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NATURAL HORIZONTAL TRANSFER OF THE *pheBA* OPERON

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original papers which will be referred to by their Roman numerals in the text.

I Peters, M., Heinaru, E., Talpsep, E., Wand, H., Stottmeister, U., Heinaru, A., and Nurk, A. (1997). Acquisition of a deliberately introduced phenol degradation operon, *pheBA,* by different indigenous *Pseudomonas* species. Appl. Environ. Microbiol. 63: 4899–4906.

II Peters, M., Heinaru, A., and Nurk, A. (2001). Plasmid-encoded catalase KatA, the main catalase of *Pseudomonas fluorescens* strain Cb36. FEMS Microbiol. Letters 200: 235–240.

III Peters, M., Jõgi, E., Suitso, I., Punnisk, T., and Nurk, A. (2001). Features of the replicon of plasmid pAM10.6 of *Pseudomonas fluorescens.* Plasmid 46: 25–36.

IV Peters, M., Tomikas, A., and Nurk, A. (2004). Adjustment of the horizontally transferred *pheBA* operon in the genomes of eight indigenous *Pseudomonas* strains. (manuscript)

ABBREVIATIONS

aa	amino acid
bp	base pair(s)
2,4-D	2,4-dichlorophenoxyacetic acid
h	hour(s)
HGT	horizontal gene transfer
IS	insertion sequence element
kb	kilo base pairs
Km	kanamycin
nt	nucleotide
ORF	open reading frame
PCR	polymerase chain reaction
PMO	phenol monooxygenase
3-POB	3-phenoxybenzoic acid
rep-PCR	repetitive extragenic palindromic sequences visualization based on polymerase chain reaction

INTRODUCTION

Horizontal gene transfer (HGT) is defined as the exchange of genetic material between bacteria other than by descent in contrast to vertical gene transfer in which information travels through the generations. During bacterial evolution, the ability of bacteria to adapt to new environments often results from the acquisition of new genes through horizontal transfer rather than in the modification of gene functions by accumulation of point mutations (reviewed in Lawrence and Hendrickson, 2003). The first reports about HGT between bacteria in natural or semi-natural habitats were published in the 1970s (Graham and Istock 1978; 1979). Today, gene transfer among microorganisms has been well demonstrated in laboratory microcosms and also in field conditions.

The increased use of chemicals in industry and agricultural techniques during last decades had lead to spread of large amounts of chemical waste in natural environments. The process of bioaugmentation using bacteria with appropriate biodegradative abilities is one of the possible mechanisms to reduce produced pollution in situ. Released foreign organisms usually do not survive in the environment for a long period. Nevertheless, the transfer of biodegradative genes from the used laboratory strains to indigenous organisms can occur under selective pressure. In this case, the genes which are necessary for successful degradation of pollutants can persist and spread in the environment, thus allowing natural microbes to adapt to the changed conditions.

The objective of the present work was to characterize the fate of the *pheBA* operon released into the environment.

1. REVIEW OF LITERATURE

1.1 Horizontal gene transfer in nature

Horizontal transfer of the genes could be detected in soil, waste-, fresh-, and seawater, in sediments and also in respiratory and digestive tracts (Goodman *et al*., 1994). In bacteria, HGT is believed to be responsible for the wide distribution of antibiotic resistance genes, genes encoding enzymes involved in biodegradation pathways and pathogenity determinants. In the case of biodegradation pathways the role of selective pressure is similar to those of antibiotic resistance but the genetic systems are usually more complex consisting from operons of genes and regulatory systems (de la Cruz and Davies, 2000).

1.2. What types of genes are susceptible to horizontal transfer?

The distinct genes have different potential to be horizontally transferred. For successful HGT the acquired genes must persist in host chromosome or be maintained autonomously. Additionally, these genes must provide a selective benefit to the recipient. Essential genes, present in all organisms, like rRNA genes, are therefore less likely objects of HGT (Lawrence, 1999).

According to the Jain *et al*. (1999), genes participating in transcription and translation processes are seldom transferred because they are members of large, complex systems, products of which are less likely function successfully in a foreign cytoplasm. On the contrary, the frequent horizontal transfer of the antibiotic resistance genes and genes participating in degradation of environmental pollutants is observed. These compounds create a strong selective pressure for the development of bacterial strains with novel or modified biodegradative capabilities. The genetic information encoding these metabolic activities is often found in mobile elements (plasmids, transposons, integrons) making possible their spreading through HGT (Top *et al*., 2002, Tsuda *et al*., 1999).

