10* is induced by DNA-damaging UV light and this is dependent on the functions of the SOS system (Eichenbaum and Livneh, 1998). Transposition of Tn*5* also seems to respond to the induction of the SOS system. However, the results obtained by different groups are controversial in respect whether induction of the SOS response enhances or inhibits Tn*5* transposition (Kuan *et al.*, 1991; Kuan and Tessman, 1991; 1992; Weinreich *et al.*, 1991).

On the other hand, transposition can course induction of SOS response. For example, transposition of Tn*10* (Roberts and Kleckner, 1988) and IS*1* (Lane *et al.*, 1994) causes induction of the SOS response. Data indicate that the signal for SOS induction is generated by transposase-induced cleavages and degradation of the transposon donor DNA molecule (Roberts and Kleckner, 1988; Lane *et al.*, 1994). Therefore, it is proposed that induction of SOS system is biologically important in helping a cell undergoing transposition to repair the transposon donor chromosome (Roberts and Kleckner, 1988).

Changing nutritional conditions can also influence the frequency of transposition. From the studies of adaptive mutations interesting implications can be drawn about transposition in starving bacteria (Shapiro and Higgins, 1989; Kasak *et al.*, 1997; Hall, 1999). For instance, carbon starvation conditions trigger induction of mutant Mu prophage (Shapiro, 1984; Mittler and Lenski, 1990; Lamrani *et al.*, 1999). Activation of Mu transposition has been shown to be dependent upon the host ClpXP and Lon proteases and the RpoS stationary phase-specific sigma factor (Gomez-Gomez *et al.*, 1997; Lamrani *et al.*, 1999). Analyses of the spectra of spontaneous growth-dependent and starvation-induced mutations in front of promoterless *pheBA* (codes for first two genes of phenol degradation pathway) operon and at *ebgR* gene (repressor of lactulose utilisation) have revealed that transposition of *Pseudomonas putida* transposon Tn*4652* and *Escherichia coli* IS*30* could be induced by starvation conditions as well (Kasak *et al.*, 1997; Hall, 1999). Naas and co-workers have studied insertion sequence-related genetic rearrangements in resting *E. coli* and they have shown high activity of IS*5* and IS*30* in agar stabs (Naas *et al.*, 1995).

Transposition of many transposons is temperature sensitive. The temperature optimum for translocation of *E. coli* transposon Tn*3* was shown to be in the range from 26 to 30°C. At temperatures above 30°C, the translocation frequency decreased rapidly and at 36°C it was only 5% of the frequency observed at 30°C (Kretschmer and Cohen, 1979). Similarly, other transposons in the Tn*3* family exhibit temperature-sensitivity of transposition (Turner *et al.*, 1990). The transposition burst of ISH27 in *Halobacterium halobium* was seen after storage of the cells at 4°C for more than two years. Upon continuous cultivation at 37°C no

transposition event could be observed, suggesting that stress factors have caused the high transposition rate (Pfeifer and Blaseio, 1990). An interesting case is IS*1086* that was isolated from *Alcaligenes eutrophus* CH34 (Dong *et al.*, 1992). *A. eutrophus* CH34, which optimal growth temperature is around 30°C, shows high degree of mortality by growth at 37°C and a high proportion of mutants has been detected among the survivors. Analysis of mutants suggested that IS*1086* transposition (as well as other mutagenic events) is activated at 37°C expressing 1000-fold higher activity than at 30°C (Dong *et al.*, 1992). Thus, transposition of IS*1086* seems to help the host to rescue from the unfavourable situation.

Altogether, different stress situations can activate the movement of transposable elements. Although most transposition-induced mutations seem to have a negative effect on host fitness, a growing body of evidence indicates that many transposon-mediated genetic changes may be beneficial for the host (Chao and McBroom, 1985; Kidwell and Lisch, 1997). Moreover, it is hypothesised that activation of transposition under stress conditions might serve as an adaptive response to overcome stress and evolve new traits (Morillon *et al.*, 2000; Capy *et al.*, 2000; Wessler, 1996). However, the exact molecular mechanisms that underlie the stress-induced transposition remain undefined.

1.5. Transposon Tn*4652* of *Pseudomonas putida*

Pseudomonas putida transposon Tn*4652* is a 17-kb-long deletion derivative of the toluene degradation *xyl* genes-carrying transposon Tn*4651* that is a part of TOL plasmid pWW0 (Tsuda and Iino, 1987). Tn*4651* harbours *xyl* genes within a 39-kb segment bounded by direct repeats of 1.4 kb in length (Fig. 6). Reciprocal recombination between these direct repeats results in the deletion of *xyl* genes and formation of Tn*4652* (Meulien *et al.*, 1981). Tn*4652* resides in the chromosome of plasmid-free strain *Pseudomonas putida* PaW85 (Meulien and Broda, 1982).

Tsuda and Iino (Tsuda and Iino, 1987) have demonstrated that, according to its transposition properties, Tn*4652* belongs to the Tn*3* family of transposons. The ampicillin resistance transposon Tn*3* was the first described transposable element encoding for antibiotic resistance (Hedges and Jacob, 1974). By now many transposons belonging to the Tn*3* family have been described. Similarly to the other members of the Tn*3* family, transposition of Tn*4652* generates 5-bp direct duplications of target DNA. Tn*4652* uses replicative transposition mechanism that involves a cointegrate as transposition intermediate molecule. For the formation of cointegrate, transposon-encoded transposase and both transposon termini are needed. Differently from the other Tn*3* family transposons, resolution of the Tn*4652*-mediated cointegrate requires intactness of two transposon-encoded genes — *tnpS* and *tnpT*. In contrast to the localisation of

resolvase gene and *res* region in close proximity to transposase gene in the Tn*3* family (Sherratt, 1989), the transposase gene and DNA region encoding for resolution functions are separated by a 9 kb DNA segment in Tn*4652* (Tsuda and Iino, 1987).

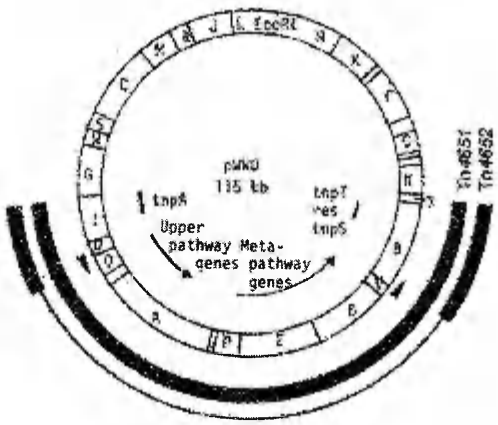

Figure 6. Map of the TOL plasmid pWW0. Arrows indicate location of the two operons of the toluene degradation *xyl* genes inside the Tn*4651* (pictured by thick line). Recombination between direct repeats (shown by triangles) results in the deletion of *xyl* genes and formation of Tn*4652* (from Tsuda and Iino, 1987).

Currently, full sequence of Tn*4652* is available (Tan et al., GeneBank Accession Number AF151431). Interestingly, analysis of this sequence could not reveal other ORFs, than ORFs for transposase and resolvase genes, revealing similarity with respective putative genes in databases. So, further experiments are needed to explore the coding capacity of the 17-kb-long sequence of Tn*4652*.

An interesting characteristic of Tn*4652* is its ability to activate silent genes by creating fusion promoters at the insertion site (Nurk *et al.*, 1993). Both terminal inverted repeats of Tn*4652* contain –35 hexamer-resembling sequences of σ^{70}-specific promoters. Therefore, insertion of Tn*4652* into the sequence similar to the –10 region of the σ^{70}-specific promoters can generate the fusion promoter. This phenomenon was discovered by studying mutational processes in starving *P. putida* PaW85. Transposition of Tn*4652* from the chromosome of *P. putida* PaW85 in front of plasmid-encoded promoterless phenol monooxygenase gene *pheA* created fusion promoters for transcription of the *pheA* gene, thereby permitting the bacteria to utilise phenol. Interestingly, transposition of Tn*4652* seems to depend on the physiological state of bacteria: transposition frequency of Tn*4652* increases during the starvation, whereas no transposition event of Tn*4652* could be detected in growing cells of *P. putida* (Kasak *et al.*, 1997). This fact indicates that starvation might increase transposition activity of Tn*4652*.

2. RESULTS AND DISCUSSION

It has been shown that different stress situations can activate the movement of several transposable elements (see section 1.4.6.). However, it is not easy to distinguish whether this activation occurs due to malfunction of host defence mechanisms under stress or this is an induced process to promote mutations that may potentially contribute to survival in unfavourable conditions. Previous results suggest that transpositional activity of Tn*4652* may increase under carbon starvation conditions (Kasak *et al.*, 1997). Therefore, the studies on regulation of Tn*4652* may enlighten the mechanisms of stress-induced transposition.

Rate of transposition is largely determined by the amount of active transposase (Kleckner, 1990). Therefore, to elucidate the regulation of transposition of Tn*4652*, studies of expression of Tn*4652*-encoded transposase were carried out. It turned out that at least two host proteins and one transposon-encoded protein are involved in transposase regulation.

2.1. Host factors involved in regulation of transposition of Tn*4652*

2.1.1. Integration host factor IHF

2.1.1.1. Transcription from the transposase *tnpA* promoter of Tn*4652* is enhanced by IHF (Reference I)

Genetic analysis on Tn*4652* has localised the putative transposase gene *tnpA* into the right end of the element (Tsuda and Iino, 1987). This region has been sequenced and analysed (Reference I). The transposase of Tn*4652* revealed a high degree of homology with the putative transposase of the mercury resistance transposon Tn*5041* (96.2% identity). Homology with other Tn*3* family transposases was only moderate — about 20–24% of identity, suggesting that Tn*4652* and Tn*5041* are distantly related members of the Tn*3* family of transposons (Fig. 3, reference I).

tnpA gene starts at 152 bp from the right end of the transposon Tn*4652*. In order to map the *tnpA* promoter and to study its regulation, two promoter probe vectors were used. Different DNA fragments of the transposon right end region were cloned upstream of the reporter genes *pheB* (encodes for catechol 1,2-dioxygenase) and *lacZ* (encodes for β-galactosidase) (Table 1 and Fig. 2; reference I). Enzyme assays (Fig. 4; reference I) and mRNA mapping (Fig. 5; reference I) revealed that the *tnpA* gene promoter is located in the 65-bp DNA segment (positions 58–122 from the transposon right end). However, the pres-

ence of the sequence of the whole right end of the transposon (including the terminal nucleotides 1–58) in the reporter plasmid enhanced transcription from the promoter approximately 4-fold (Fig. 4; reference I). Sequence analysis of the transposon right end revealed a potential binding site for integration host factor (IHF) just next to the inverted repeat sequence at the positions 44–56 bp (Fig. 2; reference I). Therefore, I controlled whether the presence of IHF-binding site upstream of the *tnpA* promoter can enhance the promoter activity. The positive role of IHF was confirmed by the finding that the enhancing effect was not detected in *P. putida ihfA*-deficient strain. On the contrary, the Tn*4652* terminal sequences affected negatively the promoter activity in the *ihfA*-defective strain (Fig. 4C; reference I). This finding indicates that the IHF site, if not occupied by IHF protein, can suppress the *tnpA* promoter activity. Actually, a study of the regulation of Tn*4652*-generated fusion promoters indicates that besides of IHF some other, so far unidentified protein (factor X) can bind to the transposon right end and may compete with IHF for the binding site (Teras *et al.*, 2000). Gel shift experiments with transposon right end DNA and cell lysates from bacteria sampled at different growth phases, suggest antagonistic nature of binding of IHF and factor X. While maximum binding of IHF was detected with cell lysates from stationary phase bacteria then binding of factor X was detected only by using lysates of exponentially grown cells (Teras *et al.*, 2000).

It is known that IHF is involved in the activation of the P_e promoter of bacteriophage Mu by a dual mechanism. IHF stimulates transcription from the P_e promoter directly and also indirectly via alleviation of the H-NS-mediated repression (van Ulsen *et al.*, 1996). I suggest analogous regulation of *tnpA* promoter of Tn*4652*: binding of IHF to the right end of Tn*4652* enhances transcription from the *tnpA* promoter not only directly but also indirectly by competing for the binding site with factor X.

2.1.1.2. Transposition of Tn*4652* depends on the level of expression of IHF (References I and IV)

IHF affects transposition mostly positively. In the Mu phage transposition, IHF acts positively both by enhancing transcription from the transposase promoter and favouring the transposase to form the stabile synaptic complex with Mu ends (Allison and Chaconas, 1992; van Ulsen *et al.*, 1996). However, there are also studies about the negative role of IHF on transposition (Gama *et al.*, 1992). An interesting case is Tn*10*, transposition of which is inhibited by IHF when the element resides on a multicopy plasmid. On the other hand, Tn*10*-promoted chromosome rearrangements are enchanced by IHF (Signon and Kleckner, 1995).

Several mobile elements are known to contain IHF binding sites at their one or both ends (Gamas *et al.*, 1987; Huisman *et al.*, 1989; Makris *et al.*, 1990; Wiater and Grindley, 1988). Therefore, after finding of IHF binding site at the

right end of Tn*4652*, the left end of transposon was studied for potential IHF binding site as well. Sequence analysis of the left terminus of Tn*4652* revealed two potential IHF-binding sites at positions 44 to 56 bp and 59 to 71 bp from the transposon left end (Fig. 2B, reference I). The possible binding of IHF to both ends of Tn*4652* was tested by gel mobility shift assay. The experiments demonstrated that both Tn*4652* ends really bind IHF (Fig. 6, reference I and Teras *et al.*, 2000). IHF-binding sites at the both ends of Tn*4652* locate just nearby the terminal inverted repeat sequences that are presumable binding-sites for the transposase. This indicates that besides activation of the *tnpA* promoter, IHF may participate in Tn*4652* transposition directly either by modulating the binding of transposase to the ends of the transposon or by influencing formation of nucleoprotein complexes needed in subsequent transposition reactions.

To elucidate the role of IHF in the regulation of transposition of Tn*4652*, the movement of Tn*4652* was examined in the wild-type and IHF-defective strains of *P. putida* KT2442 which contain a copy of Tn*4652* in their chromosome. Transposition of Tn*4652* was monitored in the test system previously used in our laboratory for the study of mutational processes in starving *P. putida* (Kasak *et al.*, 1997). In this starvation-experiment the phenol-utilising mutants were selected on phenol minimal plates. Phe^+ mutants raised due to DNA rearrangements activating transcription of the plasmid-encoded initially promoterless phenol degradation genes *pheBA* in plasmid pEST1414. In the wild-type *P. putida*, about one third of these Phe^+ mutants arose due to the insertion of Tn*4652* from the chromosome in front of the *pheA* gene by creating fusion promoters for the transcription of this gene (Kasak *et al.*, 1997; Table 2, reference IV). The insertions of Tn*4652* into the plasmid upstream of the *pheA* gene were proved by PCR-analysis. Measurement of transposition of Tn*4652* in IHF-defective *P. putida* revealed that IHF is essential for the transposition of this DNA element since no transposition activity of Tn*4652* could be detected in IHF-defective *P. putida* (Table 2, reference IV).

In order to find out whether complementation of *P. putida* IHF-defective strain with functional *ihfA* and *ihfB* genes could restore the transposition of Tn*4652*, the transposition assay was carried out in *P. putida* IHF-defective derivative strain RT31 (Teras *et al.*, 2000). This strain contains *P. putida ihfA* and *ihfB* genes under the control of *Ptac* promoter and $lacI^q$ repressor in its chromosome, enabling to change artificially the level of IHF expression. The similar starvation assay as described above, was used for the monitoring of the transposition of Tn*4652*. To provide different expression levels of IHF, the phenol minimal plates were supplied either with 0.5 mM or 0.01 mM IPTG or alternatively, no IPTG was added. Results obtained clearly demonstrate that complementation of IHF-defective strain with functional *ihfA* and *ihfB* genes can restore mobility of Tn*4652* (Fig. 1, reference IV). Interestingly, up to 10 times more Phe^+ mutants accumulated on phenol minimal plates in the presence of 0.5 mM IPTG if compared to the amount of the mutants on the other plates (Fig. 1A, reference IV). Analysis of the Phe^+ mutants raised on different plates

revealed that the frequency of transposition of Tn*4652* depended on the expression level of IHF — overexpression of *ihfAB* genes increased transposition of Tn*4652* by about one order of magnitude (Fig. 1B, reference IV). These results not only indicate that IHF is involved in the transposition of Tn*4652* but they also demonstrate that the level of IHF expression is one of the factors regulating the frequency of transposition of Tn*4652*. Abundance of IHF of *E. coli*, *P. aeruginosa* and *P. putida* is shown to be increased up to seven-fold during the transition of cells from exponential growth to the stationary phase (Ditto *et al.*, 1994; Delic-Attree *et al.*, 1996; Murtin *et al.*, 1998; Teras *et al.*, 2000; Valls *et al.*, 2002). Therefore, it is tempting to speculate that the increased concentration of IHF in stationary phase bacteria is one of the factors inducing the mobility of Tn*4652*.

Starvation assay used to monitor the frequency of transposition of Tn*4652* revealed that transposition of Tn*4652* was reduced more than 40 fold in IHF-defective strain. A study of transposase promoter activation has shown that IHF enhances the expression of the *tnpA* gene approximately 4-fold (Fig. 4, reference I). Accounting the presence of IHF binding site at both ends of transposon and more than 40-fold decrease in the frequency of transposition of Tn*4652* in the IHF-defective strain, I propose that besides of regulating the *tnpA* promoter, IHF may participate in transposition reaction of Tn*4652* also directly.

2.1.2. Stationary phase-specific sigma factor σ^S

2.1.2.1. Transposition of Tn*4652* is decreased in *P. putida* σ^S-deficient strain (Reference III)

As mentioned above, the transposition of Tn*4652* seems to be activated in bacteria starving on phenol minimal plates (Kasak *et al.*, 1997). It is known that by the adaptation of bacteria to limited nutrient availability, changes in gene regulation take place, i.e., several genes are shut down while others are induced. One of these upregulated genes, *rpoS*, codes for an alternative sigma factor, σ^S, which controls expression of multiple stationary-phase genes (Loewen and Hengge-Aronis, 1994; Hengge-Aronis, 1999). I was interested whether the RpoS could be involved in the regulation of Tn*4652*. Therefore, the transposition of Tn*4652* was studied in the wild-type *P. putida* PaW85 and in an isogenic σ^S-defective strain PKS54.

Transposition of native Tn*4652* was examined in a starvation assay as described above, except that target plasmid pEST1332 was used instead of pEST1414. Similar to pEST1414, plasmid pEST1332 (Kivisaar *et al.*, 1990) contains the promoterless *pheBA* operon. However, it is more suitable for probing the transposition of Tn*4652*, since in the experiment with pEST1332 most of the Phe^+ clones arising on phenol minimal plates emerge from the insertion of Tn*4652* (Nurk *et al.*, 1993). Comparable measurement of transposi-

tion of Tn*4652* in the wild-type and σ^S-defective strain revealed essential decrease, by more than one order of magnitude, in the transposition in the RpoS-minus strain (Fig. IB, reference III).

RpoS is known to contribute to the maintenance of bacterial cell viability during the stationary phase of growth and during nutrient starvation (Loewen and Hengge-Aronis, 1994; Ramos-Gonzalez and Molin, 1998). Survival of *rpoS*-defective *P. putida* strains KT2440 and PaW85 in liquid minimal media has been demonstrated to decrease by two orders of magnitude during one week (Ramos-Gonzalez and Molin, 1998; A. Tover, unpublished results). Therefore, I controlled whether the decrease in transposition is apparent and caused by reduced viability of σ^S-defective strain. For that, the viability of *P. putida* PaW85 and isogenic σ^S-defective strain, starving on phenol minimal plates, was estimated. Indeed, results obtained show that viability of the σ^S-defective strain decreases slowly during 14 days of starvation on phenol plates. By the end of the second week, the number of viable cells of σ^S-defective strain had decreased by 2 orders of magnitude. However, during the first 5 days of starvation (the period when transposition frequency was monitored), survival of σ^S-defective strain dropped only twofold. This probably cannot explain the more then tenfold lower accumulation of Phe^+ mutants in σ^S-defective strain. Therefore, I conclude that σ^S acts as a positive regulator in transposition of Tn*4652*.

2.1.2.2. Transcription from the transposase promoter of Tn*4652* is σ^S-dependent (Reference III)

How can RpoS control transposition of Tn*4652*? Transposition is mostly regulated by the amount and activity of transposase (reviewed in Kleckner, 1990). Therefore, the possibility, that the amount of transposase of Tn*4652* (TnpA) could be under the control of RpoS, was tested. Western blot analysis with an anti-TnpA polyclonal antiserum demonstrated that the abundance of TnpA was greatly decreased in *P. putida* σ^S-defective strain. While TnpA (overexpressed by cloning the *tnpA* gene into a plasmid) was easily detected in the cell lysates of wild-type bacteria, no TnpA protein could be detected in the isogenic σ^S-defective strain (Fig. 2, reference III).

In order to test whether the promoter of *tnpA* could be controlled by σ^S, the transcriptional activity of *tnpA* promoter was examined in PaW85 and σ^S-defective strain PKS54. The *tnpA* promoter is positively affected by IHF (section 2.1.1.1. and reference I). It has been shown that σ^S is involved in the regulation of the expression of IHF in *Escherichia coli* (Aviv *et al.*, 1994). Therefore, the *tnpA* promoter constructs either containing or lacking the IHF binding site were tested in σ^S-defective background. It turned out that transcription from the *tnpA* promoter was entirely dependent on the growth phase of bacteria. Both reporter plasmids (with and without IHF binding sites in front of

tnpA promoter) tested in RpoS wild-type background exhibited clear stationary-phase-specific induction of the *tnpA* promoter activity (Fig. 3B, reference III). However, the same reporter plasmids revealed only slightly detectable levels of β-galactosidase activity in σ^S-defective *P. putida* strain revealing no induction of the *tnpA* promoter even in stationary phase bacteria. Thus, these data indicate that stationary-phase-specific activation of the *tnpA* promoter requires specifically σ^S.

RpoS may act either directly on the *tnpA* promoter or indirectly by activation of some transcription factor operating on the *tnpA* promoter. Although σ^S- and σ^{70}-dependent promoters are generally quite similar, some subtle but essential differences in the promoter sequences exist to ensure the selectivity between these two major sigma factors. σ^S-dependent promoters contain mostly the sequence CTATACT in the conserved –10 region (Espinosa-Urgel *et al.*, 1996), while σ^{70} preferentially recognises promoters with the sequence TATAAT. The –10 region CTATGCT of the *tnpA* promoter of Tn*4652* contains the sequence determinants suggested to be important for σ^S-dependent transcription, the C nucleotide upstream of the –10 hexamer and the C at the fifth position in the –10 hexamer (Fig. 3A, reference III). Therefore, I suppose that RpoS recognises the *tnpA* promoter and is directly involved in the stationary-phase-specific expression of TnpA.

