DISSERTATIONES BIOLOGICAE UNIVERSITATIS TARTUENSIS
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STRESS-INDUCED TRANSPOSITION OF Tn*4652* IN *PSEUDOMONAS PUTIDA*

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on following original publications, which will be referred by the relevant Roman numeral in the text:

I **Ilves, H., Hõrak, R. and Kivisaar, M. 2001.** Involvement of sigma(S) in starvation-induced transposition of *Pseudomonas putida* transposon Tn*4652*. *J Bacteriol* **183**: 5445–8.

II **Ilves, H., Hõrak, R., Teras, R. and Kivisaar, M. 2004.** IHF is the limiting host factor in transposition of *Pseudomonas putida* transposon Tn*4652* in stationary phase. *Mol Microbiol* **51**: 1773–85.

III **Hõrak, R., Ilves, H., Pruunsild, P., Kuljus, M. and Kivisaar, M. 2004.** The ColR-ColS two-component signal transduction system is involved in regulation of Tn*4652* transposition in *Pseudomonas putida* under starvation conditions. *Mol Microbiol* **54**: 795–807

ABBREVIATIONS

ACP	acyl carrier protein
bp	base pairs
Dam	DNA adenine methylase
DnaA	replication initation protein
DDE	conserved amino acid motif of two aspartic acid residues and one glutamic acid residue in the active site of transposase
DR	direct repeats
ER	transposition intermediate complex of Mu phage that contains Mu enchancer sequence and right end
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
IR	terminal inverted repeat of transposable element
IS	insertion sequence element
kb	kilo base pairs
LER	transposition intermediate complex of Mu phage that contains Mu left end, enhancer sequence and right end
LTR	long terminal repeat sequence at the each end of retrotransposons or retroviruses
ORF	open reading frame
RBS	ribosome binding site
RR	response regulator protein of bacterial two-component signal system
TE	transposable element
TnpA	transposase

INTRODUCTION

Bacteria live in ever-changing and mostly growth-limiting environmental conditions. To cope with diverse stress situations bacteria have evolved several strategies for detection and appropriately responding to different environmental signals. In situation when bacteria are unable to grow because nutrients are exhausted or cannot be used, mechanisms that increase genetic variation could allow some members of population to achieve a phenotype that enables them to survive and proliferate. One of the mechanisms leading to the increased genetic variability is transposition.

Transposons are widespread in the genomes of prokaryotic and eukaryotic organisms. Transposition may produce either major effects on phenotypic traits or small changes detectable only at the DNA sequence level, depending on the location of target site. For example, the transposon insertion within a gene inactivates it, but insertion into upstream region in some cases is able to activate transcription of this gene. In addition to that, transposons also promote inversions and deletions in the host genomes by providing regions of homology for DNA recombination apparatus of host. Besides promoting DNA rearrangements many bacterial transposons have clearly selective values for host by encoding additional functions such as antibiotic resistance, virulence determinants or unusual catabolic properties. Therefore, the dissemination of transposons (usually by plasmids) among bacterial species can contribute to either catabolic versatility of soil bacteria or emergence of pathogens with multiple resistances to antibiotics.

As a potentially destructive process, transposition occurs usually at low level, roughly 10^{-3} to 10^{-8} per element per generation (Kleckner, 1990; Craig, 1996). Yet, several reports claim that the transposition frequency of some transposable elements can transiently increase during stress. It has been hypothesised that the elevated transposition of mobile elements increases genetic variability to help host to overcome harsh stress conditions and at the same time promote expression of new genetic traits (Capy *et al*., 2000; Morillon *et al*., 2000). However, the molecular mechanisms responsible for the stress-induced transposition have remained poorly studied.

Previous results obtained from our laboratory have revealed that transposition of *Pseudomonas putida* specific transposon Tn*4652* is inducible under conditions of carbon starvation (Kasak *et al*., 1997). Activation of Tn*4652* transposition under nutrient starvation makes it a suitable object to study the phenomenon of stress-induced transposition. Therefore, the main aim of the current thesis was to enlighten molecular mechanisms underlying stress-induced transposition of *P. putida* transposon Tn*4652*. I was particularly interested in whether an activation of Tn*4652* transposition in carbon starved *P. putida* cells is caused by malfunctioning of host control mechanisms or instead, is a host-induced process in response to stressful environmental conditions.

1. REVIEW OF LITERATURE

1.1. General features of transposons

A transposable element (TE) or a transposon is a discrete DNA segment that is capable of inserting itself into new genomic location. In the simplest instance, TE is a DNA sequence with defined specific inverted repeats (IR) at its both ends and it encodes a transposition catalysing protein, transposase. Such bacterial elements are for example, IS (insertion sequence) elements that mostly have only these determinants and are thereby relatively small (<2.5 kb) (Fig. 1). More complex and larger transposons encode some additional determinants, such as catabolic genes and antibiotic or metal resistance genes. Some of them, called composite transposons, contain this additional genetic load between two IS elements, from which both can individually be mobile as well (Tn*5* and Tn*10* are the best-characterised examples of the composite transposons). Therefore, composite and non-composite transposons are highly variable in size and genetic organisation.

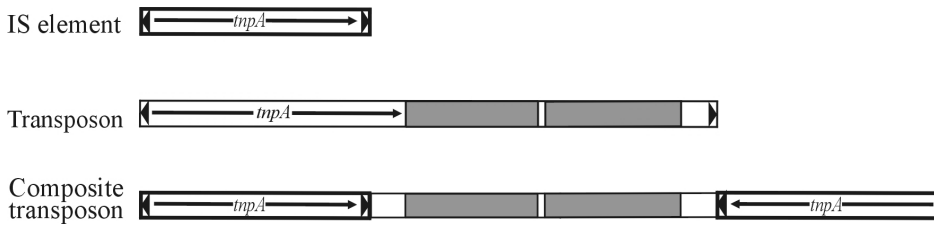

Figure 1. Simplified genetic organisation of transposable elements. The transposase gene (*tnpA*) of element is located between terminal inverted repeats (black triangles). Grey boxes represent the different additional genes carried by transposons.

Some bacteriophages also use transposition as a mechanism for multiplication of their genomes. For example, *Escherichia coli* phage Mu combines properties of a temperate phage and a TE. Among transposons, Mu is one of the most extensively studied elements, probably because its life cycle involves two transposition modes: non-replicative mode resulting in lysogeny and replicative mode leading to the lytic growth. During lytic phase Mu is able to produce approximately 100 copies of its DNA by replicative transposition within an hour (Pato, 1989). Because of this feature, Mu is one of the exceptions to the general rule that transposition occurs at low frequency.

Historically the TEs have been classified based on several criteria, but to date the most appreciated way to categorise them is by their transposition chemistry, which is determined by their transposition mediating proteins. Based on the features of transposition catalysing enzymes, the TEs can be divided into

five groups. The major group of TEs (this extremely widespread class includes many transposons, IS elements, transposing phages and LTR retroelements) encodes so-called DDE transposases. DDE transposases were named after the highly conserved amino acid triplet (Asp, Asp, Glu) in the active site of enzyme. Transposition of DDE transposons involves DNA cleavage at the 3´OH ends of element that is followed by the joining of 3´OH termini to opposite strands of target DNA at the insertion site. Some of DDE transposons undergo additional transposon 5´end cleavage before the target capture (see the section 1.3) (reviewed in Haren *et al*., 1999). The other groups of TEs encode enzymes that are more related to the serine site-specific recombinases, tyrosine site-specific recombinases, rolling circle replicases or encode a combination of reverse transcriptase and endonuclease activities (reviewed in Curcio and Derbyshire, 2003).

In my thesis, I will primarily focus on bacterial DDE transposons, their transposition and regulatory mechanisms.

1.2. Transposition pathways

Bacterial TEs use mainly two modes of transposition: replicative and non-replicative (called cut-and-paste) (Fig. 2). Generally, these two pathways are different from each other by the mode of transposase-performed DNA cleavage at the ends of transposon, which will lead to the different outcomes of transposition. In the replicative transposition, only single stranded nicks are made at the ends of transposon and during the target capture, the transposon is remained linked with its donor site. Both target and donor molecules will carry a copy of transposon after replicative transposition. In contrast, in non-replicative transposition the transposon is entirely excised from the donor molecule and then is inserted into a target molecule. Common to both transposition types is the generation of short duplications of target sequence, called direct repeats (DR) that are located adjacent to the element in the new target site. These short duplications with a length of 2 to 14 bp are created by replication or gaps repair following to transposase introduced staggered cuts in the target DNA.

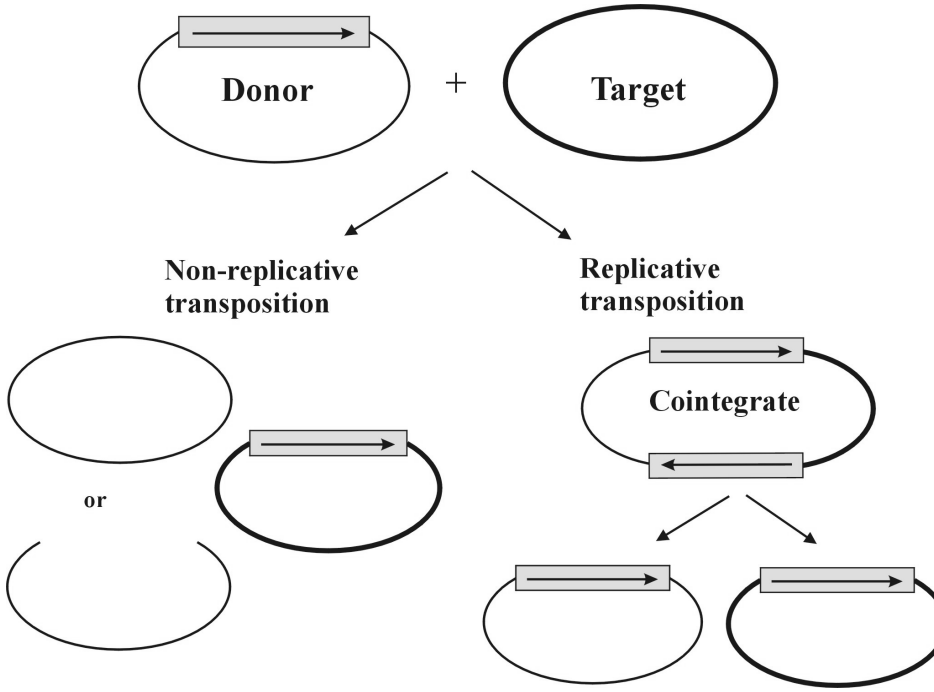

Figure 2. Two major types of transposition: non-replicative and replicative transposition. Grey box represents the transposable element (after Craig, 1996).

1.2.1. Replicative transposition

In replicative transposition, the TE insertion into the same molecule (intramolecular transposition) can cause either element-adjacent deletion or inversion, whereas transposition into another molecule (intermolecular transposition) ends in the formation of intermediate structure, known as cointegrate (Arthur and Sherratt, 1979; Shapiro, 1979). In cointegrate the donor and target sequences are joined by direct repeat copies of the transposon in each point of connection. Subsequently cointegrate is resolved into two separate DNA molecules by recombination between the two transposon copies. Thus, the outcome of replicative transposition is the restored original donor molecule and the target molecule carrying a copy of the transposon (Fig. 2) (reviewed from Grindley, 2002; Haren *et al*., 1999).

The type of transposition pathway is determined by the mode of transposase-introduced cut. In the case of replicative transposition, the DNA is cleaved by transposase only at the 3´ends of transposon. Thereafter the released 3´ends of the element attack the target DNA, followed by the covalent joining of transposon to the cleaved 5´ends of the target DNA and leaving the 3´OH ends of the target strands unjoined. This structure is named a Shapiro

intermediate, based on proposed model by James Shapiro (Fig. 3) (Shapiro, 1979). The existence of this intermediate structure in Mu transposition was proved by experiments of Craigie and Mizuuchi (Craigie and Mizuuchi, 1985). In the Shapiro intermediate, the gaps are located in the target DNA adjacent to transposon as the target sequence was cleaved by staggered fashion. The free 3´OH ends flanking the transposon are used as primers for replication initiation. The duplication of the whole transposon through replication leads to the formation of donor-target cointegrate.

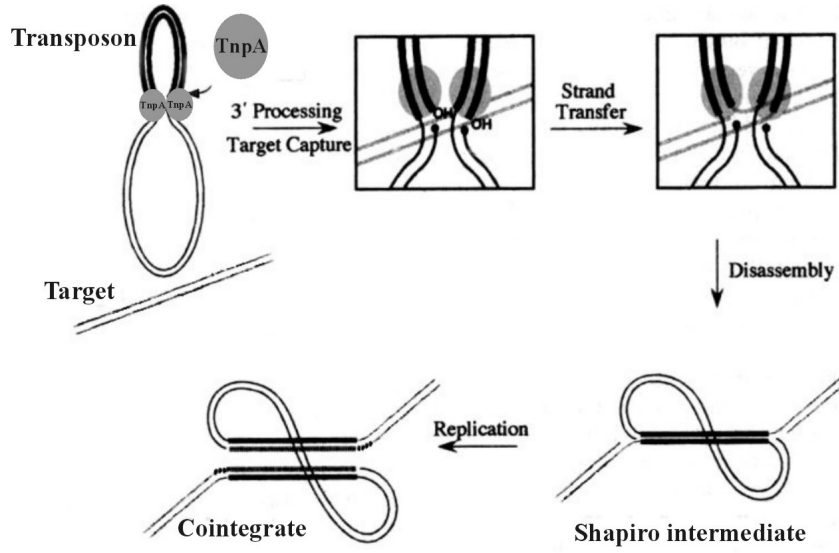

Figure 3. Formation of Shapiro intermediate in replicative transposition. Transposase (represented by a grey oval) binds to the ends of transposon and introduces single-stranded breaks at both 3´ends of the element (3´processing). The released 3´OH ends of the element directly attack the target sequence, followed by joining them to the cleaved 5´ends of the target and leaving the 3´OH ends of the target strands free (Shapiro intermediate). These free 3´OH ends of the target sequence are used by replication machinery to duplicate the transposon, resulting in cointegrate (modified from Grindley, 2002).

The following step is the resolution of cointegrate either by site-specific or homologous recombination, depending on TE (reviewed from Hallet and Sherratt, 1997 and Grindley, 2002). For example, the Tn*3* family transposons have a site-specific recombination system (either a serine recombinase of the resolvase/DNA invertase family or a tyrosine recombinase of the integrase family) for cointegrate resolution. The resolvase function is to disassemble the cointegrate structure by catalysing the site-specific recombination between the *res* sites present in both copies of transposon (Grindley *et al*., 1982; Grindley,

2002). The cointegrate resolution by host-encoded system is used, for example, by the members of IS*6* family and Mu phage (Chandler and Mahillon, 2002; Mitkina, 2003).

