DISSERTATIONES BIOLOGICAE UNIVERSITATIS TARTUENSIS
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REGULATION OF TRANSCRIPTION FROM THE FUSION PROMOTERS GENERATED BY TRANSPOSITION OF Tn*4652* INTO THE UPSTREAM REGION OF *pheBA* OPERON IN *PSEUDOMONAS PUTIDA*

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I **Teras, R., Hõrak, R. and Kivisaar, M.** 2000. Transcription from fusion promoters generated during transposition of transposon Tn*4652* is positively affected by integration host factor in *Pseudomonas putida*. J. Bacteriol. **182**, 589–598.

II **Ojangu, EL., Tover, A., Teras, R. and Kivisaar, M.** 2000. Effects of combination of different –10 hexamers and downstream sequences on stationary-phase-specific sigma factor σ^S-dependent transcription in *Pseudomonas putida*. J. Bacteriol. **182**, 6707–6713.

III **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.

LIST OF ABBREVIATIONS

bp	base pairs
CAP	catabolite gene activation protein
DDE motif	conserved motif of two aspartatic acid residues and a glutamic acid residue in the active site of transposase
$E\sigma^{54}$	σ^{54}-programmed RNAP
$E\sigma^{70}$	σ^{70}-programmed RNAP
$E\sigma^{S}$	σ^{S}-programmed RNAP
Fis	factor for inversion stimulation
H-NS	histone-like nucleoid protein
HU	heat-unstable nucleoid protein
IHF	integration host factor
IPTG	isopropylthio-β-D-galactoside
IR	terminal inverted repeat of transposable element
kb	kilo base pairs
kDa	kilo Daltons
IS	insertion sequence element
Mb	mega base pairs
ppGpp	guanosine tetraphosphate – the effector molecule of the stringent response
RNAP	RNA polymerase

INTRODUCTION

Due to the complexity, biological processes in a bacterial cell are often studied separately, like in the case of transcription and transposition. It helps to understand details of the mechanism, but the "big picture" of bacterial life still remains fragmented.

The initiation of transcription is precisely regulated in bacteria and has therefore gained much interest by molecular biologists (Ishihama, 2000, Magnusson *et al*., 2005; Travers and Muskhelishvili, 2005). Generally, two kinds of factors are involved in regulation of the transcription: 1) the global ones such as sigma factors affect many regulons (stimulons) and have the major role in transcriptional regulation; 2) specific regulators ensure the precise gene (operon) expression (Ishihama, 2000; Martinez-Antonio and Collado-Vides, 2003). It was shown lately that transcriptional regulators controlling the physiological state of bacteria are in hierarchical connections (Martinez-Antonio and Collado-Vides, 2003; Babu *et al*., 2004).

The transposition of mobile DNA elements in the genome of bacteria is well known process and is commonly described as propagation of parasitic DNA. Although involvement of transposable elements in the activation of host genes has been known for long time (Chandler and Mahillon, 2002) it's impact on life of a cell has most probably been underestimated.

Often the transposons carry outwardly directed σ^{70}-type promoters that may activate bacterial genes by transposing into upstream region (Chandler and Mahillon, 2002). Transposition can also generate the fusion promoters which only a part of the promoter sequence originates from the transposon, and the second part is found from the target DNA (Chandler and Mahillon, 2002). Thereby, the proteins that regulate transposition by binding to the ends of transposable element may also have influence on transcription initiation from newly created promoters. Usually a new promoter formation is only admitted at the upstream region of a gene by transposition (Chandler and Mahillon, 2002), but the connection between transposition and the global transcription regulation is not commonly studied.

A good example of transposable elements that can activate genes upon transposition is the transposon Tn*4652* that contains a DNA sequence similar to –35 consensus of σ^{70}-dependent promoters in both inverted repeats (Tsuda and Iino, 1987; Nurk *et al*., 1993). To generate a functional promoter for transcriptional initiation this transposon has to be inserted at a correct distance from the existing –10 hexamer in a target DNA. Thus, the insertion of Tn*4652* into upstream sequence of *pheA* gene encoding the key enzyme of phenol degradation pathway resulted in expression of this gene and growth of *Pseudomonas putida* on phenol (Nurk *et al*., 1993). *P. putida* is a cosmopolitan soil bacterium able to degrade many secondary carbon-sources (Timmis, 2002).

Since *P. putida* acquires new genes often by horizontal gene transfer (Timmis, 2002) it is a good model system to study the involvement of transposable elements in gene activation under starvation conditions. As Tn*4652* can generate only fusion promoters, global regulators that control transposition of this element and transcription from created fusion promoters can be predicted.

In the first part of my thesis, I will give an overview of promoters dependent on sigma factors' σ^{70}- and σ^{S}, and will discuss differences in their DNA sequences using data on well-studied bacterium *Escherichia coli* and the soil bacterium *P. putida*. In addition, I will describe possibilities for the activation of down-regulated or silent bacterial genes by transposition, and will concentrate on a global regulator IHF that is involved in regulation of both, transcription and transposition. In the second part of my thesis, I will focus on regulation of the transcription from the fusion promoters created by the transposition of Tn*4652* in *P. putida* and will show that IHF regulates positively the transcription from these fusion promoters. Additionally, I will show that some fusion promoters are the sigma factor σ^{S}-dependent and try to find explanation to that fact.

1. REVIEW OF LITERATURE

1.1. Regulation of transcription initiation in bacteria

Bacteria react instantly to changes in environment by altering gene expression pattern, which is ensured by a cascade of mechanisms. The regulation of genes in bacteria is controlled at different levels – transcriptional, post-transcriptional, translational and post-translational level. Obviously, the most important regulation step of gene expression is transcription, because controlling the first stage of gene expression saves energy that bacteria must spend for the synthesis of mRNA and protein. The initiation of transcription is the most tightly regulated level of gene expression and it is the complicated process involving several different phases: promoter recognition by RNA polymerase (RNAP), formation of a competent initiation complex and promoter clearance – synthesis of the initial posphodiester bonds and movement of RNAP into elongation phase (deHaseth *et al*., 1998). The enzyme for carrying out transcription in eubacteria is the single RNA polymerase comprising of one β, one β', two α and one ω subunits (Helmann and Chamberlin, 1988; Ebright, 2000). Promoters are recognised by the holoenzyme (Eσ), where the core enzyme of RNAP is reversibly associated with a σ subunit. The σ subunit is necessary for promoter selection and transcription initiation. After transcription initiation, σ subunit is released and elongation is prolonged by core RNAP (Ishihama, 2000). Although most of the work concerning the selection of σ factors by RNAP has been done in *Escherichia coli*, it seems that this mechanism is conserved in eubacteria. Seven different σ subunits are known in *E. coli*, which can associate with the single core RNAP (Ishihama, 2000), whereas *P. putida* and *P. aeruginosa* contain 24 putative sigma factors (Martinez-Bueno *et al*., 2002). Switches in the use of σ factors allow the specific regulation of subsets of genes (Ishihama, 2000). In general, each σ factor recognises different type of promoter sequence and, therefore, the control of different regulons does not overlap. The exceptions are σ^S and σ^{70}-dependent promoters that can be recognised by both sigma factors (Ding *et al*., 1995; Kolb *et al*., 1995; Tanaka *et al*., 1995).

The sigma factor σ^{70} is the most important one in the fast-growing bacteria, but bacteria are often exposed to nutrient limitation and other stressful conditions, leading to reduction of growth speed. Due to the stress, bacteria grow and divide slowly or not at all; bacteria are in stationary phase. The physiology and morphology of stationary phase cell is determined by the general stress response, which is controlled at molecular level mainly by RNAP subunit σ^S, the second important sigma factor in bacterial cells (Mulvey and Loewen, 1989; Nguyen *et al*., 1993; Tanaka *et al*., 1993). The sigma factor σ^S is involved in survival of bacteria under starvation conditions (Lange and Hengge-

Aronis, 1991b; Schuster *et al*., 2004; Weber *et al*., 2005), modulation of bacterium shape in stationary phase (Lange and Hengge-Aronis, 1991a) and protection against osmotic, acidic and oxidative stress (Loewen and Hengge-Aronis, 1994). In addition, the stationary phase sigma factor σ^S is important in activation of genes for degradation of secondary carbon sources. For example, a Pm promoter of alkylbenzoate degradation genes from TOL plasmid pWW0 in *P. putida* is σ^S- and σ^H- (sigma factor of heat shock) dependent (Marques *et al*., 1999).

Up to now, it has been commonly considered that most changes in gene expression pattern which are caused by sudden stress conditions, are controlled by the stationary phase sigma factor – σ^S, notwithstanding that the control over the gene expression pattern is more complex (Travers and Muskhelishvili, 2005).

1.1.1. What makes promoters σ^S-dependent?

1.1.1.1. DNA sequence of –10 and –35 hexamers of σ^{70}- and σ^S-dependent promoters in *E. coli*

Although the sigma factors σ^S and σ^{70} have different missions in *E. coli*, the structure of σ^S and even its target promoters sequence are similar to those of main sigma subunit, σ^{70}. These two sigma subunits have similar domains for interactions with the –10 and –35 consensus region of promoter (Lonetto *et al*., 1992). The –10 hexamer with consensus sequence TATAAT is centred approximately 10-bp upstream of transcriptional start point and it is recognised by the 2.3 and 2.4 regions of σ^{70}. The –35 hexamer with consensus sequence TTGACA locates 16–18-bp upstream from the –10 element and interacts with the region 4.2 of σ^{70} (Gross *et al*., 1998). It is described that σ^S and σ^{70} show overlapping specificities *in vitro* conditions. However, many promoters are recognised solely by one of these sigma subunits *in vivo*. In addition, many promoters are recognisable by both sigma subunits (Nguyen *et al*., 1993; Tanaka *et al*., 1993; Ding *et al*., 1995; Hengge-Aronis, 1999). Consequently, it is difficult to define the consensus of σ^S-dependent promoter because the genes that are dependent on $E\sigma^S$ *in vivo* are often transcribed *in vitro* by $E\sigma^{70}$ and *vice versa* (Becker and Hengge-Aronis, 2001; Weber *et al*., 2005).

Compared to the σ^{70}-dependent promoters, the transcription initiation from σ^S-dependent promoters is less affected by various fluctuations from the typical promoter consensus sequence. For example, the –35 hexamer is not so important for transcription initiation by $E\sigma^S$ compared to transcription initiation by $E\sigma^{70}$. Therefore, it has been proposed that promoters recognised by $E\sigma^S$ may lack the sequence of –35 consensus, but instead of that have curved DNA

region (Kolb *et al*., 1995, Tanaka *et al*., 1995, Espinosa-Urgel *et al*., 1996). Kolb *et al.* (1995) showed with the promoters of *gal*P1 and *gal*P2 that the absence of –35 sequence but existence of curvature gave the promoters σ^S-dependence, whereas the presence of –35 sequence caused preferentially recognition by $E\sigma^{70}$ (Kolb *et al*., 1995). Espinosa-Urgel *et al.* (1996) compared the 33 σ^S-dependent promoters of *E. coli*. They were not able to find any conserved sequence upstream of –10 hexamer of σ^S-dependent promoters (Espinosa-Urgel *et al*., 1996). Weber et al. (2005), who analysed 481 promoter regions of *E. coli* genes that were activated by osmotic upshift, acid stress or starvation, supported this idea. The 140 genes of those constituted core group that were found to be $E\sigma^S$ controlled under all three growth and stress conditions. They analysed these promoter regions *in silico* and no conserved sequence for –35 hexamer was found (Weber *et al*., 2005). However, some authors have discussed about the similarity between –35 consensuses of σ^S- and σ^{70}-dependent promoters. For example, it was suggested that σ^S-dependent promoters have either CTGCAA consensus (Bohannon *et al*., 1991) or CCGACA consensus (Wise *et al*., 1996) instead of the $E\sigma^{70}$-specific –35 consensus sequence TTGACA.

The –10 consensus sequence of σ^S-dependent promoters does not differ very much from the consensus sequence recognised by $E\sigma^{70}$. Nevertheless, some differences were proposed in 1993 by Hengge-Aronis. She suggested that the –10 consensus sequence of *E. coli* σ^S-dependent promoters is TATACT, instead of TATAAT which is recognised by $E\sigma^{70}$ (Hengge-Aronis, 1993). Moreover, some years later it was proposed that the –10 consensus sequence of σ^S-dependent promoters is longer than six nucleotides – CTATACT (Espinosa-Urgel *et al*., 1996). Recently, the new extended consensus sequence for –10 region of σ^S-dependent promoters was offered – KCTAYRCTTAA, where K stands for T or G, Y stands for T or C and R stands for A or G. This data was calculated by comparing 140 promoter regions of genes, which expression was altered by σ^S in stationary phase cells. CTA nucleotides at positions –13 to –11 and T nucleotide at position –7 were most conserved (Weber *et al*., 2005).