2.2. Role of σ^S and IHF in starvation-induced transposition of Tn*4652* (References III and IV)

Activity of several transposable elements increases in response to different kind of stresses (see section 1.4.6.). There could be two different explanations for this phenomenon. Activation of transposition by stress could be a consequence of the loosened control over the transposition frequency (malfunction of regulation). Alternatively, induction of transposition could occur just in response to stress (channelled regulation). Actually, these two explanations are not mutually exclusive since transposition of a certain mobile element is mostly controlled by several regulatory mechanisms, which can respond to stress differently. Several authors have proposed that elevated transposition during stress may reflect a survival strategy while promoted mutagenesis processes may potentially contribute to survival in unfavourable conditions (Capy *et al.*, 2000; Chao *et al.*, 1983, Kidwell and Lisch, 1997, 2001; Wessler, 1996). However, not very much is known about molecular mechanisms involved in stress-induced transposition. Results obtained in this study indicate that activation of Tn*4652* in response to starvation might be an induced process. I suggest that at least two factors could be responsible for starvation-induced transposition of Tn*4652*. First, in starving

bacteria, transposition of Tn*4652* is elevated due to direct control of *tnpA* promoter by the stationary phase sigma factor σ^S that is induced in order to improve the survival of cells under stressful conditions. In addition to RpoS, IHF may be involved in the enhancement of transposition of Tn*4652* in the stationary phase *P. putida* as well. Transposition of Tn*4652* was shown to be elevated by overexpression of IHF (Fig. 1, reference III). Amount of IHF is known to increase near to one order of magnitude during the transition of the bacteria 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). Therefore, it is tempting to speculate that the increased concentration of IHF in stationary phase bacteria is another factor (besides of σ^S) inducing the mobility of Tn*4652*.

Summing up, I believe that transposition of Tn*4652* is regulated by physiological conditions of the host. Stationary phase-specific regulation of both the expression of TnpA (by σ^S and IHF), and potentially transposition reaction (by IHF) may explain the increased frequency of transposition of Tn*4652* in stationary phase bacteria.

2.3. Regulation of transposition of Tn*4652* by transposon-encoded TnpC

2.3.1. The abundance of Tn*4652* transposase is downregulated by TnpC (Reference II)

Transposition is a reaction potentially deleterious for the host. Therefore, the movement of transposons is strictly downregulated. This is generally achieved by tight control over the amount of active transposase in the bacterium. As a consequence, the transposase is never synthesised at a high level (reviewed in Kleckner, 1990).

Our study of regulation of the transposase of Tn*4652* revealed that expression of TnpA is downregulated. I could not detect TnpA by Western blot analysis in the cell lysate of *P. putida* PaW85 that carries Tn*4652* in its chromosome. Similarly, TnpA was not detectable in bacteria harbouring increased copy number of Tn*4652* (Fig. 2, reference II). Yet, TnpA protein was detected when the *tnpA* gene was subcloned from the transposon DNA. In order to test whether the TnpA expression is downregulated by a Tn*4652*-encoded factor, I generated different subclones of this transposon and tested TnpA expression in these subclones by Western blot analysis. It turned out, that for downregulation of TnpA, intactness of an ORF, located just next to the *tnpA* gene and named as *tnpC*, was necessary. The presence of *tnpC* gene decreased the abundance of TnpA in cell

lysate about 10-fold as judged by quantitative Western blot analysis (Fig. 2, reference II).

tnpC encodes for an 120-amino-acid-long protein, with the calculated molecular mass of 13.0 kDa. Comparison of the deduced amino acid sequence of the TnpC with the translated sequences of genes in the EMBL database revealed striking similarity of TnpC to a putative 120-amino-acid-long polypeptide encoded by the mercury resistance transposon Tn*5041* (Fig. 4, reference II). Hereby I wish to note that also TnpA of Tn*4652* is very similar to TnpA of Tn*5041* (Fig. 3, reference I; Kholodii *et al.*, 1997). Up to now, no data is available about the regulation of TnpA of Tn*5041*. However, considering the similarity between TnpC of Tn*4652* and the putative 120-amino-acid-polypeptide of Tn*5041*, I suggest similar regulation of these two transposons.

The question about the checkpoint of the TnpC action in the regulation of the TnpA cannot be answered unambiguously. However, results obtained support the possibility that TnpC operates in the regulation of the transposase of Tn*4652* at the post-transcriptional level. First, TnpC does not interfere with the transcription initiation from the *tnpA* promoter. Exchanging the *tnpA* promoter with another one did not affect the ability of TnpC to downregulate expression of TnpA (Fig. 2, reference II). Second, testing the effect of TnpC on transcription throughout the *tnpA* gene revealed that transcription elongation was not affected by TnpC (Fig. 6; reference II). Third, experiments with translational fusions of the *tnpA* gene 5' end with the reporter gene *gusA* showed that TnpC could not affect either the transcriptional or the translational initiation of the *tnpA* gene (Fig. 5, reference II). On the basis of these results, I suggest that TnpC functions in the regulation of TnpA post-translationally. One may speculate that TnpC can alter the transposase folding and/or transposase stability. However, I cannot exclude the possibility that TnpC is involved in the regulation of *tnpA*-specific mRNA stability.

2.3.2. Overexpression of TnpC *in trans* cannot affect the transposition of Tn*4652* (Reference IV)

To study the effect of TnpC on the frequency of transposition of Tn*4652*, the extra-copy of *tnpC* gene under the control of *Ptac* promoter and *lacI*^{q} repressor was introduced into the chromosome of *P. putida* strain KT2442 to obtain strain KT2442C. The ability of this extra-copy of *tnpC* to downregulate the abundance of the plasmid-encoded TnpA was tested with the aid of Western blot analysis. Overexpression of TnpC in the presence of 0.5 mM IPTG reduced the amount of TnpA below the level of Western blot analysis detection limit. Thus, TnpC was able to act in *trans* in the regulation of amount of plasmid-encoded TnpA in

this control experiment. Therefore, I expected that elevated level of TnpC should lead to the decrease in the frequency of transposition of Tn*4652*.

Transposition of Tn*4652* was tested in TnpC-overexpressing *P. putida* KT2442C by using the starvation-assay. To manipulate the level of expression of TnpC in bacteria, the phenol minimal plates were supplied with different concentrations of IPTG or no IPTG was added. Interestingly, no differences were observed in the number of Phe^+ mutants accumulating on phenol minimal plates either in the presence of different concentrations of IPTG or on the plates without IPTG. By using PCR analysis, the percentage of the Tn*4652*-linked Phe^+ mutants was examined. Surprisingly, this analysis revealed that increased expression of TnpC did not affect the percentage of Tn*4652*-linked mutants among Phe^+ mutants (Table 2, reference IV). Thus, although the overexpressed TnpC effectively downregulated the cellular amount of the plasmid-encoded TnpA in the control experiment, it was not able to influence the transposition frequency of Tn*4652* in the starvation assay.

2.3.3. Transposition of miniTn*4652* is effectively inhibited by *cis*-encoded TnpC (Reference IV)

Unexpected results obtained in TnpC-overexpressing strain may be explained by different functionality of TnpC acting either *in cis* or *in trans*. Therefore, to further address the question about the effect of TnpC *in cis* on the frequency of transposition of Tn*4652*, a miniTn*4652* system was designed. MiniTn*4652* carries kanamycin resistance gene from Tn*903* between the ends of Tn*4652* (Fig. 2A, reference IV). In order to test the effect of *tnpC* on transposition of miniTn*4652*, either *tnpA* or *tnpAC* with their native promoter(s) were cloned into the same plasmid as miniTn*4652* (Fig. 2B, reference IV). A plasmid carrying a frameshift mutation in the *tnpC* gene was constructed as an additional control to test whether the frequency of transposition would be affected by TnpC protein.

The frequency of transposition of miniTn*4652* was tested in Tn*4652*-free *P. putida* strain PRS2000 harbouring conjugative plasmid R751tet and different plasmids with miniTn*4652* by using the mating-out transposition assay. Results obtained clearly demonstrate that TnpC operates as an inhibitor in the regulation of transposition of miniTn*4652*. Frequency of transposition of miniTn*4652* into the conjugative plasmid was very high when TnpA alone was coded in the miniTn*4652* donor plasmid, reaching up to 10^{-1} transpositions per conjugation event (Table 3, reference IV). However, co-expression of TnpA with TnpC lowered the transposition activity of miniTn*4652* drastically — by 4 orders of magnitude. I suppose that this is due to the TnpC-caused downregulation of the cellular amount of transposase.

Thus, TnpC strongly inhibited the transposition of miniTn*4652* when encoded *in cis*. Why the TnpC overexpressed *in trans* could not interfere in transposition regulation of native Tn*4652* in the starvation assay carried out with *P. putida* KT2442C?

Several transposable elements encode for a *trans*-acting negative regulators to keep the control over the multiplication of the mobile element (reviewed in Kleckner, 1990). The effectiveness of these negative regulators to downregulate transposition may depend on these concentrations. For example, the IS*10*-encoded negative regulator is an antisense RNA effectiveness of which to inhibit transposition increases with its increasing concentration, i.e. with increasing transposon copy number (Simons and Kleckner, 1983). However, the IS*50*-encoded inhibitor protein (Inh) is effective even in a single copy and only very large increases in inhibitor protein (Inh) are needed to see additional inhibition of transposition of Tn*5* below the natural level (Johnson and Reznikoff, 1984b; Yin and Reznikoff, 1988). We measured transposition of native Tn*4652* under the conditions of overexpression of inhibitor protein TnpC as well. Our results revealed that this extra-amount of TnpC could not affect the frequency of transposition of native Tn*4652* (Table 2, reference IV). One explanation to this phenomenon might be that TnpC operates differently either acting *in cis* or *in trans*. However, this is not very plausible since the overexpressed TnpC can effectively act *in trans* in the control experiment by downregulating the plasmid-encoded transposase TnpA. The other reason for insensitivity of transposition of native Tn*4652* toward to overexpression of TnpC might ground on finding that the *cis*-encoded TnpC is very effective inhibitor as was revealed in experiments with miniTn*4652*. Presuming the non-linear (for example hyperbolic) relation between the concentration of TnpC and transposition of Tn*4652*, I suppose that concentration of *cis*-encoded TnpC is high enough to near fully inhibit the transposition (Fig. 7). Therefore, I favour the speculation that the *cis*-encoded TnpC is able to reduce the transposition of Tn*4652* to the basal level and this could be the reason why no additional effect of *trans*-overexpressed TnpC on transposition of Tn*4652* in starvation-experiment can be detected. Accordingly, it is hypothesised for Tn*5* that inhibitor dose — transposition response curve is not a linear plot but rather hyperbolic in shape (Yin and Reznikoff, 1988).

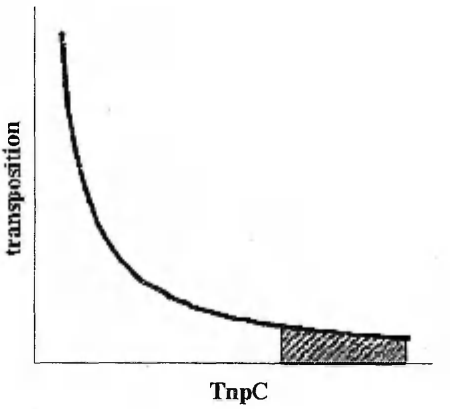

Figure 7. Hypothetical curve of non-linear relation between transposition of Tn*4652* and concentration of inhibitor TnpC. Transposon-encoded TnpC effectively inhibits transposition of Tn*4652* near basal level. Therefore, additional expression of TnpC *in trans* (hached region) could not influence transposition frequency.

However, as we established in the experiments with native Tn*4652* and miniTn*4652*+AC, some transposition still occurred at this basal level (Tables 2 and 3, reference IV). Therefore, one can speculate that if some molecules of TnpA escape the action of *cis*-encoded TnpC, the transposition reaction is carried out. Furthermore, there may be some additional regulatory mechanisms that can channel this rescued transposase molecule into transposition reaction.

CONCLUSIONS

For now, the biochemical reaction of transposition is well characterised. Also, many regulatory mechanisms controlling the frequency of transposition are described. However, some aspects in transposition regulation are not well understood. These include, for example, the fluctuations in frequency of transposition due to different extracellular (environmental) and intracellular signals. Our study of regulation of *Pseudomonas putida* transposon Tn*4652* enlightens the regulation of transposition under stress conditions.

The present work can be briefly summarised as follows: transposition of *Pseudomonas putida* transposon Tn*4652* is positively regulated by host factors σ^S and IHF and negatively by transposon-encoded TnpC.

For more detail, following conclusions can be drawn.

1. Binding of IHF to the right end of Tn*4652* can moderately enhance transcription from the transposase promoter. IHF binds also to the transposon left end just next to the presumed binding site of transposase. Transposition frequency of Tn*4652* decreases essentially in IHF-defective *P. putida* strain by declining under the detection limit of the assay used. Therefore, I propose that IHF has a dual role in regulation of transposition of Tn*4652*. First, it enhances transposition by elevating the concentration of transposase. Second, IHF binding to the ends of the transposon may directly interfere in transposition reaction. IHF might either favour productive binding of transposase and/or it might be needed in nucleoprotein complex formation for subsequent strand nicking and transfer reactions.

2. Transcription from the transposase promoter of Tn*4652* is strictly controlled by growth phase of bacteria due to σ^S-dependent regulation of this promoter. Transposition of Tn*4652* decreases about one order of magnitude in the σ^S-defective strain. Evidently, σ^S-dependent expression of transposase gene primarily explains the starvation-induced transposition of Tn*4652* observed by our group previously. However, I suppose that also IHF may commit to the increased transposition of Tn*4652* under starvation conditions since the concentration of IHF is known to increase in stationary phase bacteria and the data presented in this study show that frequency of transposition of Tn*4652* increases with increased expression of IHF.

3. Transposase expression is downregulated by Tn*4652*-encoded TnpC. Results obtained indicate that TnpC operates in the regulation of the TnpA at the post-transcriptional level. TnpC is a very effective inhibitor: the transposition of an artificial miniTn*4652* decreased about 4 orders of magnitude when

TnpC was coexpressed with TnpA. Therefore, I suppose that TnpC is the major factor that ensures the low rate of transposition of Tn*4652*.

Consequently, the results presented in current thesis, suggest that Tn*4652* and its host *P. putida* are in mutual relationships. Transposition frequency of Tn*4652* is low but it is induced by the host factors under starvation conditions when it may be potentially advantageous for the host. Debate about the selfish and/or "junk" nature of transposable elements has continued already several decades. Regulation of Tn*4652* indicates that it is well domesticated by its host and Tn*4652* itself downregulates its "selfish" characteristics. On the light of the results obtained, it is tempting to speculate that the host uses the potential of transposon to promote mutagenesis process in conditions in which it could be potentially useful, for example, under conditions of starvation.

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PSEUDOMONAS PUTIDA TRANSPOSOONI TN*4652* TRANSPOSITSIOONI REGULATSIOON

Kokkuvõte

Vaatamata genoomide näilisele muutumatusele toimub DNA-s pidevalt mitmesuguseid ümberkorraldusi. Peale DNA polümeraasi põhjustatud kopeerimisvigade võivad DNA rekombineerumisel tekkida ulatuslikud inversioonid, duplikatsioonid, insertsioonid, deletsioonid ja translokatsioonid. Rekombinatsioon võib toimuda kas homoloogia alusel või transpositsiooni teel. Homoloogilise rekombinatsiooni korral vahetuvad DNA lõigud kahe homoloogilise piirkonna vahel. Transpositsiooni käigus liigub (translokeerub) diskreetne DNA segment, transposoon, uude kohta, vajamata homoloogiat nn. doonorlookuse ja märklaudlookuse vahel. Transposooni ehk mobiilse DNA elemendi ümberpaiknemine võib oluliselt muuta geenide avaldumise taset. Transponeerumine geeni sisse inaktiveerib selle geeni. Samas võib transposooni insertsioon geeni ette lülitada seni "vaikinud" geeni tööle. Transpositsiooni tulemusena tekkinud suuremad DNA inversioonid, deletsioonid ja replikonide liitumised võivad mõjutada paljude geenide ekspressiooni. Seega toimivad transposoonid genoomide ümberkujundajatena, olles olulised elemendid genoomide evolutsioonis.

Transpositsiooni toimumiseks on vajalikud transposooni otstes paiknevad pöördkordusjärjestused ja transposooni kodeeritud valk transposaas, mis seondub spetsiifiliselt pöördkordusjärjestustele ning viib läbi transpositsioonireaktsiooni. Lihtsamad bakteriaalsed transposoonid, IS-elemendid, kodeerivadki vaid transposaasi. Suuremad mobiilsed elemendid kannavad sageli lisaks transposaasi geenile ka teisi geene. Transposoonide koostises võivad olla resistentsusgeenid (Tn*10* — tetratsükliin; Tn*3* — ampitsilliin; Tn*21* — ampitsilliin, streptomütsiin, elavhõbe), aga ka mitmesugused "eksootilisemad" geenid, näiteks toksiinide produtseerimist või spetsiifiliste substraatide degradatsiooniensüüme kodeerivad geenid. Näiteks kannab *Pseudomonas putida* transposoon Tn*4651* tolueeni katabolismiraja geene.

Transposoone on leitud peaaegu kõigist seni uuritud organismidest alates bakteritest kuni kõrgemate eukarüootideni. Viimastes võib transposoonset päritolu DNA hulk ulatuda kuni 50%-ni kogu genoomist (nt. maisis või inimeses). Samas on enamus eukarüootsetest transposoonidest defektsed, st. ei ole võimelised genoomis ümber paiknema. Tõepoolest, transpositsioon on rakule potentsiaalselt hukatuslik protsess. Seepärast on transpositsioonisagedus enamasti madal. Bakteriaalsed mobiilsed elemendid transponeeruvad keskmiselt sagedusega 10^{-5}–10^{-8} sündmust rakugeneratsiooni kohta, mis tähendab, et enamasti on transposoonid inaktiivsed. Transponeeruvate elementide avastaja Barbara McClintock oli üks esimesi, kes arvas, et transposoonid võivad aktiveeruda vastusena mitmesugustele keskkonnamuutustele. Kirjanduses on andmeid

mõnede bakteriaalsete transposoonide aktiveerumisest stressitingimustel (nt. süsinikunäljas, mitteoptimaalsel temperatuuril, UV-kiirguse tagajärjel). On koguni spekuleeritud, et transpositsiooni suurenemine stressitingimustes on rakule adaptiivne protsess, mis võib soodustada stressi üleelamist ja/või stressist väljatulemist.

Tänaseks on transpositsioonimehhanismist palju teada. Samuti on palju uuritud erinevate transposoonide regulatsiooni. Neid tulemusi käsitleb käesoleva töö kirjanduse ülevaade. Teisalt on senini väga vähe teada signaalide kohta, mis rakus toimuvate muutuste korral võivad viia transposooni aktiveerumisele või inaktiveerumisele. Meie uurimisgrupi varasemad tulemused näitasid, et *Pseudomonas putida* transposooni Tn*4652* transpositsioonisagedus suureneb stressitingimustes süsinikunälja korral. Seepärast on Tn*4652* sobivaks mudelobjektiks, et selgitada transpositsiooni aktiveerumise mehhanisme stressitingimustes. Et transpositsiooni sagedus sõltub eelkõige aktiivse transposaasi hulgast, siis keskendusingi eelkõige transposaasi ekspressiooni regulatsioonile. Saadud tulemusi käsitleb töö eksperimentaalne osa. Järgnevalt on kokkuvõtvalt toodud peamised tulemused ja nende põhjal tehtud järeldused.

1. *P. putida* IHF (*integration host factor*) valk seondub spetsiifiliselt Tn*4652* mõlemale otsale potentsiaalsete transposaasi seondumissaitide kõrvale. IHF-i seondumine Tn*4652* paremale otsale soodustab transkriptsiooni transposaasi (*tnpA*) promootorilt. Lisaks ilmnes, et Tn*4652* transpositsioonisagedus oli *P. putida* IHF-miinustüves langenud allapoole detekteerimispiiri. Saadud tulemuste põhjal järeldan, et IHF võib mõjutada Tn*4652* transpositsiooni kahel tasemel. Esiteks, IHF soodustab transpositsiooni transposaasi kontsentratsiooni suurendamise tõttu. Teiseks võib IHF-i seondumine transposooni mõlemale otsale mõjutada otseselt transposaasi poolt läbiviidavat transpositsioonireaktsiooni.

2. Transkriptsioonitase transposaasi promootorilt on tugevasti mõjutatud bakterite kasvufaasist — eksponentsiaalselt kasvavates rakkudes on *tnpA* promootor "vaikiv" ja transkriptsioon algab alles bakterite jõudmisel statsionaarsesse kasvufaasi. Selle põhjuseks on *tnpA* promootori aktiivsuse tugev sõltuvus statsionaarse kasvufaasi spetsiifilisest sigmafaktorist, σ^S. Tn*4652* transpositsioonisagedus langes σ^S-miinus tüves ligikaudu ühe suurusjärgu võrra.

3. Tn*4652* transpositsioonisagedus bakterites suureneb süsinikunäljast põhjustatud stressi korral. Selle peamiseks põhjuseks on Tn*4652* transposaasi ekspressiooni sõltuvus statsionaarse faasi sigmafaktorist σ^S. Kuna ka IHF-i tase suureneb statsionaarse faasi rakkudes ja käesolevas töös saadud tulemused näitavad, et Tn*4652* transpositsioonisagedus tõuseb IHF-i üleekspresseerimisel, võib arvata, et transpositsiooni aktiveerimisel stressitingimustes võib lisaks σ^S-le osaleda ka IHF.

4. Transposaasi ekspressiooni inhibeerib Tn*4652* kodeeritud inhibiitorvalk TnpC. Kuigi TnpC põhjustatud inhibitsiooni mehhanism pole täiesti selge, lubavad saadud tulemused järeldada, et TnpC toimib transposaasi regulatsioonis pärast transkriptsiooni. Kanamütsiini resistentsust kandva miniTn*4652* trans-

positsioonisageduse mõõtmine näitas, et transpositsioon langes nelja suurusjärgu võrra (jäädes vaid veidi kõrgemale basaalsest tasemest), kui TnpC ekspresseerus rakkudes koos TnpA-ga.