1.2.2. Non-replicative transposition

In non-replicative (cut-and-paste) transposition, the TE is excised from its initial position by double-strand breaks at the both ends and is inserted into new target locus as a simple insertion (Fig. 2). After the excision of transposon, similar events to replicative transposition take place. The transposon is transferred to the target site and a staggered cut, with a 5´overhang is introduced into the target site by transposon-bound transposase. The 3´ends of the transposon are connected to the 5´ends of the staggered target site and gaps flanking the transposon are refilled by host repair system, giving rise to the short target sequence duplications next to the inserted element (reviewed in Haren *et al*., 1999). Additionally, this type of transposition produces a gapped donor molecule, which might be differentially processed, either degraded or repaired. When the host cell contains a second copy of the transposition donor site, then the recombinational repair system of host can restore the pre-transpositional state of the gapped donor site using the second copy as a template. For instance, this situation can occur when the replication of transposon-containing donor molecule (either plasmid or chromosome) takes place before excision of the transposon. Therefore, under certain circumstances the non-replicative transposition may also lead to an increase in transposon copy number as the replicative transposition (reviewed in Craig, 1996).

The TEs known to transpose via non-replicative pathway are Tn*5* (Bhasin *et al*., 1999; Davies *et al*., 2000), Tn*7* (Bainton *et al*., 1991), Tn*10* (Sakai and Kleckner, 1997; Kennedy *et al*., 1998), several IS elements (Chandler and Mahillon, 2002) and phage Mu during its integration into new host chromosome to establish the lysogenic phase of its life cycle (Harshey, 1984). In contrast, during lytic growth the phage Mu replicates its DNA via multiple rounds of replicative transposition using its own and host proteins (Mizuuchi, 1983; Craigie and Mizuuchi, 1985).

As alluded to above, the exceptional example is Mu phage that is able to transpose replicatively or non-replicatively, whereas the first steps of the non-replicative pathway are identical to the replicative one. In either case the nicks are made only at the 3´ends of the phage DNA, the 3´ends are then joined to the target DNA to generate the Shapiro intermediate in which the donor and target DNA are covalently joined through transposon DNA. The next step is important in making a difference between two transpositional pathways of Mu. Namely, the Shapiro intermediate can yield both non-replicative and replicative transposition products (Craigie and Mizuuchi, 1985). In the case of non-replicative pathway the 5´ends of Mu are cleaved in the Shapiro intermediate and then

joined to the target DNA, resulting in a simple insertion in the target molecule. In the replicative pathway the next step to Shapiro intermediate is the initiation of replication from the free 3´OH of target DNA, resulting in the formation of cointegrate (Craigie and Mizuuchi, 1985; Craigie and Mizuuchi, 1987; Craig, 1996). Thus, not all non-replicative transposition reactions involve an excised TE prior to target capture.

1.3. Detailed view of the transposition reaction

Five protein families have been described that mediate transposition reactions among prokaryotes and eukaryotes. The proteins from these families have different catalytic mechanisms to carry out transposition reaction (Curcio and Derbyshire, 2003). The well characterised and probably the biggest transposon family, on which I will concentrate, is the DDE-transposon family. This family includes several mobile elements from prokaryotes and eukaryotes, for example, several IS elements, Tn*3*-like elements, Mu phage, *Hermes* transposon of insects, *Ac/Ds* elements of maize, *Tc/Mariner* elements. Even the vertebrate immune system V(D)J joining process has presumably evolved from a DDE transposon. Retroviruses (such as human immunodeficiency virus and avian sarcoma virus) and LTR-retrotransposons also use enzyme with closely overlapping catalytic DDE domain, called an integrase that performs reaction similar to transposition to integrate the DNA copy of their RNA genomes into the host chromosome. The proteins of these mobile elements have in common a conserved amino acid motif of two aspartic acid residues and one glutamic acid residue known as DDE or D,D35E motif that is located in the active site and is essential for catalytic activity (reviewed in Curcio and Derbyshire, 2003).

The invariant DDE-motif residues have shown to be essential for transposition *in vitro* and *in vivo* (Baker and Luo, 1994; Bolland and Kleckner, 1996; Kennedy and Haniford, 1996; Naumann and Reznikoff, 2002). The function of this triad in the active site is to bind and coordinate divalent metal ions required for catalysis of transposition reaction. Additionally, this triad is responsible for proper positioning of the transposon DNA in the transposase active site (Bujacz *et al*., 1997; Allingham *et al*., 1999; Davies *et al*., 2000; Steiniger-White *et al*., 2004). The divalent metal ions in the transposase active site might be responsible for proper orientation of the nucleophile for catalysis, as have been revealed from crystal structure of Tn*5* transposase in complex with transposon termini (Davies *et al*., 2000; Steiniger-White *et al*., 2004). Based on the crystal structure of Tn*5* transposase, the transposase can be divided into three domains: an N-terminal domain, the DDE-motif containing central catalytic domain and a C-terminal domain. The function of N-terminal domain is primarily to bind to DNA (recognising the transposon termini) while the C-terminal domain forms protein-protein interactions with a C-terminal domain of the second transposase

molecule and interacts with the DNA. Although these transposase domains have some primary functions, they do not have independent functions from each other as they all participate in DNA binding and play a role in forming a stable synaptic complex or transpososome (reviewed in Davies *et al*., 2000).

All chemical steps of transposition occur within transpososome, which contains two transposon ends (in some cases the target DNA as well) that are brought together by oligomerised transposase molecules. As it has been shown for Mu and Tn*5* transposases (but probably is the common feature for all DDE transposases), a transposase monomer (or dimer in the case of Mu phage) bound at one transposon end catalyses cleavage and joining of the opposite end, which means that transposases are enzymes operating *in trans* (Aldaz *et al*., 1996; Savilahti and Mizuuchi, 1996; Naumann and Reznikoff, 2000). These biochemical observations are now confirmed by analysis of co-crystal structure of Tn*5* transposase bound to transposon end DNA. Namely, the dimerisation between two transposase molecules places the cleavage site at one end of Tn*5* into active site of the other transposase monomer bound to the opposite end of this transposon (Fig. 4) (Davies *et al*., 2000; Steiniger-White *et al*., 2004). Thus, the architecture of the transpososome ensures that Tn*5* DNA cleavage occurs *in trans* and can happen only after formation of the transpososome. Therefore, the correctness of transpososome assembly determines the occurrence of the whole transposition event.

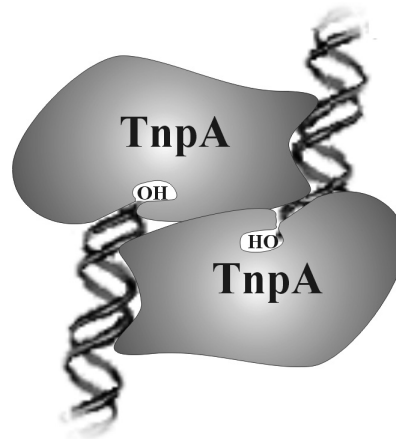

Figure 4. Tn*5* transposase-DNA complex. Within formed transpososome, transposase molecule bound to one end of transposon catalyses the cleavage reaction of the other end of transposon (after Williams and Baker, 2000).

Though the transposition/integration reactions carried out by DDE transposases/ integrases have variations in details, the fundamental mechanism of the chemical catalysis is the same. As illustrated in Figure 2 and 3, the transposition

is a multi-step process. First, transposase molecule(s) binds specifically to both terminal sequences of transposon and bends the transposon end sequences. Then, the end sequences are brought together through oligomerisation of the bound transposase molecules to form a transpososome. Within transpososome, all chemical steps of the transposition reaction occur in a defined order for each transposon end. To release the 3´OH termini of the TE, the transposase–coordinated divalent metal ions activate a water molecule that gives a nucleophilic attack on the phosphodiester backbone between transposon and flanking DNA. Thereby generated 3´OH groups attack the target DNA (called DNA strand transfer) in the case of replicative transposons (the members of Tn*3* family, Mu phage) or the opposite DNA strand in the case of non-replicative transposons (Tn*5*, Tn*10*). In the latter case, the hairpins at both transposon ends are created and transposon is excised from the flanking DNA (Goryshin and Reznikoff, 1998; Bhasin *et al*., 1999). The hairpin intermediate is resolved by a second activated water molecule that regenerates the free 3´OH at the excised element ends, which then attack phosphodiester bonds of the target in the staggered fashion. Subsequently, the transposon is integrated by formation of a covalent bond between the 3´OH groups of the transposon ends and the 5`phosphate groups of the target (reviewed in Mizuuchi, 1992; Rice and Baker, 2001; Reznikoff, 2003). Remarkably, both chemical steps, the DNA cleavage and joining reactions, are common for all characterised members of this transposase/retroviral integrase superfamily and are performed only by the single active centre of these proteins. Noteworthy, during catalysis the transposases do not require external energy source and do not form covalent protein-DNA intermediates, which means that the reaction is probably driven forward only by product binding energy (reviewed in Rice and Baker, 2001). This suggestion is supported by the facts that transposition is irreversible, the net number of high-energy bonds remains the same during the transposition reaction and that the formed transpososome becomes more stable as the reaction progresses (Rice and Baker, 2001; Liu *et al*., 2005).

In addition to transposase and defined ends of the element, mobile elements often require host encoded accessory proteins to aid the transpososome formation (see the section 1.4.2). However, there is no structure-based information about how such accessory proteins interact with the core transposition machinery and affect transposase activity.

1.4. Transposition regulation of prokaryotic transposable elements

To avoid deleterious consequences for host, TEs usually are tightly regulated and have evolved astute regulatory mechanisms. These regulatory mechanisms can act at various steps of transposition process, but most of them influence the

regulation of transposase: its transcription and translation; stability and activity; DNA binding and catalysis. Generally, the regulatory mechanisms of TEs are divided into two major groups: intrinsic control mechanisms and host-mediated regulatory mechanisms.

1.4.1. Intrinsic control mechanisms

With the term intrinsic mechanisms, I mean the regulatory mechanisms employed by the TE itself. The generic purpose of these transposon-encoded mechanisms is to keep the transposition activity in a restrained state. The following sections demonstrate that the low expression of transposase gene (usually named as *tnpA*) and very low transposition frequency is usually achieved not by one certain mechanism but by several overlapping mechanisms.

1.4.1.1. Regulatory mechanisms acting on transposase transcription

Many transposase promoters are weak and thereby limit TnpA expression at the transcriptional level. This trait has been observed for several elements: Tn*1721*, IS*1*, IS*2*, IS*30*, IS*911*, IS*200*, IS*10* (Altenbuchner and Schmitt, 1983; Nagy and Chandler, 2004; Beuzon *et al*., 2004; Lewis *et al*., 2004). For example, IS*2* native transposase promoter lacks a recognisable –35 motif and appears to rely on an "extended –10 motif", with a TG sequence located 1 bp upstream of the –10 hexamer. As a consequence, the transposase promoter activity is about 7% of p_{lacUV5} activity (Lewis *et al*., 2004). Another remarkable example is IS*10* transposase promoter p_{IN}, which activity is about 5% of p_{lacUV5} but the presence of the second IS*10* promoter, p_{OUT}, located within the transposase open reading frame (ORF) and transcribed in the opposite direction, reduces p_{IN} activity to undetectable level (Simons *et al*., 1983).

Transposase promoters are often partially located in the terminal IRs, presumably allowing regulation by transposase binding or by binding of truncated transposase derivatives, which probably are competing with the transposase for binding to the element ends (IS*1*, IS*2*, IS*911*). For example, in the case of IS*1* element, transcription of two partly overlapping ORFs (*insA* and *insB*) is initiated from a weak promoter, p_{IRL}, located partially in left IR. A transcript from this promoter can be translated to two proteins: InsA or InsAB´. InsA protein is more abundantly encoded than transposase InsAB´, which production requires a programmed translational frameshift between *insA* and *insB* frames (see the section below). Truncated form of transposase, InsA, which contains only N-terminal DNA binding domain of transposase, binds specifically to the ends of IS*1*, thereby repressing transcription from the promoter p_{IRL} and competing with InsAB´ transposase. The InsA/InsAB´ ratio determines the overall transposition frequency of IS*1* (Zerbib *et al*., 1990;

Escoubas *et al*., 1991; Chandler and Mahillon, 2002). Additionally, it has been found that IS*1* and several other IS elements (e.g. IS*2*, IS*3*, IS*5*, IS*30*) contain sequences similar to transcription termination signals within their transposase ORF. This strategy probably allows the premature transcription termination and leads to the production of N-terminal part of TnpA, which can act only as a regulatory protein (Nagy and Chandler, 2004). Actually, such truncated forms are a hallmark of many transposases and appear to bind more avidly than transposases itself (e.g. IS*30*: Stalder *et al*., 1990; IS*911*: Haren *et al*., 1998).

For Tn*3* and many other transposons belonging to the Tn*3* subclass it seems that transcription from *tnpA* promoter is inhibited by an element encoded site-specific resolvase, which is involved in resolution of cointegrates in replicative pathway of transposition. Tn*3* resolvase and transposase genes are adjacent to each other but are transcribed in opposite directions. Therefore, the binding of Tn*3* resolvase (TnpR) to the *res* sites, which overlap the divergent *tnpR* and *tnpA* promoters, represses transcription from both of them. The disruption of *tnpR* gene of Tn*3* has been shown to result even in a 10- to 100-fold increase in expression from the *tnpA* promoter (Sherratt, 1989).

Another possibility how to regulate the initiation of transcription may happen via methylation. IS*3*, IS*4*, IS*5*, Tn*10*/IS*10*, Tn*5*/IS*50* and Tn*903*/IS*903* all contain (GATC) sites for DNA adenine methylase (Dam) close to or overlapping with the *tnpA* promoters and in a *dam* defective host their *tnpA* promoter activities as well as transposition activities are increased (Roberts *et al*., 1985; Yin *et al*., 1988; Chandler and Mahillon, 2002). It is thought that Dam methylation is used to couple transposition to host replication process, allowing it to occur in a narrow time window when replication fork has passed across the element leaving it in a hemimethylated state (see also the section 1.4.2.2.) (Roberts *et al*., 1985; Chandler and Mahillon, 2002).

In spite of the fact that *tnpA* promoters are usually weak and down-regulated, many IS elements (IS*2*, IS*21*, IS*3*, IS*30*, IS*150*, IS*186*, IS*256,* IS*911*) have adopted a strategy to increase their transposase expression for short-term by creating strong transient promoter known as p_{junc} (Reimmann *et al*., 1989; Duval-Valentin *et al*., 2001; Chandler and Mahillon, 2002; Szeverenyi *et al*., 2003). In the case of IS*2*, the formed p_{junc} promoter can be nearly as strong as p_{lacUV5} and about 14-fold stronger than its native transposase promoter (Lewis *et al*., 2004). The formation of the transient promoter is based on following. The above mentioned IS elements carry an inwardly directed –10 hexamers in their left terminal ends and an outwardly directed –35 hexamers in the right ends. If two IS ends are juxtaposed by formation of head-to-tail dimers or of minicircles of the IS, then an "active" junction promoter p_{junc} is created and oriented to induce transposase expression. Synthesised transposase binds to the active junction for repression of transcription from p_{junc} and for cleaving at both ends of the element leading to transposition event and destroying the active junction (Chandler and Mahillon, 2002). Why some IS elements need the formation of stronger promoter in their transposition pathway remains largely unclear.