1.1.1.2. σ^S-dependent promoters in *Pseudomonas*

The response of *E. coli* to stress conditions and involvement of σ^S in reorganisation of gene expression pattern are well studied (Weber *et al*., 2005). Additionally, there is a lot of information about σ^S-dependent gene regulation in pseudomonads as well (Venturi, 2003; Schuster *et al*., 2004). Both groups of bacteria, enterobacteria and pseudomonads, have the alternative sigma factor σ^S up regulated in stationary phase cells (Lange and Hengge-Aronis, 1991b; Schuster *et al*., 2004; Weber *et al*., 2005). σ^S is an important factor for changes

in cellular morphology and physiology thus ensuring protection against a large variety of stresses in *E. coli*. It also modulates the expression of genes involved in the control of cell cycle and synthesis of cell wall components (Lange and Hengge-Aronis, 1991a; Muffler *et al*., 1997; Ishihama, 2000; Weber *et al*., 2005). In contrast to the functions of σ^S in *E. coli* it has less important role in general stress response in pseudomonads (Jorgensen *et al*., 1999; Suh *et al*., 1999; Espinosa-Urgel and Ramos, 2004; Schuster *et al*., 2004). This can be a reason why the σ^S-defective strains of *P. aeruginosa* are more vital and stress tolerant than those of *E. coli* (Jorgensen *et al*., 1999). In σ^S defective strain of *P. aeruginosa* expressions of 772 genes are altered (Schuster *et al*., 2004). On the contrary to *E. coli*, σ^S is largely involved in the regulation of chemotaxis, two-component regulatory systems and quorum sensing (Schuster *et al*., 2004). The effect of σ^S on expression of such a big number of genes could be explained by the fact that in pseudomonads three global regulatory systems – σ^S, quorum-sensing and two-component system GacA-GacS – control very large set of genes. Regulation of these genes can overlap because the systems are mildly cross-regulating each other (Bertani and Venturi, 2004; Schuster *et al*., 2004).

Schuster *et al.* (2004) tried to identify consensus sequence of σ^S-dependent promoters in *P. aeruginosa*. They chose 16 genes which expression was obviously regulated directly by σ^S. They revealed a consensus sequence CTATACT for the –10 region that is identical to the –10 consensus sequence of σ^S-dependent promoters in *E. coli* (Schuster *et al*., 2004).

Although, approximately 50 genes in *P. putida* are known to be σ^S-dependent under C-limitation conditions (Ramos-Gonzalez and Molin, 1998), there is no offered consensus sequence for σ^S-dependent promoter in this bacterium. On the other hand, Dominguez-Cuevas and Marques (2004) have compiled consensus sequence of σ^{70}-dependent promoters in *P. aeruginosa* and *P. putida*. They found that in general, both bacteria have similar consensus sequences to σ^{70} consensus of *E. coli* (Fig. 1; Dominguez-Cuevas, 2004). Interestingly, the –10 consensus sequence of σ^{70}-dependent promoters contained weakly conserved (33%) C nucleotide in *P. putida* at the same position that was described in *E. coli* –10 consensus sequence TATACT of σ^S-dependent promoters where C nucleotide was highly (73%) conserved (Weber *et al*., 2005). *E. coli* σ^{70}-dependent promoters contain the C nucleotide at the same position much seldom than among the studied 300 promoters (only 20%; Lisser and Margalit, 1993).

σ^{70}-dependent promoters	-35						-10					
Consensus in *E. coli*[1]	**T**	**T**	**G**	**A**	**C**	**A**	**T**	**A**	**T**	**A**	**A**	**T**
	69	79	61	56	54	54	77	76	60	61	56	82
Consensus in *P. aeruginosa*[2]	**T**	**T**	**G**	**A**	**C**	**C/a**	**T**	**A**	**T**	**A**	**A**	**T**
	54	56	52	33	52	31/27	86	71	33	40	46	75
Consensus in *P. putida*[2]	**T**	**T**	**G**	**A**	**C**	**C**	**T**	**A**	**T**	**A**	**C/a**	**T**
	74	44	56	59	38	41	82	72	44	41	33/30	82

σ^{S}-dependent promoters										
Consensus in *E. coli*[3]	**C**	**T**	**A**	**T/c**	**A**	**C**	**T**	**T**	**A**	
	100	95	100	47/44	59	73	100	81	64	
Consensus in *P. aeruginosa*[4]	**C**	**T**	**A**	**T**	**A**	**C**	**T**			
	100	100	56	50	75	69	100			

Figure 1. The consensus and degree of conservation of the –35 and –10 elements of σ^{70}- and σ^{S}-dependent promoters in the different species. For each element, the degree of conservation (in percents) of the consensus nucleotides is indicated.

[1] (Lisser and Margalit, 1993)
[2] (Dominguez-Cuevas, 2004)
[3] (Weber *et al*., 2005)
[4] (Schuster *et al*., 2004)

1.1.2. ppGpp alters transcription initiation in stressed cells

A signal molecule ppGpp, known as a global alarmone, is responsible for the regulation of transcription initiation via a discriminator region in *E. coli*. ppGpp is produced in response to amino acid and other nutrient limitations and under circumstances that cause growth arrest (Travers, 1980; Xiao *et al*., 1991). It is one of the most important signal molecules, which allows *E. coli* and *P. putida* to sense changes in the environment. ppGpp is involved in regulatory networks that push bacteria from logarithmical growth to stationary growth phase (Venturi, 2003; Magnusson *et al*., 2005).

As it was described above, the σ^{70}- and σ^{S}-dependent promoters have specific conserved regions that allow RNAP holoenzyme to recognise specific promoter and to initiate transcription. Actually, the initiation of transcription is regulated by more DNA elements in promoter region. For example, the existence of G/C-rich sequence (designated as a discriminator) between –10 hexamer and transcriptional start-point ensures the sensitivity of transcription from the concentration of ppGpp (Travers, 1984; Magnusson *et al*., 2005). The transcription from these promoters is decreased under stress conditions, when ppGpp are accumulated (Travers, 1984; Magnusson *et al*., 2005).

The alarmone ppGpp can influence transcription by affecting the open complex formation. When RNAP starts to transcribe a gene, it has to conduct several steps before elongation phase. One important stage is open complex formation – melting of DNA strands in –10 hexameric region and so-called transcriptional bubble formation. The bubble formation depends on the DNA sequence that locates downstream from the –10 hexamer. When this sequence is G/C-rich, then the DNA melting is retarded and more time is needed for initiation of transcription. At the same time the binding of ppGpp with β- and β'-subunits of RNAP destabilises the RNA polymerase (Artsimovitch *et al*., 2004) and the formed open complex will be destabilised resulted in stopped transcription (Raghavan and Chatterji, 1998; Barker *et al*., 2001; Paul *et al*., 2004). The promoters that have intrinsically unstable open complex with RNAP (e.g. promoters of tRNA and rRNA genes) are thought to be specifically sensitive to further destabilization and therefore transcription initiation from such promoters is reduced in the presence of ppGpp (Raghavan and Chatterji, 1998; Barker *et al*., 2001; Paul *et al*., 2004). It is important to note, that at the same time when bacterial growth rate decelerates and ppGpp concentration increases, the global DNA supercoiling is changed to be more relaxed causing negative effect on DNA melting (Peter *et al*., 2004; Travers and Muskhelishvili, 2005).

The positive effect of ppGpp on transcription has been supposed to be indirect (Paul *et al*., 2004, Schuster *et al*., 2004; Magnusson *et al*., 2005). When bacterial growth rate slows down and σ^S related promoters have become preferred, ppGpp will releases many RNA polymerase molecules from synthesis of tRNA and rRNA. The balance between $E\sigma^{70}$ and $E\sigma^S$ is changed to a favour of $E\sigma^S$ and the interactions between σ^S and RNAP are facilitated (Paul *et al*., 2004; Magnusson *et al*., 2005). Recent findings suggest that ppGpp regulates binding of different sigma factors to core RNAP thus being required for the function of many alternative sigma factors (Kvint *et al*., 2000; Carmona *et al*., 2000; Jishage *et al*., 2002).

1.1.3. DNA supercoiling has an influence on transcription initiation

Changes in DNA topology may be also a reason of gene expression alteration. The negative supercoiling of DNA decreases in the stationary phase cells and it lessens the probability of RNAP to recognise the promoters that have 16 or less unconserved nucleotides between the –35 and –10 hexamers in *E. coli* (Travers and Muskhelishvili, 2005). Even quite similar promoters can response differently to altered topology of DNA. For example, the promoter of *fis* (TTCATC N_{16} TAATAT) is activated at the higher negative superhelical density comparing to the promoter of tyrosyl tRNA (*tyrT*) (TTTACA N_{16} TATGAT) in *E. coli* (Schneider *et al*., 2000; Auner *et al*., 2003). Already small changes in the promoter region can cause noticeable effects. Thus, the insertion

of a single base pair in the spacer region between –10 and –35 hexamers made the promoter of *tyrT* insensitive to supercoiling changes *in vivo* (Auner *et al*., 2003). Besides the stretching DNA, supercoiling of the DNA sequence locating upstream and/or downstream of the promoters can affect transcription as well (Peter *et al*., 2004). Peter *et al.* (2004) found that 7% of *E. coli* genes had altered expression in response to changes in supercoiling of chromosome. Even more, the genes whose expression was activated by the relaxed DNA conformation contained an A/T-rich sequence downstream and upstream from the promoter. It was hypothesised that the A/T-richness helps DNA to melt in stationary phase when DNA negative supercoiling is reduced and DNA melting is retarded. The A/T richness downstream of promoter may also enhance the promoter clearance and thereby enhance the expression of gene (Peter *et al*., 2004).

Changes in supercoiling that cause imperfect spacer distance between –35 and –10 hexamers of σ^{70}-type promoters, competition of sigma factors for free RNAP, amount of free RNAP and changes in the pool of transcriptional regulators – all these cause the remodelling of gene expression pattern that is needed under stress conditions.

1.2. Transposition

McClintock first suggested the mobile DNA elements in the late 1940s as genetic determinants in maize chromosomes that could move from place to place within the genome and thereby control gene expression (Craig, 2002). Very soon, the specific DNA elements designated as transposable elements were also found in other organisms including bacteria. Transposons are bounded by terminal inverted repeats (IR) and may "jump" in the genome by a mechanism that is called transposition, the recombination reaction mediating the movement of discrete DNA segments between many non-homologous sites. The terminal inverted repeats are binding sites for the transposase that is usually encoded by the mobile element. The transposase binds to the element's inverted repeats and target DNA by mediating synapses of the transposon ends and the target DNA. Thereafter the transposase executes the DNA cutting reaction that frees the element from flanking DNA in the donor site and joins transposon to the new insertion site. The ends of transposon attack the target at staggered position gaps of both sides of inserted transposon. The reparation of these gaps by bacterium produces the target sequence duplication that is called directed repeats (Fig. 2). Transposition can result either in two copies of transposable element present in donor and target DNA-molecule (ensured by replicative transposition) or only in one copy in the target DNA molecule leaving the donor DNA molecule without transposon (ensured by 'cut and paste' transposition; reviewed Craig, 2002).

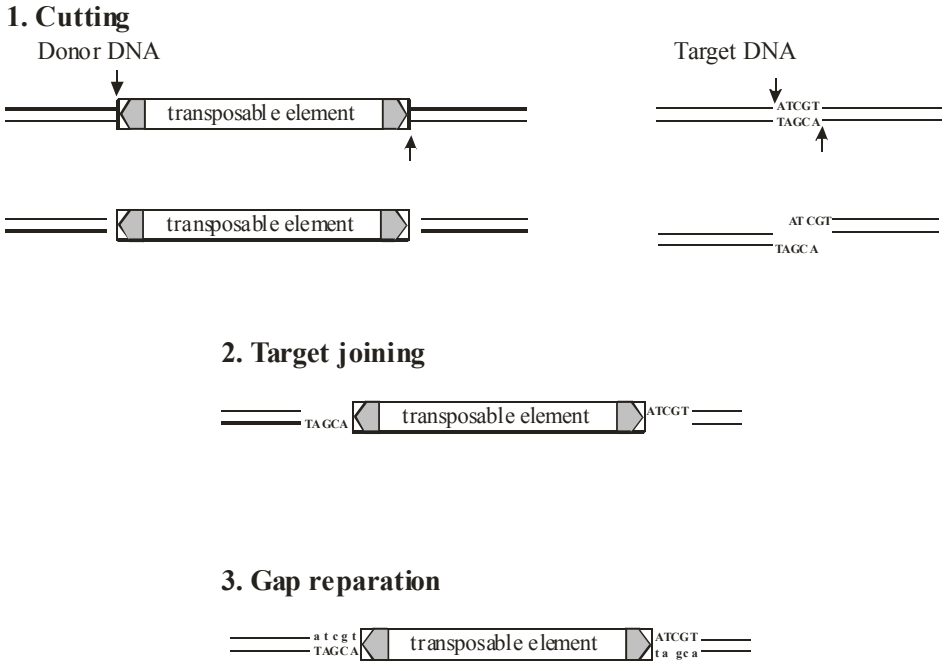

Figure 2. A simplified scheme of transposition. The transposable element (white box) has inverted repeats (grey symbols) at the ends of the element. The transposase binds to inverted repeats and target DNA, causing DNA cutting and joining of transposable element into target sequence. Bacterial DNA polymerase fills in generated gaps resulting in target sequence duplication. These duplications are named as directed repeats (Craig, 2002).