Eespooltoodu põhjal võib järeldada, et Tn*4652* transponeerumist reguleerivad nii peremehe kui ka transposooni enese kodeeritud faktorid. Eelkõige "hoolitseb" transposoon ise selle eest, et transpositsioonisagedus oleks madal (transposooni kodeeritud TnpC-vahendatud inhibitsioon). Teisalt võib teatud tingimustes, näiteks stressi korral, transpositsioonisageduse tõus bakterile kasulikuks osutuda. Transpositsiooni suurenemist stressitingimustes on eeldatud pikemat aega ja mõningate bakteriaalsete transposoonide puhul on seda ka näidatud. Seni ei olnud aga kuigi palju teada stressist indutseeritud transpositsiooni molekulaarsete mehhanismide kohta. Seepärast arvan, et käesolevas töös saadud tulemused Tn*4652* transpositsioonisageduse sõltuvuse kohta statsionaarse faasi faktoritest (σ^S ja ka IHF) võimaldavad paremini mõista peremehe ja transposooni suhteid. Juba paarkümmend aastat on kestnud debatt transposoonide "iseka" ja/või "prügiliku" olemuse üle. Käesolevas töös saadud tulemuste põhjal julgen spekuleerida, et Tn*4652* on peremehe poolt hästi kodustatud. Tn*4652* transpositsiooni aktiveerimine peremehe stressispetsiifiliste valkude abil viitab transposooni võimalikule kasutamisele mutaatorelemendina tingimustes, kus mutatsiooniprotsessis tekkivad geneetilised ümberkorraldused võivad rakule kasulikuks osutuda.

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PUBLICATIONS

I

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Expression of the Transposase Gene *tnpA* of Tn*4652* Is Positively Affected by Integration Host Factor

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Tn*4652* is a derivative of the toluene degradation transposon Tn*4651* that belongs to the Tn*3* family of transposons (M. Tsuda and T. Iino, Mol. Gen. Genet. 210:270–276, 1987). We have sequenced the transposase gene *tnpA* of transposon Tn*4652* and mapped its promoter to the right end of the element. The deduced amino acid sequence of *tnpA* revealed 96.2% identity with the putative transposase of Tn*5041*. Homology with other Tn*3* family transposases was only moderate (about 20 to 24% identity), suggesting that Tn*4652* and Tn*5041* are distantly related members of the Tn*3* family. Functional analysis of the *tnpA* promoter revealed that it is active in *Pseudomonas putida* but silent in *Escherichia coli*, indicating that some *P. putida*-specific factor is required for the transcription from this promoter. Additionally, *tnpA* promoter activity was shown to be modulated by integration host factor (IHF). The presence of an IHF-binding site upstream of the *tnpA* promoter enhanced the promoter activity. The positive role of IHF was also confirmed by the finding that the enhancing effect of IHF was not detected in the *P. putida ihfA*-deficient strain A8759. Moreover, the Tn*4652* terminal sequences had a negative effect on transcription from the *tnpA* promoter in the *ihfA*-defective strain. This finding suggests that IHF not only enhances transcription from the *tnpA* promoter but also alleviates the negative effect of terminal sequences of Tn*4652* on the promoter activity. Also, an in vitro binding assay demonstrated that both ends of Tn*4652* bind IHF from a cell lysate of *E. coli*.

Transposons are discrete DNA segments that can move from one genetic location to another. This process does not involve homologous recombination systems of the host but requires a gene product encoded by the moving element itself—transposase. Transposase interacts site specifically with the ends of the transposon, cleaves the DNA at both termini of the element, and carries out the strand transfer reaction (reviewed in references 22 and 34).

Transposition of a mobile element is precisely controlled and depends on the availability of the active transposase. Moreover, in several cases the transposition reaction itself is controlled and modulated by some other transposon-encoded protein(s) and/or host factors (29). One of the host factors participating in the transposition is integration host factor (IHF) (17, 32, 41, 42).

IHF is a sequence-specific sharply DNA bending heterodimeric protein which is involved in a variety of cellular processes including λ site-specific recombination, transposition, replication, and positive and negative control of gene expression (15). IHF has been found to regulate gene expression in a number of gram-negative bacteria (21). IHF genes from diverse bacterial species are well conserved (8, 12). In most cases, the role of IHF is architectural: it facilitates the formation of nucleoprotein complexes through strong bending of DNA. However, activation of transcription from λ pL1 and Mu phage Pe promoters involves direct interaction of IHF with RNA polymerase (20, 44).

Many mobile DNA elements carry IHF-binding sites at one or both termini (14, 18, 25, 32, 46). For γδ (Tn*1000*), it was shown that binding of IHF to the ends of the transposon facilitates binding of transposase (46). Mostly, IHF affects transposition positively (10, 35, 42). For example, in the well-studied Mu phage transposition, IHF acts positively both by enhancing transcription from the early promoter Pe and favoring the stabile synaptic complex formation that is required in the initial step of transposition (2, 44). However, there are also reports about the negative role of IHF on transposition (17, 41).

According to Kleckner (28), transposable elements from bacteria can be divided into three classes. Class II contains evolutionarily related elements mostly belonging to the Tn*3* family of transposons. Tn*3* family transposons translocate replicatively and generate 5-bp direct duplications of the target DNA (40). Members of the Tn*3* family exhibit similar inverted repeats 35 to 48 bp in length and similar transposases. Comparison of the Tn*3* family transposases showed their clustering into three subgroups (26). Tn*3* and Tn*21* subgroups associate transposons from gram-negative bacteria, while transposons from gram-positive bacteria belong to the third subgroup. Transposases of IS*1071* and recently characterized mercury resistance transposon Tn*5041* are more diverse and cannot be included to any of these three subgroups (26).

Pseudomonas putida PaW85 carries in its chromosome transposon Tn*4652*, a 17-kb derivative of the 56-kb toluene degradation transposon Tn*4651* coding for *xyl* genes (43). Tsuda and Iino (43) have shown that Tn*4652* belongs to the Tn*3* family of transposons, as determined from its transposition properties. Genetic analysis on Tn*4652* localized the putative transposase gene to a 3.0-kb segment at the end of the right arm of the element (43). However, regulation of the Tn*4652* transposase gene as well as the mechanism of transposition reactions of Tn*4652* have remained unexplored.

This study aims to elucidate the regulation of the Tn*4652* transposase gene. We sequenced the Tn*4652* transposase gene *tnpA* and localized the promoter of the gene to the right end of the element. Analysis of the deduced amino acid sequence of the *tnpA* gene revealed highest homology (96.2% identity) with

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Genotype or construction	Source or reference
E. coli		
HB101	*subE44 subF58 hsdS3*($r_B^-m_B^-$) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*	6
WM2015	*subE thi* Δ(*lac-pro*)	31
WM2017	WM2015 *himA*::Tc^r *himD*::Cm^r	31
P. putida		
PaW85	Tn*4652*	4
PRS2000	Tn*4652*-free	45
KT2442	Tn*4652 xylRS* Pu-*lacZ* Rif^r Sm^r	8
A8759	KT2442 Tn*4652 ihfA*::Km^r Pu-*lacZ*	8
Plasmids		
pBluescript KS	Cloning vector (Ap^r)	Stratagene
pEST1332	Plasmid pAYC32 carrying promoterless *pheBA* operon	27
p1332S/C	PCR-generated 122-bp Tn*4652* right-end fragment (primers Osac and Ocla) cloned into pEST1332	This work (Fig. 2)
p1332IHF/C	PCR-generated 83-bp Tn*4652* right-end fragment (primers Oihf and Ocla) cloned into pEST1332	This work (Fig. 2)
p1332D/C	*Sac*I-*Dra*I deletant of p1332S/C	This work (Fig. 2)
p1332S/N	*Nhe*I-*Cla*I deletant of p1332S/C	This work (Fig. 2)
pKT240	Cloning vector (Ap^r Km^r)	3
pKRZ-1	Cloning vector (Ap^r Km^r)	37
pKTlacZ	Promoter probe vector containing *lacZ* gene from pKRZ-1 cloned into pKT240	This work (Fig. 1)
pKTlacZS/C	PCR-generated 122-bp Tn*4652* right-end fragment (primers Osac and Ocla) cloned into pKTlacZ	This work (Fig. 2)
pKTlacZIHF/C	PCR-generated 83-bp Tn*4652* right-end fragment (primers Oihf and Ocla) cloned into pKTlacZ	This work (Fig. 2)
pKTlacZD/C	*Sac*I-*Dra*I deletant of pKTlacZS/C	This work (Fig. 2)
pHNβα	Plasmid carrying *E. coli* IHF genes *ihfA* and *ihfB*	31
pUC18	Cloning vector (Ap^r)	48
pUCPu130	129-bp *Dpn*I fragment of Pu promoter region of *xyl* genes in TOL plasmid cloned into pUC18	This work

the transposase of Tn*5041*. Study of the regulation of the *tnpA* promoter from Tn*4652* demonstrated that (i) the promoter was active in *P. putida* but silent in *Escherichia coli* and (ii) the IHF-binding site at positions −73 to −85 relative to the transcription start point affected transcription from the *tnpA* promoter in *P. putida* positively. Gel mobility shift experiments with cell lysates of *E. coli* and *P. putida* were carried out to examine binding of IHF to the ends of Tn*4652* in vitro.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are described in Table 1. Construction of the new broad-host-range promoter-probe vector pKTlacZ is depicted in Fig. 1. Bacteria were grown on LB medium (33). Antibiotics were added at the indicated final concentrations: for *E. coli*, ampicillin at 100 μg/ml and tetracycline at 15 μg/ml; for *P. putida*, carbenicillin at 1,500 μg/ml and streptomycin at 500 μg/ml. *P. putida* was incubated at 30°C, and *E. coli* was incubated at 37°C. Early-stationary-phase cultures were used for enzyme assays. *E. coli* was transformed with plasmid DNA as described by Hanahan (23). *P. putida* was electrotransformed by using the protocol of Sharma and Schimke (39).

DNA manipulations and mRNA mapping. DNA sequencing was performed with a Sequenase version 2.0 DNA sequencing kit (Amersham). Subclones of the *tnpA* promoter region (Table 1) were obtained by cloning PCR products. The following oligonucleotides, containing suitable restriction sites (*Sac*I and *Cla*I; boldfaced) and complementary to nucleotides (nt) 1 to 21, 40 to 63, and 101 to 122 relative to the right end of the Tn*4652*, were used in cloning: Osac (5′-CG **TGAGCTC**GGGGTTATGCCGAGATAAGGC-3′), Oihf (5′-**CGTGAGCTCT** GTAAATATATGATTTAAAAGG-3′), and Ocla (5′-**CGTATCGATC**AGCAT AGACGGCTAGCCAG-3′). Locations of these oligonucleotides are shown in Fig. 2A.

A reverse transcriptase reaction was carried out to identify the 5′ end of mRNA initiated from the *tnpA* promoter by a procedure described previously by our group (36). Total RNA (20 μg), purified from *P. putida* PaW85, *P. putida* PRS2000, and *E. coli* HB101 cells as described by Blomberg et al. (5), was used as the template. Oligonucleotide 5′-GTATGCTTGGCAGTCGT-3′, complementary to nt −120 to −136 relative to the start codon of the reporter gene *pheB*, was used in the primer extension analysis.

Enzyme assays. The catechol 1,2-dioxygenase (C12O) assay was carried out as described by Hegeman (24). The β-Galactosidase (β-Gal) assay was performed as specified by Miller (33). Protein concentration in cell lysates was measured by the Bradford method (7).

Gel mobility shift assay. Cell lysates used in gel shift assays were prepared from 30-ml early-stationary-phase cultures. The cells were pelleted and sonicated in 1× binding buffer (25 mM Tris-HCl [pH 7.5], 0.05 mM EDTA, 5 mM dithiothreitol, 25 mM NaCl, 50 mM KCl, 5% glycerol). Protein concentration in cleared lysates was 15 to 20 mg/ml; 1 to 3 μl of undiluted lysate or lysate diluted in 1× binding buffer was used in gel shift assays.

The following DNA fragments were used in gel shift binding assays: (i) a 108-bp DNA restriction fragment containing the right end of transposon Tn*4652* up to the *Nhe*I restriction site (Fig. 2A); (ii) a 140-bp DNA restriction fragment containing the left end of the transposon up to the *Bpu*1102I restriction site (Fig. 2B); and (iii) a 140-bp DNA restriction fragment containing a 129-bp *Dpn*I segment of the Pu promoter region cloned into pUC18 (Table 1). These DNA

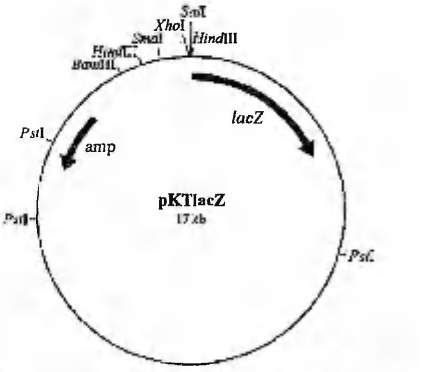

FIG. 1. Map of the broad-host-range promoter probe vector pKTlacZ. An about 5-kb *Hin*dIII-*Pst*I fragment carrying the *lacZ* gene originates from plasmid pKRZ-1 (37). After this fragment was cloned into pBluescriptSK(+) it was recut with *Xho*I and *Sma*I and subcloned into pKT240 opened with *Xho*I and *Ecl*136II. Suitable cloning sites are *Bam*HI, *Hin*dIII (two sites), *Sma*I, *Xho*I, and *Sal*I.

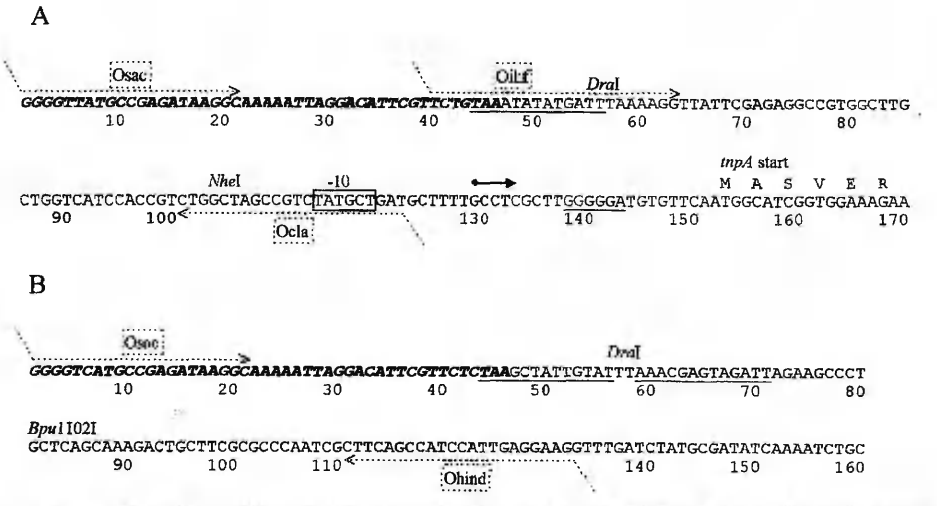

FIG. 2. Sequence analysis of the right end (A) and left end (B) of Tn*4652*. The 48-bp inverted repeats are in boldface italics. Potential IHF-binding sites resembling the *E. coli* IHF-binding consensus sequence WATCAANNNNTTR and ribosome-binding site of the *tnpA* gene are underlined. The transcription start of *tnpA* is indicated by the solid arrow, and the putative −10 hexamer of the promoter is boxed. The deduced amino acid sequence of the *tnpA* gene is presented starting from the second ATG. The first six amino acids are shown. Locations of primers used in PCR for cloning of the *tnpA* promoter and for generating DNA fragments for the gel mobility shift assay are indicated by dotted-line arrows. 5′ ends of the oligonucleotides not complementary to the termini of Tn*4652* are indicated by sloping dotted lines. Primers Osac, Oihf, and Ocla contain restriction site *Sac*I or *Cla*I for cloning of the *tnpA* promoter.

fragments were end labeled with [α-^{32}P]dCTP, using the Klenow fragment of DNA polymerase I, and subsequently purified through an polyacrylamide gel. The binding reaction was carried out in a volume of 20 μl. About 1 ng (1,000 cpm) of DNA probe was incubated at 20°C for 20 min with different cell lysates in 1× binding buffer containing 1 μg of bovine serum albumin and 5 μg of salmon sperm DNA. The following specific nonlabeled competitor DNAs containing IHF-binding sites were generated by PCR: (i) a 122-bp fragment of the right end of Tn*4652*, amplified by using primers Osac and Ocla (Fig. 2A); (ii) a 132-bp fragment of the left end of Tn*4652*, amplified by using primers Osac and Ohind (5′-CGTAAGCTTCCTCAATGGATGGCTGAAG-3′ [Fig. 2B]); and (iii) a 250-bp DNA fragment including a 129-bp *Dpn*I segment of the Pu promoter region cloned into pUC18 (Table 1), amplified by using pUC18 reverse and forward primers. When the specific competitor DNA was used, the cell lysate was added last to the binding reaction. After incubation, the reaction mixture was loaded on a 1-h-prerun 5% nondenaturing polyacrylamide gel. Electrophoresis was carried out at room temperature in 0.5× Tris-borate-EDTA buffer at 10 V/cm for 2 h. The gels were dried and autoradiographed or exposed to a phosphorimager screen.

Nucleotide sequences accession numbers. The 3,348-bp sequence of the right arm of Tn*4652* has been assigned accession no. X83686 in the EMBL database. The accession number of the 604-bp-long sequence of the left end of Tn*4652* is X83687.

RESULTS

Sequence of the Tn*4652* transposase shows highest homology with the putative transposase of Tn*5041*. Genetic analysis has localized the transposase gene of Tn*4652* to the right arm of the transposon (43). A 3.2-kb *Dra*I-*Hin*dIII fragment from Tn*4652* DNA, known to contain the transposase gene *tnpA*, was subcloned into the pBluescript KS(+) vector. Sequencing of the DNA fragment revealed a single 3,012-bp open reading frame (ORF) directed inward from the right end of the transposon. The ORF has two potential ATG start codons, separated by 6 bp (Fig. 2A). Since the potential ribosome-binding site overlaps the first ATG, initiation of translation of *tnpA* from the second ATG is more likely. The predicted protein, starting from the second ATG, is 1,001 amino acids long, with a calculated molecular mass of 114 kDa. Sequence comparison with the translated sequences of genes in the EMBL database by using the FASTA and BLAST programs revealed a high degree of homology of the Tn*4652 tnpA* with the putative transposase of the mercury resistance transposon Tn*5041* (96.2% identity). Homology with other transposases of Tn*3* family transposons (Tn*501*, Tn*1721*, Tn*1546*, Tn*21*, Tn*4430*, Tn*3926*, Tn*2501*, Tn*3*, Tn*4556*, Tn*1000*, and IS*1071*) was much lower (about 20 to 24% identity and 30 to 36% similarity). In most of the Tn*3* family transposons, the 3′ ends of the transposase genes terminate within one of the terminal repeats of the element (40). Contrary to that, the direction of the *tnpA* gene of Tn*4652* is opposite, starting from the right end of the transposon. Multiple alignment of Tn*3* family transposase sequences homologous to Tn*4652* transposase was performed via the CBRG server (http://cbrg.inf.ethz.ch/) by using the Darwin program. Alignment revealed stronger conservation in C termini of these proteins (data not shown). The phylogenetic tree of the entire protein sequences demonstrated that the Tn*4652* transposase is quite distantly related to other members of the Tn*3* family and might constitute a new Tn*3* family subgroup together with Tn*5041* (Fig. 3).

Mapping of the *tnpA* promoter. The ORF of *tnpA* gene starts at 152 bp from the right end of transposon Tn*4652*. To map the *tnpA* promoter, we constructed plasmid p1332S/C by cloning the 122-bp DNA segment covering the right end of the transposon upstream of the promoterless *pheBA* operon in plasmid pEST1332 (Table 1 and Fig. 2A). In addition, plasmids p1332D/C and p1332S/N, containing Tn*4652* right-end DNA from nt 58 to 122 and from nt 1 to 104, respectively, were constructed (Table 1 and Fig. 2A). *E. coli* HB101 and *P. putida*

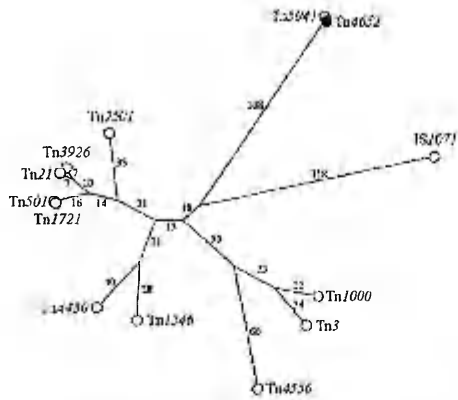

FIG. 3. Unrooted phylogenetic tree of the Tn*3* family transposase proteins. Multiple alignment of transposase sequences and construction of the phylogenetic tree were carried out via the CBRG server as described in the text. PAM distances are indicated at branches of the tree. DNA accession numbers and hosts (in parentheses): Tn*2501* (*E. coli*), Y00502; Tn*3926* (*E. coli*), X14236; Tn*21* (*E. coli*), X04891; Tn*501* (*P. aeruginosa*), X03406; Tn*1721* (*E. coli*), X61367; Tn*4430* (*Bacillus thuringiensis*), X07651; Tn*1546* (*Enterococcus faecium*), M97297; Tn*4556* (*Streptomyces fradiae*), M29297; Tn*3* (*E. coli*), V00613; Tn*1000* (*E. coli*), X60200; IS*1071* (*Alcaligenes* sp. strain BR60), M65135; Tn*5041* (*Pseudomonas* sp.), X98999; Tn*4652* (*P. putida*), X83686.

PaW85 and PRS2000 were transformed with these plasmids. As many transposase promoters are downregulated by transposon-encoded repressor proteins (29), the transposon Tn*4652*-free *P. putida* strain PRS2000 was used as a reference strain to distinguish potential effects of chromosomally encoded transposon protein(s) on the promoter activity in strain PaW85.

The C12O assay was carried out to study expression of the reporter gene *pheB* in the plasmids constructed. *P. putida* PaW85 and PRS2000 harboring plasmids p1332S/C and p1332D/C revealed promoter activity (Fig. 4A), but no C12O activity was detected in bacteria carrying plasmid p1332S/N, indicating that the *tnpA* promoter was disrupted in this construct (data not shown). Data in Fig. 4A show that C12O activities measured in *P. putida* PRS2000 were more than twofold higher than those measured in *P. putida* PaW85. Additionally, bacteria harboring p1332S/C revealed about twofold-higher enzyme activities than bacteria containing p1332D/C. None of the promoter constructs studied revealed activity in *E. coli* (Fig. 4A).