However, for IS*2* (and for IS*911*) which transposes via a circular transposition intermediate, the burst of transposase expression from p_{junc} is probably needed for efficient insertion into a target sequence, because with the native level of TnpA expression at least 90% of the IS*2* minicircles failed to insert into the target DNA (Lewis *et al*., 2004). Thus, for some IS elements (IS*2*, IS*911*) the temporary induced transposase expression from transient promoter helps to complete their transposition reaction and thereby minimise the loss of the element.

Taken together, the existence of a weak promoter seems to be a feature common to most transposase genes. However, this mechanism is not universal and in such cases when the transcription from the transposase promoter is quite high, TnpA expression is restricted by other means, for example, by presence of poor ribosome binding site (see below).

1.4.1.2. Regulatory mechanisms acting on transposase translation

For some TEs transcription from *tnpA* promoter can be relatively efficient but it does not mean that TnpA production is thereby high. For instance, IS*186* promoter appears to be quite strong one (38% of p_{lacUV5}) but the translation rate of this transcript is extremely low, probably because of the lack of obvious ribosome binding site (RBS) (Nagy and Chandler, 2004). Tn*3* has also been characterised by presence of poor RBS preceding the ATG start codon (Casadaban *et al*., 1982). The estimated translation efficiency of the Tn*3* *tnpA* gene is about 5 to 15% of the *lacZ* gene (Sherratt, 1989).

An effective mechanism for controlling transposase expression is a programmed translational frameshift between two consecutive and partially overlapping open reading frames. This strategy is used by several IS elements (mainly the members of IS*1* and IS*3* families) to synthesise transposase from two open reading frames and is based on –1 frameshifting event. In this case, the translating ribosome slides 1 base backward at the so-called slippery codons (most commonly A AAA AAG heptanucleotide sequence) at the end of the first reading frame and continues in another frame, resulting in production of fusion protein – transposase. Ribosome sliding at this position is facilitated by structures that tend to block the ribosome movement along the mRNA, such as potential RBS upstream or secondary structures downstream of the slippery codons (Mahillon and Chandler, 1998). However, the slippage of ribosome between two consecutive reading frames is not a frequent event - it can occur at a frequency of approximately 1% as has been shown for IS*1* (Escoubas *et al*., 1991). Thus, the upstream reading frame appears to be translated more abundantly and usually the product from upstream frame (for example *insA* frame of IS*1*) contains the DNA binding domain while downstream frame (for example *insB´* frame of IS*1*) includes the catalytic site. Thus, the frequency of frameshift is crucial in determining the transpositional activity, while the

product of upstream frame (InsA of IS*1*) alone can act as a modulator of transposition (Mahillon and Chandler, 1998).

Another possibility how the transposase expression can be regulated at the translational level is described for IS*10*, the active element in composite transposon Tn*10*. IS*10* encodes a small antisense RNA (RNA OUT) from a promoter p_{OUT}, which opposes the transposase promoter p_{IN} (Simons and Kleckner, 1983). This small (~70 nt) RNA molecule is unusually stable *in vivo* (half-life 60 min) and pairs complementarily with the 5′end of transposase mRNA, thereby blocking the ribosome binding to transposase mRNA (Case *et al*., 1989; Ma and Simons, 1990). In addition, the formed RNA duplex is sensitive to the cleavage by double-strand specific endoribonuclease (RNaseIII) leading to the destabilisation of the transposase mRNA, which half-life is estimated to be only ~30 s *in vivo* (Case *et al*., 1990).

TEs insert nearly randomly into DNA and can therefore sometimes insert into highly expressed genes and so be activated by strong external promoters. For protection from increased external transcription, certain IS elements have evolved mechanisms to sequester the translation initiation signals of transposase gene in a RNA secondary structure. These elements carry the potential RNA stem-loop forming sequences close to the left end. If the transcription originating from strong external promoter passes the left end of the element, then the translation initiation signals for transposase will be hidden in the forming RNA secondary structure (Chandler and Mahillon, 2002). This mechanism has been shown experimentally for IS*10* and IS*50* but potentially might be used by several other IS elements as well (Halling *et al*., 1982; Davis *et al*., 1985; Krebs and Reznikoff, 1986; Schulz and Reznikoff, 1991; Ma *et al*., 1994).

1.4.1.3. Transposase stability and *cis*-activity

An additional stage of regulation for many TEs is a control over transposase stability and activity. In general, transposases are active *in cis*, i.e., they perform their functions near the site of their synthesis. Presumably the transposase preferential *cis*-activity can prevent activation of other elements in the same cell. This generic phenomenon is usually assured by the consequence of several overlapping mechanisms. For example, in the case of IS*903* the *cis*-activity of transposase is based on its limited biosynthesis and protein instability (Derbyshire and Grindley, 1996). Similarly, IS*10* transposase *cis* preference depends on transcript release from its template, on the half-life of mRNA and on translation efficiency (Jain and Kleckner, 1993). In addition to IS*903* and IS*10*, the transposase preference to act *in cis* has also been observed for IS*1* and IS*50*. Depending on given IS element the extent of *cis*-activity can vary several orders of magnitude (Chandler and Mahillon, 2002).

There have been considerations that the domain structure of transposases may also contribute to preferential *cis*-activity. This idea has arisen from notifications that C-terminally truncated transposase derivatives can function also *in trans* and bind more efficiently to the ends of the element than full-length transposase (Mahillon and Chandler, 1998). In most cases, the DNA-binding domain is located at the N-terminal end of transposase and this would allow binding of nascent polypeptide to the binding sites located nearby if the folding of N-terminal domain occurs before complete translation of C-terminal domain (Mahillon and Chandler, 1998). For Tn*5*, it has been suggested that subsequent folding of C-terminally located catalytic domain of transposase may sterically mask the N-terminal part of protein and inhibit so the site-specific binding activity of full-length transposase molecule (Weinreich *et al*., 1993). This suggestion also correlates with observations that wild-type Tn*5* transposase is inactive *in vitro*. The inactivity of wild-type transposase is eliminated by the L372P mutation, which allows the N-terminus to move away from the C-terminus and bind DNA (Goryshin and Reznikoff, 1998; Davies *et al*., 2000; Steiniger-White *et al*., 2004).

Transposases usually function as multimeric proteins. For instance, IS*50* and IS*10* transposases work as dimers and Mu transposase is active as a tetramer. Therefore, if a truncated transposase variant is able to dimerize with transposase, it results in formation of the inactive heterodimers and in diminishing the active transposase pool. This kind of transposition down-regulation is characteristic to Tn*5*/IS*50*, which encodes from an alternative promoter a shorter version of transposase, called Inh (Yin and Reznikoff, 1988). Inh is *trans*-acting inhibitory protein with enhanced ability to dimerize with transposase. The formed Inh-Tnp heterodimer binds to the ends of the element resulting in a complex inactive for transposition (de la Cruz *et al*., 1993). It has been shown that the blocking of Inh protein synthesis increased the trans-position frequency about 10-fold (Wiegand and Reznikoff, 1992).

An important characteristic contributing to transposase *cis*-activity is the instability of protein. The transposase of IS*903* has been shown to be unstable with the physical half-life of about 3 min (Derbyshire *et al*., 1990). An important determinant in its instability is Lon protease, because in protease-defective *lon* strains the transposase was stabilised and its *trans*-action was increased 10- to 100-fold (Derbyshire *et al*., 1990). In addition, the temperature-sensitive transposition of Tn*3*, IS*911* and IS*30* may reflect the natural instability of transposase (Kretschmer and Cohen, 1979; Haren *et al*., 1997; Nagy and Chandler, 2004). At present, the exact molecular mechanism behind this phenomenon is largely unknown, but *in vivo* and *in vitro* transposition studies of IS*911* have shown that the incubation of transposase at 42ºC resulted in irreversible loss of activity, which could be overcome by mutation of the protein (Haren *et al*., 1997). Two point mutations providing the temperature-resistance of IS*911* transposase were located in the region suggested to be important for correct oligomerisation. Thus, the natural temperature-sensitivity of the

transposases might be caused by the defect in correct multimerisation at high temperature (Haren *et al*., 1998).

1.4.1.4. Regulation of transpososome assembly

The correct assembly of highly organised nucleoprotein complex, termed transpososome or synaptic complex is prerequisite for the transposase catalytic activity. This complex includes the transposon ends and transposase, and depending on the element, it might contain also a target DNA or some accessory proteins. The requirement of correct assembling before catalytic activity reduces the possibility of non-productive cleavages and strand transfer events, which otherwise would lead to the damage of host genome integrity. Therefore, this stage can be considered as an important checkpoint in transposition pathway (Nagy and Chandler, 2004).

First of all, the correct assembly of transpososome requires both ends of transposon. Usually the both ends of the element are very similar to each other but almost always not perfectly identical. On the other hand, the slight differences between two transposon ends might be needed to distinguish them in catalytic steps of transposition. For instance, phage Mu and Tn*7* are characterised as having structurally and functionally asymmetric ends (Chaconas and Harshey, 2002; Craig, 2002). In the case of Mu phage there are three transposase MuA binding sites at the each end, but these binding sites are organised differently (Craigie *et al*., 1984; Zou *et al*., 1991). The different arrangement of Mu transposase binding sites may reflect the distinct roles of Mu ends in transpososome assembly. Indeed, it has been shown that the assembly of Mu transpososome is initiated by the interactions between the Mu right end and enhancer element to establish the right end-enhancer complex (ER) (Pathania *et al*., 2003). Thereafter HU protein binds to the Mu left end and directs it to the ER complex to form another transient intermediate complex LER (Mu left end, the enhancer sequence and the right end) (Watson and Chaconas, 1996; Kobryn *et al*., 2002; Pathania *et al*., 2003). The LER complex is converted into a more stable synaptic complex (SSC) (or type 0 complex), in which MuA transposase obtains its active tetrameric form and thereby is ready to carry out the reaction chemistry of transposition (Mizuuchi *et al*., 1992; Watson and Chaconas, 1996). Similarly to Mu phage, Tn*7* has not only structurally but also functionally distinct ends. Namely, Tn*7* prefers to insert into target *attTn7* site in a particular orientation, with the right end oriented towards the bacterial *glmS* gene that encodes protein involved in cell wall biosynthesis (Lichtenstein and Brenner, 1981; Bainton *et al*., 1991). The assumption of functional difference between Tn*7* ends is also supported by the fact that if Tn*7* element contains two left terminal ends then it is transpositionally inactive (Arciszewska *et al*., 1989).

Next strategy for preventing the non-productive cleavage at the ends of TE is assured by the architecture of transpososome, as has been shown for phage Mu and Tn*5*. For example, in Mu transposition, the catalytically active site of transposase is assembled from four different MuA monomers within the transpososome (Lavoie *et al*., 1991; Baker and Mizuuchi, 1992). Moreover, it has been shown for Mu and Tn*5* transposases (but probably is common to all DDE transposases) that they are operating *in trans*, which means that an active site assembled at one end catalyses cleavage and joining of its opposite end (Aldaz *et al*., 1996; Savilahti and Mizuuchi, 1996; Naumann and Reznikoff, 2000). Thus, these restraints ensure that the cleavage reaction does not occur until the two transposon ends are paired within the transpososome.

Another important point, which may determine the occurrence of cleavage reaction within transpososome, is the requirement for target sequence. The transposon Tn*7* is one of the examples that exploits this strategy. Namely, the specific target site *attTn7* has to be included in the formed transpososome to activate the Tn*7* transposase and to initiate the cleavage reaction at both ends of the element (Bainton *et al*., 1991; Bainton *et al*., 1993). In contrast to Tn*7*, most of the other TEs interact with the target DNA after nicking the first strand at the element ends or even after excision from the donor molecule.

Taken together, the previous examples demonstrated that even if the production of transposase is favoured under appropriate conditions, then for efficient transposition reaction the transposase must obtain its catalytic activity through the sequential steps of transpososome assembly. Therefore, the correct formation of transpososome can be considered as a significant precondition for transposition to take place.

1.4.1.5. Target site selection and transposition immunity

The wellness of transposon and its host depends on how astutely transposon selects its target sequence, because an insertion into an essential gene of host can have serious implications for both of them.

The target sites differ significantly among elements, but the selection process itself is mainly determined by the direct interaction between transposon-encoded transposase and the target DNA. Alternatively to transposase, the element-encoded accessory proteins might also capture the target DNA as it occurs in the case of Tn*7* and Mu (Craig, 1997). It has been considered that TEs can insert into many different sites in the genome. However, at the nucleotide level most of them exhibit some degree of selectivity. This means that target site selection does not occur totally randomly and TEs have strategies for selecting their targets (Craig, 1997). For example, the transposition of Mu phage has been shown to exhibit a strong target site preference for all single-nucleotide mismatches (Yanagihara and Mizuuchi, 2003). Some IS elements (IS*911*, IS*30*) prefer to insert next to the sequences resembling their terminal

ends (Polard *et al*., 1994; Loot *et al*., 2002; Olasz *et al*., 1997), some other IS elements have strong preference for insertion into other mobile elements (e. g., IS*231* prefers the terminal ends of the transposon Tn*4430*, IS*2* favours a phage P1 region for insertion) (Sengstag and Arber, 1983; Hallet *et al*., 1994). Conversely to the above-mentioned elements, some elements prefer target sites locating more distantly from their own positions. This strategy helps them to avoid insertions into the pre-existing copy of itself or even nearby it. The latter process is called transposition immunity or target immunity, which will be reviewed below in this section.

Several TEs exhibit a preference for specific target sequence, although their preference can be variable in its stringency. For example, Tn*7* has strict target preference to transpose into unique chromosomal site *attTn7* in the case of one of its two transposition pathways. The selection of *attTn7* pathway is mediated by TnsABC+D proteins of Tn*7*. The TnsD protein recognises the site before the end of the highly conserved gene *glmS* among bacteria and directs Tn*7* insertion at high frequency downstream to the *glmS* gene without disrupting it (Bainton *et al*., 1993). The alternative pathway of Tn*7* occurs at low frequency and leads to transposition into DNA undergoing lagging strand synthesis, such as conjugating DNA (Wolkow *et al*., 1996; Peters and Craig, 2001). Some other examples of specific target preference are ISPpu9 and ISPpu10 from *Pseudomonas putida*, IS*1397* from *Escherichia coli* and ISKpn*1* from *Klebsiella pneumoniae* that selectively target the REP (repetitive extragenic palindromic) sequences located in intergenic regions throughout chromosomes of these organisms (Wilde *et al*., 2001; Nelson *et al*., 2002; Wilde *et al*., 2003). Thus, for TE, the high level of target specificity might represent a strategy of propagation that avoids harming the essential host genes and thereby assures the "safe place" for future generations. On the other hand, the preference of long and conserved target sites may potentially restrict the selection of target sites, which in turn can limit the host range of these elements.