1.3. Alteration of bacterial gene expression by transposition

It is believed that mobile DNA elements are extremely degraded parasites unable to exist outside of a host DNA and jump rambunctiously around the host genome. However, besides the so-called "parasitic lifestyle", transposable elements can be handled as bacterial mutagenic agents. Even more, transposable elements are involved in evolutionary processes by reorganising the genome of their host organism. Considering that, we cannot see transposable elements only as unbridled selfish parasites but as "beneficial tools" that ensure genomic flexibility for hosts (Kidwell, 2002; Biemont and Vieira, 2005).

The transposition of a mobile DNA element mostly happens indiscriminately and generally, insertions do not have beneficial value for bacteria. Even more, bacteria cannot tolerate the modifications of expression of "housekeeping" genes; the vitality of bacteria will decrease or even the death of bacteria may be

caused. Therefore, the modification of expression of the second group of genes by transposition is mostly characterised. These genes are usually the genes that ensure antibiotic resistance, the genes that are involved in pathogenesis and the genes that are needed for degradation of an alternative carbon source (reviewed Kidwell, 2002).

The gene activation by transposition may happen in two ways. Firstly, transposable elements can cause directly the activation of gene expression by bringing or generating a strong promoter upstream of a gene. Secondly, the gene activation may happen indirectly, by disrupting of a repression system involved in regulation of gene expression. This may happen by insertion of transposon into the repressor gene or target sites of the negative regulators. As a rule, new promoters are selected for inefficiently expressed genes without a good activation mechanism and a good response to signals from environment. Often these are genes, which have been picked up by bacterium through horizontal gene transfer (Kidwell, 2002; Chandler and Mahillon, 2002).

1.3.1. Promoter insertion is the simplest way for gene activation by transposition

The simplest way to activate a silent gene by transposition is to insert a promoter into upstream region of the gene. Many transposable elements carry a complete outwardly directed promoter in the inverted repeat of the element and the transcription of a downstream located gene may happen from this promoter after transposition (Mahillon and Chandler, 1998; Chandler and Mahillon, 2002). Already in the beginning of 70's the "controlling elements" that reactivated silent genes in *E. coli* were reported and these transposable elements were named mobile promoters (Saedler *et al*., 1974; Charlier *et al*., 1982). Activation of adjacent genes was supposed to be happening via transposing a functional promoter in composition of IS*2* upstream from *gal* operon (Saedler *et al*., 1974). However, very soon it was demonstrated that IS*2* could not activate the genes in all cases studied. Therefore, it remained questionable whether IS*2* carries the full outwardly directed promoter at its end or not. Later, the lack of the full promoter in the inverted repeat of IS*2* was approved. Only the –35 hexameric element was identified in the right inverted repeat, and this explained why the transposition of IS*2* activated adjacent genes only occasionally (Ghosal *et al*., 1979). IS*2* was able to activate adjacent genes only by generating a fusion promoter with the –10 hexamer found in the target sequence (Hinton and Musso, 1982).

In 1982, the activation of *argE* expression in *E. coli* by the transposition of IS*3* was reported in a strain where the natural promoter of this gene was deleted. IS*3* was inserted approximately 150-bp upstream from the *argE* gene and transcription began from the promoter carried by IS*3* (Charlier *et al*., 1982). The

outwardly directed promoters do not have to be always located in the inverted repeat, but these may lie inside of the transposable element nearby to its inverted repeat. For example, IS*6110* carries a functional promoter OP6110 within 110-bp fragment adjacent to the right terminal inverted repeat (Safi *et al*., 2004). IS*6110* is important marker in identification of *Mycobacterium tuberculosis* strains (van Embden *et al*., 1993) and this IS element can activate several genes of this bacterium. The transcription from this promoter is up regulated in *M. tuberculosis* during the growth in human monocytes and in late growth phases in broth (Safi *et al*., 2004). The growth and the survival of *M. tuberculosis* depend on the successful pathogenesis in human monocytes and macrophages. Therefore, the macrophage environment sensitive promoter ensures this bacterium a mechanism for generating potentially advantageous phenotypes. Activity of this promoter is controlled by the repeated sequences present upstream of IS*6110* promoter that may function as transcription factor binding sites (Beaucher *et al*., 2002; Safi *et al*., 2004).

1.3.2. Formation of fusion promoters

Although transposable elements may carry an outwardly directed promoter within the inverted repeat or near to it, many characterised transposable elements which activate bacterial genes, carry only outwardly directed –35 hexamer and form a fusion promoter by joining with –10 hexamer or "–10-like" region in the upstream gene of target DNA (Mahillon and Chandler, 1998).

Many of these elements contain an inwardly directed –10 hexamer of transposase gene in the left inverted repeat. The expression of transposase gene is ensured by circularisation of the particular element, which generates a functional promoter P_{junc} between the –35 hexamer from right end and the –10 hexamer from left end of element. Such structure of IS element guarantees the control over the transposition, since the transposase gene expression is down regulated by the absence of the full functional promoter (Polard and Chandler, 1995; Mahillon and Chandler, 1998). The circularisation has been observed for several IS elements from different IS families including IS*1*, IS*3,* IS*21*, IS*30*, IS*110*, IS*256* and ISL*3* families (Chandler and Mahillon, 2002). Obviously, after insertion into bacterial DNA these IS elements are able to form fusion promoters with –10 hexamers present in target DNA upstream of the gene.

There are number of transposable elements that have outwardly directed –35 hexamer, but they do not circularise or the circularisation has not been described (Chandler and Mahillon, 2002). One of the examples is IS*1490* from *Burkholderia cepacia* AC1100 that created a fusion promoter upstream of *tftAB* genes (Hubner and Hendrickson, 1997). These genes were obtained by the bacterium due to the horizontal gene transfer and they lacked functional promoter sequence in spite of a –10 hexamer present upstream of the genes. The new fusion promoter was formed between the terminal sequences of IS*1490* and the

mentioned –10 hexamer, resulting in growth of bacteria on herbicide 2,4,5-trichlorophenoxyacetic acid (2,4,5-T; Kilbane *et al*., 1983; Hubner and Hendrickson, 1997).

The outwardly directed –35 hexamer can locate at different distance from the terminus of transposable elements. Therefore, the generation of functional fusion promoter is a result of the insertion of particular element at individual distance from the –10 hexamer in target DNA. For example, the –35 hexamer can exist precisely at the end of inverted repeat, (see IS*256* in Fig. 3; Chandler and Mahillon, 2002) and in this case, the transposable element has to insert 16 to 18-bp upstream from the –10 hexamer present in target DNA (Fig. 3; Maki and Murakami, 1997). At the same time, IS*1490* has the –35 hexamer located 7-bp inward from the end of element. Therefore, the –10 hexamer in target DNA has to be located a few nucleotides away from the insertion site of IS*1490* (Hubner and Hendrickson, 1997). The third possibility is that the inverted repeat of transposable element contains the outwardly directed –35 hexamer precisely 17-bp inside from the end of element, which is optimal distance between –35 and –10 hexamers. For activation of silent genes, these elements, for instance Tn*4652*, have to "jump" precisely next to the –10 hexamer of the target DNA (Fig. 3; Nurk *et al*., 1993).

Figure 3. The fusion promoters created by transposable elements. Since the –35 hexamer may locate in different distances from the right terminus, the transposable elements have to insert into different positions from the –10 hexamer to create functional promoter. Uppercase letters show the sequence of inverted repeats of the right end of the transposable elements. Lowercase letters show target sequence of transposition. The –35 and –10 hexamers are marked by boxes. The direct repeats are in italic and the gene name follows the arrow. Product of *llm* gene is a lipophilic protein affecting *Staphylococcus aureus* lysis and ensures methicillin resistance to bacterium, the *tftA* encodes 2,4,5-trichlorophenoxyacetic acid oxygenase 1 in *B. cepacia* and *pheA* encodes phenol monooxygenase in *P. putida*.

[1] (Maki and Murakami, 1997)
[2] (Hubner and Hendrickson, 1997)
[3] (Nurk *et al*., 1993)

1.3.3. Insertion of transposable element into regulatory area upstream of promoter can activate gene expression

Besides carrying promoters or promoter elements in the ends of transposon and activating genes directly, transposons can alter the expression of bacterial genes by insertion into the regulatory area of present promoter of target gene. The insertion can maintain functional promoter but reorganise DNA sequence where binding sites locate for local or global transcription regulators. A good example of this kind of influence of transposition upon the gene expression is an activation of *bgl* operon in *E. coli*. Wild-type strains of *E. coli* have *bgl* operon in silent state, but sometimes Bgl^+ spontaneous mutants arise that are able to grow on aryl-β-glycosides such as arbutin and salicin (Schaefler and Malamy, 1969). It was found that more than 98% of the Bgl^+ mutants had an IS*1* or IS*5* insertion in the regulatory region of the promoter of the *bgl* operon, whereas the other kind of mutations (mostly deletions) were presented by less than 2% of the cases (Schnetz and Rak, 1988; Schnetz and Rak, 1992; Schnetz, 1995).

The *bgl* operon was activated in growing cells of *E. coli* mostly due to disruption of a DNA sequence for H-NS binding, while these insertions of IS elements did not disrupt a binding site for CAP. H-NS represses the expression of *bgl* operon at two levels: it represses the transcription from the *bgl* promoter and blocks the elongation of transcription of *bglG*, the first gene of the operon (Dole *et al*., 2004). On the other hand, the activator protein CAP-binding site on DNA locates adjacent to the H-NS binding site and CAP cannot activate transcription from the *bgl* operon in the presence of H-NS (Schnetz and Rak, 1988; Schnetz and Rak, 1992; Schnetz, 1995). Differently from growing bacteria where 98% of the Bgl^+ mutants had an insertion in the regulatory area of the *bgl* operon, only 80% of mutants acquired in stationary-phase arose via transposition of insertion elements and 20% of them had an insertion in the *hns* gene (Hall, 1998).

It is not clear why bacteria need so tightly down-regulated operon, which is activated only by mutations in the regulatory region. The complex regulation suggests that the expression of this operon is disadvantageous for bacteria under some conditions, whereas certain physiological and environmental conditions can favour its activation (Dole *et al*., 2004).

1.3.4. Disruption of repressor genes by transposition

The activation of gene expression can occur also through the disruption of repressor gene. It tends to be more common for genes responsible for defending of bacteria against growth inhibiting factors. For example, the expression of genes for antibiotic resistance or efflux transport genes is regulated by a specific mechanism that enables induction of transcription of these genes up to 1000

fold (Lodge and Piddock, 1991). In hospitals where bacteria live under strong antibiotic pressure, the normal regulation of antibiotic resistance genes may not be enough to allow bacteria to grow. Although the regulatory mechanism of antibiotic resistance allows bacteria to express appropriate genes at the maximum possible level, the higher fitness of bacteria will be reached when the negative regulation of the genes for antibiotic resistance is eliminated. For instance, the disruption of the *ampD* reading frame resulted in constitutive β-lactamase expression causing highly resistant *P. aeruginosa* strains arising (Bagge *et al*., 2002). Six *P. aeruginosa* clinical isolates that stably produced β-lactamase carried IS*1669* insertions at the same position, 74 bp downstream of the start codon of *ampD* gene (Bagge *et al*., 2002). Such disruption of the reading frame of *ampD* produced of a non-functional AmpD repressor protein (Langaee *et al*., 1998; Bagge *et al*., 2002). Thus, bacterium that had eliminated repression of β-lactamase synthesis developed the high resistance phenotype for β-lactams. The clinical strains of *P. aeruginosa* without functional *ampD* gene were able to grow in the presence of over 300-times higher concentration of β-lactam than isolates that had functional *ampD* gene (Bagge *et al*., 2002).

1.3.5. Specific regulation of gene expression by transposable elements

The gene activation or the repression elimination by mobile DNA elements is commonly indiscriminate in bacteria (Craig, 2002), but in certain cases, the alteration of gene expression by insertion and excision of the same mobile DNA element may happen repeatedly at the same target DNA position (Bartlett and Silverman, 1989; Hilse *et al*., 1996; Mitchell *et al*., 2003). It is ensured by transposase of particular element that recognises a specific DNA sequence. This kind of regulation of gene expression is uncommon and it is known only few examples, which are mostly involved in pathogeneses of bacteria or biofilm generation. (Bartlett and Silverman, 1989; Hilse *et al*., 1996; Mitchell *et al*., 2003).