To map the 5′ end of the mRNA initiated from the *tnpA* promoter, primer extension analysis was carried out. Total RNA extracted from *E. coli* HB101, *P. putida* PaW85, and *P. putida* PRS2000 carrying plasmid p1332S/C, which exhibited *tnpA* promoter activity, or pEST1332 as a negative control was used as a template for the reverse transcriptase reaction. The results are presented in Fig. 5. Consistent with enzyme assays, no specific transcript was initiated from the *tnpA* promoter in *E. coli* (Fig. 5, lane 5). Easily detectable primer extension products could be established by using total RNA extracts both from cells of *P. putida* PRS2000(p1332S/C) and PaW85(p1332S/C) (Fig. 5, lanes 1 and 3). Primer extension assay localized the putative transcription start point 23 bp upstream of the *tnpA* gene start codon. The sequence TATGCT, resembling the σ^{70}-recognized promoter consensus TATAAT, was found 10 bp upstream of the transcription start point. However, the −35 region of the promoter was not homologous with σ^{70}-recognized consensus hexamer TTGACA.

The IHF-binding site affects positively transcription from the *tnpA* promoter. Results presented in Fig. 4A suggest that the region from bp 1 to 56 bp of the right end of Tn*4652* has a positive effect on the transcription from the *tnpA* promoter (compare p1332S/C and p1332D/C). Sequence analysis of the transposon right end revealed a potential IHF-binding site flanking the *Dra*I site in p1332S/C (Fig. 2A). To test the effect of the presence of an IHF-binding site upstream of the *tnpA* promoter on expression of the reporter gene, the enzyme assay using the widely used β-Gal reporter system was performed. For that purpose, we constructed plasmids pKTlacZS/C, pKTlacZIHF/C, and pKTlacZD/C by cloning different DNA fragments from the *tnpA* promoter region (bp 1 to 122, 39 to

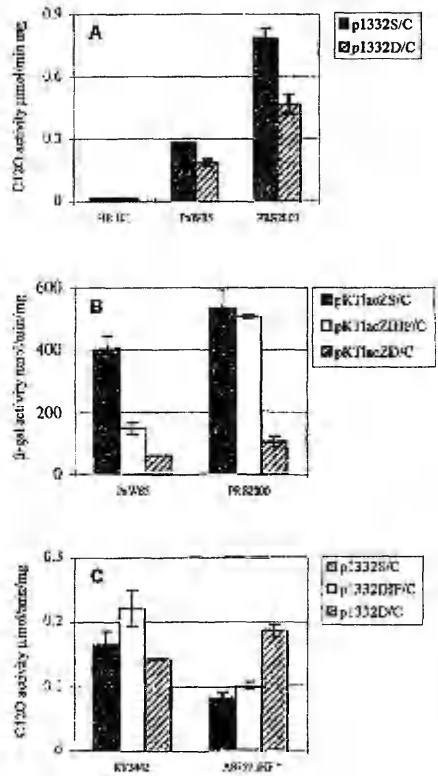

FIG. 4. C12O (A and C) and β-Gal (B) activities measured in *E. coli* HB101 and different *P. putida* strains carrying different *tnpA* promoter constructs. *P. putida* PaW85 carries in the chromosome a copy of Tn*4652*, and strain PRS2000 is Tn*4652* free. *P. putida* A8759 is an *ihfA*-deficient derivative of strain KT2442. Bacterial strains and *tnpA* gene promoter constructs are listed in Table 1. Data (means ± standard deviations) of at least five independent experiments are presented. For plasmid pEST1332, the basal level of expression of C12O is less than 0.01 μmol/min/mg; for pKTlacZ, the level of expression of β-Gal is less than 2 nmol/min/mg.

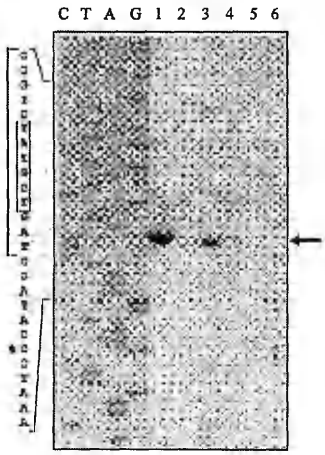

FIG. 5. Mapping of the 5′ end of mRNA initiated from the *tnpA* promoter. The primer extension product is indicated by the arrow. Lanes 1 to 6 present primer extension reactions carried out with total RNA prepared from *P. putida* PRS2000 (lanes 1 and 2), *P. putida* PaW85 (lanes 3 and 4), and *E. coli* HB101 (lanes 5 and 6) carrying *tnpA* promoter-containing plasmid p1332S/C (lanes 1, 3, and 5) or pEST1332 (lanes 2, 4, and 6) as a negative control. Lanes C, T, A, and G show DNA sequencing reactions of plasmid p1332S/C; 26 nt of this sequence is presented at the left, and the transcription start point of the *tnpA* gene is marked by a diamond. DNA originated from the right end of Tn*4652* in p1332S/C is indicated by the vertical bold line, and the −10 region of the *tnpA* promoter is boxed.

122, and 58 to 122, respectively) upstream of the β-Gal gene *lacZ* in the broad-host-range vector pKTlacZ (Table 1, Fig. 1, and Fig. 2). Plasmid pKTlacZIHF/C, which contains an IHF site adjacent to the *Dra*I site, lacks the last 39 bp from the transposon end. Results of the β-Gal assay presented in Fig. 4B confirmed previous data obtained with the C120 reporter system (Fig. 4A): the presence of the Tn*4652* terminal sequences (bp 1 to 57) upstream of the *tnpA* promoter enhances transcription from the promoter. Moreover, while in the C120 reporter system the positive effect was nearly twofold, the β-Gal system exhibited five- to sixfold enhancement. In Tn*4652*-free *P. putida* PRS2000, the presence of an IHF-binding site upstream of the *Dra*I site was sufficient to complement the positive effect of the transposon right end to the *tnpA* promoter activity (Fig. 4B; compare pKTlacZIHF/C with pKTlacZD/C and pKTlacZS/C). However, in Tn*4652*-containing *P. putida* PaW85, the positive effect of an IHF-binding site in plasmid p1332IHF/C was lower than in p1332S/C (Fig. 4B).

Analogously to the C120 reporter, no promoter activity was detected if the β-Gal reporter was used in *E. coli* (data not shown).

***tnpA* promoter activity in *ihfA*-deficient *P. putida* A8759.** To elucidate the role of IHF in the *tnpA* promoter activity, a C120 assay using *P. putida* KT2442 and in its *ihfA*-deficient derivative *P. putida* A8759 was carried out. Usage of the β-Gal reporter system was excluded since both of these strains carry a copy of the *lacZ* gene under the control of the Pu promoter in the chromosome (Table 1). In addition to plasmids p1332S/C and p1332D/C characterized before, plasmid p1332IHF/C was constructed analogously to pKTlacZIHF/C (Table 1, Fig. 1, and Fig. 2). Figure 4C shows that enzyme activities in *ihfA*-deficient *P. putida* A8759 harboring either p1332S/C or p1332IHF/C were about twofold lower than in bacteria carrying plasmid p1332D/C. Thus, the DNA region containing the IHF-binding site had no enhancing effect on the *tnpA* promoter activity in the *ihfA*-deficient *P. putida* strain. In contrast, an obvious negative effect of terminal sequences of Tn*4652* on transcription from the *tnpA* promoter could be seen in the *ihfA*-deficient *P. putida* strain A8759.

***E. coli* IHF specifically binds to both ends of Tn*4652*.** Sequence analysis of the left terminus of Tn*4652* revealed two potential IHF-binding sites from bp 44 to 56 and from bp 59 to 71 (Fig. 2B). To test the possibility that IHF can bind to both ends of the transposon, a gel mobility shift assay was carried out. For binding reactions, crude lysates prepared from both *E. coli* and *P. putida* PaW85 cells were used. Figure 6 demonstrates that IHF from *E. coli* specifically retards DNA fragments containing either the left end (Fig. 6A) or right end (Fig. 6B) of the transposon. No probe retardation was detected when cell extract from *E. coli* WM2017 defective for IHF was used (Fig. 6, lanes 3, 8, and 9). Complementation of this IHF-negative strain with plasmid pHNβα carrying *ihfA* and *ihfB* restored the shift (Fig. 6, lanes 4, 10, and 11). However, we could not detect any specific shift with cell lysate from *P. putida* PaW85 either with the right end or with the left end of the transposon (data not shown). Additionally, a gel shift assay with the DNA fragment of the Pu promoter region known to contain an IHF-binding site (1, 13) was carried out as a control to test whether this site binds IHF from cell lysate of *P. putida*. However, although the DNA segment of the Pu promoter region specifically bound IHF from *E. coli* (Fig. 6C, lanes 13 and 14), no probe retardation was detected in the cell lysate from *P. putida* (Fig. 6C, lanes 17 and 18).

Recently, it has been reported that the IHF content of *Pseudomonas aeruginosa* is about 30 times lower than that in *E. coli* (12). To test whether the amount of IHF was too low to detect the shift (up to ~4 μg of total cell protein per reaction was used), we repeated the gel mobility shift assay with more concentrated *P. putida* cell lysates. Indeed, 20 μg of total protein from *P. putida* PaW85 retarded the Tn*4652* right-end DNA probe and revealed the presence of two distinct complexes, C1 and C2 (Fig. 7A, lanes 3 and 4). C1 moved as fast as the complex containing *E. coli* IHF (Fig. 7A, lane 2), which suggested that C1 could represent *P. putida* IHF bound to a

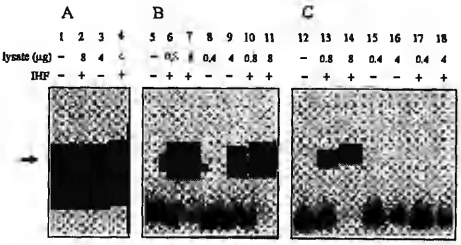

FIG. 6. Gel shift assay of in vitro binding of IHF from cell lysates of *E. coli* and *P. putida* PaW85 to the left end of Tn*4652* (A), to the right end of Tn*4652* (B), and to the DNA fragment containing the Pu promoter region (C). Cell lysates used were from *E. coli* WM2015 (lanes 2, 6, 7, 13, and 14), *E. coli* WM2017 defective in the *ihfA* and *ihfB* genes (lanes 3, 8, 9, 15, and 16), *E. coli* WM2017 complemented with plasmid pHNβα (lanes 4, 10, and 11), and *P. putida* PaW85 (lanes 17 and 18). No cell lysate was added to reaction mixtures in lanes 1, 5, and 12. The specific IHF-DNA complex is indicated by the arrow.

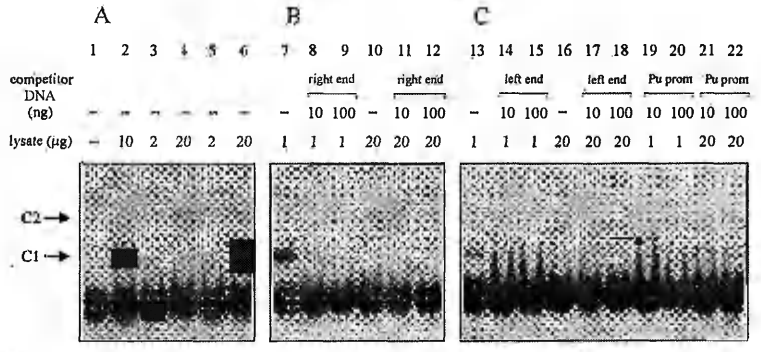

FIG. 7. (A) Gel shift assays demonstrating specific binding of some unknown factor(s) of *P. putida* to the right end of Tn*4652*; (B) competition with nonlabeled right-end DNA; (C) competition with DNA fragments containing either the left end of Tn*4652* or the Pu promoter region. The two complexes (C1 and C2) formed are indicated by arrows; C1 in lanes 2, 7, 8, 13, 14, and 19 represents binding of IHF from *E. coli* HB101 cell lysate to the DNA probe. Cell lysates used were from *E. coli* HB101 (lanes 2, 7 to 9, 13 to 15, 19, and 20), *P. putida* PaW85 (lanes 3, 4, 10 to 12, 16 to 18, 21, and 22), and *P. putida* A8759 defective in the *ihfA* gene (lanes 5 and 6). No cell lysate was added to the reaction mixture in lane 1. In some experiments, a weak band between C1 and C2 was detected when *P. putida* crude lysate was used.

probe. However, two complexes were also seen if lysate from the *P. putida ihfA*-defective strain A8759 was used in the gel shift assay (Fig. 7A, lanes 5 and 6). To test whether these complexes were specific for the right end of Tn*4652*, competition experiments with nonlabeled DNA probes were carried out. Addition to the binding reaction of the right-end DNA as a competitor suppressed the formation of CI effectively, while suppression of C2 needed more competitor DNA (Fig. 7B, lanes 11 and 12). In contrast, DNA fragments of the left end of Tn*4652* and from the Pu promoter (which were shown to bind IHF from *E. coli*) did not compete out either C1 or C2 (Fig. 7C, lanes 17, 18, 21, and 22). Both of these competitor DNAs successfully suppressed complex formation of the *E. coli* IHF with the *tnpA* promoter region (Fig. 7C, lanes 14, 15, 19, and 20).

DISCUSSION

Many transposons require bacterial host proteins for transposition. IHF is known to participate in transposition of several transposons (32, 41, 42, 47), and it also modulates transposase expression in some cases (44). The experiments presented in this report show that transcription from the Tn*4652* transposase promoter is positively affected by IHF.

We found that both ends of Tn*4652* contain sequences similar to the IHF-binding consensus sequence (Fig. 2). The putative IHF-binding site at the right end of the transposon is located at positions from −73 to −85 relative to the transcription start point of the *tnpA* gene. Transposase promoter constructs carrying sequences of the right end of Tn*4652* including an IHF-binding site revealed enhanced activity of the reporter gene *pheB* or *lacZ* in *P. putida* in comparison with the constructs lacking the IHF site upstream of the *tnpA* promoter (Fig. 4A and B). Enzyme assay using the *P. putida ihfA*-defective strain A8759 confirmed that IHF was involved in stimulation of transcription from the *tnpA* promoter. No positive effect of the IHF-binding site on promoter activity was detected in this strain (Fig. 4C). In contrast, the right end of the transposon had a negative effect on *tnpA* promoter activity when IHF was absent: both constructs p1332S/C and p1332IHF/C containing the IHF site exhibited even lower enzyme activity than p1332D/C in the *ihfA*-defective strain A8759 (Fig. 4C). This finding indicates that the IHF site, if not occupied by IHF protein, can suppress the *tnpA* promoter activity. It is known that IHF is involved in activation of the Pe promoter of bacteriophage Mu by a dual mechanism. IHF stimulates transcription from the Pe promoter directly and also indirectly via alleviation of the H-NS-mediated repression (44). Analogously, we suggest that binding of IHF to the right end of Tn*4652* enhances transcription from the *tnpA* promoter not only directly but also indirectly by competing with some unknown negatively acting factor for the binding site.

Enzyme assay demonstrated that transcription from the *tnpA* promoter was higher in the Tn*4652*-free *P. putida* strain PRS2000 than that in strains PaW85 and KT2442, which contain a copy of Tn*4652* in the chromosome (Fig. 4A and C). We propose that the chromosomally located copy of Tn*4652* may code for functions affecting the *tnpA* promoter activity in *P. putida* PaW85 and KT2442. Since terminal sequences of transposons are presumed to bind transposase, it is possible that transcription from the *tnpA* promoter is modulated by the transposase of Tn*4652*, too.

Enzyme assay revealed that the Tn*4652 tnpA* gene promoter is silent in *E. coli* (Fig. 4A). Comparison of promoter specificities of RNA polymerases from *E. coli* and *Pseudomonas* spp. revealed that they transcribe similarly well different promoters of both species (16, 19). Considering these experiments, we do not believe that the difference between the *E. coli* and *P. putida* polymerases causes the silence of the *tnpA* promoter in *E. coli*. The possibility that activation of the transcription from the promoter needs some Tn*4652*-encoded factor could be also eliminated because the promoter is functional in Tn*4652*-free *P. putida* strain PRS2000. Thus, the presence of some host factor specific to *P. putida* is required for the promoter function. We propose two alternative explanations for the silence of the *tnpA* promoter in *E. coli*. First, transcription initiation from the *tnpA* promoter needs an activator protein that is missing in *E. coli*. Many σ^{70}-dependent promoters lacking a well-conserved −35 region are known to be subjected to activation by the regulatory proteins (9). Correspondingly, the

−35 region of the *tnpA* promoter revealed no homology with the σ^{70}-recognized −35 consensus hexamer although the −10 region TATGCT of the *tnpA* promoter was considerably homologous with the σ^{70}-recognized −10 hexamer consensus sequence TATAAT. On the other hand, the *tnpA* promoter might not be necessarily recognized by σ^{70}. Therefore, an alternative sigma factor, absent in *E. coli*, might be required for promoter activation. This possibility is illustrated by the fact that alternative sigma factors of pseudomonads, not complemented in *E. coli*, are essential for the expression of several iron-regulated promoters of *Pseudomonas* strains (11, 38).

Up to now, there had been no reports about in vitro binding experiments with *P. putida* IHF. Using the gel mobility shift assay, we demonstrated that both ends of Tn*4652* can bind IHF from cell lysate of *E. coli* (Fig. 6A and B). However, we could not detect an IHF-caused shift under the same conditions when the cell extract of *P. putida* PaW85 was used. We have also carried out gel shift experiments with lysate of an *E. coli* IHF-defective mutant complemented with plasmids carrying cloned IHF genes of *P. putida*. However, we did not detect IHF-caused retardation of a DNA fragment containing the right end of the transposon or the Pu promoter region of the TOL plasmid as a control (data not shown). This indicates that the properties of *P. putida* and *E. coli* IHF are different, and the experimental conditions used in in vitro binding assay were not optimal for the binding of *P. putida* IHF. However, in vivo experiments with hybrid IHF protein containing *P. putida* and *E. coli* subunits have showed that the hybrid protein efficiently functioned as a regulator of the pL promoter in *E. coli* (8), which suggests that in vivo binding properties of the hybrid IHF protein may be similar to those of *E. coli* IHF. Nevertheless, it would be interesting to compare the properties of IHF purified from *P. putida* with that from *E. coli*.

Gel mobility shift experiments with the transposon right-end DNA probe and crude lysate of *P. putida* PaW85 confirmed formation of two specific complexes (C1 and C2). Probably neither of them corresponded to IHF bound to the probe (Fig. 7), because these complexes were also detected by using cell lysate from *P. putida ihfA*-defective strain A8759 (Fig. 7A). Also, neither the Tn*4652* left-end nor the Pu promoter-region DNA containing an IHF site suppressed formation of these complexes. Therefore, we consider that complexes detected by using the right end of the transposon represent some other protein(s) bound to the probe. Since C1 and C2 were formed with cell lysate from Tn*4652*-free *P. putida* PRS2000 as well (data not shown), we suggest that some *P. putida* host protein(s) participates in these complexes. Although the identity of the protein(s) is not established, it is tempting to speculate that complexes C1 and C2 contain the repressor protein which acts negatively on transcription from the *tnpA* promoter in a *P. putida ihfA*-deficient strain (Fig. 4C). Still, we cannot exclude the possibility that an activator, essential for the activity of the *tnpA* promoter in *P. putida*, was bound to the right end of Tn*4652* in the gel shift assay. However, further experiments are needed to test these possibilities.

Our results demonstrate that IHF from *E. coli* binds specifically to both ends of Tn*4652*, just adjacent to the terminal inverted repeats that are presumed to bind the transposase. Other mobile elements are also known to contain IHF-binding sites at one or both ends (18, 25, 32, 46). It is known that γδ transposase of γδ (Tn*1000*) transposon and IHF bind cooperatively to both ends of the element (46). Additionally, IHF is required in in vitro reactions of IS*10* transposition (35). Therefore, we suggest that besides activation of the *tnpA* promoter, IHF may participate in Tn*4652* transposition also either by modulating the binding of transposase to the ends of the transposon or by influencing formation of nucleoprotein complexes needed in subsequent transposition reactions.

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Regulation of the Transposase of Tn*4652* by the Transposon-Encoded Protein TnpC

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Transposition is a DNA reorganization reaction potentially deleterious for the host. The frequency of transposition is limited by the amount of transposase. Therefore, strict regulation of a transposase is required to keep control over the destructive multiplication of the mobile element. We have shown previously that the expression of the transposase (*tnpA*) of the *Pseudomonas putida* PaW85 transposon Tn*4652* is positively affected by integration host factor. Here, we present evidence that the amount of the transposase of Tn*4652* in *P. putida* cells is controlled by the transposon-encoded protein (TnpC). Sequence analysis of the 120-amino-acid-long TnpC, coded just downstream of the *tnpA* gene, showed that it has remarkable similarity to the putative polypeptide encoded by the mercury resistance transposon Tn*5041*. As determined by quantitative Western blot analysis, the abundance of TnpA was reduced up to 10-fold in the intact *tnpC* background. In vivo experiments using transcriptional and translational fusions of the *tnpA* gene and the reporter gene *gusA* indicated that TnpC operates in the regulation of the transposase of Tn*4652* at the post-transcriptional level.

Transposition is a DNA rearrangement process in which a discrete DNA sequence is inserted into a new location in the genome. This reaction is performed by an element-encoded protein called transposase. Mobility of bacterial transposons is strictly regulated to a very low level (10^{-3} to 10^{-8} reactions per element per generation [18]) to maintain the balance between their propagation and the potential destructive mutagenic effect on their hosts. The rate of transposition is largely determined by the amount of active transposase. Many of the mechanisms that limit transposase gene expression or transposase protein activity have been described (reviewed in reference 18). These downregulation mechanisms frequently operate coordinately at different levels of transposase expression and help maintain precise control over the amount and activity of transposase in bacteria.

Most of the transposase promoters are weak and often downregulated by transcriptional repressors that may be both transposon-encoded proteins (8, 19, 22) and host factors (13, 21). DNA methylation is also shown to modulate transposase expression in some cases. IS*10*, IS*50*, and IS*903* carry GATC methylation sites in their transposase promoter regions, and absence of methylation results in increased activity of these promoters (28, 36).

For many transposons, the level of transposase expression is determined by the efficiency of transposase gene translation. Inefficient translation, inhibition of translation by antisense RNA, and programmed translational frameshifting have been described as post-transcriptional mechanisms to regulate transposase expression (7, 9, 31). For example, translation of mRNAs of the transposases of IS*10* and IS*30* is inhibited by antisense RNAs (2, 31). For synthesis of full-length transposase of several insertion elements, programmed translational frameshifting between the two sequential open reading frames (ORFs) is needed (reviewed in reference 7). Also, transposase stability may be related to control of transposition activity. For instance, IS*903* transposase is demonstrated to be sensitive to the *Escherichia coli* Lon protease (9).