1.3. Genetic interactions among bacteria

Genetic interactions among bacteria are mediated by one of the three distinct gene-transfer mechanisms: conjugation, transformation and transduction. Gene transfer has been detected not only in laboratory conditions, but more importantly, in field studies. The later provides direct evidence that all three gene transfer mechanisms occur in nature. In some cases DNA transfer frequencies observed in the environment differed considerably from those obtained under

laboratory conditions (reviewed in Dröge *et al*., 1999). These studies have identified several natural habitats of bacteria as "hot spots" of gene transfer among bacteria such as the rhizosphere (van Elsas *et al*., 1988; Dröge *et al*., 1998) and the gut of some soil organisms (Hoffmann *et al*., 1998) where nutrient availability supports locally high bacterial cell densities and positively influences metabolic activities. Recent findings also show that gene transfer via conjugation and transformation occurs frequently within biofilms (Björklöf *et al*., 2000, Springael *et al*., 2002; Hendrickx *et al*., 2003). Moreover, conjugation mechanism may stimulate biofilm development (Ghigo, 2001).

Horizontal transfer of genetic material between different bacteria has been detected in a wide variety of different bacterial species and genera. The transfer of part of the Ti plasmid DNA from *Agrobacterium tumefaciens* to plants (Zupan and Zambryski, 1995) and to yeast (Bundock *et al*., 1995) demonstrates the horizontal transfer of genes between different phylogenetic kingdoms. Also, under laboratory conditions, a kanamycin resistance gene integrated in the DNA of transgenic plant could transform *Acinetobacter* sp. to Km^r (Gebhard and Smalla, 1998).

1.3.1. Transformation

In natural transformation, competent bacteria pick up extracellular DNA and incorporate it into their genome. Bacteria are the only organisms capable for natural transformation. Studies have shown that the process of gene transfer by free DNA depends on several requirements. These are a) presence and persistence of free DNA in the environment b) development of competent cells c) interactions of cells with DNA and the uptake of DNA and d) stable maintenance and expression of the acquired genes (Lorenz and Wackernagel, 1994).

1.3.1.1. DNA in nature

The extracellular DNA in the environment is derived from the lysis of cells following the death of host organisms (plants, animals and microorganisms) or as a result of the active excretion of plasmid or chromosomal DNA by some microorganisms (Ogram *et al*., 1987, rev. in Paget and Simonet, 1994; Steinmoen *et al.*, 2002). Usually the extracellular DNA is rapidly degraded, but some portion of DNA escapes the enzymatic activity of the DNases present in the soil (Lorenz and Wackernagel, 1994). This protection would be related to an adsorption of DNA molecules onto clay or sand particles (Paget *et al*., 1992). Clay minerals are highly reactive: one gram of pure montmorillonite, for example, is able to absorb up to 30 mg of DNA (Paget *et al*., 1992). Divalent cations and a low pH facilitate adsorption, and several studies have

demonstrated that adsorbed DNA is still available to transform bacterial cells (Khanna and Stotzky, 1992; Paget *et al*., 1992). Studies of different habitats indicated that free DNA is usually more rapidly degraded in aquatic than in terrestrial environments. Romanowski with coworkers (1992, 1993) demonstrated that DNA seeded into soil was rapidly degraded but the traces of DNA were still detectable after more than 2 months after inoculation. In phosphorous limited seawater added DNA was hydrolyzed more rapidly (10 times) than in non phosphorous-limited seawater (Turk, 1992). DNA inoculated into oligotrophic marine surface water was nearly completely degraded after less than 12 h of inoculation (Paul, 1987); whereas in marine sediments half-lives of DNA molecules were approximately 140 h (Maeda and Taga, 1974).