A good example of regulation of biofilm genes is IS*492* in *Pseudoalteromonas atlantica* DB27. The IS*492* insertion in *eps* locus switches off the extracellural polysaccharide production and causes nonmucoid EPS^- phenotype of bacteria (Bartlett and Silverman, 1989; Perkins-Balding *et al*., 1999). Although IS*492* generates 5-bp long directed repeats, these will be removed when IS*492* excises from the *eps* locus (Bartlett and Silverman, 1989; Perkins-Balding *et al*., 1999) thus restoring the polysaccharide production and biofilm formation. The accurate excision of IS*492* from the *eps* locus is ensured by circularisation of this IS element and P_{junc} promoter formation for transposase expression. The 5-bp long DNA sequence (the generated directed repeat) is needed to obtain correct distance between the –35 hexamer from the left end

and the –10 hexamer from the right end of IS*492*. This 5-bp long DNA sequence (CTTGT) is highly conserved and is used as the target for insertion (Perkins-Balding *et al*., 1999).

Similarly, IS*1301* is involved in a regulation of capsulation of *Neisseria meningitidis*. When this bacterium is attacking a host, it has to pass the epithelial tissue of human organism. To be more adhesive and undetectable for the host's immune system, bacteria have to lose encapsulation. The production of capsule building blocks, the polysaccharides, was stopped by the insertion of IS*1301* into a structural gene *siaA* (Hilse *et al*., 1996). The transposase of IS*1301* recognises a DNA sequence for insertion, which is AYTAG (Y represents nucleotides C or T). Hilse *et al.* in 1996 screened the genome of *N. meningitidis* and found that IS*1301* needs for insertion an additional secondary structure of DNA around the target sequence consensus. The stable target DNA bending is caused by palindromic symmetry of long AT tracts (Perez-Martin *et al*., 1994; Hilse *et al*., 1996).

A few years ago IS*1301* was found in *Actinobacillus actinomycetemcomitans*, where it has been inserted upstream of the *ltx* operon resulting in enhanced synthesis of leukotoxin. *A. actinomycetemcomitans* is a gram-negative bacterium that uses the leukotoxin for killing the lymphocytic and monomyelocytic cells. The *ltx* operon contains four genes, but only the *ltxA* gene encodes the toxin, whereas the rest of the genes are needed for the activation and secretion of the toxin (reviewed Mitchell *et al*., 2003). The expression of toxin genes is complexly regulated by two *cis*-sequences. The *ltx* operon has an AT-rich region immediately upstream of the promoter. This region behaves as a positive regulator, but region in position –87 to –111-bp functions as a negative regulator (Mitchell *et al*., 2003). The insertion of IS*1301* in 71 nucleotides upstream of the transcription start point of the *ltx* genes disrupts the original regulation of transcription – the negative *cis*-element is moved farther from promoter and therefore the repression is abolished. Moreover, in this organism IS*1301* uses for insertion ACTAA sequence that is similar to the sequence AYTAG described in *N. meningitidis*. Unlike *N. meningitidis* where IS*1301* regulates gene expression by disruption of the coding sequence of a structural gene and restoration of initial situation by precise excision, in *A. actinomycetemcomitans* the *ltx* expression is enhanced by destroying local negative regulation site (Mitchell *et al*., 2003).

1.4. Formation of fusion promoters by transposition of Tn*4652* in *P. putida*

1.4.1. The transposon Tn*4652*

The transposon Tn*4652* was discovered in 1987 by Tsuda and Iino as a fragment of pWW0 DNA that had inserted onto the plasmid pACYC184 (Tsuda and Iino, 1987). pWW0 (117-kb, Fig. 4) is the first-described toluene degradative catabolic plasmid which was found in *P. putida* mt-2 (PaW1) and it specifies a set of enzymes for degrading of toluene and related compounds. All the genes essential for toluene degradation (*xyl* genes) have been localised into a 39-kb long segment (Williams and Murray, 1974; Greated *et al*., 2002) which is flanked by 1,275-bp long directly repeated copies of IS*1246* (Meulien *et al*., 1981; Reddy *et al*., 1994). The plasmid pWW0 belongs to IncP-9 incompatibility group and 46-kb long sector of this plasmid contains all the IncP-9 core functions. The rest of the area is occupied by transposons (Greated *et al*., 2002). The biggest transposon Tn*4653* (71-kb long) contains a smaller transposon Tn*4651* (56-kb long) which probably arose after an insertion of the *xyl* genes into an ancestral transposon of Tn*4652* (17-kb long). The two copies of IS*1246* flank the toluene and the xylene degrading genes and most likely these elements have brought the *xyl* genes into the ancestral transposon Tn*4652* (Tsuda and Iino, 1987; Tsuda and Iino, 1988; Greated *et al*., 2002).

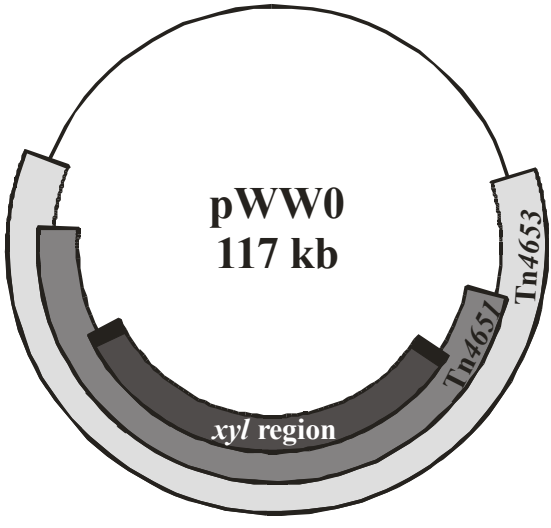

Figure 4. A simplified map of the plasmid pWW0 (117 kb). The transposons Tn*4653* (71 kb) and Tn*4651* (56 kb) are shown with grey boxes. Two copies of IS*1246* (1.3 kb) are shown with black boxes and they flank *xyl* region (39 kb) that contains genes of toluene and xylene degradation.

On the other hand, the pWW0 plasmid can lose the *xyl* genes by recombination between the directly repeated copies of IS*1246*, leading to formation of the transposons Tn*4652* and Tn*4654* from Tn*4651* and Tn*5653*, respectively. This occurred after growing of *P. putida* mt-2 (PaW1) on benzoate (Meulien and Broda, 1982). The authors described loosing of all plasmid DNA or only *xyl* genes from pWW0 that caused forming of the smaller, but still conjugative, 78-kb plasmid pWW0-8. They also described plasmid-free strains, which contained Tn*4652* in the bacterial chromosome (PaW85; Meulien and Broda, 1982).

The transposons Tn*4653* and Tn*4651* can transpose independently from each other but they both belong to the Tn*3* family (class II transposons; Tsuda *et al*., 1989). Tn*3*-family transposons have many common features that distinguish them from other transposons and IS-elements. Firstly, they have an unusually large transposase (approximately 1000 amino acid residues). Secondly, they contain similar and quite long inverted repeats, generally 40-bp long. Thirdly, they transpose by a replicative mechanism forming an intermediate, called cointegrate. Tn*3*-family transposons encode site-specific recombination system that is responsible for resolution of cointegrate into two separate molecule, target and donor. The fourth, a duplication of 5-bp long target sequence usually occurs during transposition. At last, usually a target molecule containing a transposon of Tn*3* family is immune to further insertions of the same transposon (Grindley, 2002). Based on the homology of the *tnpA* gene it is grouped into a subfamily with Tn*5041* and Tn*4556* (Grindley, 2002). Tn*4652* and the related elements differ from the other members of the Tn*3* family also by organization of the transposon having the *tnpA* gene located adjacent to the terminal right inverted repeat and transcribed inwardly. The transposase genes of other elements are transcribed outwardly from central *res* site, which is used for separation of cointegrate. In addition, Tn*4652* has extra genes that are involved in or have influence on transposition – *tnpC*, *tnpS* and *tnpT* (Tsuda *et al*., 1989; Hõrak and Kivisaar, 1999). Tn*4652* needs TnpC for the down regulation of transposition activity via repression of *tnpA* expression. TnpC does not affect transcription or translation of *tnpA* gene (Hõrak and Kivisaar, 1999). It is supposed that TnpC affects TnpA post-translationally by altering transposase folding or transposase stability. However, the regulation of stability of mRNA of *tnpA* is not excluded (Hõrak and Kivisaar, 1999).

1.4.2. The activation of the *pheA* gene by the transposition of Tn*4652*

Tn*4652* can generate fusion promoters by joining the –35 hexamer from the inverted repeat of the element and the –10 hexamer from target DNA (Nurk *et al*., 1993). Both IR-s of Tn*4652* carry only a part of the σ^{70}-type promoter – outwardly directed sequence TTGCCT that matches well with the –35-hexamer

consensus TTGACA and lack the –10 hexamer. The localization of the –35 hexamers is significant – they position exactly 17 nucleotides inward the transposon ends (Fig 5). This is an ideal distance between –10 and –35 hexamers of σ^{70}-dependent promoters and requires transposon to be inserted exactly adjacent to –10-hexameric sequence present on a target DNA. The 5-bp long target sequence of Tn*4652* is not conserved, but under selective pressure when expression of certain genes has positive value for growth, insertion of Tn*4652* adjacent to the potential –10 hexamers will be selected. For example, Nurk *et al.* (1993) described six fusion promoters upstream of the phenol monooxygenase gene *pheA* that were formed between the –35 hexamer present in Tn*4652* inverted repeat and potential –10 hexamers flanking the transposon insertion in the target DNA. All six target-sequences that were described had good matches to the consensus of –10 element TATAAT. In those particular cases, the expression of *pheA* gene was switched on resulting in the growth of bacteria on selective plates containing phenol as the only carbon source (Nurk *et al*., 1993).

It was studied how *P. putida* was able to activate the expression of the promoterless *pheBA* operon under the phenol selection conditions (Nurk *et al*., 1993). The plasmid pEST1332 carrying the *pheBA* operon without its original promoter mimicked "unwanted" result of horizontal gene transfer between species, when bacteria had acquired the genes that were unable to express. Phenol monooxygenase encoded by *pheA* was needed for *P. putida* initial cleavage phenol, whereas the rest of enzymes for phenol catabolism were chromosomally encoded. The mutants able to grow on phenol plates appeared in a few days of selection. Firstly, only one particular target sequence TATCA locating 1259-bp upstream of *pheA* gene was identified (see PRA1 in Fig. 5). When this target site was removed, other targets were observed as well. Among these five, a target TATGA locating 312-bp upstream of the *pheA* gene was strongly preferred over the others (see PRA2 in Fig. 5). The rest of the target sequences were unique (Fig. 5). As already mentioned above, both inverted repeats of Tn*4652* carry identical –35 hexamers. Therefore, both ends of the transposon could be able to generate fusion promoters. However, the right end was clearly preferred. Only PLA1 from six detected fusions was generated by the left end of Tn*4652*. However, not all target sequences had good matches to the consensus of –10 element, for formation the fusion promoters like PRA3, PRA7 and PLA1 an additional point mutation (base substitutions or insertions) was needed in the target DNA. (Fig. 5; Nurk *et al*., 1993).

The transpositional activity of Tn*4652* is not constant. It has been shown that Tn*4652* is activated in stressed cells of *P. putida* and the expression of its tranposase depends on the stationary phase sigma factor σ^S (Ilves *et al*., 2001).

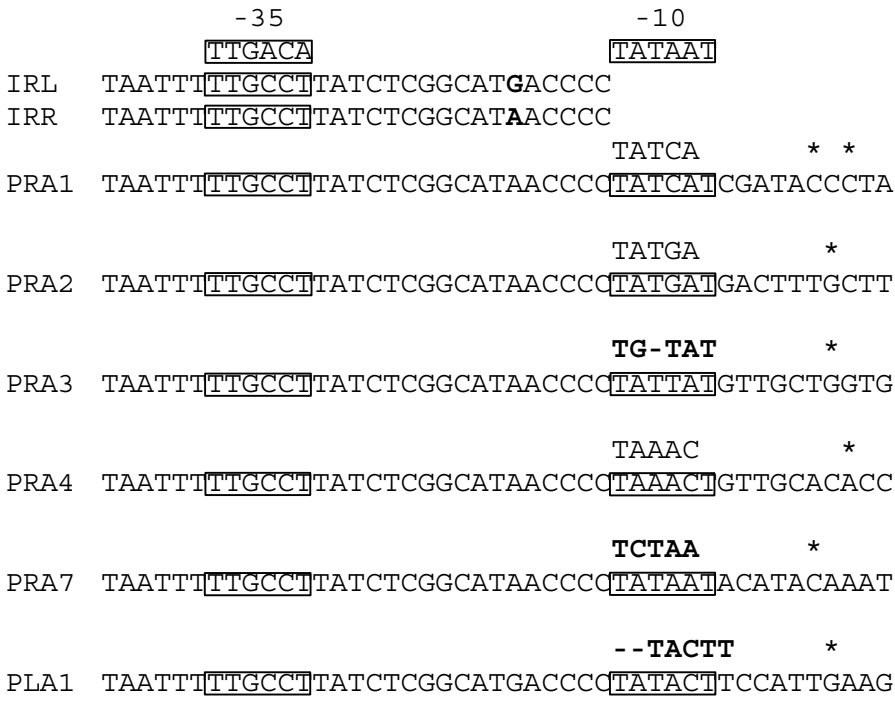

Figure 5. The sequences of fusion promoters PRA1, PRA2, PRA3, PRA4, PRA7 and PLA1. The 29 terminal nucleotides of the right and the left end of Tn*4652* inverted repeats and 16 nucleotides adjacent to the insertion sites are presented. The sixth nucleotide of the inverted repeat, which differs in the right and the left end, is shown with bold letter. Two hexamers homologous to the *E. coli* σ^{70} dependent promoter –35 and –10 consensus sequences are boxed. The initial target sequences of Tn*4652* are shown above of –10 hexamer sequences. The target sequences with point mutation are shown in boldface. The first nucleotides of transcription are marked with asterisks (from Nurk *et al*., 1993).