Transposition of several transposons is controlled by regulation of transposase catalytic activity. IS*1* and Tn*5* modulate transposase catalytic activity with inhibitor proteins coded from the same ORF as the transposase (20, 22). Additionally, many transposases are known to require bacterial host proteins for their activity. Integration host factor (IHF), which is known to alter the conformation of DNA, is the host factor most usually involved in transposition (1, 30, 35). Recently, activity of the transposase of Tn*3* was demonstrated to be stimulated by a quite different type of host factor, acyl carrier protein (23).

Pseudomonas putida PaW85 carries transposon Tn*4652* in its chromosome. Tn*4652* is a 17-kb-long deletion derivative of the toluene degradation *xyl* gene-carrying transposon Tn*4652*. Tsuda and Iino (33) have shown that, according to its transposition properties, Tn*4652* belongs to the Tn*3* family of transposons. We have sequenced the transposase gene *tnpA* of Tn*4652* and shown that transcription from the *tnpA* promoter is positively affected by IHF (12).

In this study, we demonstrate that the amount of Tn*4652* transposase (TnpA) is downregulated by the Tn*4652*-encoded protein TnpC. The ORF encoding the 120-amino-acid protein TnpC begins just downstream of *tnpA* and exhibits striking similarity to an ORF of Tn*5041* encoding a putative 120-amino-acid-long polypeptide. In vivo experiments using transcriptional and translational fusions of the *tnpA* gene and the reporter gene *gusA* indicate that TnpC interferes with the regulation of TnpA at the post-transcriptional level.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* TG1 (6) was used for the DNA cloning procedures. Bacteria were grown on Luria-Bertani medium (24). Antibiotics were added, with final concentrations as follows: ampicillin, 100 μg/ml for *E. coli*; carbenicillin, 1,500 μg/ml for *P. putida*. *P. putida* was incubated at 30°C. Early-stationary-phase cultures were used for enzyme assays. *E. coli* was transformed with plasmid DNA as described by Hanahan (11). *P. putida* was electrotransformed according to the protocol described by Sharma and Schimke (29).

DNA manipulations. DNA sequencing was performed with the Sequenase version 2.0 DNA sequencing kit (Amersham). For cloning of the *tnpA* gene into pET19b, the *Xba*I and *Nde*I restriction sites were designed in the 5′ end of *tnpA*

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or construction	Source or reference
E. coli		
TG1	*supE hsd*Δ*5 thi* Δ(*lac-proAB*) F′ (*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15)	5
BL21(DE3)	*hsdS gal* (λcI*ts*857 *ind*1 *Sam*7 *nin*5 *lac*UV5-T7 gene *1*)	32
P. putida		
PaW85	Tn*4652*	4
PRS2000	Tn*4652* free	34
Plasmids[a]		
pBluescript KS	Cloning vector (Ap^r)	Stratagene
pET19b	Protein expression vector (Ap^r)	Stratagene
pET19-tnpA	*tnpA* is fused with histidine tag in pET19b	This work
pKT240	Cloning vector (Ap^r Km^r)	3
pEST1354	Plasmid containing Tn*4652* upstream of the *pheBA* operon	17
pKTtnpA(D/H)	Tn*4652 tnpA* gene within the 3.2-kb *Dra*I-*Hin*dIII fragment cloned into pKT240	This work (Fig. 3)
pKTtnpA(D/P)	Tn*4652 tnpA* and *tnpC* genes within the 3.7-kb *Dra*I-*Pvu*I fragment cloned into pKT240	This work (Fig. 3)
pKTtnpA(D/P)*	pKTtnpA(D/P) with the *tnpC* gene disrupted by frameshift	This work
pKTGC/tnpA	pKT240 plus pDEL2-GC promoter region plus the promoterless *tnpA* gene	This work
pKTGC/tnpAC	pKT240 plus pDEL2-GC promoter region plus the promoterless *tnpA* gene with *tnpC*	This work
pGUS102	Promoter probe vector containing the *gusA* gene cloned as a 1.8-kb *Eco*RI fragment into pBR322	A. Eriksson
pKTGUS	Vector for translational fusions containing the *gusA* gene without translation initiation codon ATG in pKT240; *Hin*dIII restriction site designed at the 5′ end of the *gusA* is suitable for in-frame cloning	This work
pTr1	PDEL2-GC promoter region plus 42 nt of the coding region of *tnpA* fused with *gusA* in pKTGUS	This work (Fig. 5B)
pTr2	PDEL2-GC promoter region plus 546 nt of the coding region of *tnpA* fused with *gusA* in pKTGUS	This work (Fig. 5B)
pTr3	PDEL2-GC promoter region plus 1,166 nt of the coding region of *tnpA* fused with *gusA* in pKTGUS	This work (Fig. 5B)
pKT-ACG	*gusA* is cloned downstream of *tnpC* in pKTtnpA(D/P)	This work (Fig. 6B)
pKT-AdelCG	*Cla*I-*Cfr*10I deletion derivative of pKT-ACG	This work (Fig. 6B)
pKT-a1CG	*Dra*I-*Cla*I deletion derivative of pKT-ACG	This work (Fig. 6B)
pKT-a2CG	*Dra*I-*Nru*I deletion derivative of pKT-ACG	This work (Fig. 6B)
pKT-CG	*Dra*I-*Cfr*10I deletion derivative of pKT-ACG	This work (Fig. 6B)

[a] Oligonucleotides used for construction of the plasmids are described in Materials and Methods.

by using oligonucleotide pETtnpA (5′-CC*TCTAGA*[*Xba*I]*CATATG*[*Nde*I]TGTT CAATGGCATCGGTGG-3′). For amplification and cloning of the PDEL2-GC promoter from plasmid pEST1414 (15), oligonucleotides YrgHind (5′-CCA*AAG CTT*[*Hin*dIII]TGTTTACGATCCAGGC-3′) and AB (5′-GTATGCTTGGCAG TCGT) were used. The *Cla*I restriction site just flanking the −10 hexamer of the PDEL2-GC promoter was suitable for cloning of *tnpA-gusA* translational fusions. To design the *Cla*I restriction site in the 5′ end of the *tnpA*, oligonucleotide GCtnpA (5′-CTA*ATCGAT*[*Cla*I]TTTGCCTCGCTTGGGGGAT-3′) was used. For construction of vector pKTGUS for translational fusions, oligonucleotides Gus1 (5′-CTA*AAGCTT*[*Hin*dIII]ACGTCCTGTAGAAACCCCAA-3′) and Gus2 (5′-ACTGATCGTTAAAACTGCCTGG-3′) were used. For construction of translational fusions of *tnpA* with the reporter gene *gusA*, oligonucleotide GCtnpA and either oligonucleotide Tr1 (5′-GGT*AAGCTT*[*Hin*dIII]CTGGGCA AGATAGGGTAGGCT-3′), Tr2 (5′-ACC*AAGCTT*[*Hin*dIII]GGCGCTCGAGT CACGACTA), or Tr3 (5′-GA*GAAGCTT*[*Hin*dIII]TCCCGAATCAGGCTGCC AG) were used. For construction of the plasmids pTr1, pTr2, and pTr3 with the *tnpC* gene, the *tnpC* under the control of the benzoate-inducible P_1 promoter of the *pheBA* operon (14) was cloned downstream of the *tnpA-gusA* translational fusions. The *tnpC* expression cassette was initially designed in pBluescript and was subsequently cloned into plasmids pTr1, pTr2, and pTr3. Inducible expression of the *tnpC* gene under control of the P_1 promoter was tested in plasmid pKTtnpA(D/H) by the ability of TnpC to downregulate TnpA.

For cloning of *gusA* downstream of *tnpC* in transcription fusion *tnpAC-gusA* in plasmid pKT-ACG, an *Eco*RI restriction site was designed in the 3′ end of *tnpC* by using oligonucleotide TnpCEco (5′-CCA*GAATTC*[*Eco*RI]CCAAGTGCTTA CTGTTCGTG-3′).

Overexpression and purification of His-TnpA. To obtain soluble His-TnpA, *E. coli* BL21(DE3)(pET19-tnpA) was grown at 22°C in 200 ml of Luria-Bertani medium. Expression of His-TnpA was induced for 3.0 h by adding isopropyl-β-D-thiogalactopyranoside (IPTG; final concentration, 0.4 mM) when the culture optical density at 590 nanometers reached about 1.0. Cells were pelleted and sonicated in buffer A (100 mM Tris-HCl [pH 7.5], 0.25 mM EDTA, 5 mM β-mercaptoethanol, 1 M NaCl, 0.1% Triton X-100, 10% glycerol). The cell lysate was centrifuged at 15,000 × *g* for 20 min. Imidazole (100 mM) was added to the supernatant before it was loaded into the Ni^{2+}-immodiacetic acid-activated chelating Sepharose 6B column previously equilibrated with buffer A. The column was washed with 8 volumes of buffer A supplemented with 100 mM imidazole (pH 6.5). Purified His-TnpA was eluted with buffer A containing 500 mM imidazole. Imidazole and excess salt were removed by dialyzing the eluate against buffer B (75 mM Tris-HCl [pH 7.5], 0.2 mM EDTA, 5 mM β-mercaptoethanol, 200 mM NaCl, 0.1% Triton X-100, 10% glycerol), and the purified protein was stored at −75°C.

Preparation of cell lysates and immunoblotting of TnpA. Cell lysates were prepared from 30-ml early-stationary-phase cultures. Cells were pelleted and sonicated in 500 μl of 0.5× buffer B. Protein concentration in cleared lysates was estimated as described by Bradford (5). Equal amounts of total protein (40 μg) were used for a Western immunoblotting assay. Proteins were separated by sodium dodecyl sulfate-8% polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (BA 85; Schleicher & Schuell). For Western blotting, the membranes were probed with mouse anti-TnpA polyclonal serum diluted 1:5,000, followed by alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (LabAS Ltd., Tartu, Estonia) diluted 1:5,000. The blots were developed with bromochloroindolyl phosphate and nitroblue tetrazolium.

Enzyme assays. β-Glucuronidase (GUS) activity was assayed by using *p*-nitrophenyl β-D-glucuronide as the substrate (26). The degradation product of *p*-nitrophenyl β-D-glucuronide, *p*-nitrophenol, was detected at 405 nm and GUS-specific activities were measured in nanomoles of *p*-nitrophenol per minute per optical density unit of cell culture at 590 nm.

Nucleotide sequence accession numbers. The nucleotide sequences of *tnpA* and *tnpC* have been deposited in the EMBL database under the accession no. X83686.

RESULTS

Overexpression and purification of the transposase of Tn*4652*. To investigate the regulation of the Tn*4652*-encoded transposase TnpA, the transposase protein was overexpressed and purified to obtain antibodies against it. Coding sequence

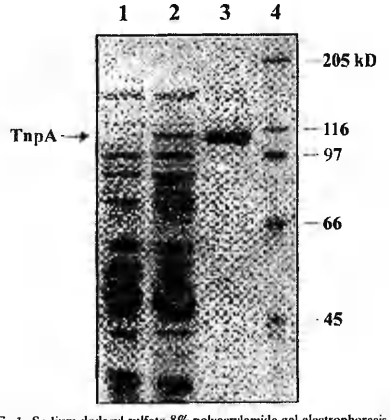

FIG. 1. Sodium dodecyl sulfate-8% polyacrylamide gel electrophoresis demonstrating overexpression and purification of His-tagged TnpA in *E. coli* BL21(DE3). Lane 1, crude extract from *E. coli* BL21(DE3)(pET19-tnpA); lane 2, as described for lane 1, but induced with 0.4 mM IPTG; lane 3, purified His-TnpA; lane 4, standard molecular weight markers.

of the *tnpA* gene was fused with N-terminal histidine tag in the protein expression vector pET19b. The His-tagged TnpA was overexpressed in *E. coli* BL21(DE3) and purified by single-step Ni^{2+}-chelate affinity chromatography. Purification yielded near-homogeneous TnpA protein (Fig. 1, lane 3). The molecular mass of TnpA was estimated to be approximately 114 kDa, which is consistent with the predicted molecular mass of 114.3 kDa suggested by the results of the *tnpA* gene sequence analysis (12).

Amount of TnpA is downregulated by the Tn*4652*-encoded factor. To observe TnpA expression in different genetic backgrounds, we used Western blot analysis with anti-TnpA polyclonal antiserum. We could not detect TnpA in the cell lysate of *P. putida* PaW85 that carries Tn*4652* in its chromosome (data not shown). Similarly, TnpA was not detectable in the cell lysates of *P. putida* PaW85 and PRS2000 (free of Tn*4652*) which harbored Tn*4652*-containing plasmid pEST1354 (17) (Fig. 2, lanes 2 and 4). In order to test whether TnpA expression is downregulated either by some *P. putida* host factor or by a Tn*4652*-encoded factor, we generated a subclone of this transposon. The *tnpA* gene with its native promoter was cloned into the broad-host-range vector plasmid pKT240 to obtain the plasmid pKTtnpA(D/H). This plasmid contained the 3.2-kb fragment of the right arm of Tn*4652* from the distal *Dra*I restriction site up to the *Hin*dIII site (Fig. 3 and Table 1). Western blot analysis of crude lysates prepared from the cells of *P. putida* PaW85 and PRS2000 harboring the plasmid pKTtnpA(D/H) allowed detection of the TnpA protein (Fig. 2, lanes 3 and 5). This result pointed to a transposon-encoded regulator of transposase located outside of the *Dra*I-*Hin*dIII restriction fragment of Tn*4652*.

Localization and sequencing of the DNA region of Tn*4652* influencing the expression of TnpA. In order to localize the DNA region that controls the accumulation of TnpA, deletion analysis of Tn*4652* was performed. The amount of TnpA was tested in lysates of Tn*4652*-free *P. putida* PRS2000 cells carrying plasmids which contained the *tnpA* gene linked to different regions of Tn*4652*. Plasmid pKTtnpA(D/P) carried the right arm of DNA of Tn*4652* (including also the *tnpA* gene) from the distal *Dra*I restriction site up to the *Pvu*II site (Fig. 3 and Table 1). Results of the Western blot analysis presented in Fig. 2 show that TnpA was detectable in the cell lysates of bacteria

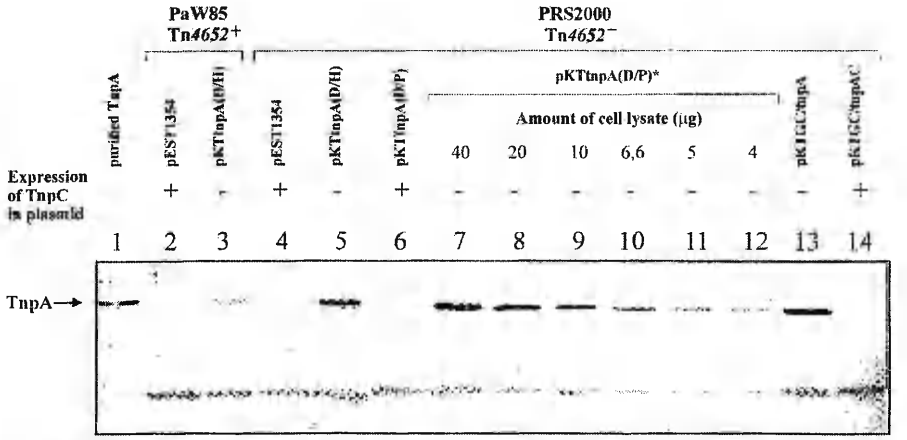

FIG. 2. Western immunoblot analyses of *P. putida* PaW85 and PRS2000 cell lysates by using anti-TnpA polyclonal antibodies. Lane 1, purified TnpA protein; lane 2, crude extract from *P. putida* PaW85(pEST1354); lane 3, crude extract from *P. putida* PaW85[pKTtnpA(D/H)]; lane 4, crude extract from *P. putida* PRS2000(pEST1354); lane 5, crude extract from *P. putida* PRS2000[pKTtnpA(D/H)]; lane 6, crude extract from *P. putida* PRS2000[pKTtnpA(D/P)]; lanes 7 through 12, gradual dilutions of crude extracts of *P. putida* PRS2000[pKTtnpA(D/P)*]; lane 13, crude extract from *P. putida* PRS2000(pKTGC/tnpA); lane 14, crude extract from *P. putida* PRS2000(pKTGC/tnpAC). The amount of crude lysate was 40 μg per lane except that for lanes 8 to 12, gradual dilutions of cell lysate of *P. putida* PRS2000[pKTtnpA(D/P)*] were used.

FIG. 3. Genetic organization of *tnpA* and *tnpC* in the right arm of the Tn*4652*. Right inverted repeat of Tn*4652* is marked by a black triangle. Restriction sites relevant to this study are indicated. The arrows indicate the direction of transcription of the *tnpA* and *tnpC* genes. The promoter of the *tnpA* gene is designated p_{tnpA}.

harboring the plasmid pKTtnpA(D/H) (Fig. 2, lane 5), but not in cell lysates of bacteria harboring the plasmid pKTtnpA(D/P) (Fig. 2, lane 6). According to these results, the putative regulator of TnpA was localized just downstream of the *tnpA* gene, in the DNA region extending to the *Pvu*II site.

Sequence analysis of the DNA region downstream of the *tnpA* gene revealed a 360-nucleotide (nt)-long ORF starting 8 nt apart from the stop codon of the *tnpA* (Fig. 3). The predicted protein encoded by this ORF is 120 amino acids long, with a calculated molecular mass of 13.0 kDa. Comparison of the deduced amino acid sequence of the putative regulator (TnpC) of the transposase of Tn*4652* with the translated sequences of genes in the EMBL database with the BLAST program revealed homology of TnpC to the putative 120-amino-acid-long polypeptide encoded by the mercury resistance transposon Tn*5041* (Fig. 4). Amino acid sequence identity of 52% and similarity of 75% were demonstrated.

Intact ORF of *tnpC* is needed for the downregulation of TnpA. In order to test whether the TnpC protein indeed acts on the expression of the *tnpA* gene product, we disrupted the ORF of TnpC in the plasmid pKTtnpA(D/P). The unique *Hin*dIII restriction site in *tnpC* was used to generate a +1 frameshift into the coding sequence of the *tnpC* gene [plasmid pKTtnpA(D/P)*]. Western blot analysis of the crude lysates prepared from the cells of *P. putida* PRS2000 harboring either pKTtnpA(D/P) or pKTtnpA(D/P)* demonstrated that in-frame *tnpC* was needed to decrease the amount of TnpA (Fig. 2, compare lane 6 to lane 7).

However, downregulation of TnpA by TnpC was not complete. We could also detect a small amount of TnpA in the cell lysates of *P. putida* PRS2000 while intact *tnpC* was present (not visible in Fig. 2, but seen in overdeveloped filters). To quantify the extent of downregulation of TnpA by TnpC, gradual dilutions of cell lysates of *P. putida* PRS2000[pKTtnpA(D/P)*] were tested on a Western blot and compared to the amount of TnpA detected in cells containing pKTtnpA(D/P). Four independent measurements with different preparations of cell lysates indicated that the presence of TnpC decreased the abundance of TnpA about 10-fold (Fig. 2, compare lane 6 to lanes 7 through 12).

Testing the effect of TnpC on transcriptional and translational initiation of *tnpA*. Quantification of the *tnpA*-specific mRNA in both *tnpC*-expressing and *tnpC*-deficient backgrounds could answer the question of whether TnpC would affect the expression of the *tnpA* gene product at the transcriptional or at the post-transcriptional level. Since we failed to detect *tnpA*-specific mRNA in both primer extension and Northern blot analyses, alternative approaches were used to solve this problem. In order to test whether TnpC represses transcription initiation from the *tnpA* promoter, we replaced the native promoter of the *tnpA* gene with the constitutive promoter PDEL2-GC described by members of our group previously (15). The promoter of the *tnpA* gene was earlier localized into the terminal 122-nt DNA region of the right end of Tn*4652*, and the transcription starting point of the *tnpA* gene was mapped at 129 nt from the end of this transposon (12). The DNA fragments lacking the terminal 125 nt from the right end of Tn*4652* and containing either gene *tnpA* or *tnpAC* were fused with the PDEL-GC promoter. The fusions were designed without altering the 5′ end of the *tnpA*-specific mRNA (Table 1 and Materials and Methods). Obtained plasmids pKTGC/tnpA and pKTGC/tnpAC were introduced into *P. putida* PRS2000, and Western blot analysis of the cell lysates was performed. Data presented in Fig. 2, lanes 13 and 14, demonstrated that although the promoter of the *tnpA* gene was replaced with another one, the expression of TnpA was still downregulated by TnpC.

To investigate whether TnpC affects the expression of the *tnpA* gene at the level of initiation of transcription or translation, we constructed different translational fusions of the 5′ end of the *tnpA* gene (up to one-third of the gene) with the reporter gene *gusA* (encodes GUS) (Fig. 5B). Plasmids pTr1, pTr2, and pTr3 contained 42, 546, and 1,143 nt of the coding region of the *tnpA* gene, respectively, fused with the *gusA* gene. The control plasmid for translational fusions was designed by substituting transposase start codons (there are two potential ATG start codons of *tnpA* separated by 6 nt) for ATC in translation fusion plasmid pTr3. The obtained plasmid was introduced into *P. putida* PRS2000, but no GUS activity was detectable in an enzyme assay using this strain. Thus, this control experiment confirms that translation of the *tnpA* and *gusA* fusion starts from the ATG of the *tnpA* gene. All translational fusions were expressed under the PDEL2-GC promoter (Fig. 5B; Table 1). The *tnpC* gene, if present, was expressed in the same plasmids under the control of the benzoate-inducible P_i promoter of the *pheBA* operon (14) (see Materials and Methods). No negative effect of TnpC on GUS activity was observed when expression of these translational fusions was tested in *P. putida* PRS2000 cells in either the presence or absence of benzoate (Fig. 5A). On the basis of these experiments and considering the results of the promoter change experiment described above, we suggest that TnpC could influence the accumulation of TnpA after either the transcriptional or translational initiation of the *tnpA* gene.