1.3.1.2. Competent bacteria

Natural transformation has been identified in more than 40 species of bacteria belonging to either Gram-positive or the Gram-negative bacterial groups, as well as some archae (Lorenz and Wackernagel, 1994). However, the large number of viable but uncultivable bacteria, compared to the cultivable bacterial species tested for competence, suggests that this phenomenon may be more wide-spread than has been previously assumed. Competent bacteria have been isolated from a variety of environments, including water, soil and sediment. Some bacteria are permanently competent (e.g., *Neisseria gonorrhoeae*) (Lorenz and Wackernagel, 1994) and some (e.g., *Acinetobacter calcoaceticus*) become competent during their life cycle (Palmen *et al*., 1993). In *Streptococcus pneumoniae*, the competent state is induced by a peptide pheromone through a quorum-sensing mechanism (Håvarstein *et al*., 1995). Some experiments indicate that the development of competence is dependent from limitation of nutrients and microelements in growth medium (Lorenz and Wackernagel, 1994). Naturally competent *Pseudomonas stutzeri* needs the presence of specific nutrients and nitrogen and phosphate deficiencies were required for competence development (Lorenz and Wackernagel, 1991). Competence development in *Azotobacter vinelandii* requires an optimum concentration of Ca^{2+} (0.5 to 1 mM) and neutral pH (Page and Doran, 1981).

Little is known about what portion of bacteria of a given community are naturally transformable. Cultivable heterotrophic bacteria of various marine environments were tested for their ability to take up DNA in filter transformation assays (Frischer *et al*., 1994). Three out of 30 and 15 out of 105 tested isolates were transformed by plasmid and chromosomal DNA, respectively. Isolates transformed by plasmid DNA included members of the genera *Vibrio* and *Pseudomonas* and moreover, isolates of unknown identity displayed the capability of natural transformation (Frischer *et al*., 1994).

1.3.1.3. Cellular barriers to transformation

The gene transfer is dependent on the fate of the transferred DNA once it attaches and enters to the recipient cell.

Adsorption of DNA at the cell surface. The mechanisms of interaction between DNA and the cell wall of some bacteria depend on the presence of a recognition nucleic acid sequence in the donor DNA. For example, in *Haemophilus influenzae* Rd and *N. gonorrhoeae*, the specific sequences are 9 bp (Smith *et al*., 1995) and 10 bp (Elkins *et al*., 1991), respectively. The presence of specific recognition sequence can be considered as a strategy to optimize gene transfers between cells belonging to the same species and restrict the uptake of foreign DNA. Other naturally competent bacteria do not exhibit such specific adhesion mechanism and can be transformed by any type of DNA (Dubnau, 1991).

Restriction/modification systems. The bacterial restriction-modification systems destroy foreign DNA to protect their own DNA. This system involves two enzymes, a restriction endonuclease that cleaves specific 4–8 bp DNA sequence and a methyltransferase that specifically methylates A or C residue at the same sequence protecting the host DNA from the cleavage (reviewed in Bickle and Krüger, 1993). However, in most naturally competent bacteria transforming DNA is able to escape the restriction systems, permitting a successful transformation of the bacterium. Bacteriophages and wide-host-range plasmids have evolved ways to manage with these restriction systems by reducing the number of restriction cleavage sites (Brunel and Davison, 1979; Wilkins *et al*., 1996). Inefficient restriction systems could also lead to successful transformation of the bacterial cells (Bickle and Krüger, 1993).

Internalization of transforming DNA. During transformation, the DNA must be efficiently internalized either due to autonomous replication or homologous recombination. A plasmid must be capable to replicate in the new host. The genes carried by a transposon need to be successfully integrated into the host chromosome (Davison, 1999).

The role of the SOS system. The SOS system plays a major role in homologous recombination. Single-strand DNA, known to be an SOS-system-inducing signal, could lead to an increase in the RecA protein concentration, thus providing help for the transforming DNA to be integrated (Matic *et al*., 1995).

1.3.1.4. Integration of foreign DNA during natural transformation

With sufficient DNA-sequence similarity between transforming DNA and the recipient genome, integration can occur by homologous recombination (Strätz *et al*., 1996). Homologous recombination can only occur when the donor DNA and the host genome share DNA sequence similarities required to form DNA heteroduplexes. With a similarity decrease from 100 to 90%, the recombination

frequency is reduced over 40-fold in *E. coli* (Shen and Huang, 1986). Bacteria differ by the required length of the DNA region sharing similarity between the donor DNA and the recipient host genome. The lengths of these minimal segments are 70, 27 and 30 bp in *B. subtilis*, *E. coli* and *Saccharomyces cerevisiae*, respectively. The effectiveness of homologous recombination varies between the DNA sources. It decreases when DNA originates from phylogenetically more remote microorganisms (reviewed in Bertolla and Simonet, 1999).