1.5. Involvement of multifunctional proteins in regulation of transcription and transposition

As it was discussed above, transposons can activate genes by providing functional promoter sequences, but this is not the end of the story. The transcription from newly generated promoters does not depend only on the sequence of promoter. The DNA sequences surrounding the promoters' play role as well. Of course, the sigma factors are the most important in recognising the promoters and providing expression in the specific conditions. However, the regulators that bind to the sequence present around the promoter ensure tighter control

over gene expression and activate them only when they are needed (Martinez-Antonio and Collado-Vides, 2003).

The more accurate control of the transcription occurs due to regulators that allow RNAP to transcribe gene only in the presence of a right signal. There are two types of regulators ensuring precise expression of genes, local and global. The local regulators control only few genes or operons and usually the regulation is specific. On the other hand, the global regulators, whose influence is general, are involved in many different processes in bacteria as transcription, replication, translation, transposition etc. They determine expression of many operons and regulons (stimulons) and may influence transcription from promoters generated by transposition. These proteins are important in admitting and responding different signals from environment (Martinez-Antonio and Collado-Vides, 2003; Martinez-Antonio *et al*., 2003; Venturi, 2003).

Since the transposition of a particular element is mostly regulated by the proteins that bind to the end of the element, it is logical to suggest that transcription from the promoters generated by transposition may be also controlled by these proteins. For instance, the global regulators IHF, Fis, HU and H-NS have important role both in regulation of transcription initiation and in regulation of transposition of different mobile DNA elements (Chandler and Mahillon, 2002; Martinez-Antonio and Collado-Vides, 2003; Travers and Muskhelishvili, 2005). Several mobile DNA elements like IS*1*, IS*903*, IS*10*, Tn*10* (Mahillon and Chandler, 1998) and Tn*4652* (Hõrak and Kivisaar, 1998) carry the specific binding sites for IHF within or close to the terminal inverted repeats. Liu *et al.* (2005) reported the involvement of IHF in transpososome assembly of Tn*10* (Liu *et al*., 2005). The Tn*10* transpososome is DNA-protein complex where two transposon ends, transposase dimer and IHF are assembled in an asymmetrical complex. IHF is essential only in the first step for generation of needed DNA conformation. After first cleavage of the transposon end the affinity for IHF decreases dramatically resulting in the ejection of IHF from the transpososome and the unfolding of the complex (Liu *et al*., 2005). IHF binds to the ends of γδ, a Tn*3*-family element, cooperatively with transposase and stimulates transpositional immunity of γδ as well (Wiater and Grindley, 1988; Wiater and Grindley, 1990). On the other hand, IHF can modulate transposition by regulating transposase expression. The DNA sequence for IHF binding locates in all above-mentioned mobile DNA elements adjacent to the *tnpA* promoter (Hõrak and Kivisaar, 1998; Mahillon and Chandler, 1998). In the case of Tn*4652*, IHF affects the transcription from the promoter of *tnpA* positively. IHF binds to the DNA sequence locating 73 to 85 bp upstream from this promoter with well-conserved DNA core-sequence for IHF binding (Hõrak and Kivisaar, 1998).

The global regulators, which influence transposition, can be involved in regulation of transposase expression, formation of transpososome, ensure target specificity and influence supercoiling of DNA (Mahillon and Chandler, 1998; Travers and Muskhelishvili, 2005).

The influence of global regulator in transposition can also be indirect. For instance, the transposition of Tn*5* and IS*50* is enhanced by Fis in exponentially growing *E. coli* cells, but it is supposed that this effect is caused by other host factor whose expression is under the control of Fis (Weinreich and Reznikoff, 1992). The composite transposon Tn*5* consists of two IS*50* elements oriented as inverted repeats. The inner ends of both IS*50* contain specific DNA sequence for Fis binding and the transposition activity is significantly reduced in *fis*$^-$ background. However, the transposition of a truncated Tn*5* element, which lacks internal Fis sites, is still stimulated by Fis (Weinreich and Reznikoff, 1992).

The inhibitory effect of Fis on IS*50* transposition has been shown in conditions where DNA is semimethylated in *E. coli*. Fis binds at inner ends of IS*50* and affects probably transpososome complex formation. Therefore, it is supposed that Fis damps the transposition activity of IS*50* under semimethylated conditions when transposition is not acceptable to the cell. At the same time, Fis also activates transposition of IS*50* and Tn*5* in logarithmically growing cells by indirect way (Weinreich and Reznikoff, 1992).

Another small global regulator, H-NS, is also involved in transposition. It is associated with transposition of IS*1*, but its role is not clear yet (Shiga *et al*., 2001). While at least one H-NS binding site is shown to be adjacent of the right inverted repeat of IS*1*. Shiga and co-workers supposed that H-NS promotes the formation of an active IS*1* DNA-transposase complex (Shiga *et al*., 2001). At the same time, it is assumed that H-NS may act post-translationally protecting the proteolysis of IS*1* transposase (Rouquette *et al*., 2004).

1.5.1. The global regulator IHF (integration host factor)

Integration host factor (IHF) is a small (20-kDa) basic protein that belongs to the histone-like family proteins that have several functions in bacteria (Friedman, 1988). IHF has been demonstrated to be involved in DNA replication, recombination, transposition, gene expression but also in phage DNA packing and partition in *E. coli* (Friedman, 1988; Freundlich *et al*., 1992; Goosen and van de Putte, 1995). IHF is a sequence-specific DNA-binding and – bending heterodimeric protein that is abundant in *E. coli* and *P. putida* cells. Its two subunits are encoded by the *ihfA* and *ihfB* genes (Weisberg *et al*., 1996) and are structurally similar. Both of them have a helix-turn-helix domain for dimerization that makes a "body" of IHF, and two antiparallels β-sheets that form so-called "arm" for the binding with DNA (Rice *et al*., 1996). IHF binding sites in *E. coli* consist of a core sequence WATCAA N_4 TTR (where W is A or T and R is A or G) and a less conserved 4–6 bp long A/T-rich track which locates 6–8 bp upstream from the core sequence (Goodrich *et al*., 1990; Lee *et al*., 1991). It has been shown by footprint analysis that DNA sequence protected by

IHF is at least 35-bp long, which is longer than the length of the IHF-binding core sequence and the A/T-rich tract taken together (Yang, 1989). However, IHF binds most strongly to the core sequence and the A/T track while the binding to the flanking DNA is less specific and weaker (van Rijn *et al*., 1991). IHF makes contact only with DNA minor groove by its flexible β-ribbon "arms" and produces DNA U-turn that can exceed up to 180° (Rice *et al*., 1996). A highly conserved proline residue at the tip of each arm induces and/or stabilizes DNA bending by intercalating between base pairs. The "body" of IHF makes additional contact with the TTR motif in the core sequence and the A/T rich tract. This stabilizes binding and causes wrapping of DNA around the protein (Lee *et al*., 1992; Hales *et al*., 1994; Lynch *et al*., 2003; Swinger and Rice, 2004).

The IHF proteins of *P. putida* and *E. coli* are similar. Both the *ihfA* and *ihfB* genes of *P. putida* code for 100-amino-acid-long polypeptides. The IhfA subunit is one residue longer and IhfB is six residues longer than the subunits of *E. coli* IHF. Compared to IHF of *E. coli*, both *P. putida*'s IHF subunits have some different amino acid residues that locate mostly in the region that is not so important for recognition of the DNA (Calb *et al*., 1996). Moreover *P. putida* IhfA and IhfB subunits can interact with *E. coli* subunits and form the active hybrid heterodimers *in vivo* (Calb *et al*., 1996). The hybrid proteins were able to restore λ phage growth in IHF-defective *E. coli* strains (Calb *et al*., 1996).

In both bacteria amount of IHF in cells depends on growth rate. Approximately 8,500 – 12,000 molecules of IHF can be detected in logarithmically growing cells of *E. coli* and its maximum is in the early stationary phase cells with 55,000 – 60,000 molecules (Ditto *et al*., 1994; Murtin *et al*., 1998; Ali Azam *et al*., 1999). During prolonged incubation of *E. coli* in stationary phase, the level of IHF decreases again to less than one-half of the maximum level. However, IHF becomes the second most abundant global regulator in stationary phase cells (Ali Azam *et al*., 1999). Valls *et al.* (2002) have shown that the number of IHF molecules in *P. putida* arises from the less than 2000 molecules in fast growing cells over to 14,000 molecules in early stationary phase cells.

IHF can either activate or repress transcription, depending on the location of IHF binding site in relation to the promoter. In the case of *ompB* (the *omp* operon contains genes of osmoregulators in *E. coli*), two IHF binding sites in DNA overlap with the –10 and –35 hexamers of the promoter. In this case, the binding of IHF physically inhibits the RNA polymerase interaction with the promoter (Goosen and van de Putte, 1995). IHF can also inhibit transcription indirectly through the modulation of interaction of activators with RNAP. IHF may block the binding of the activator to DNA or obstruct its function (e.g. the promoter region of *ompF* gene; Goosen and van de Putte, 1995). However, it is well known that IHF can activate transcription from promoters as well. It can stabilize the formation of close or open complexes of RNA polymerase with promoter. For instance, $E\sigma^{54}$ recognises promoter and often forms the closed

complex by ministration of IHF. Additionally, the open complex is not formed without the $E\sigma^{54}$ interaction with activator protein. Usually, IHF plays only an architectural role by binding to the DNA between the promoter and activator-binding site, thus bounding the DNA and bringing the activator and RNAP together (Giladi *et al*., 1990). This can happen for example, in the case of the upper pathway promoter Po of *dmp* genes (Sze *et al*., 2001).

IHF can also activate transcription directly, without the involvement of other regulators. This was shown *in vivo* and *in vitro* for the P_L1 promoter of phage λ (Giladi *et al*., 1990), the P_G2 promoter of *ilvGMEDA* (Pagel and Hatfield, 1991) and the early promoter Pe of bacteriophage Mu (Krause and Higgins, 1986). P_L1 and Pe promoters contain IHF binding site 81 bp upstream from transcriptional initiation point, but P_G2 has IHF binding site 95 bp upstream of the transcriptional initiation start point. The activation of transcription from P_L1 and Pe promoters requires the interaction of IHF and the RNA polymerase C-terminal domain of α subunits (Giladi *et al*., 1992; van Ulsen *et al*., 1996). At the same time, the activation of transcription from the P_G2 promoter of *ilvGMEDA* operon in *E. coli* does not need specific IHF and RNA polymerase interaction but IHF alters DNA supercoiling at downstream promoter region and facilitates open complex formation (Parekh and Hatfield, 1996).

The enhancing effect of transcription by IHF may be a result of avoiding the repressor binding with the promoter region. IHF competes with H-NS for the binding to the Pe promoter region. H-NS represses transcription from Pe by binding with relatively large region overlapping this promoter. The binding of IHF alleviates H-NS inhibition, by interfering with the formation of a repressive H-NS-DNA complex (van Ulsen *et al*., 1997).

Only the role of IHF in transposition and regulation of transcription was described here. Several other multifunctional proteins exist in bacteria that are involved both in regulation of transposition and transcription (Azam and Ishihama, 1999). There exist myriad of different transposable elements with different features that can alter transcription of bacterial genes in particular ways.

2. RESULTS AND DISCUSSION

2.1. Aims of the study

The formation of new promoters by transposition is a possibility to activate silent or weakly expressed genes, especially those acquired by horizontal gene transfer. Nurk *et al.* described six fusion promoters that were generated by transposition of Tn*4652* into upstream region of promoterless *pheA* gene (Nurk *et al*., 1993). They all differ by location, sequence and strength that caused dissimilar expression level of *pheA* (Nurk *et al*., 1993). Surprisingly, the *pheBA* genes-carrying plasmid pRA1 (Nurk *et al*., 1993) that contained 34 nucleotides from the right end of Tn*4652* upstream of the –35 hexamer of the fusion promoter PRA1 did not support growth of *P. putida* PaW85 on phenol minimal plates. This result was unexpected, considering the fact that the original hybrid plasmid pEST1354 (Nurk *et al*., 1993), where the fusion promoter PRA1 was subcloned, gave bacteria the ability to degrade phenol. Hence, the question was raised – how the transcription from the created fusion promoters is controlled.