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Tn4652  MIEIPPATFRVTPYGEVDGVALDKLRASFDTSQLLRLVDQFDACLAELGGVVAVRDELLK   60
          + IPPA+FRVTPYG+VD    LD LRAS+DT+QLL LVD+ DACLAE+GGV ++R++ L+
Tn5041  MHIPPASFRVTPYGDVDTKVLDSLRASYDTAQLLNLVDRLDACLAEIGGVGSIREDFLR

Tn4652  LHAMALTLVEGVPLTVPTENA-CIWTEAESLQQNLETLAGWVRSAQAGIIPLVNLAPDHEQ 120
          LH MA+T++EG PLTV T +   IW +A +LQ+++  L   +++     + PL  LAPD++
Tn5041  LHGMAMTVLEGFPLTVATSDVDSIWAQAMALQEDTSALCSCLQAGSKHVAPLAALAPDYQD
```

FIG. 4. Alignment of the deduced amino acid sequence of TnpC of Tn*4652* with the putative 120-amino-acid-long polypeptide encoded by Tn*5041* (16). Identical amino acids are indicated between the two aligned sequences in boldface. Similar amino acids are marked by plus signs.

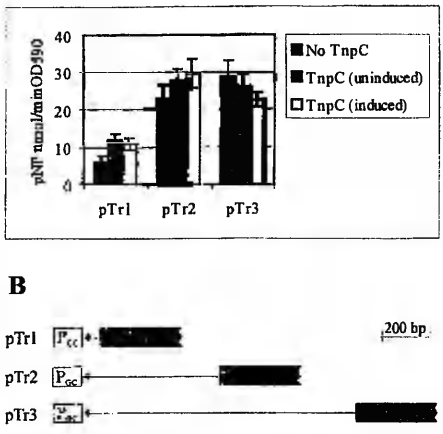

FIG. 5. (A) GUS activities measured in *P. putida* PRS2000 carrying different translational fusion plasmids either together with the *tnpC* gene or without the *tnpC* gene. Na-benzoate (10 mM) was used for the induction of *tnpC*. Data (means ± standard deviations) of at least five independent experiments are presented. (B) Schematic presentation of the translational fusions of the 5′ end of the *tnpA* gene with the reporter gene *gusA*. For each fusion, the PDEL2-GC promoter is indicated by an open box, the 5′ region of *tnpA* is marked by a line, and the translation initiation codon ATG of *tnpA* is indicated by a black diamond.

Localization of the *tnpC* promoter region. The *tnpC* gene lies just downstream of the transposase gene *tnpA*. Thus, the transcription of *tnpC* could be initiated from its own promoter(s), or it could be cotranscribed with the *tnpA* gene from the *tnpA* promoter. In order to measure transcription of the *tnpC* gene, we constructed the plasmid pKT-ACG that contained the native *tnpAC* gene cassette and the reporter gene *gusA* just downstream of the *tnpC* gene (Fig. 6B; Table 1; Materials and Methods). Additionally, deletion derivatives of pKT-ACG lacking different amounts of the sequence from the 5′ end of *tnpA* were generated (pKT-a1CG, pKT-a2CG, and pKT-CG) (Fig. 6B). GUS activity in the cells of *P. putida* PRS2000 harboring these plasmids was estimated (Fig. 6A, white bars). The highest level of GUS activity was detected in *P. putida* PRS2000 cells carrying the plasmid pKT-ACG (contains the full-length *tnpA* gene together with its promoter upstream from *tnpC*). Plasmids pKT-a1CG and pKT-a2CG with deletions from the 5′ sequences of the *tnpA* gene revealed levels of GUS activity 65 to 75% of that measured in cells carrying pKT-ACG (Fig. 6A). Bacteria containing plasmid pKT-CG (lacks *tnpA* but harbors all of *tnpC*), used as a control; showed significantly lower levels of GUS activity. Thus, the estimated GUS activity in our test system represents the sum of the function of the *tnpA* promoter and the internal promoters of the *tnpA* gene.

TnpC does not affect transcription elongation of the *tnpA* gene. Results of the experiments using the *tnpA-gusA* translational fusions revealed that TnpC affected TnpA expression after transcriptional initiation of the *tnpA* gene. To investigate if TnpC operates at the transcription elongation of the *tnpA* gene, we compared the expression of the reporter gene *gusA* in

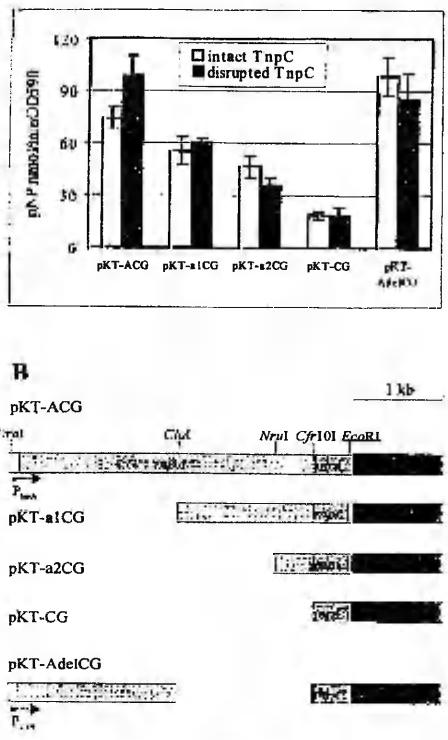

FIG. 6. (A) GUS activities measured in *P. putida* PRS2000 carrying the different transcriptional fusions of the *tnpAC* region with the reporter gene *gusA*. Plasmids with disrupted *tnpC* are marked by asterisks in the text. Data (means ± standard deviations) of at least five independent experiments are presented. pNP, *p*-nitrophenol; OD590, optical density at 590 nanometers. (B) Schematic depiction of plasmids with transcriptional fusions employed in GUS activity assays. Restriction sites used for construction of deletion derivatives of pKT-ACG are indicated. The *Eco*RI restriction site in the 3′ end of *tnpC* is artificial, designed by using oligonucleotide TnpCEco (Materials and Methods). The direction of transcription from the *tnpA* promoter is indicated by an arrow.

plasmids pKT-ACG, pKT-a1CG, and pKT-a2CG (Fig. 6A) and in their TnpC-defective derivatives pKT-AC*G, pKT-a1C*G, and pKT-a2C*G [Fig. 6A; the same strategy employed in the construction of pKTtnpA(D/P)* was used for designing them]. No differences in levels of GUS activity were established in the cells of *P. putida* PRS2000 harboring the 5′ deletion derivatives of the full-length *tnpAC+gusA* gene cassette either with intact *tnpC* or with disrupted *tnpC* (Fig. 6A). A modest repressive effect of intact *tnpC* on GUS activity (approximately 25%) appeared in the pKT-ACG-containing cells of *P. putida* PRS2000 compared to the GUS activity in pKT-AC*G-carrying bacteria (Fig. 6A). To control whether this effect is real and whether it might be obscured by the downstream transcription, plasmids pKT-AdelCG and

pKTAdelC*G lacking the second half of the *tnpA* gene (DNA region between the restriction sites *Cla*I and *Cfr*10I) (Fig. 6B) were constructed. GUS activity levels measured in *P. putida* PRS2000 containing plasmid pKT-AdelCG with either intact or disrupted *tnpC* were similar (Fig. 6A). Therefore, we suggest that instead of influencing the transcription of the *tnpA* gene, TnpC affects TnpA expression post-transcriptionally.

DISCUSSION

A high level of transposition activity would be harmful for the host. Therefore, every transposon must have regulatory mechanisms that keep the level of transposition low. Most of these regulatory mechanisms are developed to control the level of active transposase, the protein that carries out the transposition reaction (reviewed in references 7 and 18). Data presented in this paper show that the abundance of the Tn*4652* transposase TnpA in *P. putida* is downregulated by the transposon-encoded protein TnpC.

The amount of the Tn*4652* transposase in bacterial cell lysates was monitored by Western blot analysis with polyclonal antibodies against the TnpA protein of Tn*4652*. The analysis revealed that this protein was not detectable in either Tn*4652*-containing *P. putida* PaW85 or Tn*4652*-free *P. putida* PRS2000 complemented with Tn*4652* in the plasmid pEST1354 (Fig. 2, lanes 2 and 4). However, subcloning of the *tnpA* gene together with its native promoter allowed us to detect the TnpA protein in both the *P. putida* PaW85 and PRS2000 backgrounds (Fig. 2, lanes 3 and 5). This indicated that some factor encoded by Tn*4652* must be involved in TnpA downregulation. A DNA region affecting the amount of TnpA in bacteria was located just downstream of the *tnpA* gene, where an ORF encoding a 120-amino-acid-long polypeptide was discovered. Disruption of this ORF demonstrated that the protein encoded by the ORF and named TnpC by us was functioning as a regulator of the TnpA protein (Fig. 2, lanes 6 and 7).

Notably, the level of TnpA was elevated in *P. putida* PRS2000 compared to the concentration of TnpA in *P. putida* PaW85 (Fig. 2, lanes 3 and 5). *P. putida* PaW85 contains Tn*4652* in its chromosome. Therefore, we suggest that chromosomally encoded TnpC may act in *trans* and decrease the amount of plasmid-encoded TnpA. For many transposons encoding both transposase and its inhibitor, it has been shown that transposase can function effectively only in *cis* but the inhibitor can act in *trans* as well (22, 27, 31). This mechanism is believed to have evolved to limit the rate of accumulation of transposable elements in the genome (18).

Investigation of TnpC expression revealed that TnpC is expressed from multiple promoters located inside of the *tnpA* gene (Fig. 6A). Part of *tnpC* expression is promoted by the first half of the coding sequence of *tnpA* and possibly also from the *tnpA* promoter. However, a larger amount of the transcription of *tnpC* was initiated from the 3′ terminal half of the *tnpA* gene. Interestingly, data presented in Fig. 6A showed that when the 3′ terminal half of the *tnpA* gene was eliminated (plasmid pKT-AdelCG), the GUS activity was about the same as in the case of the full-length *tnpAC+gusA* cassette (plasmid pKT-ACG). This was approximately twice as high as could be expected on the basis of the simple arithmetical subtraction of downstream promoter activities from the upstream ones. This finding could be interpreted as a diminishing effect of the DNA sequences located in the 3′ terminal half of the *tnpA* gene on the transcription initiated in the first half of *tnpA*. Concerning the expression of *tnpA*, one may speculate that transcription elongation of the *tnpA*-specific mRNA might be influenced by this region. However, we point out that this silencing effect of the downstream region of *tnpA* was not related to the intactness of *tnpC* (Fig. 6A). Therefore, we suspect that besides the TnpC-specific downregulation of TnpA, expression of *tnpA* could also be influenced by a restraint on the rate of transcription elongation of the transposase gene. Indeed, the transcription elongation rate is not constant and there are multiple examples for retardation of transcription elongation due to certain DNA sequences or the nature of nascent RNA (reviewed in reference 25).

The question about the checkpoint of the TnpC action in the regulation of the concentration of TnpA cannot be answered unambiguously. However, our results support the possibility that TnpC operates in the regulation of the transposase of Tn*4652* at the post-transcriptional level. First, it does not interfere with the transcription initiation from the *tnpA* promoter. Exchanging the *tnpA* promoter with another one revealed no effect on the ability of TnpC to downregulate expression of TnpA (Fig. 2, lanes 13 and 14). Second, translational fusions of the *tnpA* gene 5′ end with the reporter gene *gusA* exhibited no sensitivity to the expression of TnpC (Fig. 5A). Thus, TnpC affected neither the transcriptional nor the translational initiation of the *tnpA* gene. Third, testing the effect of TnpC on transcription throughout the *tnpA* gene revealed that transcription elongation was also not altered by TnpC (Fig. 6A). On the basis of these results, we suggest that TnpC functions in regulation of TnpA post-transcriptionally. Moreover, TnpC seems to act after translation initiation, as determined by results obtained from experiments with translational fusions. Herein, it should be noted that it is improbable that translation elongation would be controlled by protein repressors (10). Therefore, it is possible that TnpC acts post-translationally by altering transposase folding and/or transposase stability. However, we cannot exclude the possibility that TnpC is involved in the regulation of *tnpA*-specific mRNA stability.

Comparison of TnpC with the translated sequences of genes in the EMBL database showed a striking similarity between TnpC and a putative 120-amino-acid-long polypeptide encoded by the mercury resistance transposon Tn*5041* (Fig. 4). We have previously shown that TnpA of Tn*4652* is very similar to TnpA of Tn*5041* (12). Up to now, there are no data about the regulation of TnpA of Tn*5041*. However, considering the similarity between TnpC of Tn*4652* and the putative 120-amino-acid polypeptide of Tn*5041*, we suggest that a regulatory mechanism similar to that described for TnpC of Tn*4652* may also regulate the transposase of Tn*5041*.

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Involvement of σ^S in Starvation-Induced Transposition of *Pseudomonas putida* Transposon Tn*4652*

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Transpositional activity of mobile elements can he induced by different environmental stresses. Here, we present evidence that transposition of Tn*4652* is elevated in stationary-phase *Pseudomonas putida* and suppressed in an isogenic σ^S-defective strain. We demonstrate that transcription from the Tn*4652* transposase promoter is controlled by the stationary-phase-specific sigma factor σ^S. To our knowledge, this is the first example of direct stationary-phase-specific regulation of a mobile element transposase. Data presented in this report support the idea that activation of transposition under stressful conditions could be an inducible process.

Transposons are widespread in genomes and have important roles in evolution. Transpositional activity of a mobile element is generally maintained at a low level, yet a high frequency of transposition may occur in response to certain environmental stimuli. It has been shown that different stresses, such as carbon starvation (17), temperature effects (16, 21), and UV light (7), can enhance transposition of bacterial mobile elements. Moreover, it is hypothesized that activation of transposition under stress conditions might serve as an adaptive response to overcome stress and permit new traits to evolve (4, 24). However, the exact molecular mechanisms that underlie stress-induced transposition remain undefined.

Transposon Tn*4652* is a 17-kb-long deletion derivative of the toluene degradation transposon Tn*4651*. *Pseudomonas putida* strain PaW85 harbors Tn*4652* in the chromosome. Mutational processes in *P. putida* PaW85 have been previously studied in starving conditions on phenol minimal plates (13). That work showed the emergence of phenol-utilizing mutants due to the activation of transcription of plasmid-encoded promoterless phenol degradation genes *pheBA* in the plasmid pEST1414. About one-third of the starvation-induced Phe^+ mutants appeared as a result of insertion of Tn*4652* in front of the phenol monooxygenase gene *pheA* (13) (Fig. 1A). The transposition resulted in the formation of a fusion promoter between the transposon-inverted repeat and target DNA (13, 19). Interestingly, transposition of Tn*4652* seemed to depend upon the physiological state of bacteria: transposition frequency increased with time of starvation, whereas no Tn*4652*-linked Phe^+ mutants were detected among growing cells of *P. putida* (13). This indicated that starvation might increase transposition activity of Tn*4652*.

By the adaptation of bacteria to limited nutrient availability, changes in gene regulation take place, i.e., several genes are shut down while others are induced. One of the upregulated genes, *rpoS*, codes for an alternative sigma factor, σ^S, which controls expression of multiple stationary-phase genes (10, 18). In order to examine the potential role of σ^S in the regulation of Tn*4652*, we measured transposition of Tn*4652* in the wild-type *P. putida* PaW85 and in an isogenic σ^S-defective strain.

Transposition of Tn*4652* is decreased in the *P. putida rpoS* mutant strain. Transposition of native Tn*4652* was examined in a starvation assay as described previously (13), except that target plasmid pEST1332 was used instead of pEST1414. Similar to pEST1414, plasmid pEST1332 (15) contains the promoterless *pheBA* operon. However, it is more suitable for probing transposition of Tn*4652* since most of the Phe^+ clones arising on phenol minimal plates emerge from the insertion of Tn*4652* (19). Plasmid pEST1332 contains a specific target site that is preferred over the other sites present in both pEST1332 and pEST1414. To study the effects of σ^S on transposition of Tn*4652*, plasmid pEST1332 was introduced into *P. putida* PaW85 and into its *rpoS*-defective derivative PKS54. Bacteria were grown overnight (ON) in Luria-Bertani medium at 30°C and washed with M9 solution. Approximately 10^9 cells of ON cultures of PaW85 and PKS54 were spread onto five phenol minimal plates, and the accumulation of mutant Phe^+ colonies was monitored upon incubation of the plates at 30°C for 7 days. Results presented in Fig. 1B demonstrate that the emergence of Phe^+ mutants in the *rpoS*-defective *P. putida* was strongly suppressed. Appearance of Phe^+ mutants in the *rpoS*-defective strain was reduced 5 to 10 times compared to that in the wild-type *P. putida*. In order to test the insertions of transposon Tn*4652* into pEST1332, Phe^+ mutants were analyzed by PCR with oligonucleotides pheA, TnR, and TnL (Table 1). PCR analysis of Phe^+ colonies of the *P. putida* wild-type strain revealed that more than 95% of these mutants contained a Tn*4652* insertion upstream of the *pheA* coding region. In contrast, only about 20 to 30% of the Phe^+ colonies that appeared in the *P. putida* σ^S-defective strain carried a Tn*4652* insertion in pEST1332 (Fig. 1B). Thus, the absence of σ^S protein decreased transposition substantially, by more than 1 order of magnitude, but did not prevent it entirely. Here, we want to point out that the Phe^+ colonies revealed similar patterns of insertions in both the wild-type and *rpoS*-defective strains. Also, previous results indicate that RpoS is not obligatory for

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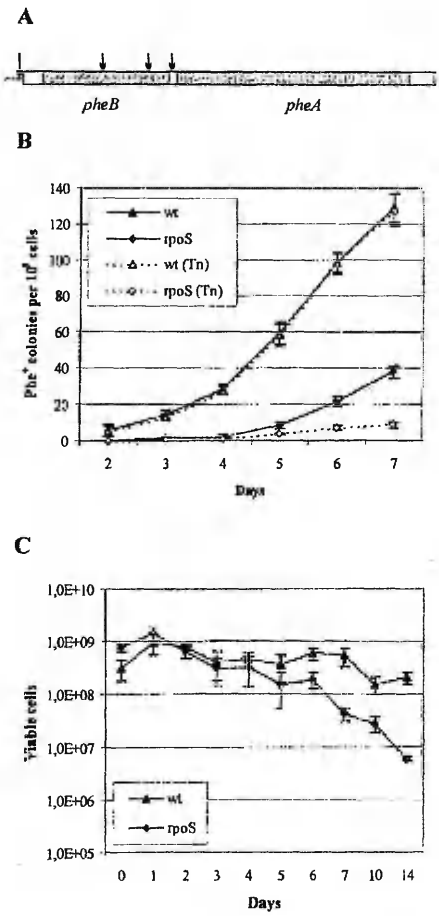

FIG. 1. (A) Schematic presentation of transposition target region in plasmid pEST1332. Catechol 1,2-dioxygenase (*pheB*) and phenol monooxygenase (*pheA*) genes are indicated by grey boxes. Vector DNA of pAYC32 is depicted with a line. Different insertion sites of Tn*4652* are indicated with arrows. (B) Accumulation of Phe^+ mutants on phenol minimal plates is indicated for *P. putida* strain PaW85 (wt) and isogenic *rpoS*-defective strain PKS54 (rpoS) containing plasmid pEST1332. Each point represents the mean of five independent determinations, and error bars represent standard deviations. Dashed lines indicate the theoretical appearance of Tn*4652*-linked Phe^+ mutants deduced from the results of PCR analysis of Phe^+ colonies. Up to 30 Phe^+ mutants were subjected to analysis on each day. (C) Viability of *P. putida* PaW85 (wt) and PKS54 (rpoS) carrying plasmid pAYC32 on phenol minimal plates. Each point represents the mean of five independent measurements, and error bars represent standard deviations. 1,0E + 08, e.g., marks 10^8 viable cells.

transcription from the fusion promoters created by Tn*4652* insertions (20).

RpoS is known to contribute to the maintenance of bacterial cell viability during the stationary phase of growth and during nutrient starvation (18, 22). Survival of *rpoS*-defective *P. putida* strain KT2440 has been demonstrated to decrease by 2 orders of magnitude during 1 week in liquid minimal medium (22; our unpublished results). Therefore, we estimated the viability of starving *P. putida* PaW85 and PKS54 on phenol minimal plates. In this experiment, *P. putida* PaW85 and PKS54 carrying plasmid pAYC32 (which differs from pEST1332 by its lack of the *pheBA* genes) were used in order to avoid the accumulation of Phe^+ mutants. About 5×10^8 to 8×10^8 bacteria of PaW85(pAYC32) and PKS54(pAYC32) were plated onto five phenol minimal plates, and small plugs were cut from the agar on each starvation day. Bacteria from the plugs were suspended in M9 solution, and the number of colony-forming units was determined on glucose minimal plates supplied with carbenicillin. Data in Fig. 1C show that viability of the σ^S-defective strain decreases slowly during 14 days of starvation on phenol plates; by the end of the second week, the number of viable cells of PKS54(pAYC32) had decreased by 2 orders of magnitude. However, during the first 6 days of starvation, survival of the σ^S-defective strain dropped only twofold. This cannot explain how Tn*4652*-linked Phe^+ mutants had an accumulation rate in PKS54(pEST1332) that was more than 10-fold lower than that in PaW85(pEST1332) (Fig. 1B). Therefore, we conclude that σ^S can act as a positive regulator in transposition of Tn*4652*.

Expression of transposase of Tn*4652* is σ^S dependent. How can RpoS control transposition of Tn*4652*? Transposition is mostly regulated by the amount and activity of transposase, the protein that performs the transposition reaction. Therefore, we evaluated the amount of transposase (TnpA) of Tn*4652* in a σ^S-defective background by Western blot analysis with an anti-TnpA polyclonal antiserum. TnpA is downregulated by the Tn*4652*-encoded TnpC, and therefore, the concentration of TnpA in the Tn*4652* background is not detectable by Western blot analysis (12). Yet, TnpA protein can be shown by this method if the copy number of the *tnpA* gene is increased by cloning the *tnpA* into plasmid pKT240 [plasmid pKTtnpA(D/H)] (12). Thus, we performed Western blot analysis with cell lysates prepared from ON cultures of *P. putida* PaW85 and PKS54 carrying plasmid pKTtnpA(D/H). We found that expression of plasmid-encoded TnpA was substantially decreased in the σ^S-defective strain; no TnpA protein could be detected by Western blot analysis in PKS54 (Fig. 2).

Transcription from the transposase *tnpA* promoter of Tn*4652* is growth phase controlled and σ^S dependent. In order to test whether the Tn*4652*-encoded transposase could be under the control of σ^S, the transcriptional activity of the *tnpA* promoter (Fig. 3A) was examined in *P. putida* strains PaW85 and PKS54. Previously, transcriptional fusions of the *tnpA* promoter region with the reporter gene *lacZ* have been constructed, and it has been demonstrated that the *tnpA* promoter is positively affected by integration host factor (IHF) (11). It has been shown that σ^S is involved in the regulation of the expression of IHF in *Escherichia coli* (1). Therefore, the *tnpA* promoter constructs either containing or lacking the IHF binding site (plasmids pKTlacZS/C and pKTlacZD/C, respectively)