There are identified two possibilities for the integration of foreign DNA with low sequence similarity in bacteria. First, short specific nucleotide sequences are recognized by transposases or integrases that can cut and paste DNA at these sites (Mahillon and Chandler, 1998). Second, illegimate (nonhomologous) recombination not requiring RecA protein can occur. Illegimative recombination leads often to the duplication, insertion, deletion and translocation of target DNA (Kokontis *et al*., 1988).

Illegimative recombination could occur *via* short homologous sequences like 8 bp in the case of *B. subtilis* (Bashkirov *et al*., 1987) or without any homology like described in *Campylobacter coli* where no significant target site duplication had occurred. Moreover, analyzes of integrates exhibited deletions in the integrated sequence, suggesting involvement of nucleases in recombination (Richardson and Park, 1997). Wackernagel and coworkers (2002, 2003) have demonstrated in *Acinetobacter* and *P. stuzeri* that illegimate recombination is enhanced when homologous sequence to the recipient DNA was present on the otherwise heterologous DNA providing a recombination anchor. The effectiveness of illegimate recombination was correlated with length of homologous sequence; shortest functional anchors were detected to be at least 183 and 311 nucleotides, respectively (De Vries and Wackernagel, 2002; Meier and Wackernagel, 2003).

1.3.2. Mobile genetic elements

It has been shown that mobile genetic elements have an important role in evolution of bacterial genomes and adaptation of microbial populations to specific environmental changes e.g. the spread of antibiotic resistant genes (Davies, 1994) and adaptation of bacteria to environments contaminated with toxic xenobiotic compounds (Top *et al*., 2002). These elements include plasmids and transposable elements (Tsuda *et al*., 1999). In both cases catabolic genes are often boarded by insertion sequences (IS elements) increasing the potential of exchange of the genes between different hosts (Wyndham *et al*., 1994).

1.3.2.1. Transposons

Bacterial transposons are discrete DNA elements that can move from one site to another without the need for genetic homology between the donor and target sites. Although transposons are not involved directly in HGT, they are able to group different genes into one replicon and transfer them between chromosomal and plasmid DNA influencing expansion of the gene pool (Stephenson and Warnes, 1996). Bacterial transpsosns have been classified into three major classes: conjugative transpsosons (described below), Class I insertion sequences and composite transposons and Class II transposons (Berg and Howe, 1989).

1.3.2.1.1. Insertional sequences

IS elements are up to 2.5 kb long DNA elements encoding functions necessary for their translocation into genomes. They can be found in the genomes of a wide range of bacteria. Most of them integrate randomly and integration occurs independently of homologous recombination. IS can cause chromosomal re-arrangements like deletions, duplications, insertions and translocations and also disrupt genes and cause mutations (reviewed in Mahillon and Chandler, 1998).

1.3.2.1.2. Composite transposons

Class I composite transposons consist of variety of genes (catabolic pathways, antibiotic and toxin resistance genes) boarded by two copies of very similar or identical IS in direct or inverted orientation (Mahillon and Chandler, 1998).

Class II transposons commonly lead to a 5-bp duplication of the target sequence after transposition and carry short terminal inverted repeats (Berg and Howe, 1989). Each element carries two recombinase genes: transposase and resolvase, catalyzing respectively formation and resolution of cointegrate of donor and target DNA molecules. As in the case of class I composite transposons, the class II transposons encode various genetic traits not related to transposition. Such catabolic transposons are often located on plasmids (Mahillon and Chandler, 1998, Tsuda *et al*., 1999). For example, mercury resistant genes (*mer*) are often found on different transposons and horizontal transfer of these *me*r determinants in Gram-negative (and also in Gram-positive) bacteria has been described (Yurieva, 1997). The genes for chlorobenzoate catabolism are often associated with IS*1071*. Peel and Wyndham (1999) suppose that horizontal transfer of the composite transposon Tn*5271* is the primary mode of dissemination of these genes. Furthermore, as IS*1071* is linked with a diverse collection of biodegradative genes (Di Gioia *et al*., 1998), important role for mobilization of catabolic genes by this element is suggested (Peel and Wyndham, 1999).