Nevertheless, the primary sequence of the target is not the only determinant. In most cases, the global features of the target DNA may additionally influence (or be the primary determinants) the element insertion into particular target site. These can be, for example, the degree of DNA supercoiling (Lodge and Berg, 1990), DNA bending (Hallet *et al*., 1994), presence of some specific host proteins on target site (so-called protein-mediated targeting) (Swingle *et al*., 2004), transcription (Bernardi and Bernardi, 1988; Casadesus and Roth, 1989; Wang and Higgins, 1994) and replication (Bernardi and Bernardi, 1987; Wolkow *et al*., 1996; Peters and Craig, 2000; Peters and Craig, 2001a).

As mentioned above, the transposition immunity plays a key role for some mobile elements in determining which target sites will be selected for. So far the transposition immunity is observed only for more complex transposons such as Tn*7* and Mu phage, as well as members of Tn*3* family (Arciszewska *et al*., 1989; Darzins *et al*., 1988; Adzuma and Mizuuchi, 1988; Lee *et al*., 1983; Amemura *et al*., 1990; Goto *et al*., 1987; May and Grindley, 1995). In these

cases, the immunity signals are provided by the transposase bound sequences at the ends of transposon DNA (Lee *et al*., 1983; Adzuma and Mizuuchi, 1988; Stellwagen and Craig, 1997). Unlike to Tn*3* transposon, Mu and Tn*7* have special proteins in addition to transposase involved in transposition immunity. Namely, the target DNA is captured and channelled into assembled transpososome by Mu phage protein MuB and Tn*7* protein TnsC. However, when MuB or TnsC are occasionally bound to the target DNA containing Mu or Tn*7* ends, respectively, then transposase bound transposon ends of Mu and Tn*7* will trigger the ATP hydrolysis dependent dissociation of MuB and TnsC from the target DNA leaving it "immune" to further insertions (Adzuma and Mizuuchi, 1988; Greene and Mizuuchi, 2002; Stellwagen and Craig, 1997; Skelding *et al*., 2003). As have been shown for Tn*7*, the target immunity relies on DNA spacing while the magnitude of immunity is greater at close DNA sites than DNA sites farther away (DeBoy and Craig, 1996). This phenomenon is explained by high local concentration of transposase on target DNA that contains transposase-binding sites, thereby inhibiting the binding of TnsC to the immune target DNA (Stellwagen and Craig, 1997). Tn*7* target immunity has been shown to act over quite long distances. For example, the presence of Tn*7* ends prevented Tn*7* insertions into large (60 kb) plasmids (Arciszewska *et al*., 1989) and in the chromosome Tn*7* ends reduced insertions into sites 190 kb away, but insertions into sites 1900 kb away were not affected (DeBoy and Craig, 1996).

What is the purpose of transposition immunity? Probably it helps to prevent the formation of DNA molecules containing two nearby located copies of element that could be then substrates for homologous recombination leading to deleterious events, like deletion of the sequence between two copies of element. In addition, it prevents the self-destruction events of particular element. Thus, transposition immunity not only protects the transposon and its host but also ensures transposon dispersion (Peters and Craig, 2001b; Craig, 2002).

1.4.2. Host mediated regulatory mechanisms

Host factors may have important roles in regulation of transposition. They can implicate several stages throughout transposition, including transposase expression, transpososome assembly, target site selection and are clearly required in the stage of DNA repair. The combination of host factors involved in transposition differ among elements, each element can have its own set of host factors and may have its own way to use a given host factor. In this section I do not intend to cover all the host factors found in regulation of transposition, instead I will stop only at the most common ones. In this connection, I would like to point out that despite the huge amount of TEs identified not many of them are studied in sufficient detail to establish these, sometimes very subtle host effects.

1.4.2.1. Nucleoid-associated proteins and DNA supercoiling status

Most frequently, involvement of several nucleoid-associated proteins, such as IHF (integration host factor), HU (heat-unstable nucleoid protein), Fis (factor for inversion stimulation) and H-NS (histone-like nucleoid structuring protein) have been demonstrated in regulation of transposition. These four proteins, according to their intracellular abundance and ability to bend DNA locally on binding, play important roles in the compaction and organisation of the bacterial chromatin. In addition to their architectural role, each nucleoid protein is involved in a variety of cellular processes, such as replication, recombination and transcription (Dorman and Deighan, 2003). The expression levels of these proteins vary throughout development of a bacterial culture, also depending on growth conditions (Ali Azam *et al*., 1999). This growth phase dependent variation in nucleoid protein levels can change the local structure of nucleoid and can modulate the global gene expression (Ali Azam *et al*., 1999; Dame, 2005). For example, Fis is the most abundant nucleoid-associated protein in growing *E. coli* cells and is needed for transcription of growth-related genes (those of tRNA and rRNA), but in the stationary phase its expression decreases about 1000-fold, even to undetectable level (Ali Azam *et al*., 1999). Conversely to Fis, the level of IHF was shown to increase about 7-fold upon entry to the stationary phase of growth, making the IHF one of the major histone-like proteins in the stationary phase cells (Ditto *et al*., 1994; Murtin *et al*., 1998; Ali Azam *et al*., 1999). The overall HU level declines with the onset of stationary phase, whereas H-NS is maintained at a more-or-less constant level throughout the growth (Ali Azam *et al*., 1999; Dorman, 2004).

All four above-mentioned nucleoid-associating proteins have been shown to be involved in the regulation of transposition of Mu phage. IHF has even dual action in it. First, it has been shown *in vitro* that IHF acts as an architectural protein (IHF can introduce a bend up to 180º) and a supercoiling relief factor promoting transpososome assembly when supercoiling of Mu DNA is decreased (Surette *et al*., 1989; Allison and Chaconas, 1992). Secondly, the binding of IHF at the enhancer site upstream of Mu p_e promoter facilitates transcription from the p_e promoter that drives expression of genes needed for Mu transposition (van Ulsen *et al*., 1996; van Ulsen *et al*., 1997). Furthermore, IHF activates p_e transcription also indirectly through the alleviation of the H-NS-mediated repression, i.e., IHF binding to its site disrupts nucleoprotein complex formed between H-NS and DNA at the region of p_e promoter (van Ulsen *et al*., 1996; van Ulsen *et al*., 1997). IHF related dimeric protein HU stimulates the Mu transposition reaction by binding to the left end between two transposase binding sites (introduces a bend of about 155º), thereby promoting interactions between transposase monomers and facilitating the assembly of transpososome (Lavoie and Chaconas, 1993; Lavoie *et al*., 1996). In particular, the HU is responsible for bringing the left end into the transposition intermediate complex LER (Kobryn *et al*., 2002; Pathania *et al*., 2003). In addition to its architectural

role, HU is able to mediate changes in DNA supercoiling to the transposition machinery of Mu phage. Namely, it has been shown by Kobryn *et al* (1999) that the level of DNA supercoiling, which varies according to growth phase and environmental conditions can dramatically modulate HU binding to its specific region within Mu left end. Interestingly, the hints about synergistic action of HU and IHF during Mu transposition can be found in the literature (Surette and Chaconas, 1989; Betermier *et al*., 1995). *In vitro* the high amounts of IHF allowed completely to eliminate HU from the reaction (Surette and Chaconas, 1989) and *vice versa*, HU was able to bend enhancer region instead of IHF and to stimulate the formation of nucleoprotein complex between Mu repressor and the enhancer site (Betermier *et al*., 1995). Finally, both H-NS and Fis have negative influence on Mu transposition, presumably by affecting phage gene expression (Falconi *et al*., 1991; Betermier *et al*., 1993; van Drunen *et al*., 1993). In addition to histone-like proteins, several observations indicate that gyrase, the enzyme that introduces negative supercoils into DNA in *E. coli* is also crucial during early stages of Mu transposition (Pato *et al*., 1990; Pato and Banerjee, 1996; Pato *et al*., 1995; Sokolsky and Baker, 2003). Thus, involvement of quite wide spectrum of host factors implies a complicated interplay between them during the regulation of Mu transposition.

Some TEs such as Tn*10*/IS*10*, IS*1* and transposon γδ (Tn*1000*) carry the binding sites of IHF in one or both terminal ends (Gamas *et al*., 1987; Morisato and Kleckner, 1987; Wiater and Grindley, 1988). In the case of Tn*10*, the affinity of IHF binding sites within outer termini of the element changes during transposition (Liu *et al*., 2005). It has been shown *in vitro* that DNA-loop introduced by IHF is required at the early stages of Tn*10* transpososome assembly (Chalmers *et al*., 1998; Liu *et al*., 2005). However, when transpososome is assembled, IHF must be ejected from the complex to promote the conformational changes needed for the cleavage steps of the reaction and for capturing the target DNA. Consecutively, the excised transposition intermediate attains again a high affinity for IHF. If IHF is reacquired at this stage of the reaction, it inhibits interactions with target DNA (Liu *et al*., 2005). IHF functions also as "supercoiling relief factor", relieving the requirement of negative supercoiling in the substrate DNA at the early stage of Tn*10* transposition (Chalmers *et al*., 1998; Liu *et al*., 2005). Thus, like in the case of Mu phage, IHF and/or DNA negative supercoiling are important factors stimulating the early steps of Tn*10* transposition. However, IHF and negative supercoiling may respond in the opposite directions during environmental changes. For instance, with the onset of stationary phase the negative supercoiling decreases while the amount of IHF increases (Ditto *et al*., 1994; Murtin *et al*., 1998). These variations are offering an additional level for regulation and meanwhile probably couple the Tn*10* transposition rate with the host cell physiology (Liu *et al*., 2005).

Both terminal ends of transposon γδ contain a binding site for IHF immediately adjacent to transposase binding site (Wiater and Grindley, 1988).

Although IHF and γδ transposase bind cooperatively to the ends of γδ, the role of IHF in γδ transposition seems to be only modulatory (Wiater and Grindley, 1988; Wiater and Grindley, 1990a). Namely, the γδ transposon transposed equally well with or without terminal IHF binding sites (Wiater and Grindley, 1990a). However, Wiater and Grindley (1990) found that the major effect of IHF was the stimulation of transposition immunity through facilitating binding of transposase to the elements ends (Wiater and Grindley, 1990a; Wiater and Grindley, 1990b). Likewise to γδ transposon, the role of IHF in IS*1* transposition is quite unclear as IS*1* transposition was not notably changed in an IHF mutant host (Gamas *et al*., 1985; Gamas *et al*., 1987). Instead of IHF, H-NS has been shown to be required for IS*1* transposition and its role has been proposed to be stimulation of transpososome formation (Shiga *et al*., 2001).

In addition to their role in transpososome formation, it has been shown that the nucleoid-associated proteins can influence the target selection, presumably either by making DNA regions more accessible for the transposition machinery or stabilising the binding of transpososome to the captured target DNA (Gamas *et al*., 1987; Swingle *et al*., 2004). For example, IS*1* has a preference to insert into the region containing IHF binding sites (Gamas *et al*., 1987). Quite recently it has been found that H-NS can affect the targeting of IS*903* and Tn*10* (Swingle *et al*., 2004).

Thus, the dependence of transposition on nucleoid-associated proteins and DNA supercoiling status is pointing to the astute strategy how transposition rate of certain elements might be coupled to the physiological state of the cell.

1.4.2.2. Other host factors

Dam methylase is also quite common host protein involved in transposition regulation. In addition to its role in regulation of transposase expression (see above in the 1.4.1.1. section) it influences directly the activity of transposon ends (IS*50*/Tn*5*, IS*10*/Tn*10*) as hemimethylated ends are transpositionally more active than fully methylated ends (Roberts *et al*., 1985; Yin *et al*., 1988). It is assumed that the methylation of element ends inhibits transposase binding due to a steric block of interactions between transposase and its binding region (Jilk *et al*., 1996; Reznikoff, 2002; Steiniger-White *et al*., 2004). Considering that Tn*10* and Tn*5* transpose via non-replicative pathway, this arrangement is obviously advantageous to transposon as it ensures element replication before transposition takes place.

Besides Dam methylase, there are several other host proteins such as IHF, Fis and replication initiation protein DnaA implicated in modulation of Tn*5* transposition, however their exact molecular action remains unknown (Yin and Reznikoff, 1987; Makris *et al*., 1990; Weinreich and Reznikoff, 1992). Yet, based on the facts that DnaA has a binding site within the Tn*5* outer ends (OE) (Fuller *et al*., 1984) and it stimulates transposition of Tn*5* at least 10-fold *in vivo*

(Yin and Reznikoff, 1987), it is hypothesised that DnaA could help to disconnect transposase from the final transposition products (Reznikoff, 2002). In addition to above-listed factors, Tn*5* transposition is also stimulated by topoisomerase I, which presumably relaxes donor DNA and facilitates target capture, and by gyrase that possibly generates the negatively supercoiled target for Tn*5* (Sternglanz *et al*., 1981; Isberg and Syvanen, 1982; Yigit and Reznikoff, 1999; Reznikoff, 2002).

The proteases and the chaperone-linked proteases such as Lon, ClpX and ClpP also play important roles in modulating transpositional activity. Lon protease is involved in degradation of the IS*903* transposase as indicated in the section 1.4.1.3. (Derbyshire *et al*., 1990; Derbyshire and Grindley, 1996). The molecular chaperone ClpX is known to intervene in two distinct stages of the Mu phage life cycle. First, it is essential for Mu growth, while it remodels the transpososome strand transfer complex trough destabilising Mu transposase MuA tight grip on DNA and thereby promotes initiation of Mu DNA synthesis by specific replication enzymes (Mhammedi-Alaoui *et al*., 1994; Levchenko *et al*., 1995; Kruklitis *et al*., 1996). In the second stage, ClpX together with the protease component ClpP can stimulate Mu entry to the lytic cycle of development by degrading the Mu repressor protein, which downregulates Mu transposition functions and maintains Mu lysogenic state (Geuskens *et al*., 1992; Mhammedi-Alaoui *et al*., 1994; Welty *et al*., 1997; Jones *et al*., 1998).

Additionally, small proteins with clearly distinct primary roles can function as transposition cofactors. In the case of Tn*3*, the acyl carrier protein (ACP) has been shown to stimulate 3′end cleavage of the transposon (Maekawa *et al*., 1996). Whereas in the case of Tn*7* transposition ACP together with the ribosomal protein L29 has been reported to facilitate the binding of TnsD protein to the target site *attTn7* (Sharpe and Craig, 1998). However, the exact molecular mechanisms of their action have remained unknown.

1.5. Transposition under stress conditions

Transposition as a potentially mutagenic process for host is strictly controlled by various host- and transposon-encoded mechanisms (exemplified in the previous sections). However, despite these strict control mechanisms there are several observations about the transient bursts of transposition under certain stress conditions. In this connection, it has been hypothesised that TEs might be a major source of genetic diversity in response to environmental changes (Kidwell and Lisch, 1997).