TABLE 1. Bacterial strains, plasmids, and oligonucleotides

Strain, plasmid, or oligonucleotide	Description	Reference
Strains		
P. putida		
PaW85	Tn*4652*	3
PKS54	Tn*4652 rpoS*::Km	20
Plasmids		
pAYC32	Broad-host-range vector (Ap^r)	6
pEST1332	Plasmid pAYC32 carrying promoterless *pheBA* operon	15
pKT240	Cloning vector (Ap^r Km^r)	2
pKTtnpA(D/H)	Tn*4652 tnpA* gene cloned into pKT240	12
pKTlacZS/C	122-bp Tn*4652 tnpA* promoter region with IHF binding site cloned into pKTlacZ	11
pKTlacZD/C	65-bp Tn*4652 tnpA* promoter region lacking IHF binding site cloned into pKTlacZ	11
Oligonucleotides		
pheA	5′-TGCTCAAGATTATCATTACGCT-3′ (positions 11–32 in the *pheA* coding region)	
TnR	5′-ATCAGCATAGACGGCTAGCCAG-3′ (positions 101–122 from Tn*4652* right end)	
TnL	5′-CTTCCTCAATGGATGGCTGAAG-3′ (positions 111–132 from Tn*4652* left end)	

were tested in the σ^S-defective background. The results presented in Fig. 3B demonstrate that the transcription from the *tnpA* promoter is entirely dependent on the growth phase of the bacteria. Both reporter plasmids, pKTlacZS/C and pKTlacZD/C, tested in PaW85 exhibited stationary-phase-specific induction of the *tnpA* promoter. Also, as demonstrated previously (11), an about five- to sixfold-higher positive effect became apparent in the presence of the IHF binding site upstream of the *tnpA* promoter (Fig. 3B). Measurement of the β-galactosidase expression in the σ^S-defective *P. putida* strain PKS54 revealed that no obvious increase could be detected with either pKTlacZS/C or pKTlacZD/C during growth (Fig. 3B). Bacteria harboring either plasmid pKTlacZS/C or pKTlacZD/C showed similar and only slightly detectable levels of β-galactosidase activity that remained 50- or 10-fold lower, respectively, than that estimated in the wild-type strain, and no positive effects of the IHF binding site could be detected. Thus, these data indicate that stationary-phase-specific activation of the *tnpA* promoter specifically requires σ^S.

RpoS may act either directly on the *tnpA* promoter or indirectly by activation of some transcription factor operating on the *tnpA* promoter. Although σ^S- and σ^{70}-dependent promoters are generally quite similar, some subtle but essential differences in promoter sequences exist to ensure the selectivity between these two major sigma factors. σ^S-dependent promoters harbor mostly the sequence CTATACT in the conserved −10 region (8), while σ^{70} preferentially recognizes promoters with the sequence TATAAT. The −10 region CTATGCT of the *tnpA* promoter of Tn*4652* contains the sequence determinants suggested to be important for σ^S-dependent transcription, the C nucleotide upstream of the −10 hexamer and the C at the fifth position in the −10 hexamer (Fig. 3A). Therefore, we suppose that RpoS recognizes the *tnpA* promoter and is directly involved in the stationary-phase-specific expression of TnpA.

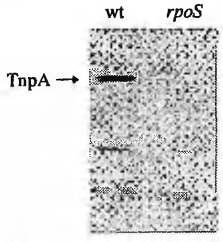

FIG. 2. Western immunoblot analyses of Tn*4652* TnpA in *P. putida* strain PaW85 (wt) and *rpoS*-defective strain PKS54 (*rpoS*) containing TnpA-expressing plasmid pKTtnpA(D/H). About 40 μg of crude cell lysate was loaded per lane.

Up to now the role of σ^S in regulation of transposition has been studied only in experiments with the mutant bacteriophage Mu. It has been shown that carbon starvation conditions trigger induction of mutant Mu prophage, resulting in formation of the *araB-lacZ* coding sequence fusions (17). Appearance of the *araB-lacZ* fusion clones on lactose-selective plates was completely abolished in a σ^S-negative *E. coli* strain (9). Since the transposase promoter of Mu was demonstrated to be not under the direct control of σ^S, it was supposed that σ^S could regulate Mu activation indirectly (17). Thus, according to our knowledge, σ^S-dependent upregulation of the transposase of Tn*4652* is the first example of direct stationary-phase-specific regulation of a mobile element transposase.

It is well known that plenty of mutations and other types of genetic variation are associated with the activity of mobile elements. Transpositional activity of most mobile elements is greatly suppressed, yet there are several examples of transposons that are activated under the conditions in which fast genetic changes are needed, i.e., under different stresses (5, 14, 23). An interesting question arises: does the activation occur due to malfunction of host defense mechanisms or is this an induced process to promote mutations that may potentially contribute to survival in unfavorable conditions? According to the results presented in this report, we prefer the latter version. Our results demonstrate that transposition of Tn*4652* is regulated by physiological conditions of the host. In starving bacteria, transposition of Tn*4652* is elevated due to direct control

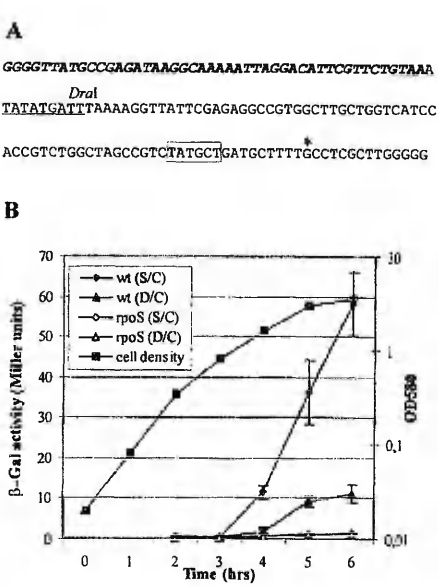

FIG. 3. (A) Sequence of right end of Tn*4652* containing promoter region of *tnpA*. The 46-bp inverted repeat is in boldface italics. The −10 hexamer of the *tnpA* promoter is boxed, and the transcription start of *tnpA* (11) is indicated by an asterisk. The potential IHF binding site is underlined. (B) Growth-dependent expression of *tnpA* promoter. *P. putida* wild-type strain PaW85 (wt) and its *rpoS* mutant PKS54 (rpoS) carrying either pKTlacZS/C or pKTlacZD/C were grown on Luria-Bertani medium. Plasmid pKTlacZD/C lacks the 57 nucleotides (up to the *Dra*I restriction site; for details, see the description for panel A) of the Tn*4652* right end sequence. Data (mean ± standard deviation) of at least four independent experiments are presented. OD580, optical density at 580 nm.

of the stationary-phase sigma factor σ^S that is induced just for better survival of cells in stressed conditions. Therefore, we believe that Tn*4652* serves as a good example of transposons that are activated under stressful conditions to increase the overall mutation rate and to generate new and potentially useful mutations.

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IV

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Regulation of transposition of Tn*4652*:
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Regulation of transposition of Tn*4652*: involvement of *Pseudomonas putida* integration host factor and transposon-encoded inhibitor TnpC

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Running title: IHF and TnpC in regulation of transposition of Tn*4652*

Keywords: transposon Tn*4652*, *Pseudomonas putida*, integration host factor, regulation of transposition

SUMMARY

Transposition of a mobile DNA element is potentially deleterious for the host. Therefore, the rate of transposition must be precisely controlled. The control is often realised through co-ordinate regulation both by the transposon-encoded factors and host factors. Here, we have shown that the transposition of *Pseudomonas putida* transposon Tn*4652* is positively regulated by integration host factor (IHF) and inhibited by transposon-encoded TnpC. Results obtained indicate that IHF is involved in the transposition of Tn*4652* as an activator while no transposition activity of native Tn*4652* was detected in IHF-defective *P. putida* A8759. Additionally, we show that changes in the IHF concentration can alter the frequency of transposition of Tn*4652* — overexpression of *ihfAB* genes increases transposition of Tn*4652* by about one order of magnitude. Contrary to the positive action of IHF, the inhibition of transposition of Tn*4652* was demonstrated by transposon-encoded TnpC. TnpC efficiently downregulated transposition of the artificial miniTn*4652* in the mating-out assay. We suppose that TnpC is the major factor that ensures the low rate of transposition of Tn*4652*.

INTRODUCTION

Transposons are discrete mobile DNA segments that have important roles in evolution. Chromosome mutagenesis and gene transfer are often promoted by the movement of transposable DNA elements. The requirement to keep transposition at low levels (10^{-3} to 10^{-8} per element per generation; Kleckner, 1990) is common to all transposons. This is needed to maintain the balance between their propagation and potential destructive mutagenic effects to their hosts. The frequency of transposition of one particular mobile element is not constant. Although transposition activity is generally maintained at a low level, high frequency of transposition can still occur under certain circumstances (Lamrani *et al.*, 1999; Chow & Tung, 2000). Indeed, transposition of a particular transposon

is mostly controlled both by negatively and positively acting factors. Depending upon the expression of these regulators the activity of transposition may vary largely.

Transposition frequency can be modulated both by mobile element-encoded factors and by various host factors. Several transposons are known to encode for special inhibitor molecules which downregulate the synthesis of transposase or repress the transposition reaction. For example, IS*10*-encoded antisense RNA prevents initiation of transposase translation by pairing with 5' end of the transposase mRNA (Simons & Kleckner, 1983). IS*1* and Tn*5* modulate transposition using inhibitor proteins coded by the same ORF as the transposase (Johnson & Reznikoff, 1984; Yin & Reznikoff, 1988; Machida & Machida, 1989). IS*1* specifies for the inhibitor protein InsA that binds to the ends of IS*1*, and regulates both transposase expression and transposition of IS*1* (Machida & Machida, 1989). Tn*5*-encoded Inh protein inhibits transposition by forming transpositionally inactive heterooligomers with transposase (de la Cruz *et al.*, 1993). One important feature that decreases transposition activity of a particular transposon is that while transposases are preferentially *cis* acting proteins then inhibitors are effective also *in trans* (Simons & Kleckner, 1983; Yin & Reznikoff, 1988; Machida & Machida, 1989). Moreover, the effectiveness of the inhibitor may be positively correlated with its concentration, like in the case of IS*10* (Simons & Kleckner, 1983).

Up to now, many host factors are described that modulate the frequency of transposition (reviewed in Mahillon & Chandler, 1998). Integration host factor (IHF) that is known to alter the conformation of DNA is the most usual host factor involved in transposition (Wiater & Grindley, 1988; Allison & Chaconas, 1992; Signon & Kleckner, 1995). In the Mu phage transposition IHF acts positively both by enhancing the transcription from the transposase promoter and favouring the transposase to form the stable synaptic complex with Mu ends (Allison & Chaconas, 1992; van Ulsen *et al.*, 1996). Transposase of γδ transposon (Tn*1000*) and IHF bind co-operatively to both ends of the element (Wiater & Grindley, 1988). However, since the wild-type γδ transposon transposes in equal rates both with or without the IHF binding sites (Wiater & Grindley, 1990; May & Grindley, 1995) the role of IHF in transposition of Tn*1000* seems to be only modulatory. Additionally, there are also reports about the negative role of IHF on transposition (Gama *et al.*, 1992; Signon & Kleckner, 1995). An interesting case is Tn*10* transposition of which is inhibited by IHF when an element resides on a multicopy plasmid. On the other hand, Tn*10*-promoted chromosome rearrangements are enhanced by IHF (Signon & Kleckner, 1995).

Tn*4652* is a 17-kb-long derivative of the toluene degradation transposon Tn*4651* that belongs to the Tn*3* family of transposons. Transposition of Tn*4652* requires transposon terminal sequences and element-encoded transposase (Tsuda & Iino, 1987). We have previously established that the expression of the transposase of Tn*4652* is positively affected by IHF in *P. putida* (Hõrak & Kivisaar, 1998). Actually, both ends of the transposon can bind IHF (Hõrak & Kivisaar, 1998; Teras *et al.*, 2000) which indicates that besides modulating the transcription of *tnpA* gene IHF may also function in the transposition reaction directly.

TnpC is a regulator protein coded just downstream of the transposase *tnpA* gene in the right arm of Tn*4652* (Hõrak & Kivisaar, 1999). We have demonstrated that TnpC reduces the abundance of TnpA up to 10-fold in *P. putida*. Previous experiments indicated that TnpC operates in the downregulation of the transposase of Tn*4652* at post-transcriptional level (Hõrak & Kivisaar, 1999). While TnpC reduces the concentration

of transposase in *P. putida* it is reasonable to suppose that it would also inhibit transposition of Tn*4652*.

The aim of this work was to study regulation of transposition of Tn*4652*. We monitored the transposition of native Tn*4652* in IHF-defective and in IHF-overexpressing *P. putida* strains. Additionally, artificial miniTn*4652* was constructed and frequency of its transposition was measured in *tnpC*-free and in *tnpC*-expressing backgrounds. Results obtained indicate that IHF is a positive and TnpC an effective negative factor in transposition of Tn*4652*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are listed in Table 1. Plasmids used in mating-out transposition assay are pictured in Fig. 2B. Plasmid pBluescript KS(+) was used for subcloning and *E. coli* strain TG1 (Carter *et al.*, 1985) was used as a host in cloning procedures.

Bacteria were grown on either Luria-Bertani (LB) or M9 minimal medium (Miller, 1992) containing glucose as carbon source. Antibiotics were added at the indicated final concentrations: ampicillin, 100 μg/ml and tetracycline, 10 μg/ml for *E. coli*; carbenicillin, 1500 μg/ml and tetracycline 40 μg/ml for *P. putida*. Kanamycin was added at the final concentration of 50 μg/ml both for *E. coli* and *P. putida*. *P. putida* was incubated at 30°C. *E. coli* was transformed with plasmid DNA as described by Hanahan (1983). *P. putida* was electrotransformed according to the protocol of Sharma & Schimke (1996).

Construction of plasmids and strains. For construction of *tnpC*-overexpressing *P. putida* strain KT2442C, mating between *E. coli* S17-1 λ*pir* (Miller & Mekalanos, 1988) carrying pUTKm-tactnpC and *P. putida* KT2442 (Calb *et al.*, 1996), was performed. Selection of kanamycin-resistance transconjugants on glucose minimal plates enabled to obtain *P. putida* KT2442C. The presence of the *lacI-Ptac-tnpC* cassette in the chromosome of *P. putida* KT2442C was verified by PCR by using oligonucleotides Prtac (5'-AATTAATCATCGGCTCGTATAA-3') and TnpCBam (5'-CC**AGGATCC**CCAAGTGCTTACTGTTCGTG-3') complementary to the *Ptac* promoter and to the 3' end of *tnpC*, respectively. For construction of miniTn*4652* (see Fig. 2A), *Sac*I and *Eco*RI sites were designed at the right and the left ends of Tn*4652*, respectively using following oligonucleotides: Osac (5'-CGT**GAGCTC**GGGGTTATGCCGAGATAAGGC-3') and Oeco (5'-CGT**GAATTC**CCCAGTACGGCTCTATTCCG-3'). Frameshift in the coding sequence of *tnpC* in plasmid pMini*4652*+AC* was generated by blunting and ligating *Hin*dIII-generated ends. Conjugative plasmid R751 (Pansegrau & Lanka, 1987) was tagged with tetracycline resistance marker by using the mini-Tn*5*Tc delivery plasmid pUTmini-Tn*5*Tc (de Lorenzo *et al.*, 1990) and three subsequent matings. First, R751 was conjugatively transferred from donor *E. coli* strain J53 (Sambrook *et al.*, 1989) into kanamycin resistance *E. coli* WM2016 (provided by W. Messer). WM2016[R751] was used as a recipient in subsequent mating with *E. coli* S17-1 λ*pir* carrying plasmid pUTmini-Tn*5*Tc. Finally, mating of mixed population of tetracycline resistance *E. coli* WM2016[R751]tet with recipient *P. putida* PRS2000 was carried out to conjugatively transfer the R751tet into PRS2000.

Transposition assays. Transposition assays were performed by using two different methods: transposition of the native Tn*4652* was monitored in the starvation assay and frequency of transposition of the miniTn*4652* was measured in the mating-out assay.

Starvation assay was carried out as described previously (Kasak *et al.*, 1997). *P. putida* strains KT2442, A8759, RT31 or KT2442C carrying promoterless *pheBA* genes on plasmid pEST1414 were grown in a liquid culture on M9 minimal medium containing glucose as a carbon source. Samples were taken from the culture, pelleted, and washed with M9 solution. Approximately 10^8 washed cells were spread onto phenol-minimal plates. To obtain different expression level of IHF and TnpC in the cells of *P. putida* RT31 and KT2442C, respectively, the phenol-minimal plates were supplied either with 0.01 mM or 0.5 mM IPTG or no IPTG was added. Tn*4652* can activate transcription of the phenol monooxygenas gene *pheA* by creating fusion promoters (Nurk *et al.*, 1993). Phe^+ mutants that accumulated on phenol plates were analysed by PCR to detect the insertion of chromosomal Tn*4652* upstream of the *pheA* gene in plasmid pEST1414. We have shown previously that fusion promoters activating *pheBA* genes were created preferentially by the right-end sequence of the Tn*4652* (Nurk *et al.*, 1993). Therefore, the fusions of the transposon right end with the upstream sequences of the *pheA* were probed by PCR by using oligonucleotides OcIa (5'-CGTATCGATCAGCATAGACGGCTAGCCAG-3') and OpheA (5'-GCTCAAGATTATCATTACGCT-3'), complementary to the right end of Tn*4652* and 5' region of the *pheA*, respectively.

A mating-out transposition assay was performed to estimate the frequency of transposition of kanamycin resistance-conferring miniTn*4652*. *P. putida* PRS2000[R751tet] was electrotransformed with plasmids carrying miniTn*4652* (Fig. 2B). Three colonies from each transformation were tested in mating-out assay by using *P. putida* PaW340 (Franklin & Williams, 1980) as a recipient strain. Donor strains and recipient strain were grown overnight at 30°C in LB. Dilutions (1:100) of these cultures were grown to the mid-exponential growth phase without any antibiotic. Equal amounts of donor and recipient cultures were mixed in eppendorf tube and 100 µl of the mixture was spotted onto the LB plate. After 24 hours of incubation at 30°C half of the mating spot was suspended in 1 ml of M9 and serial dilutions of each mating mixture were plated onto different selective media selecting for recipient cells (streptomycin), R751tet conjugal transfer (streptomycin, tetracycline) and transposition events into conjugatively transferred R751tet (streptomycin, tetracycline, kanamycin). Frequency of conjugation was expressed as the ratio of transconjugants (Sm^rTet^r) to recipient cells (Sm^r). Frequency of transposition was determined as the ratio of transposition events ($Sm^rTet^rKm^r$ transconjugants) to conjugation events (Sm^rTet^r transconjugants).

RESULTS

Transposition of Tn*4652* is not detectable in IHF-defective *P. putida*. Previously, we have demonstrated that IHF moderately (approximately 4-fold) activates transcription from the *tnpA* promoter (Hõrak & Kivisaar, 1998). Additionally, both ends of Tn*4652* have been shown to bind IHF of *P. putida* (Teras *et al.*, 2000). *P. putida* KT2442 contains Tn*4652* in its chromosome (Table 1). To elucidate the role of the host factor IHF in the regulation of transposition of Tn*4652*, we examined the movement of Tn*4652* in

the strain A8759, an IHF-defective derivative of *P. putida* KT2442. We measured the transposition of Tn*4652* in the system previously used by us in the study of mutational processes in starving *P. putida* (Kasak *et al.*, 1997). In this starvation-experiment (described in Materials and Methods) we selected for phenol-utilising mutants, which rose due to the activation of transcription of the plasmid-encoded initially promoterless phenol degradation genes *pheBA* in plasmid pEST1414. In the wild-type *P. putida* about one third of these Phe^+ mutants were generated due to the insertion of Tn*4652* from the chromosome in front of *pheA* creating fusion promoters for the transcription of this gene (Kasak *et al.*, 1997, Table 2). To address the question about the role of IHF on transposition, we analysed by PCR the Phe^+ mutants accumulating during starvation of *P. putida* IHF-defective strain A8759 carrying plasmid pEST1414. 99 Phe^+ mutants emerging on day 5 on phenol plates were tested by PCR but none of those carried Tn*4652* insertion in the plasmid (Table 2). This indicates that IHF is necessary for the transposition of Tn*4652* at natural level.