Two newly generated fusion promoters, PRA1 and PLA1, were chosen for the study of the regulation of transcription activation. PRA1 represents the fusion promoters where the –35 hexamer was derived from the right end of transposon and PLA1 was chosen as a unique fusion promoter where the –35 hexamer was derived from the left end of transposon.

2.2. The regulation of transcription from fusion promoter PRA1

2.2.1. The DNA sequence of Tn*4652* right end enhances the transcription from PRA1

The plasmid pRA1 contained 57 nucleotides of the right-end-sequence of Tn*4652* (up to the DraI restrictase site; Fig. 6) and pEST1354 contained entire transposon upstream of the *pheBA* operon. This indicated that transcription from the fusion promoter, at least in the case of PRA1, was altered by transposon DNA present upstream of the fusion promoter. To localise the region influencing fusion promoter activity we constructed two plasmids pRA1-7 and pRA1-12 containing fusion promoter PRA1 together with different lengths of transposon right-end DNA (Fig. 6). Compared to pRA1 the plasmid pRA1-7 contained additional 18 nucleotides from the right end of Tn*4652* flanking the DraI restrictase site at the end of the transposon. At the same time, the plasmid pRA1-12 contained 222 extra nucleotides upstream from the DraI site (Fig. 6).

To estimate activity of the fusion promoter PRA1 we measured the specific activity of product of the first gene of *pheBA* operon, catechol 1,2-dioxygenase (C12O).

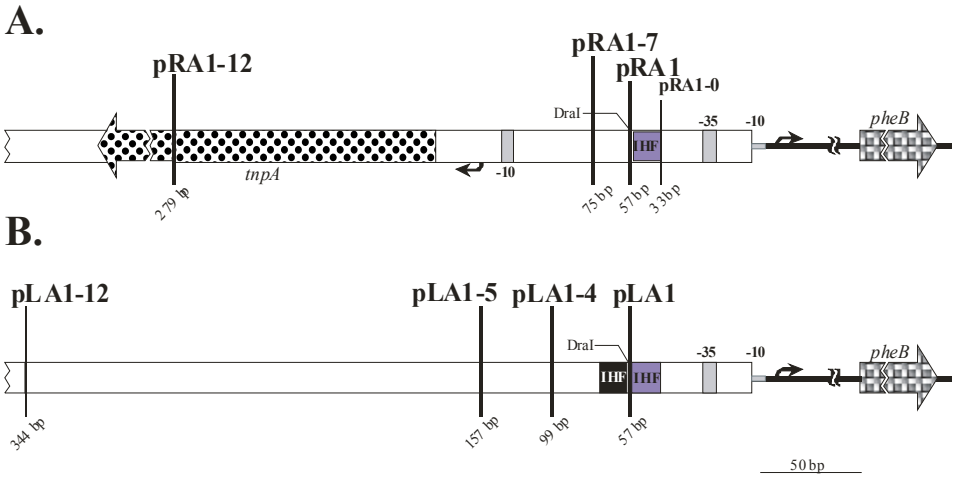

Figure 6. A scheme of PRA1 and PLA1 constructs. The plasmids pRA1-0, pRA1, pRA1-7 and pRA1-12 carry the fusion promoter PRA1 that is based on the right end of the transposon Tn*4652* (A). The plasmids pLA1, pLA1-4, pLA1-5 and pLA1-12 carry the fusion promoter PLA1 that is based on the left end of the transposon Tn*4652* (B). The length of DNA derived from transposon ends in the particular plasmids is shown for every plasmid separately below the schemes. Open boxes designate noncoding sequences of Tn*4652* and thick lines show noncoding sequences of plasmid pEST1332. The large spotted arrow represents the *tnpA* gene of Tn*4652*, and checked arrows represent the reporter gene *pheB*. The hexamers of fusion promoters and *tnpA* promoter, as well as the core sequence for IHF binding are shown by grey boxes. Another hypothetical core sequence for IHF binding is shown by black box. The DraI restrictase site is shown.

The transcription from the fusion promoter PRA1 depended on the length of DNA of the transposon Tn*4652* end. The extension of the transposon Tn*4652* right end-DNA only by 18 nucleotides upstream of the DraI site increased the level of transcription from PRA1 to the level similar to that observed in the case of full-length Tn*4652* containing plasmid pEST1354. Compared to pRA1, the specific activity of C12O was 3 times higher in bacteria with the plasmid pRA1-7 (Fig. 3 in Ref. I). Therefore, quite short DNA sequence from the Tn*4652* right end was missing from the PRA1 promoter to achieve the maximum level of transcription. Location of *cis*-acting enhancing element into such short DNA sequence indicated that this DNA region could contain an additional promoter element or some DNA binding site for transcription activators. Analysing of the upstream sequence of the fusion promoter PRA1

did not reveal any additional σ^{70}-dependent promoter sequence TTGACA $N_{16–18}$ TATAAT. The absence of additional potential overlapping promoter was excluded by primer extension analysis (Ref. I). Therefore, the possibility remained that host proteins that were involved in transposition could activate transcription from PRA1.

2.2.2. IHF affects transcription from the fusion promoter PRA1 positively

It has been previously shown that *E. coli* IHF binds to both ends of transposon Tn*4652* (Hõrak and Kivisaar, 1999). In this study, the approximate positions of the core sequences for IHF binding were predicted. Based on DNA sequence analysis, it was proposed that the right end of Tn*4652* contains one core sequence for IHF binding at the position 44 to 56-bp inside the right end. Moreover, the expression of *tnpA*, which encodes Tn*4652* transposase, was modulated by IHF (Hõrak and Kivisaar, 1998). The promoter of this gene was inwardly directed and the –10 hexamer of *tnpA* promoter located 115 nucleotides inside at the Tn*4652* right end. The core sequence AATCATATATTTA for IHF binding in right end of Tn*4652* had good matches to *E. coli* IHF binding core consensus WATCAA N_4 TTR (where W can be A or T and R can be A or G). The left end of Tn*4652* contains two flanking putative IHF-binding sequences at the positions 44 to 56 and 59 to 71 bp inside the transposon end (Hõrak and Kivisaar, 1999; Fig. 6).

All mentioned binding sites locate near to the fusion promoters and, therefore, it was reasonable to suppose that the binding of IHF to both ends of Tn*4562* DNA could have an effect on transcription from the fusion promoters. To examine the possible influence of IHF on transcription from these promoters, the C12O specific activity was measured in crude lysates of *P. putida* wild-type KT2442 cells and *ihfA* defective strain A8759 cells. The positive effect of IHF on transcription activation from PRA1 was clearly seen by using the plasmids pRA1-7 or pRA1-12 (Fig. 3A in Ref. I). At the same time, the transcription from PRA1 did not differ in the wild type and the *ihfA*$^-$ strain of *P. putida* if they contained the plasmid pRA1. The more detailed investigation of the sequence of the Tn*4652* right end revealed that the PRA1 promoter in the plasmid pRA1 contained only the core sequence for IHF binding, but it lacked the A/T-rich DNA sequence necessary for efficient binding of *E. coli* IHF to DNA. Unlike pRA1, the plasmids pRA1-7 and pRA1-12 contained the potential 5-bp-long A/T-rich sequence six nucleotides upstream from the core sequence of IHF binding. Both constructs exhibited three to fourfold higher reporter gene expression in the wild type than in the *ihfA*$^-$ mutants that was not seen in the case of pRA1. Additionally, the gel shift experiments with *P. putida* crude cell extracts supported the idea that the A/T-rich tract upstream from the core

sequence was essential for the binding of *P. putida* IHF to the right end of Tn*4652* (Fig. 4 and 5 in Ref. I). Therefore, the role of IHF on the modulation of transcription from PRA1 became obvious.

2.2.3. Another protein besides IHF is involved in the transcription regulation from the fusion promoter PRA1

Another DNA-protein complex that moved faster than the IHF-DNA complex was detected in gel shift assay by using a DNA fragment containing the right end of Tn*4652* (Fig. 7 in Ref. I). This protein-DNA complex was formed with crude cell lysates prepared from the exponentially growing *P. putida* cells and it disappeared when the bacterial growth rate started to decelerate (Fig. 7 in Ref. I). In addition, same effect of this complex was seen with the crude cell lysates of *ihfA*-defective *P. putida* (unpublished data). Thus, the transcription from PRA1 might be regulated more complexly than firstly assumed. An interesting phenomenon appeared when the sequence of Tn*4652* right end was shortened upstream from the PRA1 promoter. The plasmid pRA1-0 (Fig. 6) contained only 33 nucleotides from the right end of the transposon and the core sequence for IHF binding was completely deleted in this construct. However, this plasmid expressed 2.5–3 times higher reporter gene activity than pRA1 in the wild type *P. putida* (Fig. 7).

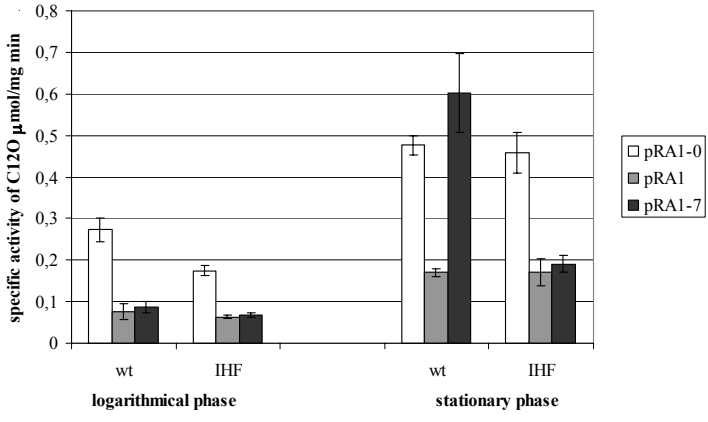

Figure 7. Study of the effects of upstream sequences and IHF on transcription from the fusion promoter PRA1 by comparison of the specific activity of C12O in the wild type *P. putida* (wt) and its *ihfA* knockout derivate A8759 (IHF). C12O specific activity was measured in exponentially growing and stationary phase cells of *P. putida*. Data (means ± standard deviations) of at least three independent experiments are presented.

The enhancing effect appeared already in the exponentially growing cells and as expected, because the sequence for IHF binding was missing, the increased expression level was seen in the *ihfA*-defective strain A8759. At the same time, the dependency from the growth rate of bacteria remained. The level of transcription from PRA1 in pRA1-0 was approximately 1.5-times higher in stationary-phase cells than in exponentially growing cells (Fig. 7). This enhancement was connected with the deletion of 24 nucleotides of the Tn*4652* right end DNA upstream from the –35 hexamer of PRA1 in the plasmid pRA1. This indicates that we have most likely deleted at least partially a DNA sequence, which is necessary for binding of another protein that may act as a repressor. In exponentially growing cells where the concentration of the IHF is relatively low, the influence of hypothetical repressor prevails and transcription from PRA1 is reduced. When the growth of *P. putida* decelerates and the amount of IHF increases, the binding of hypothetical repressor is avoided. The finding that the expression level of the reporter gene from the PRA1 promoter was comparable in the stationary phase of *P. putida* wild type cells that harboured either the construct pRA1-0 or pRA1-7 (Fig. 7) supported this idea. At the same time, pRA1-0 lacked influence of both regulators, whereas, the pRA1-7 needed binding of IHF for full level of transcription from PRA1.

The possibility that some protein that is encoded by Tn*4652* (e.g., Tn*4652*-encoded transposase) binds to ends of the transposon and affects negatively the transcription from the fusion promoters was excluded. Firstly, the expression ratios of pRA1-0, pRA1 and pRA1-7 were the same in the *P. putida* strain A3.12 that is Tn*4652* free (unpublished data). Secondly, it was shown that the transposase of Tn*4652* binds to the ends of the transposon only in the presence of IHF (Ref. III) but both constructs pRA1-0 and pRA1 lack the functional IHF binding site.

2.3. The regulation of transcription from the fusion promoter PLA1

2.3.1. The upstream region from PLA1 contains only one site for IHF binding

Two putative flanking sequences for the binding of IHF upstream from the PLA1 promoter were predicted (Hõrak and Kivisaar, 1999; Fig. 1 and 2 in Ref. I). The first one, ATACAATAGCTTA, located at 44 to 56-bp inside the left end of the transposon (Fig. 6). The second one, AATCTACTAGTTT, located at positions 59 to 71 bp inside the left end (Fig. 6). These sequences differed from *E. coli* core consensus WATCAA N_4 TTR (where W can be A or T and R can be A or G) by two nucleotides but in different positions. Although the DNA sequence preceding the IHF binding core sequences at the left end of Tn*4652*

was not A/T-rich, the longer DNA region than the core sequence was still needed for the binding of IHF *in vitro* (Fig. 5 in Ref. I). Only the usage of DNA fragment that contained transposon DNA up to 99 nucleotides inside the left end (pLA1-4 in Fig. 6) enabled to detect the IHF-DNA complex from crude cell lysates of *P. putida* wild-type strain. Initially, it was supposed that this fragment contains two hypothetical sequences for IHF binding (Hõrak and Kivisaar, 1999), but the results of the next studies (Ref. III) did not support this idea.