The transposition rate has been shown to increase in response to different sources of stress. For example, transposition frequency can respond to temperature effects. The higher temperature can activate several IS elements (e.g., IS*401*, IS*402*, IS*406*, IS*407*, IS*408*, IS*Bmu2* and IS*Bmu3*) in *Burkholderia*

multivorans as has been reported recently by Ohtsubo *et al*. (2005). These findings are in accordance with the earlier report by Dong *et al*. (1992) where they showed that IS*1086* transposition as well as other mutagenic events in *Alcaligenes eutrophus* are activated about 1000-fold by higher growth temperature (37Cº). At this temperature the *A. eutrophus* strain, whose optimal growth temperature is 30Cº, displays a high degree of mortality and a very high proportion of mutants among survivors (Dong *et al*., 1992). This indicates that under hostile environmental conditions the increase in IS*1086* transpositional activity probably has evolved to contribute to survival of the host cell population. However, these examples are in contrast to the finding that some TEs in *E. coli* such as Tn*3*, IS*1*, IS*30*, and IS*911* have decreased transposition activities at higher temperature that has been considered the intrinsic property of their transposases (see also the section 1.4.1.3.) (Kretschmer and Cohen, 1979; Haren *et al*., 1997; Nagy and Chandler, 2004). Thus, the reason why some TEs are activated while the others are repressed in response to higher temperature remains still enigmatic.

In addition to temperature, transposition can be induced when host organisms are exposed to such stress factors as UV irradiation (Aleshkin *et al*., 1998; Eichenbaum and Livneh, 1998), limited nutrient availability (Lamrani *et al*., 1999; Gomez-Gomez *et al*., 1997; Kasak *et al*., 1997; Hall, 1999), micro-aerobic conditions (Ghanekar *et al*., 1999). Even magnetic field can increase the transposition activity (Chow and Tung, 2000; Del Re *et al*., 2004).

The DNA-damaging agents like UV light or chemicals are probably quite common stress sources for microbes, because in response to DNA damages bacteria have evolved specific regulatory network, a SOS response. SOS response causes induction of DNA repair systems allowing bacteria to survive sudden increases in DNA damage. Interestingly, the involvement of SOS response in transposition enhancement is reported for IS*10.* The activity of IS*10* transposition was stimulated by the UV radiation and was shown to be dependent on the functioning of the SOS response system (Eichenbaum and Livneh, 1998). Besides IS*10*, there seems to be connection between SOS response and Tn*5* transposition, though some controversy exists. Some reports are indicating that the induction of SOS system activates Tn*5* transposition while according to another report the SOS response reduces the mobility of Tn*5* (Kuan *et al*., 1991; Kuan and Tessman, 1991; Kuan and Tessman, 1992; Weinreich *et al*., 1991). On the other hand, there are some examples of the host SOS response induction by transposition itself, as it was shown in the case of Tn*10* and IS*1* (Roberts and Kleckner, 1988; Lane *et al*., 1994). Most likely increased transposition activity results in accumulation of DNA double-strand breaks that provide an inducing signal for the SOS system (Lane *et al*., 1994).

The most common stress situation occurring in microbial world is limitation of nutrients. Although carbon starvation *per se* is not generally mutagenic (Hall, 1997), the transpositional activity of several elements seems to be increased under starvation conditions. For example, the transposition of IS elements,

particularly IS*5* and IS*30*, was highly active in agar stabs stored at room temperatures for 30 years (Naas *et al*., 1994; Naas *et al*., 1995). The transposition burst of ISH27 element, which movement was not observable upon continuous cultivation at 37Cº, was seen after the storage of *Halobacterium halobium* cells more than two years at 4Cº (Pfeifer and Blaseio, 1990). Moreover, the induction of mutant Mu prophage transposition has shown to be triggered by prolonged carbon starvation and by the presence of stationary phase sigma factor σ^S (Shapiro and Higgins, 1989; Lamrani *et al*., 1999; Gomez-Gomez *et al*., 1997). However, the involvement of σ^S in Mu prophage transposition has been suggested to be indirect, σ^S did not affect the expression from Mu transposase promoter (Lamrani *et al*., 1999). Evidence supporting the preference for transposition in the late growth has been obtained also from the studies of stationary phase (adaptive) mutations, revealing that transposition of *P. putida* transposon Tn*4652* and *E. coli* element IS*30* is inducible under starvation conditions (Kasak *et al*., 1997; Hall, 1999). This idea is further supported by the recent observations of Coros *et al*. (2005) demonstrating that the transposition of IS*903* and Tn*552* occurred in response to both the developmental stage of the cells and to the gradient of exogenous purines across the colony, preferentially taking place at late times during colony growth. The reason for requirement of exogenous purines in IS*903* and Tn*552* transposition is unclear, but the authors have noted that the GTP-binding motif is present in the transpositional accessory protein of Tn*552* (Coros *et al*., 2005). In addition to these observations, the same group conducted a comprehensive screen for *E. coli* host factors that affect IS*903* transposition under colony environment situation (Twiss *et al*., 2005). This study revealed a range of *E. coli* host proteins involved (either directly or indirectly) in the regulation of IS*903* transposition. Most intriguing was the finding that *aspA* mutation, which prevented conversion of aspartate into fumarate under microaerophilic or anaerobic conditions, caused transposition to occur earlier than normal during colony growth (Twiss *et al*., 2005). The inference of authors was that the transposition was responding to lack of a fermentable carbon source (Twiss *et al*., 2005). Taken together, in the light of previous examples, it is obvious that several stress signals can cause increase in the frequency of movement of TEs.

While transposition is generally considered to be harmful to host fitness, why does it increase under stress conditions? It is proposed that the genetic variability resulting from the increased transposition is likely beneficial for increasing the chance for survival under unfavourable conditions and thereby for the expression of new genetic traits (Morillon *et al*., 2000; Capy *et al*., 2000). This variability could be important especially for those bacteria that have not evolved special mechanisms such as sporulation to resist starvation, but instead have to change their physiology during stressful periods. Indeed, there are several reports about the involvement of TEs in host adaptation (Chao *et al*., 1983; Chao and McBroom, 1985; Wery *et al*., 2001; Bongers *et al*., 2003; Kozitskaya *et al*., 2004; Morillon *et al*., 2000; Riehle *et al*., 2001; Edwards *et*

al., 2002), although the molecular mechanisms underlying this phenomenon are still hardly studied.

1.6. Evolutionary success of transposable elements

Since their discovery by Barbara McClintock in the 1940s (reviewed in Jones, 2005), the TEs have been found in almost all organisms examined to date (exceptional are some specialised intracellular parasites with highly degenerate genomes) (Katinka *et al*., 2001; Moran and Plague, 2004). The proportion of TEs in different genomes varies, ranging from few elements in some bacteria to more than 70% of genome in some plants (reviewed in Kidwell and Lisch, 2002). However, in contrast to bacteria, most elements are no longer active in large eukaryotic genomes and in host's point of view do not carry obvious selectable values as some bacterial transposons do (for example genes of antibiotic resistance, etc.). Instead, the presence of TEs is known to decrease the host fitness due to their ability to insert into coding DNA sequences and cause chromosomal breaks, deletions or inversions. These findings, the abundance of TEs in eukaryotic genomes and their potential to be harmful to the host genome, have raised several questions.

Two of the main questions raised by the evolution of TEs are their origin and the relationship between the element and its host organism. Actually, these topics have been a matter of debate almost since the discovery of mobile DNA. The TEs are labelled differently over the time course. They are thought to be controlling elements (McClintock, 1984), parasitic or selfish DNA (Orgel *et al*., 1980; Doolittle and Sapienza, 1980), junk DNA (reviewed in Kidwell and Lisch, 2000 and Biemont and Vieira, 2005), semiparasitic (Hickey, 1982), parasitic and useful (Capy *et al*., 2000), maladaptive (Vinogradov, 2003) or natural components of genomes like promoters, exons, etc. (Labrador and Corces, 2002; Shapiro, 2005; von Sternberg and Shapiro, 2005). In fact, all these hypotheses do not preclude each other and may play roles in the evolution of TEs.

The parasitic or selfish DNA hypothesis originates from two essays written by Orgel (1980) and Doolittle and Sapienza (1980). The hypothesis is based on TEs ability to replicate on the host expense and to do it potentially faster than host genome (Orgel *et al*., 1980; Doolittle and Sapienza, 1980). According to "the selfish DNA hypothesis" the explanation for TEs continuing existence in the genomes is their positive selection at the DNA level (based on their replicative advantage over non-mobile sequences) without involvement of positive selection at the level of organism (Orgel *et al*., 1980; Doolittle and Sapienza, 1980). "The junk DNA hypothesis" is based on the TEs (especially the inactive ones) neutrality at the host level. According to this hypothesis the TEs continue to increase their copy numbers in the host genomes as long as the host tolerates the increased genetic load (reviewed in Kidwell and Lisch, 2000;

Biemont and Vieira, 2005). By contrast to these interpretations that increased mobile element fractions are proof of the absence of their role in host organism evolution, Henikoff *et al*. (1997) propose that mobile elements "may be a manifestation of the evolutionary benefits of genomic flexibility". Indeed, there is mounting evidence that transposon-derived genes can contribute to several cellular processes in eukaryotes (Agrawal *et al*., 1998; Hudson *et al*., 2003; Hammer *et al*., 2005), meaning that mobile elements have already influenced and may continuously influence the evolution of their eukaryotic host by being a source of genomic novelties. The well-known examples are *HeT-A* and *TART* retrotransposons which repeated transposition provides a mechanism for lengthening the shortened chromosome ends in *Drosophila melanogaster*, a function normally performed by telomerase (Biessmann *et al*., 1990; Biessmann *et al*., 1992; Biessmann *et al*., 2000; reviewed in Pardue and DeBaryshe, 2002). The V(D)J recombination responsible for the immune repertoire of vertebrates is claimed to be evolved from an ancient TE (Agrawal *et al*., 1998; Plasterk, 1998; Zhou *et al*., 2004; Kapitonov and Jurka, 2005; reviewed in Gellert, 2002). Also the evidence that TEs are involved in gene regulation by providing regulatory sequences (reviewed in Kidwell and Lisch, 2000; Kidwell and Lisch, 2002) or by epigenetic silencing of nearby genes is accumulating (reviewed in Lippman *et al*., 2004). Even the element insertion into intron sequences can have transcriptional effects on gene expression as it has been shown for retrotransposon L1 (Han *et al*., 2004). Most interestingly, the recent study performed by Bundock and Hooykaas (2005) is pointing out that abundant transposase-like sequences in the genomes may not always be just "genetic fossils", but they have potential to evolve functions that are essential for development of host organism. This hypothesis is also supported by the Nekrutenko and Li (2001) earlier notification that TEs (or sequences derived from them) can be found within a number of protein-coding genes in humans, suggesting that TE insertions can indeed contribute to creation of new genes (Nekrutenko and Li, 2001).

In spite of the ongoing intense debate about the functional significance and persistence of TEs within eukaryotes through time, it is widely accepted that TEs contribute to the evolution of bacteria. An example of wide evolutionary change is the emergence of transmissible antibiotic resistance in bacteria. In this event, the contribution of TEs and other natural mobile genetic systems (conjugative plasmids, integrons) is well documented at the molecular level. Likewise, the TEs are known to help construct pathogenicity islands and xenobiotic degradation pathways in bacteria (reviewed in Davis and Waldor, 2002; Tan, 1999; van der Meer *et al*., 1992). Thus, even if the TEs were parasitic in nature at their origin then the co-evolution and co-adaptation with their host genomes was essential for their long-term survival, and many beneficial effects of this co-existence are evident in eukaryotic and prokaryotic world.

Altogether, these examples mentioned here are reinforcing a notion that host organisms use TEs as a source of genomic variability, and the potentially harmful side-effects of transposition can be viewed as a price to pay for having a powerful mutator mechanism, which in some situations might be beneficial for survival (additional examples and comprehensive discussion about the importance of the TEs can be found in several reviews Kidwell and Lisch, 2001; Kidwell and Lisch, 2002; Labrador and Corces, 2002; Britten, 2004; Kazazian, 2004; Shapiro, 2005 and in special issue of Cytogenet Genome Res. 2005; 110 (1–4)).

1.7. *Pseudomonas putida* transposon Tn*4652*

Transposon Tn*4652*, which resides in the chromosome of *Pseudomonas putida* plasmid-free strain PaW85, is a 17 kb derivative of a bigger (56 kb) toluene degradative transposon Tn*4651*. Tn*4652* differs from its ancestral transposon Tn*4651* only by the lack of region containing toluene/xylene-degradative *xyl* genes (Tsuda and Iino, 1987; Meulien and Broda, 1982). *xyl* genes within the 39 kb region of Tn*4651* are bounded by two directly repeated copies of IS*1246* (Reddy *et al*., 1994). The occasional homologous recombination between these copies of IS*1246* may delete the entire catabolic region within Tn*4651* leading to the formation of Tn*4652* (Meulien and Broda, 1982; Greated *et al*., 2002). Thus, differently from its ancestor transposon Tn*4651*, transposon Tn*4652* does not carry any obvious selective values to the host cell.

Transposon Tn*4651* was first discovered as a part of TOL plasmid pWW0, where it was nested within another transposon Tn*4653* (Fig. 5) (Tsuda and Iino, 1987; Tsuda and Iino, 1988; Greated *et al*., 2002).

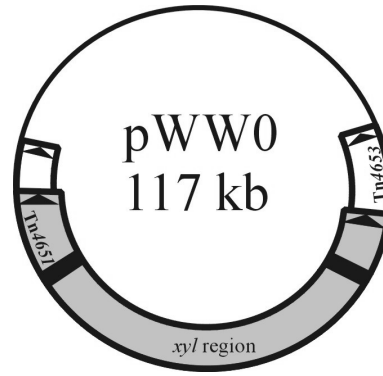

Figure 5. The simplified map of the plasmid pWW0 (117 kb). The transposon Tn*4653* (71 kb) is shown as a white box and Tn*4651* (56 kb) is shown as a grey box. Two copies of IS*1246* (1.3 kb) are represented as black boxes and they flank *xyl* region (39 kb) that encodes genes for toluene and xylene degradation.

Both transposons are shown to belong to the group of Tn*3*-like elements based on their transposition mechanism and properties (Tsuda *et al*., 1989; Grindley, 2002). Namely, they transpose by the replicative pathway forming a transposition intermediate known as a cointegrate (see the section 1.2.1.), introduce a 5 bp duplication of the target sequence and share some homology at the amino acid sequence level of their transposases (Tsuda and Iino, 1987; Tsuda *et al*., 1989; Hõrak and Kivisaar, 1998). However, Tn*4652* forms a distinct subfamily within Tn*3* family transposons, based on the following facts. Tn*4652* transposition functions are not interchangeable with Tn*4653* and other well-studied Tn*3*-like elements (Tsuda *et al*., 1989; Grindley, 2002). Contrary to the other typical transposons of this family, Tn*4652*-specific resolution system requires two gene products, TnpS and TnpT, to function as a resolvase for efficient site-specific resolution of cointegrates (Tsuda and Iino, 1987; Genka *et al*., 2002). Additional distinguishing feature of Tn*4652* is that the *tnpS* and *tnpT* genes together with the recombination site *res* do not locate next to the transposase gene (*tnpA*), but are lying 9 kb away from *tnpA* (Tsuda and Iino, 1987). TnpS has been shown to be a site-specific recombinase belonging to the integrase family and TnpT protein enhances the site-specific resolution performed by TnpS (Genka *et al*., 2002). Additional members of Tn*4652* subfamily are the mercury resistance transposon Tn*5041* with its derivatives (Kholodii *et al*., 1997; Kholodii *et al*., 2002) and quite recently discovered carbazole-degrading transposon Tn*4676* (Maeda *et al*., 2003; Nojiri *et al*., 2004; Shintani *et al*., 2005). Interestingly, the analysis of Tn*4676* implied that Tn*4676* and Tn*4651* may have evolved from a common ancestor that had both *tnpAC* and *tnpST* gene clusters (Maeda *et al*., 2003; Nojiri *et al*., 2004).