Frequency of transposition of Tn*4652* depends on the level of expression of IHF. In order to find out whether complementation of *P. putida* IHF-defective strain A8759 with functional *ihfA* and *ihfB* genes could restore the transposition of Tn*4652*, the transposition assay was carried out in *P. putida* strain RT31 that contains *P. putida ihfA* and *ihfB* genes under the control of *Ptac* promoter and $lacI^q$ repressor in its chromosome, enabling artificially to change the level of IHF expression (Teras *et al.*, 2000). The similar assay system as described above, was used for the monitoring of the transposition of Tn*4652*. To provide different expression levels of IHF, the phenol minimal plates were supplied either with 0.5 mM or 0.01 mM IPTG or alternatively no IPTG was added. Interestingly, about 10 times more Phe^+ mutants accumulated during the first 6 days on phenol minimal plates in the presence of 0.5 mM IPTG if compared to the amount of the mutants on the other plates (Fig. 1A). PCR analysis of Phe^+ mutants revealed that transposition of Tn*4652* largely depended on the expression level of IHF in bacteria. Accumulation of the Tn*4652*-linked Phe^+ mutants on the plates containing 0.5 mM IPTG was up to 10 times more elevated as compared to that on the other plates (Fig. 1B). These results not only indicate that IHF is involved in the transposition of Tn*4652* but they also demonstrate that the level of IHF expression is one of the factors regulating the frequency of transposition of Tn*4652*.

Overexpression of TnpC cannot affect the transposition of Tn*4652*. Previously we have demonstrated that Tn*4652*-encoded *tnpC* downregulates the abundance of transposase TnpA in *P. putida* cells (Hõrak & Kivisaar, 1999). However, the function of TnpC in transposition of Tn*4652* was not investigated in this paper. To study the effect of TnpC on the frequency of transposition of Tn*4652*, we constructed *P. putida* strain KT2442C by introducing the extra-copy of *tnpC* gene under the control of *Ptac* promoter and $lacI^q$ repressor into the chromosome of the strain KT2442 (Table 1, Materials and Methods). To control the effect of the inducible expression of this extra-copy of *tnpC*, the strain KT2442C was transformed with plasmid pKTtnpA(D/H) carrying *tnpA* gene (Table 1). With the aid of Western blot analysis the ability of TnpC to downregulate the amount of the plasmid-encoded TnpA was tested. Overexpression of TnpC in the presence of 0.5 mM IPTG reduced the amount of TnpA below the level of Western blot analysis detection limit (data not shown). Thus, while TnpC was able to act in *trans* in the regulation of amount of plasmid-encoded TnpA, we expected that elevated level of expression of TnpC should lead to the decrease in the frequency of transposition of Tn*4652*. Therefore, using *P. putida* KT2442C carrying plasmid pEST1414, we tested

transposition of Tn*4652* in the starvation-assay. Again, to manipulate the level of expression of TnpC in bacteria, the phenol minimal plates were supplied either with 0.5 mM or 0.01 mM IPTG or no IPTG was added. No differences were observed in the number of Phe^+ mutants accumulating on phenol minimal plates either in the presence of different concentrations of IPTG or on plates without IPTG (data not shown). Using PCR analysis, the percentage of the Tn*4652*-linked Phe^+ mutations was examined among mutants emerging on days 3 to 5. Surprisingly, results presented in Table 2 showed that increased expression of TnpC did not affect the percentage of Tn*4652*-linked mutants among Phe^+ mutants. Thus, although the overexpressed TnpC effectively downregulated the cellular amount of the plasmid-encoded TnpA in the control experiment, it was not able to change the transposition frequency of Tn*4652* in the assay used.

Construction of miniTn*4652* system for the *in vivo* transposition assay in *P. putida*. Unexpected results obtained in TnpC-overexpressing strain may be explained by different functionality of TnpC acting either *in cis* or *in trans*. Therefore, to further address the question about the effect of TnpC *in cis* on the frequency of transposition of Tn*4652*, a miniTn*4652* system was designed. We constructed miniTn*4652* which carries kanamycin resistance (Km^r) gene from Tn*903* between the ends of Tn*4652* (Fig. 2A, Materials and Methods). MiniTn*4652* was inserted into *Van*91I deletion derivative of RSF1010-based plasmid pAYC32 resulting in the plasmid pMini*4652* (Fig. 2B). This plasmid, presumably defective in mobilisation because of the deletion in *mobA* and *mobB* genes (Frey *et al.*, 1992), was used in the mating-out assay as a control plasmid. In order to test the effect of *tnpC* on transposition of miniTn*4652*, either *tnpA* or *tnpAC* with their native promoter(s) were cloned into pMini*4652* (Fig. 2B, pMini*4652*+A and pMini*4652*+AC). Plasmid pMini*4652*+AC* carrying a frameshift mutation in the *tnpC* gene was constructed as an additional control to test whether the frequency of transposition would be affected by TnpC protein. All these plasmids were examined in the mating-out assay by using the conjugative plasmid R751tet as a transposition target. R751 encodes for the resistance to trimethoprim but *P. putida* is insensitive to this antibiotic. Therefore, R751 was tagged with tetracycline resistance gene to obtain R751tet (see Materials and Methods).

Transposition of miniTn*4652* is downregulated by *tnpC* in *P. putida* strain PRS2000. We estimated the frequency of transposition of miniTn*4652* in Tn*4652*-free *P. putida* strain PRS2000. *P. putida* PRS2000 harbouring R751tet was electroporated with plasmids described in Fig. 2B and at least 3 independent clones obtained from each transformation were tested in the mating-out transposition assay. Results presented in Table 3 clearly demonstrate that TnpC operates as an inhibitor in the regulation of transposition of miniTn*4652*. Donor plasmids pMini*4652*+A and pMini*4652*+AC* allowed high frequency of transposition — 10^{-1} transposition event per conjugation event. The presence of intact *tnpC* in the donor plasmid (pMini*4652*+AC) reduced the transposition frequency by four orders of magnitude.

DISCUSSION

Transposition is a DNA reorganisation reaction strictly regulated both by the DNA-element-encoded factors and by the host where the transposon resides in. Previously, we have demonstrated that expression of TnpA of Tn*4652* is regulated positively by *P. pu-*

tida host factor IHF and negatively by element-encoded TnpC (Hõrak & Kivisaar, 1998; 1999). Here, we have investigated the role of both these factors in transposition of Tn*4652* and have shown that in regard to transposition they act in an antagonistic manner.

P. putida IHF binds specifically to the both ends of Tn*4652*, just adjacent to the terminal inverted repeats that are presumed to bind the transposase (Hõrak & Kivisaar, 1998; Teras *et al.*, 2000). For γδ transposon (Tn*1000*) it is known that γδ transposase and IHF bind co-operatively to both ends of the element (Wiater & Grindley, 1988). However, since the wild-type γδ transposon transposes equally well with or without the IHF binding sites (Wiater & Grindley, 1990; May & Grindley, 1995) the role of IHF in transposition of Tn*1000* seems to be only modulatory. Measurement of transposition of native Tn*4652* revealed that IHF is essential for the transposition of this DNA element at natural level. First, we could not detect transposition activity of Tn*4652* in IHF-defective *P. putida* A8759 (Table 2). Accounting the sensitivity of the transposition assay used it means that transposition of Tn*4652* was reduced more than 40-fold. Second, complementation of IHF-defective strain with functional *ihfA* and *ihfB* genes restored mobility of Tn*4652* (Fig. 1). Previously, we have shown that IHF enhances the expression of the *tnpA* gene approximately 4-fold (Hõrak & Kivisaar, 1998). Therefore, we suggest that more than 40-fold decrease in the frequency of transposition of Tn*4652* indicates that besides of regulating the expression of *tnpA* IHF participates in transposition of Tn*4652* directly.

Frequency of transposition of Tn*4652* depends on the expression level of IHF (Fig. 1). Previously, it have been demonstrated that induction of *ihfAB* genes under the *Ptac* promoter present in the chromosome of *P. putida* strain RT31 with 0.01 mM IPTG resulted in nearly natural expression level of IHF (Teras *et al.*, 2000). Induction of expression of IHF with 0.5 mM IPTG leads to the overexpression of *ihfAB* genes in RT31 and as presented in Fig. 1B, increases transposition of Tn*4652* by about one order of magnitude. This indicates that changes in the IHF concentration can alter the frequency of transposition of Tn*4652*. For several mobile elements it has been reported that the frequency of transposition increases in stationary phase cells (Skaliter *et al.*, 1992; Lamrani *et al.*, 1999). We have observed that transposition of Tn*4652* is also enhanced in stationary phase bacteria (Kasak *et al.*, 1997). Evidently, one reason for this is stationary phase specific regulation of the transposase of Tn*4652* by σ^S (Ilves *et al.*, 2001). However, also IHF may contribute to the activation of Tn*4652* in the stationary phase. It is shown that in *E. coli*, *P. aeruginosa* and *P. putida* the abundance of IHF is increased up to seven-fold during the transition of cells from exponential growth to the stationary phase (Ditto *et al.*, 1994; Delic-Attree *et al.*, 1996; Murtin *et al.*, 1998; Teras *et al.*, 2000; Valls *et al.*, 2002). Therefore, it is tempting to speculate that the increased concentration of IHF in stationary phase bacteria is another factor that can induce the movement of Tn*4652*.

Most of transposable elements have regulatory mechanisms ensuring strict control over the multiplication of the element and permitting only minimal level of transposition activity (reviewed in Kleckner, 1990). Here, we have demonstrated that transposition of Tn*4652* is under the effective negative control of transposon-encoded TnpC. Frequency of transposition of miniTn*4652* into conjugative plasmid was very high when TnpA alone was coded in miniTn*4652* donor plasmid, reaching up to 10^{-1} transpositions per conjugation event (Table 3). However, co-expression of TnpA with TnpC lowered the transposition activity of miniTn*4652* drastically — by 4 orders of magnitude. Thus,

TnpC strongly inhibited the transposition of Tn*4652*. We suggest that this inhibition could be achieved due to TnpC-caused downregulation of the cellular amount of transposase as shown by us previously (Hõrak & Kivisaar, 1999). Here, we want to point out that this kind of mechanism of transposition regulation might be conserved among some transposons, because *tnpA* and *tnpC* genes of Tn*4652* are highly homologous with relevant genes of mercury resistance transposon Tn*5041* (Kholodii *et al.*, 1997; Hõrak & Kivisaar, 1998; Hõrak & Kivisaar, 1999).

Several transposable elements encode for a *trans*-acting negative regulators to keep the control over the multiplication of the mobile element (reviewed in Kleckner, 1990). The effectiveness of these negative regulators to downregulate transposition may depend on transposon copy number. For example, the IS*10*-encoded negative regulator is an antisense RNA effectiveness of which to inhibit transposition increases with its increasing concentration, i.e. with increasing transposon copy number (Simons & Kleckner, 1983). However, the IS*50*-encoded inhibitor protein (Inh) is effective even in a single copy and only very large increases in inhibitor protein (Inh) are needed to see additional inhibition of transposition of Tn*5* below the natural level (Johnson & Reznikoff, 1984; Yin & Reznikoff, 1988). We measured transposition of native Tn*4652* under the conditions of overexpression of inhibitor protein TnpC as well. However, our results revealed that this extra-amount of TnpC could not affect the frequency of transposition of native Tn*4652* (Table 2). One explanation to this phenomenon might be that TnpC operates differently whether acting *in cis* or *in trans*. However, this is not very plausible because the very same overexpressed TnpC can effectively act *in trans* by downregulating the plasmid-encoded transposase TnpA. Previously, we have demonstrated that TnpC is expressed from multiple promoters located inside the *tnpA* gene (Hõrak & Kivisaar, 1999) and we believe that TnpC is expressed at higher level than TnpA. Therefore, we favour the possibility that the *cis*-encoded TnpC is able to reduce effectively the transposition of Tn*4652* to the basal level and this could be the reason why no additional effect of overexpressed TnpC on transposition of Tn*4652* in starvation-experiment can be detected. Accordingly, for Tn*5* it is hypothesised that inhibitor dose — transposition response curve is not a linear plot but rather hyperbolic in shape (Yin & Reznikoff, 1988). However, as we established in the experiments with native Tn*4652* and pMini*4652*+AC, some transposition still occurred at this basal level (Tables 2 and 3). Therefore, one can speculate that if some molecules of TnpA escape the action of *cis*-encoded TnpC, the transposition reaction is carried out. It is obvious that chances of *trans*-encoded TnpC to eliminate this particular "lucky" molecule are very low. Of course, there may be some additional regulatory mechanisms that can channel this rescued transposase molecule into transposition reaction.

Here, we have shown that transposition of Tn*4652* is controlled by negatively-operating Tn*4652*-encoded TnpC and positively-acting *P. putida* host factor IHF. However, it has been shown that in addition to IHF some other host factor binds to the right end of the transposon as well (Teras *et al.*, 2000). Interestingly, this unknown protein may counteract to IHF. While IHF binds to the Tn*4652* ends from the cell lysates prepared from the stationary phase bacteria then binding of this unknown factor to the right-end DNA was well-detectable only by using cell-lysates of exponentially grown bacteria (Teras *et al.*, 2000). Therefore, we suggest that further investigations of transposition of Tn*4652* as a function of different physiological conditions of bacteria would widen awareness about the regulation of this mobile DNA element.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or construction	Source or reference
E. coli		
TG1	*supE hsdΔ5 thi Δ(lac-proAB) F'* [*traD*36 *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	Carter *et al. (1985)*
S17-1 λ*pir*	Tp^r Sm^r *recA thi pro* (r$^-$ m$^+$) RP4::2-Tc::Mu::Km Tn*7* λ*pir*	Miller & Mekalanos (1988)
WM2016	*subE thi Δ(lac-pro) fis*::Km^r	provided by W. Messer
J53	F' *pro met*	Sambrook *et al.* (1989)
P. putida		
KT2442	Tn*4652 xylRS* Pu-*lacZ* Rif^r Sm^r	Calb *et al.* (1996)
A8759	KT2442 *ihfA*::Km^r	Calb *et al.* (1996)
RT31	A8759 *P. putida ihfA* and *ihfB* under control of P_{tac} promoter and *lacI*^{q} repressor Tc^r	Teras *et al.* (2000)
KT2442C	KT2442 *tnpC* under control of P_{tac} promoter and *lacI*^{q} repressor Km^r	This work
PRS2000	Tn*4652*-free	Wheelis & Ornston (1972)
PaW340	Tn*4652 trp* Sm^r	Franklin & Williams (1980)
Plasmids[a]		
pBluescript KS(+)	Cloning vector (Ap^r)	Stratagene
pAYC32	Broad-host-range vector (Ap^r)	Chistoserdov & Tsygankov (1986)
pEST1414	pAYC32 containing promoterless *pheBA* operon	Kasak *et al.* (1997)
pKTtnpA(D/H)	Plasmid containing *tnpA* gene of Tn*4652*	Hõrak & Kivisaar (1999)
pAYC32Van	Mobilization defective *Van*91I deletion derivative of pAYC32	This work
pBlMini*4652*	108-bp of Tn*4652* right-end plus Km resistant gene from plasmid pUTmini-Tn*5*Km2 plus 85-bp of Tn*4652* left-end (=miniTn*4652*) cloned into pBluescript KS	This work (Fig. 2A)
pMini*4652*	pAYC32Van containing miniTn*4652*	This work (Fig. 2B)
pMini*4652*+A	pMini*4652* containing Tn*4652 tnpA* gene within the 3.28-kb *Dra*I-*Hin*dIII fragment	This work (Fig. 2B)
pMini*4652*+AC	pMini*4652* containing Tn*4652 tnpAC* genes within the 3.47-kb *Dra*I-*Bam*HI[a] fragment	This work (Fig. 2B)
pMini*4652*+AC*	pMini*4652*+AC with the *tnpC* gene disrupted by frameshift	This work
R751	Conjugative plasmid of IncP1 compatibility group (Tmp^r)	Pansegrau & Lanka (1987)
R751tet	R751 containing mini-Tn*5*Tc (Tet^r)	This work
pUTmini-Tn*5* Tc	Delivery plasmid for mini-Tn*5* Tc (Ap^r Tet^r)	de Lorenzo *et al.* (1990)
pMMB208	*lacI*^{q}/*Ptac*-based broad-host-range expression plasmid (Km^r)	Morales *et al.* (1991)
pBR322	Cloning vector (Ap^r Tet^r)	Bolivar *et al.* (1977)
pBRlacItac	*Ptac* promoter and *lacI*^{q} repressor in 2.2 kb *Nru*I-*Eco*RI fragment from plasmid pMMB208 cloned into *Eco*RV-*Eco*RI cleaved pBR322	This work
pBRlacItac-tnpC	pBRlacItac containing *tnpC* in a 640 *Cfr*10I-*Pvu*II fragment under the *Ptac* promoter	This work
pUC18Not	pUC18 with *Not*I restriction sites in multicloning region (Km^r)	Herrero *et al.* (1990)
pUCNot-tactnpC	pUC18Not containing 2.9-kb *Bam*HI-*Kpn*I fragment with *lacI*^{q}-*Ptac-tnpC* cassette	This work
pUTmini-Tn*5* Km2	Delivery plasmid for mini-Tn*5* Km2 (Ap^r Km^r)	de Lorenzo *et al.* (1990)
pUTKm-tactnpC	pUTmini-Tn*5* Km2 containing 2.9-kb *Not*I fragment with *lacI*^{q}-*Ptac-tnpC* cassette	This work

[a] the *Bam*HI restriction site in the 3' end of the *tnpC* is artificial, designed by using oligonucleotide TnpCBam (see Marerials and Methods)

TABLE 2. Transposition of Tn*4652* from the chromosome of *P. putida* upstream of *pheA* in plasmid pEST1414 in starvation-experiment

P. putida strain	IPTG added	Phe^+ mutants analysed	Percentage of Tn*4652*-linked mutants
KT2442 (wt)	no	108	37%
A8759 (KT2442 *ihfA*:Km^r)	no	99	0%
KT2442C (KT2442 Ptac/*tnpC*)	no	135	33%
	0.01 mM	135	47%
	0.5 mM	135	38%

TABLE 3. Transposition frequency of miniTn*4652* in *P. putida* PRS2000

Donor plasmid	Frequency of transposition (Sm^r Tet^r Km^r/ Sm^r Tet^r)
pMini*4652*	3.3×10^{-6}
pMini*4652*+A	3.4×10^{-1}
pMini*4652*+AC	1.8×10^{-5}
pMini*4652*+AC*	2.5×10^{-1}

The frequency of conjugation was approximately 6×10^{-1}.

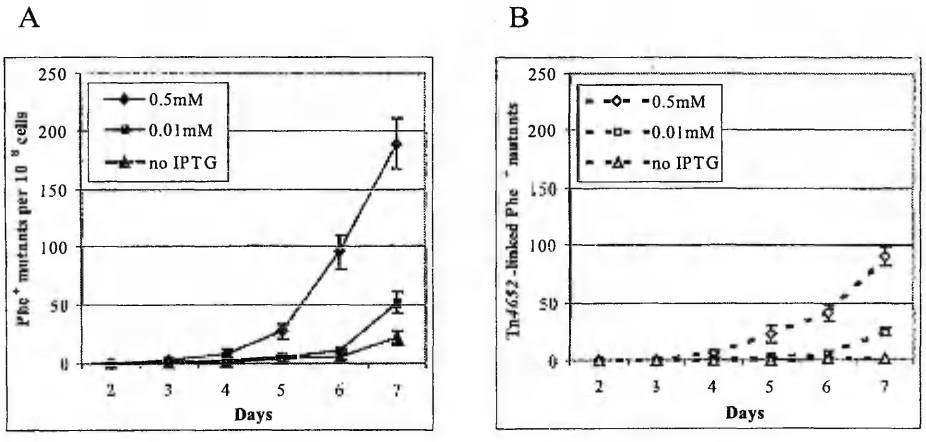

Figure 1. (A) Accumulation of Phe$^+$ mutants on phenol-minimal plates at different expression levels of IHF in *P. putida* RT31. Each point represents the mean and standard deviation of five independent determinations. (B) The theoretical appearance of Tn*4652*-linked Phe$^+$ mutants deduced from the results of PCR analysis of Phe$^+$ colonies. In first 4 days all Phe$^+$ mutants were analysed by PCR. Later, when accumulation of Phe$^+$ mutants was higher, about 30 Phe$^+$ mutants were subjected to analysis on each day.

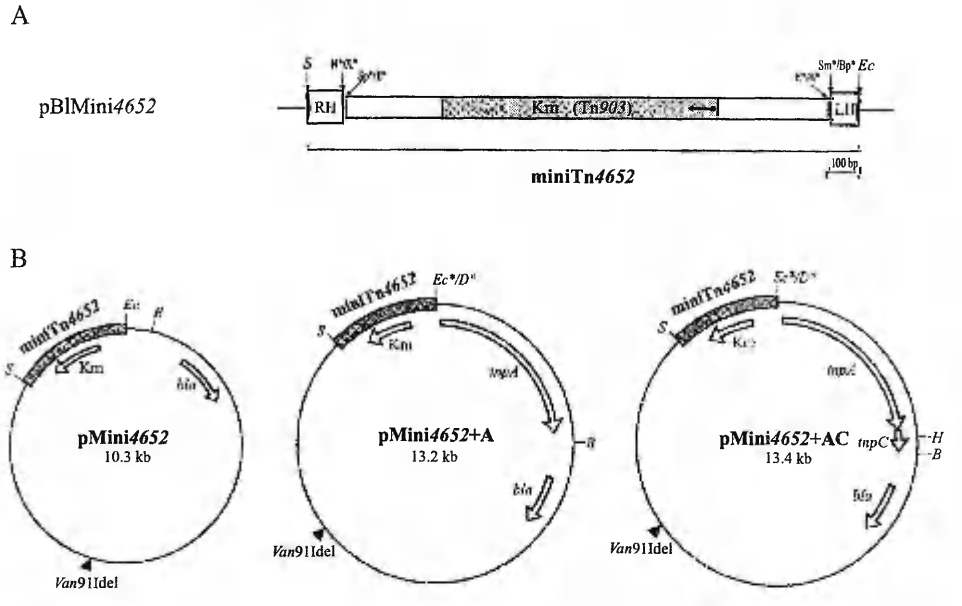

Figure 2. (A) Schematic presentation of the miniTn*4652* in the plasmid pBlMini*4652*. Open boxes represent DNA fragments cloned into pBluescript KS(+) in the following order: 108-bp DNA fragment containing the right end of Tn*4652* up to *Nhe*I restriction site (designated as RH), 1430-bp *Eco*47III fragment from plasmid pUTmini-Tn*5*Km2 containing kanamycin resistance gene (shadowed box) and 85-bp segment containing the left end of Tn*4652* up to *Bpu*1102I restriction site (LH). Regions of the pBluescript KS(+) are shown by lines. Restriction sites used in cloning are indicated as follows: *S*, *Sac*I; *N*, *Nhe*I; *X*, *Xba*I; *Sp*, *Spe*I; *E*, *Eco*47III; *B*, *Bam*HI; *Sm*, *Sma*I; *Bp*, *Bpu*1102I; *Ec*, *Eco*RI. Slash between two restriction sites indicates the junction of DNA fragments that were ligated with each other after blunting. Restriction sites marked with * were disrupted during cloning. *Sac*I and *Eco*RI restriction sites at the Tn*4652* right and left end, respectively, are artificial, designed by using oligonucleotides Osac and Oeco, respectively (Materials and Methods). (B) Maps of the plasmids carrying miniTn*4652*. MiniTn*4652*, designated by grey box, was cloned as *Sac*I-*Eco*RI fragment into pAYC32Van. Locations and orientations of kanamycin resistance gene, *bla*, *tnpA* and *tnpC* genes are shown by open arrows. Black triangle marks location of *Van*9H deletion in pAYC32 resulting in deletion of *mobA* and *mobB* genes. *D* and *H* designate *Dra*I and *Hin*dIII restriction sites, respectively. Other sites are indicated as above.

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Education and professional employment

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1982–1987	Tartu University, Faculty of Biology and Geography, graduated as biochemist
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Scientific work

Since 1987 I have been worked in the group of Dr. Maia Kivisaar. I have studied regulation of phenol degradation genes and mutational processes under carbon starvation in *Pseudomonas putida*. Since about 1996 I have been concentrated on regulation of transposition of *Pseudomonas putida* transposon Tn*4652*.

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1987–1997	Eesti Biokeskus, nooremteadur
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Teadustegevus

Olen töötanud dr. Maia Kivisaare töögrupis alates 1987 aastast. Olen uurinud fenooli lagundamist kodeerivate geenide regulatsiooni mullabakteris *Pseudomonas putida*. Samuti olen osalenud töös, mille eesmärgiks on selgitada bakterites toimuvate mutatsiooniprotsesside sõltuvust bakterite füsioloogilisest seisundist. Alates 1996 aastast olen keskendunud *Pseudomonas putida* transposooni Tn*4652* regulatsiooni uurimisele.

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