The DNase I footprint method was used to determine the possible IHF binding sequence at the ends of Tn*4652*. IHF clearly protected DNA region between nucleotides 34 to 76 bp inside the both ends of Tn*4652* (Fig. 4 and 5 in Ref. III). As *P. putida* IHF protected 42 nt at the Tn*4652* ends and *E. coli* IHF needs more than 35 nucleotides for the site-specific connection with DNA (Yang, 1989), the possibility of IHF binding to the second potential site overlapping the first one at the left end of Tn*4652* was raised. Even increasing of the IHF concentration up to 100 μM in the reaction mixture did not reveal more DNA protection at the left end of Tn*4652* similarly to the right end of Tn*4652* that contains only one site for IHF binding (Fig. 4 in Ref III). Therefore, the possibility that IHF has two binding sites at the left end of Tn*4652* was excluded.

2.3.2. IHF affects transcription from the fusion promoter PLA1 positively

Although the transcription-enhancing effect by IHF was seen for PRA1 promoter, the transcription regulation from PLA1 promoter appeared to be more complex. First, it should be admitted that IHF had about 1.5-fold positive effect on transcription from PLA1, too. However, the connection between the binding of IHF to the left end of Tn*4652* DNA and transcription enhancing effect of IHF from PLA1 promoter was not as clear as it was in the case of PRA1. Surprisingly, the absence of A/T-rich track upstream from the core sequence of IHF binding site at the Tn*4652* left end did not affect the transcription from PLA1. Even more, the highest expression of the reporter from PLA1 promoter was assured by the plasmid pLA1 where IHF was not able to bind. The plasmid pLA1 contains 58 nucleotides from the left end of Tn*4652* where is presented only the core sequence for IHF binding but not the A/T-rich tract (Fig. 6).

Although the over-expression of *P. putida*'s IHF did not enable to detect the protein-DNA complex formation *in vitro*, the weak binding of *E. coli* IHF to the same DNA region was seen (Fig. 5 in Ref. I). Considering the fact that *E. coli* produces approximately four to six times more IHF than *P. putida* (Ditto *et al*., 1994; Valls *et al*., 2002) one can speculate, that the concentration of IHF in *P. putida* crude lysates was too low for detection of IHF binding to incomplete DNA sequence. However, two other explanations could be considered as well:

the possible differences of IHF binding affinities in *P. putida* and *E. coli*; and the possibility that IHF of *P. putida* and *E. coli* may recognise a little bit different DNA sequences. Although the *pheB* gene expression was approximately 1.5 times higher in the stationary phase wild-type cells than in *ihfA*$^{-}$ strain containing pLA1, the over-expression of the *P. putida* IHF in *ihfA*$^{-}$ strain demonstrated that increasing of IHF concentration did not enhance the level of transcription from PLA1 (Fig. 6D in Ref. I). Therefore, the possibility that the cellular amount of IHF was too low to regulate transcription from PLA1 promoter is not plausible.

The binding of IHF upstream from the promoter PLA1 did not ensure the expected enhancing effect of transcription (compare the constructs pLA1 and pLA1-4 in Fig. 3C in Ref. I). Even more, extending the transposon left end by 48 nucleotides upstream from the PLA1 promoter (pLA1-5 in Fig. 6) resulted in three times lower level of transcription from PLA1 in the stationary phase cells of *P. putida ihfA*$^{-}$ strain than in the wild-type strain (Fig. 3C in Ref. I). On the other hand, if the transcription from PLA1 was modulated only by the presence of IHF, then it could be remained unchanged in the IHF-deficient strain despite the length of left-end DNA of Tn*4652* was present upstream of the PLA1 promoter. Moreover, the extending of the lengths of left end DNA reduced the expression of the reporter gene under the control of PLA1 in the exponentially growing cells of *P. putida* IHF-deficient strain A8759 as well. Apart from this, the disability of pLA1 to bind IHF ensured the higher level of transcription from PLA1 in *P. putida* cells than in the constructs with intact IHF binding sequence. The simplest explanation could be that IHF represses the transcription from PLA1 promoter, but it conflicts with data obtained in the IHF-defective strain of *P. putida* (Fig. 3C in Ref. I).

It looks probable that, the higher level of transcription from pLA1 was caused by disability of a potential repressor to bind to the region overlapping with the IHF binding site. This hypothesis is supported by the fact that the over-expression of IHF resulted in increased level of transcription from PLA1 in the stationary phase cells of *P. putida* that harboured the plasmid pLA1-12 (Fig. 6D in Ref. I) and exceeded the level that was measured from the cells with pLA1. At the same time, the levels of expression of C12O specific activity were insensitive to the over-expression of IHF when the plasmid pLA1 was used. Even more, the higher amount of specific IHF complex with Tn*4652* left end was seen in gel shift assay when IHF was over-expressed (Fig. 4 and 5 in Ref. I). Consequently, if IHF competes for binding with the hypothetical repressor then connection between the amount of IHF and increased expression of the reporter gene *pheB* from PLA1 promoter can be seen in pLA1-12. If presume that IHF increases the transcription from PLA1 in stationary phase cells when the cellular amount of IHF is higher then the lower level of transcription in logarithmically growing *P. putida* cells that harboured pLA1-4, pLA1-5 or pLA1-12 compared to pLA1 may be caused by the higher amounts of repressor and the lower amount of IHF in these cells. As both proteins compete for the

binding to the same region, the transcription rate from PLA1 depends on the cellular amounts of both proteins.

The concentration of IHF changes with growth rate of bacteria and different affinity of IHF to its different binding sites is known (Murtin *et al*., 1998; Valls *et al*., 2002). The sequences with good matches to the consensus have higher affinity, but even these sites are not completely occupied by IHF when *E. coli* cells are logarithmically growing, let alone the sequences with medium or even weak affinity for IHF (Murtin *et al*., 1998). The concentration of IHF changes in *P. putida* as well as in *E. coli* and it is the lowest in exponentially growing cells (Ditto *et al*., 1994; Valls *et al*., 2002). Possibly, the 1.5-fold higher *pheB* expression in logarithmically growing pLA1-containing *P. putida* was caused by the absence of entire binding site for the hypothetical repressor at the left end of Tn*4652*. On the other hand, the increasing concentration of IHF could inhibit binding of this protein, too. Although, the over-expression of IHF in logarithmically growing *P. putida* that harboured pLA1-12 did not assure the expression of *pheB* at same level as pLA1 did, the positive effect of increased amount of IHF was shown (Fig. 6 C in Ref. I).

It is possible that the higher concentration of IHF was needed for the elimination of negative effect of the presence of upstream Tn*4652* left end sequence on transcription from PLA1 in logarithmically growing cells than in stationary phase cells. While the 0.01 to 0.1 mM concentration of inducer molecule IPTG ensured sufficient amount of IHF for enhancement of transcription in stationary phase cells of *P. putida* this concentration of the IPTG was insufficient for exponentially growing cells. Only the amount of IHF obtained in the presence of 1 mM IPTG resulted in twofold higher expression in pLA1-12 compared to the absence of IPTG (Fig. 6C in Ref. I). In conclusion, the transcription from the fusion promoter PLA1 is complexly regulated and in addition to IHF, there are at least one more factor involved in this regulation.

2.4. The level of transcription from PRA1 and PLA1 depends on the growth rate of *P. putida*

The study of the fusion promoters activation revealed that the transcription from these promoters was growth phase dependent (Ref I and II). When we measured the specific activity of C12O from *P. putida* cells controlled by the fusion promoters PRA1 or PLA1, it become obvious that in addition to the length of Tn*4652* sequence present upstream of the fusion promoters, the growth phase of bacteria also affected remarkably the level of transcription from these promoters (Fig. 3 in Ref. I). There was no difference in the specific activity of C12O between logarithmically growing *P. putida* wild-type cells carrying the pRA1, pRA1-7 or pRA1-12 plasmids. The high expression of PRA1 on the plasmids pRA1-7 and pRA1-12 became evident only in the case when bacteria reached into stationary phase. The expression of the reporter gene *pheB* on these plasmids was

exceeded three to four times the value that was measured in the logarithmically growing cells or in the stationary phase cells with the pRA1 plasmid.

The growth phase dependency was also seen in the case of the fusion promoter PLA1. The highest specific activity of C12O was measured in the stationary phase cells of *P. putida*, but in contrary to the Tn*4652* right end sequences-carrying PRA1, the further upstream sequences of Tn*4652* left end had the negative effect on transcription from PLA1. At the same time, the level of transcription from PLA1 was up to four times higher in the stationary phase cells than in the logarithmically growing *P. putida* wild-type cells irrespective of the PLA1 construct they harboured (Fig. 3 in Ref. I).

2.4.1. The transcription from certain fusion promoters depends on σ^S

The involvement of IHF in the regulation of transcription from the fusion promoters PRA1 and PLA1 and the fact that the cellular concentration of IHF depends on the stage of growth phase of *P. putida* raised the question whether the modulation of transcription from the fusion promoters by IHF could be the only reason for the higher level of the *pheB* expression in stationary phase. Definitely, it is not explicable only by changes in the concentration of IHF, because the level of transcription from the fusion promoters increased also in the IHF-deficient strain A8759 of *P. putida.* The experimental data showed more than two times higher levels of transcription from these fusion promoters in the stationary phase cells of A8759 compared to the exponentially growing cells (Fig. 3 in Ref. I). This inspired us to examine the effect of stationary phase sigma factor σ^S on transcription regulation from the fusion promoters. The sigma factor σ^S is involved in the changes of expression pattern of many genes when the bacterial growth speed is decelerating (Mulvey and Loewen, 1989; Nguyen *et al*., 1993; Tanaka *et al*., 1993). Because the C12O-expressing reporter system was quite insensitive, we decided to clone the fusion promoters upstream of the *lacZ* reporter gene. To eliminate the effect of IHF the promoter constructs lacking full IHF binding sites at Tn*4652* ends were used. The specific activity of β-Gal was measured until the late stationary phase cells of *P. putida* wild type and σ^S-deficient strain.

Surprisingly, the presence of σ^S affected positively transcription from PLA1 but not from PRA1 (Table 2 in Ref. II). In the case of PLA1, the dependency from σ^S was seen already in 12 hours-grown *P. putida*. However, in the late stationary phase – 36 hours after the inoculation, the differences in specific activities measured in the wild type and σ^S deficient bacteria appeared to be threefold in favour of the wild type. The next question arose, whether the fusion promoter PLA1 that contains the Tn*4652* left-end DNA could be specifically σ^S-dependent.

The generation of σ^S-dependent promoters was not connected with DNA sequences locating in the left end of Tn*4652*. Since only this fusion promoter contained the left end of the transposon – one may ask an opposite, whether all the fusion promoters based on the Tn*4652* right end are σ^S-independent. We found that in addition to PLA1, PRA4 and PRA7 showed also dependency from σ^S. 2.5 times and 4.8 times lower β-Gal specific activity, respectively, were detected in the σ^S-deficient *P. putida* strain compared to that in the wild type strain (Table 2 in Ref. II). From this, we hypothesised that the –10 element of the different fusion promoters originated from the target DNA may determine whether Eσ^S preferentially recognises the promoter or not.

2.4.2. The σ^S-dependency of the fusion promoters is complex

Because the σ^S-dependency of the fusion promoters was not determined by the transposon DNA, we decided to concentrate on the analysis of the target sequences. Despite the conflicting ideas about what makes promoters σ^S-dependent, the CTATACT consensus of –10 element is proposed for the σ^S-dependent promoters both in enterobacteria and in pseudomonads (Schuster *et al*., 2004; Weber *et al*., 2005). As both ends of the transposon Tn*4652* are terminated with four C residues, all fusion promoters contain the first C nucleotide of the extended –10 element of σ^S-dependent promoter. Consequently, the specificity of fusion promoters is determined only by target sequences. Although in *E. coli* and *P. aeruginosa* the sixth nucleotide of the extended –10-consensus sequence of σ^S-dependent promoters is reported to be C and the A nucleotide is conserved in the same position among the σ^{70}-dependent promoters, it is not so obvious for promoters in *P. putida* (Lisser and Margalit, 1993; Schuster *et al*., 2004; Dominguez-Cuevas, 2004; Weber *et al*., 2005). Approximately 50 genes in *P. putida* are known to be σ^S-dependent under C-limitation conditions (Ramos-Gonzalez and Molin, 1998), but the consensus sequence for σ^S-dependent promoter in this bacterium have not been offered yet. Additionally, this position of –10-consensus of σ^{70}-dependent promoter in *P. putida* is not very conserved and can contain C as well as the A nucleotide. (Dominguez-Cuevas, 2004). Therefore, it seems that –10 consensus for σ^{70} and σ^S-dependent promoters is more flexible in *P. putida* than in *E. coli* and *P. aeruginosa*, and the C nucleotide in the sixth position of the extended –10 element is not so important for distinguishing between stationary phase sigma factor σ^S-specific promoters and σ^{70}-dependent promoters. We found three σ^S-dependent fusion promoters PLA1, PRA4 and PRA7, but only two of them had the above-mentioned C in the "right" position. Specifically, the –10 element of PLA1 was CTATA**C**T and the –10 hexamer of PRA4 was CTAAA**C**T. At the same time, the –10 element of PRA7 was CTATA**A**T and

contained A in sixth position like all other fusion promoters PRA1, PRA2 and PRA3 that expressed independently from σ^S.