Tn*4651* was sequenced and analysed by Greated *et al*. (2002) during the establishment of the complete nucleotide sequence of TOL plasmid pWW0. The analysis of Tn*4651* sequence revealed besides transposase, site-specific recombinase TnpS/TnpT and catabolic gene products also other translation products that are marked as being hypothetical proteins or proteins with putative functions. Thus, in addition to the genes necessary for its transposition the transposon Tn*4652* does not seem to carry other genes with well-known functions.

Previous studies of Tn*4652* transposition regulation, performed by our workgroup, have revealed that Tn*4652* transposase promoter is active in *P. putida* cells, but silent in *E. coli*, suggesting the requirement of some *Pseudomonas*-specific factor(s) for transcription from this promoter (Hõrak and Kivisaar, 1998). It turned out that *P. putida* IHF can bind to both ends of the transposon and moderately enhance the transcription from *tnpA* promoter located in the right end of Tn*4652* (Hõrak and Kivisaar, 1998; Teras *et al*., 2000). On the other hand transposase expression is downregulated by the small (120-amino-acid) regulator protein TnpC encoded directly downstream of *tnpA* gene (Hõrak and Kivisaar, 1999). Although Tn*4652* does not seem to carry obvious selective value to the host, it has an interesting feature to become

transpositionally active under carbon starvation conditions and potentially turn on silent genes at the insertion sites by creating fusion promoters (Kasak *et al*., 1997; Nurk *et al*., 1993). Namely, Tn*4652* carries the sequence resembling the -35 hexamer (TTGCCT) of the σ^{70}-recognised promoters of *E. coli* just 17 bp inward of its both terminal inverted repeats. The right positioning of this promoter element upon transposition in front of the potential –10 hexamer present in the target site leads to the formation of fusion promoter for transcription of downstream genes (Nurk *et al*., 1993). This phenomenon was detected by studying mutational processes in starving *P. putida*. *P. putida* phenol-utilising mutants arose due to the transposition of Tn*4652* from the chromosome into the plasmid in front of the promotorless phenol monooxygenase gene *pheA*, yielding the fusion promoters for *pheA* gene transcription and thereby allowing phenol utilisation by bacteria (Nurk *et al*., 1993; Kasak *et al*., 1997). Remarkably, no Tn*4652*-linked rearrangements were detected among growing cells (Kasak *et al*., 1997).

2. RESULTS AND DISCUSSION

2.1. Aim of the present study

It has been shown that several stress conditions can cause transient activation of TEs in the host genome (see section 1.5). However, it is not clear whether an increased transposition frequency under stressful conditions is a result of occasional inactivation of transposition control mechanisms due to malfunctioning of the host or in contrary, is a process inducible by the host organism to enhance the chance of survival. To clarify which one of the hypotheses could be valid, I started the studies to elucidate the regulation of Tn*4652* transposition. *Pseudomonas putida* specific transposon Tn*4652* is a suitable object to study the phenomenon of stress-induced transposition since it activates in carbon starved *P. putida* cells (Kasak *et al*., 1997). The previously obtained results of our group have suggested that the participation of some *Pseudomonas*-specific host factor(s) might be needed for transposition of Tn*4652* (Hõrak and Kivisaar, 1998). Therefore, the general aim of the present study was to enlighten molecular mechanisms underlying the stress-induced transposition of Tn*4652*. More precisely, I looked for the host factor(s) involved in activation of Tn*4652* transposition.

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

2.2.1. Implication of stationary phase-specific σ^S subunit of RNA polymerase

2.2.1.1. σ^S deficiency decreases the transposition rate of Tn*4652* (Reference I)

As already mentioned, Tn*4652* transposition appeared to be dependent on physiological state of the host as it was non-detectable in exponentially growing cells and was activated under carbon starvation conditions (Kasak *et al*., 1997**)**. It is well-known that because of nutrient limitation (and other stressful conditions), bacteria grow and divide slowly or not at all — they are in stationary phase. The expression of stationary phase-specific genes is mostly controlled by sigma subunit σ^S (encoded by *rpoS* and thereby also called RpoS) of RNA polymerase, which expression is induced by various stress conditions (Loewen and Hengge-Aronis, 1994; Hengge-Aronis, 2002a; Weber *et al*., 2005). In this connection, we were interested in finding out whether the increased transposition frequency of Tn*4652* under starvation conditions is linked to σ^S. Therefore, we examined the movement of native Tn*4652* in the wild type and σ^S-defective strains of *P. putida* PaW85.

We use a starvation assay to examine Tn*4652* transposition *in vivo*. This test system takes advantage of Tn*4652* property to generate fusion promoters upon insertion at the target site (Nurk *et al*., 1993). The advantage of the starvation assay is that it mimics the natural environment, in which the growth of bacteria is usually limited by lack of utilisable carbon source. In this test system *P. putida* strain PaW85, harbouring a native single copy of Tn*4652* in its chromosome and carrying the promotorless *pheBA* operon in plasmid pEST1332 (Nurk *et al*., 1993), was selected on phenol minimal plates for emergence of phenol-utilising mutants (Phe^+). During a week, most of the accumulated Phe^+ mutants arose due to the transposition of chromosomal Tn*4652* into the plasmid in front of the phenol monooxygenase gene *pheA*. Activation of the *pheA* gene by the created fusion promoter allowed *P. putida* strain PaW85 to utilise phenol as a carbon source (Tn*4652* insertion sites in front of the *pheA* gene are represented in Fig. 1 A, ref. I and Nurk *et al*., 1993). Transposition of Tn*4652* into upstream region of *pheA* was proved by PCR analyses. Monitoring of Tn*4652* transposition in σ^S-deficient *P. putida* strain revealed the decrease in transposition more than one order of magnitude (Fig. 1B, ref. I).

How can σ^S affect the transposition of Tn*4652*? There are several possibilities. First, it is known that σ^S plays a role in maintaining the viability of bacterial cells starved for carbon (Ramos-Gonzalez and Molin, 1998; Sarniguet *et al*., 1995). To examine the possibility that transposition of Tn*4652* was dropped in σ^S-deficient cells because of the drastic decrease in the viability of σ^S mutant strain, we evaluated the survival of starving σ^S mutant and its parental PaW85 strain cells on phenol minimal plates. Compared to wild type, the viability of σ^S mutant gently declined (more precisely, the viability of σ^S mutant cells was diminished 2-fold on the 6^{th} starvation day) during the first week of starvation on phenol minimal plates and by the end of the second week it had lowered about two orders of magnitude (Fig. 1 C, ref. I). However, the fact that the viability of *P. putida* σ^S mutant cells was comparable with wild type cells during the first 6 days of starvation indicates that the 10-fold drop in Tn*4652* transposition during a week is not caused by higher mortality of σ^S-deficient cells (compare Fig. 1 C and B, ref. I). Also, the possibility that σ^S could be obligatory for the transcription from fusion promoters created by Tn*4652* insertions has been previously ruled out by earlier results of our group (Ojangu *et al*., 2000). Together, these results strengthened our idea that σ^S can act as a positive regulator in transposition of Tn*4652*.

2.2.1.2. Tn*4652* transposase promoter is under the control of σ^S (Reference I)

The previous literature sections of this thesis have demonstrated that majority of the factors affecting transposition are concentrated on regulation of transposase,

the protein carrying out transposition reaction. Therefore, it was relevant to ask, whether the σ^S-deficiency influences the amount of Tn*4652* transposase. To evaluate the amount of Tn*4652* transposase in *P. putida* σ^S-deficient background we conducted a Western blot analysis with an anti-TnpA polyclonal antiserum. As native TnpA of Tn*4652* is downregulated by transposon-encoded protein TnpC (Hõrak and Kivisaar, 1999) we used a strain where gene dosage of *tnpA* was increased due to the presence of *tnpA*-expressing plasmid (Hõrak and Kivisaar, 1999). Western blot analysis revealed that in contrast to the wild type strain, the expression of plasmid-encoded TnpA was non-detectable in the σ^S-deficient strain (Fig. 2 in ref. I), pointing to the involvement of σ^S in regulation of TnpA.

To find out whether σ^S controls the transcription of Tn*4652 tnpA* promoter, we measured the activity of *tnpA* promoter in *P. putida* wild type and in its σ^S mutant strain (Fig. 3 B, ref. I). Results revealed that transcription from *tnpA* promoter is tightly related to the growth phase of wild type bacteria, being induced in stationary phase of growth. Notably, this stationary-phase-specific induction of transcription from *tnpA* promoter vanished in σ^S-deficient background; the level of reporter gene expression was hardly detectable both in growing and stationary-phase cells of σ^S mutant (Fig. 3 B, ref. I). The explanation for this effect could be that σ^S controls the transcription of Tn*4652 tnpA* promoter either directly or alternatively, it acts indirectly by activating transcription of some host factor required for expression of *tnpA* gene.

Even though σ^S- and σ^{70}-dependent promoters are quite similar to each other, some subtle but essential elements in their sequences exist that contribute to the selectivity of a promoter. In general, σ^{70}-dependent promoters of *E. coli* have TATAAT hexamer in the –10 region, while CTATACT sequence at this position is more specific to σ^S-dependent promoters (Espinosa-Urgel *et al*., 1996; Hengge-Aronis, 2002b). The transposase promoter of Tn*4652* exhibits the DNA sequence features suggested to be important for σ^S recognition of the –10 region, such as the presence of C nucleotide at the fifth position in the –10 hexamer, C nucleotide upstream of the –10 hexamer and AT-richness of the DNA region locating downstream of the –10 element (Fig. 3 A., ref. I). Therefore, we assume that the stationary-phase-specific sigma factor σ^S recognises directly Tn*4652* transposase promoter and it is responsible for stationary-phase-specific expression of Tn*4652* transposase.

According to our knowledge, the involvement of σ^S in the regulation of Tn*4652* is the first evidence for direct involvement of σ^S in regulation of transposition. Yet, the indirect involvement of σ^S has been suggested for transposition of mutant Mu prophage. Despite the fact that transposition of mutant Mu prophage was abolished in the σ^S-deficient strain, the transcription initiation from Mu *tnpA* promoter was demonstrated to not require σ^S, and it was assumed that σ^S affects transposition of Mu indirectly (Lamrani *et al*., 1999; Gomez-Gomez *et al*., 1997). To sum up, the finding that σ^S directs the expression of Tn*4652* transposase gene supports the view that activation of

mobile elements under stress conditions might be an inducible process and not just the malfunction of transposition control mechanisms.

2.2.2. Contribution of integration host factor (IHF)

Previous study in our laboratory has established that the expression of Tn*4652* transposase is positively affected by *P. putida* host factor IHF (Hõrak and Kivisaar, 1998). In addition to moderate effect of IHF (about 4-fold) on *tnpA* promoter, it has been shown that IHF binds to both ends of Tn*4652* (Hõrak and Kivisaar, 1998; Teras *et al*., 2000). In fact, the putative IHF binding sites locate within the transposon ends adjacent to terminal inverted repeat sequences, just next to the potential binding sites of transposase. This led us to hypothesise that besides facilitating transcription from *tnpA*, IHF might be involved in further steps of Tn*4652* transposition as well, either acting on binding of transposase or on subsequent transpososome formation.

2.2.2.1. The absence of IHF prevents transposition of Tn*4652* (Reference II)

To test the possible influence of IHF on Tn*4652* transposition, we examined the activity of Tn*4652* in wild type PaW85 and in its IHF knockout strain (*ihfA*$^-$) using previously described starvation assay. The results demonstrated that IHF is essential for transposition of Tn*4652*, as no transposition occurred in the *ihfA*-minus strain (Fig. 1A, ref. II). However, detection of Tn*4652* transposition in the starvation assay is based on generation of fusion promoters and these promoters have shown to be positively regulated by IHF (Teras *et al*., 2000). Therefore, we tested the possibility whether the fusion promoters in front of the *pheA* gene were strong enough to assure the growth of the Phe$^+$ mutants lacking IHF. The obtained results proved that the transcription of *pheA* gene under the control of several fusion promoters was sufficient to permit the bacteria lacking IHF to grow on phenol (data not shown). Hence, if Tn*4652* had transposed in IHF deficient strain in front of the *pheA* gene, we would have detected this in starvation assay. Yet, there is another possibility why transposition of Tn*4652* ceased in the absence of IHF. It has been shown that IHF is important for survival under starvation in liquid minimal medium as *himA* (*ihfA*) mutant strain of *E. coli* exhibited higher mortality during 4 days of glucose starvation (Nyström, 1995). Considering this, we estimated the viability of *P. putida* IHF-deficient strain starving on phenol minimal plates. The viability of IHF mutant remained fully comparable with wild type strain during 9 days of starvation (data not shown) implying that drastic reduction seen in accumulation of Tn*4652*-linked Phe$^+$ mutants is not caused by decreased survival of IHF-

defective strain. Rather, the transposition of Tn*4652* is hindered in the absence of IHF.

Further confirmation to the role of IHF in Tn*4652* transposition was obtained by complementation of *P. putida ihfA*-minus strain with functional IHF (encoded by *ihfA* and *ihfB* genes) under the control of p_{tac} promoter and $laqI^q$ repressor. Using phenol minimal plates with different IPTG concentrations (no IPTG, 0.01 mM or 0.5 mM IPTG) we were able to vary IHF expression levels in starving bacteria. According to Western blot analysis, the induction with 0.01 mM IPTG resulted in a nearly native expression level while the induction with 0.5 mM IPTG causes overexpression of IHF (data not shown). As it was expected, the complementation of *ihfA*-minus strain with IHF restored the accumulation of Tn*4652*-linked Phe^+ mutants on phenol plates, but to our surprise, the overexpression of IHF could strikingly raise the frequency of transposition in comparison with that in wild type strain (Fig. 1B, ref. II). Thus, these results confirm the role of IHF in Tn*4652* regulation and additionally demonstrate that native concentration of IHF in stationary phase cells does not saturate the potential of Tn*4652* transposition machinery, thereby being a limiting factor in Tn*4652* transposition.

2.2.2.2. IHF overexpression does not affect the expression of Tn*4652* transposase (Reference II)

Involvement of IHF in the regulation of Tn*4652* was first proposed because of its ability to moderately enhance transcription from the transposase promoter (Hõrak and Kivisaar, 1998). However, as overexpression of IHF resulted in remarkable increase in the transposition rate of Tn*4652* (Fig. 1B, ref. II), it raised the question whether IHF overproduction elevates transcription from transposase promoter over the native level. To test this, we measured *lacZ*-fused transposase promoter activities in *P. putida* wild type, $ihfA^-$ and $ihfA^-$ strain complemented with *ihfAB* genes under the control of inducible p_{tac} promoter (strain PaW85ihfAtacIHF). Modulation of IHF expression with different IPTG concentrations in the PaW85ihfAtacIHF strain revealed that although transcription from *tnpA* promoter is dependent on IHF, the transcription level from this promoter did not exceed the native level even in the case of IHF overproduction (Fig. 2 A, ref. II). To confirm IHF overexpression under the employed conditions, we carried out the gel shift experiments with the same cell lysates used in *tnpA* gene expression studies. As presented in Fig. 2 B (ref. II) the amount of IHF-DNA complex increased with both DNA fragments of Tn*4652* terminal ends when IHF was overproduced. Thus, these results convinced us that increased transposition of Tn*4652* in the case of IHF overexpression was not caused by elevated amount of transposase. Instead, IHF seemed to have an additional role in the further steps of Tn*4652* transposition.

2.2.2.3. IHF is crucial for transposase binding to both Tn*4652* ends (Reference II)

IHF functioning as an accessory protein in the regulation of transposition has been demonstrated for several transposons (see the section 1.4.2.1). However, it has not yet been reported that the presence of IHF is essential for transposition reactions *in vivo*. Even in the case of Tn*4652*-related transposon γδ, for which it is demonstrated that the γδ transposase and IHF bind cooperatively to the transposon ends *in vitro*, the absence of IHF or its binding sites did not alter the transposition rate *in vivo* (Wiater and Grindley, 1990a). Therefore, we considered the finding that Tn*4652* transposition is severely affected by IHF concentration *in vivo* very intriguing.

Which step of the transposition reaction might be affected by IHF? The first step in transposition reaction is transposase binding to terminal sequences of transposon. In spite of our efforts, we failed in demonstrating of Tn*4652* transposase binding to transposon ends. Hence, we tested whether IHF is necessary for TnpA binding to Tn*4652* terminal end sequences. Probing the transposon left- and right-end DNA fragments with purified IHF or TnpA proteins in the gel shift assay evidenced that only IHF is able to retard DNA fragments. However, when we used a mixture of purified IHF and TnpA proteins in the binding reaction, then the supershifted complex appeared in the gel, indicating the binding of TnpA (Fig. 3 A and B, ref.II). Further confirmation to necessity of preformed IHF-DNA complex for binding of TnpA was obtained by using mutated transposon left-end DNA in the gel shift assay. In this left-end DNA fragment presumably the both putative IHF binding sites (but not TnpA binding site) were disrupted by introduced mutations. Neither IHF nor TnpA could bind to the mutated left-end DNA fragment as represented in the Fig. 3 C in ref. II, suggesting that the DNA region close to the transposase binding site must be bound by IHF prior to TnpA can bind to transposon ends.

To characterise more precisely the interactions of IHF and TnpA with Tn*4652* ends, we carried out DNase I footprinting experiments, which confirmed the results of the gel shift assay. As shown in Fig. 4 lane 7, the transposase alone could not bind to either end of the transposon in contrast to IHF protein, which clearly protected the regions between 35 and 76 bases in both ends of Tn*4652* (Fig. 4 A and B, lane 3). On the other hand, when both IHF and TnpA were added to the footprinting reaction, the TnpA-specific protection across transposon ends and up to 9–10 bp into the flanking DNA were observable. TnpA binding also extended IHF protected regions inwards both termini of transposon by 2–3 bases, indicating that IHF interaction with DNA alters upon binding of TnpA (Fig. 4 and 5, ref.II). Interestingly, IHF and TnpA binding sites in the ends of Tn*4652* are organised very similarly to corresponding sites in transposon γδ of *E. coli*. Furthermore, the cooperative binding of γδ transposase and IHF with the termini of γδ is described *in vitro* (Wiater and Grindley, 1988). However, contrary to Tn*4652* transposase, the γδ

transposase could bind with γδ ends *in vitro* also without IHF. This may explain why IHF was not essential in the *in vivo* transposition of γδ (Wiater and Grindley, 1990a). Yet, another possible explanation for discrepancy of Tn*4652* and γδ transposition regulation by IHF is that the used transposition assay conditions were different, leaving the probability that we monitored Tn*4652* transposition under conditions in which the effect of IHF was more prominent. For example, it is known that the presence of IHF in the *in vitro* experiments relieves the strict requirement for DNA negative supercoiling in the case of Mu and Tn*10* transposition (Surette and Chaconas, 1989; Chalmers *et al*., 1998; Crellin and Chalmers, 2001), meaning that both IHF and DNA supercoiling act homeostatically by stimulating the early steps of transposition reaction. Therefore, in starving bacteria, where the DNA negative supercoiling is decreased (Balke and Gralla, 1987) and the level of IHF is increased (Ditto *et al*., 1994; Murtin *et al*., 1998), it is possible that the elevated IHF concentration can alleviate or compensate the DNA negative supercoiling requirement for transposition.

Thus, taking into account the results presented and discussed above, one can conclude that the preceding binding of IHF to Tn*4652* transposon ends changes the DNA structure so that it enables the binding of TnpA and allows transposition reaction to begin in the stationary phase bacteria. Consequently, IHF is another host protein besides RNA polymerase sigma factor σ^S, which adjusts the transposition of Tn*4652* according to the physiological state of the host.

2.2.3. Implication of external signals and two-component system ColRS

In order to adapt to and survive under different environmental conditions bacteria possess systems that are able to sense and respond to environmental stimuli. The predominant mean by which bacteria sense and respond to extracellular signals are two-component systems, comprised of histidine kinases and their partner response regulators (Stock *et al*., 2000). A signal in these systems is generally detected by a transmembrane histidine kinase, which is autophosphorylated in response to the detected signal and thereafter alters the phosphorylated state of its partner response regulator (RR). Usually the phosphorylated RR is able to bind with DNA and acts either as activator or as repressor of specific genes, thereby modifying cellular physiology according to prevailing environmental conditions. It has been hypothesised that signal transduction networks could guide cellular DNA engineering processes such as transposition (Shapiro, 1997).

2.2.3.1. *P. putida* ColR- and ColS-deficiency impairs the frequency of Tn*4652* transposition (Reference III)

During the studies on regulation of Tn*4652* transposition, we found that *P. putida* two-component signal system ColRS might be involved in Tn*4652* regulation. As ColRS system was first identified as an important component of root-colonising ability of *Pseudomonas fluorescens* (Dekkers *et al*., 1998), it was intriguing to clarify the role of this system in regulation of transposition. Our results demonstrated that the movement of Tn*4652* was diminished about one order of magnitude both in *colR*- and *colS*-defective *P. putida* strains under phenol selection conditions (Fig. 1A, ref. III), implying that signal transduction from the histidine kinase ColS to the response regulator ColR is important in transposition of Tn*4652*.

In order to find out why transposition of Tn*4652* is declined in *colR*- and *colS*-defective bacteria, we first checked the viability of these strains on phenol minimal plates. The results of this measurement excluded the probability that *colR*- and *colS*-defective cells of *P. putida* are dying during the transposition assay (data not shown). Thus, the decreased viability cannot be the cause of 10-fold drop in transposition frequency of Tn*4652*. We also examined the possibility that some of the factors known to be involved in regulation of Tn*4652* transposition might be under the control of ColRS signal system. Western blot analyses confirmed that proteins known to be required for Tn*4652* transposition, σ^S and IHF, are not controlled by ColRS two-component system (Fig. 2 A and B, ref. III). Furthermore, the amount of transposase was not altered in *P. putida colR*-deficient background (Fig. 2 C, ref. III) indicating that ColRS signal system does not regulate the expression of transposase. Moreover, although ColR is potentially DNA binding protein it does not bind to the ends of Tn*4652*, at least under *in vitro* conditions used by us (data not shown). Regarding the previous notifications, it seemed plausible that ColRS signal system is rather indirectly related to transposition of Tn*4652*.

2.2.3.2. The possible linkage between ColRS two-component system and Tn*4652* transposition

While transposition is a part of mutational processes, we asked whether the other types of mutations (base substitutions and frameshift mutations) could also be affected by the ColRS two-component signal system. For this purpose, we used assay systems that allowed separately detect different types of mutations (e.g., 1-bp deletions and different base substitutions) in *P. putida* stationary phase cells (corresponding test systems are described in Tegova *et al*., 2004). Like the transposition assay, these test systems are based on expression of phenol utilisation genes. However, differently from the transposition assay, the *pheA* gene locating in the test plasmids is constitutively transcribed but unable

to express functional phenol monooxygenase. The coding sequence of *pheA* gene present in these plasmids was altered so that it contained either +1 frameshift mutation for detection 1-bp deletions or stop codon (TGA, TAA, or TAG) instead of the Leu CTG codon for detection of base substitution mutations. For the detecting of Phe^+ revertants emerging either due to frameshift or base substitution mutations, the *P. putida* wild type and *colR*-deficient cells carrying these different assay systems were starved on phenol minimal plates like in the case of transposition assay. To our surprise, this experiment revealed that similarly to transposition the rate of frameshift and base substitution mutations was also remarkably diminished in the *colR*-defective strain, being at least 5-fold lower than in parental strain (data of base substitution assay are presented in Fig. 6). Based on these results we can conclude that *colR*-deficiency affects different mutational processes in resting (non-dividing) *P. putida* cells.

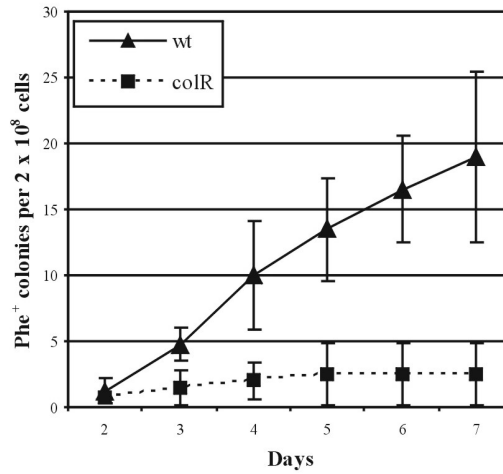

Figure 6. Accumulation of Phe^+ revertants on phenol minimal plates due to base substitutions restoring the functionality of *pheA* gene. *P. putida* wild-type strain PaW85 (wt) and its *colR*-defective (colR) derivative harbour the test plasmid pKTpheA22TGA (Tegova *et al*., 2004), which contains stop codon TGA within the coding sequence of *pheA*. Each point represents the mean of at least four independent determinations, and error bars represent standard deviations.

Next we asked whether ColR affects only mutations occurring under phenol selection conditions or influences other type of mutations as well. To analyse the appearance of spontaneous mutations in growing cells, we plated wild type and *colR*-defective *P. putida* cells on media containing rifampicin. In this assay, resistance to rifampicin can readily be acquired by single base changes in the gene encoding the β-subunit of RNA polymerase, *rpoB*, and the total number of

Rif$^+$ mutants is countable on the next day after plating (Jin and Gross, 1988; Garibyan *et al*., 2003). However, the number of emerged Rif$^+$ mutants in *colR*-derivative was comparable to that in wild type (data not shown), suggesting that not all mutations are affected in *colR*-knockout strain. This finding raised an intriguing question – what is the common link between the transposition and point mutations, which is likely affected by ColRS signal system under particular assay conditions? One possible link could be the exposure of bacteria to phenol on which mutants were selected for in both assays. The simplest way to test whether phenol can have an effect through ColR deficiency on both processes was to exploit different phenol concentrations during these assays. Indeed, the results presented in the Figure 7 revealed that in contrast to wild type strain the accumulation of transposition-linked Phe$^+$ mutants in *colR*-knockout strain depended on the phenol concentration. Namely, the reduction of phenol concentration by 10-fold (0.25 mM phenol) in growth medium could increase the frequency of emergence of Tn*4652*-linked Phe$^+$ mutants in *colR*-defective strain almost to half the level observed in the wild type strain in the presence of routinely used phenol concentration (2.5 mM phenol) (Figure 7 B). Moreover, similar effect of phenol concentration on accumulation of Phe$^+$ revertants was observed when frequency of point mutations was examined in *colR*-knockout strain (data not shown). Together these results indicate that *colR*-knockout strain is more vulnerable to phenol than wild type *P. putida.* Therefore, the role of ColRS signal system is rather related with the phenol tolerance in *P. putida* than with the direct regulation of Tn*4652* transposition.

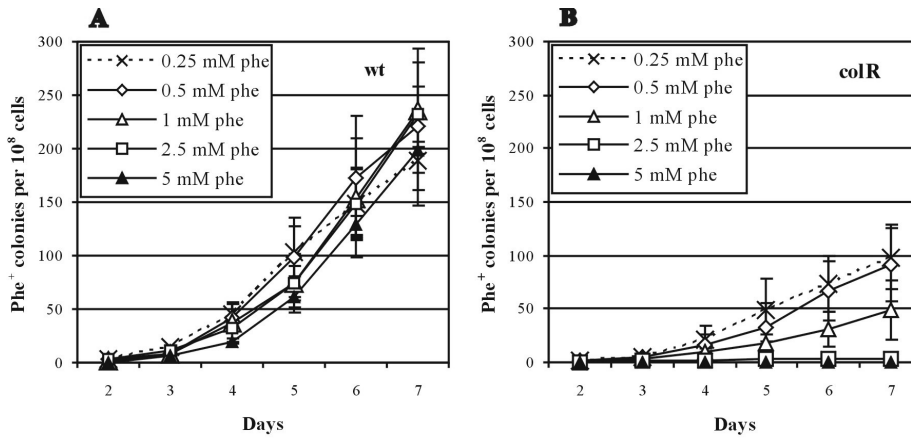

Figure 7. Accumulation of Tn*4652* transposition-dependent Phe$^+$ colonies on phenol minimal plates with different concentrations of phenol in *P. putida* wild-type strain PaW85 (A) and its *colR*-defective derivative (B). Each point represents the mean of at least four independent determinations, and error bars represent standard deviations.

It is known that phenol as a hydrophobic compound accumulates in bacterial membranes (Isken and de Bont, 1998). Moreover, it has been shown that the treatment of *E. coli* cells with phenol caused leakage of potassium ions, ATP and other nucleotides (Heipieper *et al*., 1991), meaning that due to phenol toxicity bacteria are apparently faced with energy crisis. Therefore, it is reasonable to assume that a better permeability of membranes of *colR*-knockout cells can account for their increased susceptibility to toxicity of phenol. We hypothesise that during starvation on phenol *P. putida colR*-mutant probably has to manage simultaneously to increased intracellular concentration of phenol and enhanced energy crisis due to changes in its membrane functions. Additionally, it is not clear how sensitive the cellular functions are to phenol; therefore, one can suspect that if the better entrance of phenol is permitted into the cell then different enzymatic processes, including DNA synthesis, can be inhibited. Considering that both transposition and generation of point mutations need DNA synthesis, it is tempting to speculate that during starvation on phenol the DNA synthesis might somehow be inhibited in response to the increased intracellular level of phenol in *colR*-knockout strain. Most likely, DNA synthesis could be reduced due to the decreased energetic status of the *colR*-mutant cells. Yet, the finding of target genes regulated by ColRS signal system (currently in progress) will probably help to shed light on whether or how ColRS two-component system and phenol are related to mutational processes in *P. putida*.