To examine the role of –10 sequences on the σ^S-dependency, we changed the –10 element of PLA1 with sequences of the –10 elements of the other fusion promoters in this way that all hybrid promoters contained the same downstream sequence, but had different –10 elements. We chose PLA1 (TATACT) because it had –10 element identical to the –10 element of *fic* promoter, which was reported as a specific σ^S-dependent reference promoter in *E. coli* (Tanaka *et al*., 1993; Utsumi *et al*., 1993). We measured the specific activity of β-Gal in the wild type and the σ^S-deficient *P. putida* cells that harboured the hybrid promoters upstream of the *lacZ* reporter.

Unfortunately, we did not get clear evidence that the sequence of –10 element could influence the σ^S-specificity of the fusion promoter. When we substituted the –10 element TATACT of PLA1 with the –10 element sequences of the different fusion promoters that expressed independently from σ^S, we observed different level of expression of the reporter gene. The substitution of –10 element of PLA1 with the sequences of –10 elements of PRA1 or PRA3 retained the influence of σ^S for the transcription from the hybrid promoters. However, the replacement of this sequence with the –10 element of PRA2 did not have such effect. The hybrid promoters whose –10 elements came from fusion promoters that had originally σ^S dependency also behaved differently. The –10 element from PRA4 retained σ^S dependency for the hybrid promoter but the PLA1-PRA7 hybrid promoter was only mildly, if at all, affected by σ^S (Table 3 in Ref. II).

When we substituted the –10 element of PRA1, PRA4 and PRA7 with –10 element of PLA1, the σ^S dependency retained in the case of PRA4-PLA1 or PRA7-PLA1 hybrid promoters, but not PRA1-PLA1 hybrid promoter (Table 3 in Ref. II). Therefore, it is not possible to say that the σ^S dependency of the fusion promoters is determined only by –10 element. Rather, the DNA sequence locating downstream from –10 element may also determine σ^S-specificity. Thus, the unique sequence of the –10 element in concert with the sequence downstream from –10 element could influence σ^S-dependent transcription from the fusion promoters.

Usually, the dependency of transcription from growth rate and the presence of ppGpp are connected to each other (van Delden *et al*., 2001; Hirsch and Elliott, 2002; Venturi, 2003). The promoters that are under the repression of ppGpp usually have a discriminator region (G/C-rich tract) between the –10 hexamer and transcription start point (Travers, 1984), but if the –10 element of a promoter is followed by A/T-rich DNA, the transcription from the σ^S-dependent promoters is facilitated (Mooney *et al*., 1998; Hsu, 2002; Peter *et al*., 2004). However, the fusion promoters characterised by us did not contain distinctive discriminator region downstream from the –10 element or the A/T-rich sequence that could facilitate transcription initiation. Thereby every fusion promoter has to be observed individually.

CONCLUSIONS

The formation of fusion promoters upstream of silent genes by transposition is quite common among bacteria, especially under stressful conditions when genes that have been obtained by horizontal gene transfer but are not expressed at needed level are activated. During starvation of *P. putida* on phenol minimal plates, the fusion promoters were created by transposition of Tn*4652* into upstream region of the promoterless *pheBA* operon allowing *P. putida* to grow on phenol. The fusion promoters were composed from the –35 hexamer sequence derived from the Tn*4652* inverted repeats and the –10 hexamer derived from the target DNA sequence. In my thesis, I investigated the mechanism of transcriptional activation from these fusion promoters. The results can be summarised as follows:

1. The rate of transcription from the fusion promoters depends on the growth rate of *P. putida*, being highest in stationary phase cells.
2. The transcription from the fusion promoters is complex. The proteins that bind to the ends of the transposon and thereby control the transposition of Tn*4652* control also transcription from these promoters.
3. Both ends of the transposon Tn*4652* contain one site for the IHF binding. The core sequences for IHF binding locate at a position 44 to 56 bp inside both ends of the transposon. An additional region upstream of the core sequence is essential for the binding of IHF, whereas lack of this region precludes the binding of IHF.
4. IHF affects the transcription from the fusion promoters positively; however, its positive effect appears only in the stationary phase cells.
5. In addition to IHF the transcription from the fusion promoters was affected also by other protein(s). This effect was seen in the logarithmically growing cells.
6. The fusion promoters studied differ from each other by their –10 hexameric sequences and sequences downstream from that. Three fusion promoters PLA1, PRA4 and PRA7 are σ^S-dependent, whereas PRA1, PRA2, and PRA3 are not.
7. The –10 hexameric sequences and further downstream sequences of fusion promoters determine their transcription specificity, including σ^S-dependence.

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SUMMARY IN ESTONIAN

Tn*4652* transponeerumisel *pheBA* operoni ette tekkinud liitpromootoritelt lähtuva transkriptsiooni regulatsioon *Pseudomonas putida*'s

Bakter hakkab tavaliselt esmase süsinikallika ammendumisel tarvitama alternatiivseid C-allikaid. Selleks piisab vajalike geenide ekpresseerimisest ja/või ebaoluliste vaigistamisest. Mõnikord aga pole see võimalik, sest hädavajalikke geene ei suudeta ekspresseerida kas siis transkriptsiooniks vajaliku promootori puudumise või liiga tugeva geenikspressiooni mahasurutuse tõttu. Sellised geenid võivad siiski aktiveeruda tänu vajalike mutatsioonide tekkele DNA "õiges" piirkonnas. Tihti sisenevad mobiilsed DNA-elemendid promootorita geeni(de) ette ning tagavad transkriptsiooni, kas siis kaasa toodud promootori või uue hübriidse liitpromootori moodustamise abil. Viimasel juhul on töötava promootori moodustumiseks vajalik transponeeruva elemendi täpne sisenemine –10 heksameeri ette. Selle heaks näiteks on transposoon Tn*4652*, mis aktiveeris pormootorita *pheBA* operoni *Pseudomonas putida* rakkudes, mis tagas näljas olnud bakterile fenooli omastamise võime. Vajalikud geenid aktiveeriti tänu liitpromootoritele, mis tekkisid transposooni õlas oleva –35 heksameeri ja märklaudjärjestuse baasil *pheBA* operoni ette (Nurk jt., 1993). Kuna tekkinud liitpromootorid ei taganud võrdsel määral geenide transkriptsiooni, siis tekkis küsimus, kuidas kontrollitakse tekkinud liitpromootoritelt transkriptsiooni.

Käesolevas töös on põhjalikumalt uuritud liitpromootoreid PRA1 ja PLA1. Esimene neist on valitud transposooni parema õla baasil tekkinud liitpromootori näiteks ning teine vasaku õla baasil tekkinud liitpromootori näiteks.

Kuna liitpromootorid sisaldasid Tn*4652* DNAd, siis oli mõistlik oletada, et need faktorid, mis mõjutavad selle transposooni transponeerumist, võivad mõjutada ka liitpromootoritelt lähtuvat transkriptsiooni. Teada oli, et Tn*4652* mõlemad õlad sisaldavad potentsiaalset IHF (inglise keeles, *integration host factor*) seondumisjärjestust (Hõrak ja Kivisaar, 1999). See on väike globaalne transkriptisooniregulaator, mis osaleb peale transkriptiooni reguleerimise ka paljudes teistes protsessides nagu transpositsioonis, replikatsioonis, faag λ pakkimisel jm (Friedman, 1988). Seetõttu on käesolevas doktoritöös uuritud, kuidas *P. putida* IHF mõjutab liitpromootoritelt lähtuvat transkriptsiooni. Veel on uuritud liitpromootorite sõltuvust statsionaarse faasi spetsiifilisest sigma faktorist σ^S, mis on vajalik stressitingimustes vajaminevate geenide promootorite äratundmiseks (Mulvey ja Loewen, 1989).

Käesoleva doktoritöö tulemused võib kokku võtta järgmiselt:

1. moodustunud liitpromootoritelt lähtuv transkriptsioon sõltub *P. putida* kultuuri kasvufaasist, olles kõrgem statsionaarse faasi rakkudes.
2. Transkriptsioon liitpromootoritelt on kompleksselt reguleeritud samade valkude poolt, mis osalevad ka Tn*4652* transponeerumises.
3. Mõlemad Tn*4652* õlad sisaldavad ühte IHF seondumisjärjestust, mille põhijärjestus asub positsioonis 44 kuni 56 nukleotiidi sissepoole vastavast õlast, kuid IHF seondumiseks on vajalik pikem DNA lõik transposooni õlas.
4. IHF mõjutab liitpromootoritelt lähtuvat transkriptsiooni positiivselt ning selle valgu mõju *P. putida* rakkudes ilmneb alles statsionaarses kasvufaasis.
5. Liitpromootoritelt lähtuv transkriptsioon on reguleeritud peale IHF ka mõne teise valgu poolt. Selle valgu mõju on näha logaritmiliselt kasvavates rakkudes.
6. Kuuest uuritud liitpromootorist kolmel (PLA1, PRA4, PRA7) oli transkriptioon σ^S-sõltuv, samas aga liitpromootoritelt PRA1, PRA2 ja PRA3 lähtuv transkriptisoon ei sõltunud sigma faktorist σ^S.
7. Liitpromootorite σ^S-sõltuvus on kompleksselt määratud ning ei ole põhjustatud ainult –10 heksameeri järjestusest vaid ilmneb –10 heksameeri ja sellele järgneva ala koosmõju toimel.

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PUBLICATIONS

Teras, R., Hõrak, R. and Kivisaar, M. 2000. Transcription from fusion promoters generated during transposition of transposon Tn*4652* is positively affected by integration host factor in *Pseudomonas putida*. J. Bacteriol. **182**, 589–598.

Ojangu, EL., Tover, A., Teras, R. and Kivisaar, M. 2000. Effects of combination of different –10 hexamers and downstream sequences on stationary-phase-specific sigma factor σ^S-dependent transcription in *Pseudomonas putida*. J. Bacteriol. **182**, 6707–6713.

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.

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List of Publications

1. **Ilves, H., R. Horak, R. Teras, M. Kivisaar.** 2004. "IHF is the limiting host factor in transposition of *Pseudomonas putida* transposon Tn*4652* in stationary phase" Mol Microbiol. (6):1773–85.
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Pseudomonas sp. strain ADP: effects of toxicity and adaptation" Appl Environ Microbiol. (4):1907–12.

3. **Ojangu, O., A. Tover, R. Teras, M. Kivisaar.** 2000. "Effects of combination of different –10 hexamers and downstream sequences on σ^S-dependent transcription instationary-phae-specific sigma factor *Pseudomonas putida*" J. Bacteriol. 182 (23):6707–6713.
4. **Teras, R., R. Hõrak, M. Kivisaar. 2000.** "Transcription from the fusion promoters generated at transposition of the transposon Tn*4652* is positively affected by integration host factor in *Pseudomonas putida*" J. Bacteriol. 182 (3):589–98.

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Teaduslik töö

Minu teadustöö teemadeks on Tn*4652* järjestust sisaldavatelt liitpromootoritelt lähtuva transkriptsiooni regulatsioon DNA topoloogiat mõjutavad valgud *Pseudomonas putidas*.

Publikatsioonide nimekiri

1. **Ilves, H., R. Horak, R. Teras, M. Kivisaar.** 2004. "IHF is the limiting host factor in transposition of *Pseudomonas putida* transposon Tn*4652* in stationary phase" Mol Microbiol. (6):1773–85.

2. **Neumann, G., R. Teras, L. Monson, M. Kivisaar, F. Schauer, HJ. Heipieper.** 2004. "Simultaneous degradation of atrazine and phenol by *Pseudomonas* sp. strain ADP: effects of toxicity and adaptation" Appl Environ Microbiol. (4):1907–12.
3. **Ojangu, O., A. Tover, R. Teras, M. Kivisaar.** 2000. "Effects of combination of different –10 hexamers and downstream sequences on σ^S-dependent transcription in stationary-phae-specific sigma factor *Pseudomonas putida*" J. Bacteriol. 182 (23):6707–6713.
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