2.3. Regulation of Tn*4652* transposition in the context of stationary phase (adaptive) mutations

Mutational processes in starving bacteria are different from those occurring in growing cells. Specific mechanisms of mutation are induced in bacteria under growth-limiting conditions, thereby causing genetic instability. The action of these stress-induced mechanisms, including transposition of some elements, can give rise to beneficial mutations relieving selective pressure, a phenomenon called “stationary-phase mutation” or “adaptive mutation” (reviewed in Kivisaar, 2003; Tenaillon *et al*., 2004; Foster, 2005).

Phe$^+$ mutations occurring due to Tn*4652* transposition and point mutations can be classified as stationary-phase (adaptive) mutations because they enable the appearance of Phe$^+$ cells of *P. putida* during starvation on phenol (described in 2.2.1.1. and 2.2.3.2). In fact, the emergence of stationary phase mutation in non-dividing bacterial cells has been subject to lively debate for decades. Already the early studies in 1950s demonstrated that auxotrophic bacteria starved of the necessary amino acid could mutate to prototrophy in the absence of cell division and were therefore inconsistent with a widely held view that

spontaneous mutations arise during DNA replication in dividing cells (reviewed in Bridges, 1997). Since that the evidence has been accumulating that DNA synthesis required for emergence of mutations occurs also to some extent in resting cells and may utilise breakdown products of DNA and RNA (named as DNA turnover) (reviewed in Bridges, 1997; Bridges and Ereira, 1998). If our assumption that the better entrance of phenol is the cause of suppression of DNA synthesis (DNA turnover) in starving *colR*-deficient cells of *P. putida* (discussed in previous section) is right, then this also supports the view that DNA turnover occurring under starvation conditions has a great impact on appearance of stationary phase mutations.

The knowledge of possible mechanisms responsible for occurrence of adaptive mutations is mostly obtained from studies based on *E. coli*. It has been shown that in *E. coli* cells the stress responses like SOS response, σ^S-mediated general stress response and heat-shock response are involved in the occurrence of point mutations via affecting the expression of error-prone DNA polymerases (reviewed in Foster, 2005). For example, a master regulator of general stress response, σ^S, induces expression of error-prone DNA polymerase IV (encoded by *dinB*) in *E. coli* (Layton and Foster, 2003). However, in *P. putida* the transcription of *dinB* was shown not to be dependent on σ^S (Tegova *et al*., 2004), which at the same time does not exclude the possibility that σ^S might be implicated in other ways in stationary-phase mutagenesis in *P. putida*.

The findings presented in this thesis (section 2.2.1.) show that σ^S is involved in generation of stationary phase mutations by inducing Tn*4652* transpositional activity in *P. putida* starving cells. Furthermore, also another stationary-phase-induced host protein, IHF, is assisting the movement of Tn*4652* under stress (section 2.2.2). The dependence of these two stress-induced host proteins makes Tn*4652* transposition exclusively a stationary-phase-specific event and additionally excludes the possibility that transposition of Tn*4652* could be enabled due to malfunctioning of host mechanisms. Following this line of thought, it is tempting to speculate that host *P. putida* itself up-regulates Tn*4652* transposition as a response to stressful environmental conditions to increase genetic variability and thereby enhance the chance for survival. On the other hand, for the transposon it would be also advantageous to increase its copy number in the genome of host exposed to harmful conditions. During the stress, the host bacterium is faced with higher probability to die. When it occurs, the increased copy number probably would give a greater chance to transposon to transfer its DNA into other bacteria either before or after cellular death. Thus, the increased transposition under stressful environmental conditions could be beneficial for both host and transposon.

CONCLUSIONS

Transposition as a potentially destructive process is usually controlled tightly both by transposon and by host. However, it has been observed that under certain stressful conditions the activity of some mobile elements is elevated. Since the molecular basis of this phenomenon has remained poorly studied, we undertook the investigation of molecular mechanisms underlying the stress-induced transposition of Tn*4652* in soil bacterium *Pseudomonas putida*.

The results presented in this study demonstrate that at least two stationary phase-specific host proteins such as σ^S and IHF contribute to activation of transposition of Tn*4652* during stress. This indicates that activation of transposition under stress is rather induced process, not just result of malfunctioning of host control mechanisms.

The main conclusions of the present thesis can be drawn as follows:

1. Transcription of Tn*4652* transposase promoter depends on the physiological state of host, being directly under the control of stationary phase σ^S subunit of RNA polymerase. Transposition of Tn*4652* declines about one order of magnitude in the σ^S-deficient strain indicating that σ^S-dependent expression of transposase is one of the key factors that allows induction of Tn*4652* transposition in stationary phase.
2. Besides the contribution of σ^S, the transposition of Tn*4652* is limited by the amount of host protein IHF, which concentration increases in stationary phase cells. The movement of Tn*4652* in starving cells is undetectable in the absence of IHF and increases over the wild type level in the case of IHF overexpression. *In vitro* studies revealed that the binding of host protein IHF adjacent to potential transposase binding sites is prerequisite for binding of transposase at the both ends of Tn*4652*. Thus, IHF is essential for Tn*4652* transposition reaction and affects the transposition frequency in concentration-dependent manner.
3. In the absence of two-component signal system ColRS the transposition of Tn*4652* in starving *P. putida* is sensitive to phenol concentration in the medium. We hypothesise that this effect could result from an altered membrane permeability of *colR*-knockout bacteria. The higher membrane permeability of *colR*-defective *P. putida* cells probably makes them more sensitive to phenol toxicity, which in turn may indirectly influence DNA synthesis needed for occurrence of Tn*4652* transposition.

In light of the results presented in this thesis, it may not be far-fetched to conclude that soil bacterium *P. putida* itself promotes the movement of Tn*4652* under stressful environmental conditions in order to increase its chance to survive.

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SUMMARY IN ESTONIAN

Pseudomonas putida transposooni Tn*4652* aktiveerumine stressitingimustes

Transposoonid on potentsiaalselt mutageensed DNA elemendid, mis võivad oma peremeesorganismi genoomis tekitada ulatuslikke ümberkorraldusi. Transposoonide ümberpaiknemiseks ehk transpositsiooniks ei ole vajalik homoloogilise DNA järjestuse olemasolu doonor- ja märklaudlookuses, mistõttu võivad transposoonid inserteeruda genoomi erinevatesse osadesse. Selline juhuslik ümberpaiknemine võib genoomi funktsionaalsusele rohkem kahju tekitada, kui kasu tuua ning transposoonide võimalik "kasulikkus" ilmneb tihti vaid ekstreemsetes olukordades.

Transposoonid võivad nii aktiveerida kui inaktiveerida peremeesorganismi geene olenevalt sellest, kuhu nad genoomis inserteeruvad. Insertsioon geeni kodeerivasse alasse inaktiveerib vastava geeni, kuid sisenemine geenieelsesse regiooni võib mõnel juhul põhjustada ka geeni aktivatsiooni. Mitmete transposooni koopiate olemasolu genoomis soodustab omakorda DNA ümberkorralduste (inversioonid ja deletsioonid) toimumist, sest nad võivad olla märklauaks peremeesraku homoloogilisele rekombinatsioonisüsteemile. Lisaks transposoonide võimele suurendada geneetilist mitmekesisust on neil sageli ka kasulikke lisafunktsioone. Näiteks võivad transposoonid sisaldada antibiootikumi resistentsusgeene või kataboolseid geene, mis võimaldavad bakteritele kas resistentsuse teatud antibiootikumi suhtes või uute süsinikallikate kasutamise võimaluse.

Transpositsioon toimub tavaliselt madala sagedusega, ligikaudu 10^{-3} kuni 10^{-8} sündmust bakteri ühe generatsiooni kohta (Kleckner, 1990; Craig, 1996). Samas on kirjanduses andmeid transpositsiooni sagenemisest vastusena mitmesugustele stressitingimustele nagu näiteks UV-kiirgusele, süsinikallika puudusele, temperatuuri muutusele jne. Üldiselt arvatakse, et transpositsiooni sagenemisest tingitud geneetilise mitmekesisuse suurenemine võib anda peremeesrakule rohkem võimalusi stressitingimustes ellujäämiseks ja neist väljatulemiseks tänu uutele, antud tingimustes kasulikele geneetilistele omadustele.

Kuigi tänaseks on paljude mobiilsete elementide transpositsiooni põhjalikult uuritud, on siiski vähe teada molekulaarsetest mehhanismidest, mis viivad transpositsiooni sagenemisele stressitingimustes. Meie uurimisrühma varasemad tulemused on näidanud, et mullabakter *Pseudomonas putida* transposoon Tn*4652* aktiveerub süsinikunäljast põhjustatud stressis. Seetõttu on Tn*4652* sobivaks mudelobjektiks stressi poolt aktiveeritava transpositsiooni uurimiseks. Käesoleva töö eesmärgiks oli välja selgitada millised molekulaarsed mehhanismid põhjustavad Tn*4652* transpositsioonisageduse suurenemise stressitingimustes.

Töös esitatud tulemused võib kokku võtta järgnevalt:

1. Tn*4652* transposaasi avaldumine sõltub peremeesrakkude füsioloogilisest seisundist. Saadud tulemused näitavad, et transposaasi kodeeriva geeni *tnpA* transkriptsiooni kontrollib RNA polümeraasi stressispetsiifiline sigmafaktor σ^S. Tn*4652* transpositsiooni vähenemine *P. putida* σ^S-defektses tüves ligikaudu ühe suurusjärgu võrra näitab, et σ^S-st sõltuv transposaasi ekspressioon on üks põhilistest faktoritest, mis võimaldab Tn*4652* aktiveerumise stressitingimustes.
2. Lisaks σ^S-le on Tn*4652* transpositsioon kontrollitud peremeesvalgu IHF-i (*integration host factor*) kogusega rakkudes. IHF-i rakusisene hulk sõltub bakteri füsioloogilisest seisundist, suurenedes rakkude sisenemisel statsionaarsesse kasvufaasi. IHF-i puudumisel nälgivates *P. putida* rakkudes pole võimalik Tn*4652* transpositsiooni tuvastada. Samas suurendab IHF-i üleekspresseerimine Tn*4652* transpositsiooni võrreldes algses tüves mõõdetuga, mis viitab sellele, et natiivsetes tingimustes limiteerib IHF-i hulk Tn*4652* transpositsiooni toimumist ka nälgivates *P. putida* rakkudes. *In vitro* katsetest selgus, et transposaasi seondumiseks mõlemale Tn*4652* otsale on vajalik IHF-i eelnev seondumine transposaasi seondumiskohaga külgnevale DNA-le. Seega on IHF oluline Tn*4652* transpositsiooni reaktsioonis, mõjutades oma rakusisese hulgaga transposaasi seondumise efektiivsust transposooni otstele ja selle kaudu Tn*4652* transpositsiooni sagedust.
3. Kahekomponendilise signaalsüsteemi ColRS-i defektsuse korral on nälgivates *P. putida* rakkudes toimuvad mutatsiooniprotsessid, nii Tn*4652* transpositsioon kui ka punktmutatsioonid, tundlikud fenooli kontsentratsioonile söötmes. Meie tulemused viitavad sellele, et *P. putida colR*-defektse tüve tundlikkus fenoolile on põhjustatud muutustest membraani läbilaskvuses. Seega võib püstitada hüpoteesi, et *colR*-defektsus põhjustab *P. putida* rakumembraanis muutusi, mis kergendavad fenooli tungimist rakku. Fenooli suurenenud kontsentratsioon rakkudes võib halvendada rakkude energeetilist seisundit või otseselt inhibeerida nii Tn*4652* transpositsiooniks kui ka teiste mutatsiooniprotsesside toimumiseks vajalikku DNA sünteesi.

Käesoleva töö tulemused näitavad, et vähemalt kaks *Pseudomonas putida* statsionaarse kasvufaasi-spetsiifilist valku, σ^S ja IHF, osalevad Tn*4652* aktiveerumises stressitingimustes. Sellest võib järeldada, et Tn*4652* transpositsioonisageduse suurenemine pole tingitud stressis oleva peremeesorganismi kontrollmehhanismide nõrgenemisest vaid on pigem *P. putida* enda poolt soodustatud protsess stressitingimustega kohanemiseks.

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PUBLICATIONS

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Scientific work

Since 1998 I have been interested in regulation of transposition of Tn*4652* in soil bacterium *Pseudomonas putida*. During my graduate studies, I got attracted to two-component signal transduction systems and currently my interests are more related to the identification of target genes for *P. putida* two-component signal system ColRS.

Additional information

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List of Publication

1. Hõrak, R., Ilves, H., Pruunsild, P., Kuljus, M. and Kivisaar, M. (2004) The ColR-ColS two-component signal transduction system is involved in regulation of Tn*4652* transposition in *Pseudomonas putida* under starvation conditions. *Mol Microbiol* **54**: 795–807.
2. Ilves, H., Hõrak, R., Teras, R. and Kivisaar, M. (2004) IHF is the limiting host factor in transposition of *Pseudomonas putida* transposon Tn*4652* in stationary phase. *Mol Microbiol* **51**: 1773–85.
3. Ilves, H., Hõrak, R. and Kivisaar, M. (2001) Involvement of sigma(S) in starvation-induced transposition of *Pseudomonas putida* transposon Tn*4652*. *J Bacteriol* **183**: 5445–8.

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Teaduslik töö

Minu põhiliseks uurimisteemaks on olnud alates 1998. aastast Tn*4652* transpositsiooni aktiveerivate faktorite väljaselgitamine mullabakteris *Pseudomonas putida*. Seoses kahekomponendilise signaalsüsteemi *colRS* kaudse mõjuga Tn*4652* transpositsioonile, osalen ka töös, mis käsitleb *colRS* märklaudgeenide leidmist *P. putida* genoomist.

Lisainformatsioon

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Publikatsioonide nimekiri

1. Hõrak, R., Ilves, H., Pruunsild, P., Kuljus, M. and Kivisaar, M. (2004) The ColR-ColS two-component signal transduction system is involved in regulation of Tn*4652* transposition in *Pseudomonas putida* under starvation conditions. *Mol Microbiol* **54**: 795–807.
2. Ilves, H., Hõrak, R., Teras, R. and Kivisaar, M. (2004) IHF is the limiting host factor in transposition of *Pseudomonas putida* transposon Tn*4652* in stationary phase. *Mol Microbiol* **51**: 1773–85.
3. Ilves, H., Hõrak, R. and Kivisaar, M. (2001) Involvement of sigma(S) in starvation-induced transposition of *Pseudomonas putida* transposon Tn*4652*. *J Bacteriol* **183**: 5445–8.