1.3.2.1.3. Integrons

Gene acquisition by bacterial genomes can occur also by site-specific integration events mediated by DNA integrases (Hall and Stokes, 1993). Integrons are DNA elements which include a gene encoding a site-specific DNA recombinase, a DNA integrase, and an adjacent site where a wide variety of antibiotic resistance and other genes have been inserted. The integration of gene cassettes is mediated by the enzyme integrase which recognizes and recombines at specific small recombination sites, which give rise to integron structures that are shown to be mobile DNA elements (Hall and Stokes, 1993; Tschäpe, 1994).

Poelarends *et al*. (2000) have described and analyzed the flanking sequences of the *dhaA* (haloalkane dehalogenase) genes from different hosts. The presence of a highly conserved *dhaA* gene in the three phylogenetically different organisms *Rhodococcus rhodochrous*, *Pseudomonas pavonaceae* and *Mycobacterium* sp. suggests horizontal distribution of *dhaA* among these bacteria. An open reading frame encoding integrase was found upstream of the *dhaA* gene and therefore gene spreading in constitution of integrons was suggested (Poelarends *et al*., 2000).

1.3.3. Conjugation

Bacterial conjugation is a cell-contact-dependent parasexual process, whereby specific plasmids or transposons transfer from donor to recipient cells. Most of the reports about gene transfer in the environment concern conjugation.

The potential of conjugation for the exchange of genetic information among bacteria in nature is indicated by the widespread occurrence of conjugative elements, such as self-transmissible plasmids and plasmids transferable with a help of conjugative plasmids (mobilizable plasmids). The conjugative transfer of plasmids is reported concerning gram-positive and gram-negative bacteria in various ecosystems: different soil types, sludges, rhizosphere, plant leaves, aquatic ecosystems and animal intestinal tracts (reviewed in Davison, 1999; Dröge *et al*., 1999). For occurring successful conjugal transfer certain requirements must be balanced. Dröge *et al*. (1999) have concluded these requirements as following i) plasmid transfer is dependent on the physiological status of donor cells, while the starvation of donor makes conjugation less probable; ii) plasmid must express essential transfer (*tra*) genes. The plasmid incompatibility specific requirements for transfer medium have also been shown. For example transfer frequency for IncP group plasmids is higher in solid surfaces than in liquid medium (Thomas, 1989). The positive effect of rhizosphere to conjugal transfer is reported obviously due to root exudates available as nutrients to bacteria (van Elsas *et al*., 1988).

1.3.3.1. Plasmids

The conjugative (self-transmissible) plasmids encode the functions necessary for both mating pair formation and the transfer of DNA. Very important feature of the conjugative plasmids is the presence of the *oriT* (origin of transfer) sequence. Conjugative plasmids are belonging often to broad host range plasmids group and are usually transferred to and maintained within most gram-negative bacterial species (Thomas, 1989). Among conjugative plasmids there are also antibiotic resistance plasmids allowing wide spread of antibiotic resistance in nature. Dröge *et al*. (2000) have demonstrated existence of wide set of conjugative broad-host-range antibiotic resistance plasmids isolated from activated sludge. Most of them belonged to IncP incompatibility group confirming the widespread occurrence of this plasmid type in nature (Dröge *et al*., 2000).

Some plasmids, called mobilizable plasmids, lack the genes needed to create the mating bridge and therefore cannot transfer themselves. However, if they carry genes encoding the proteins that nick the plasmid at its *oriT* they can be transferred with help of the self-transmissible plasmid (Amabile-Cuevas and Chicurel, 1992).

1.3.3.2. Conjugative transposons

A second type of self-transmissible gene transfer element is a conjugative transposon. Conjugative transposons are discrete DNA elements found in Gram-negative and-positive bacteria carrying antibiotic resistance or other genes that are normally integrated into bacterial genome. Like transposons, they excise and integrate into DNA (although by different method than well-studied transposons), but conjugative transposons have covalently closed circular transfer intermediate and do not cause target site duplication, like true transposons. Like plasmids, they are transferred by conjugation but the circular intermediate is not self-replicative (Bedzyk *et al*., 1992; Salyers *et al*., 1995).

1.3.4. Transduction

In the process of transduction, bacterial genes are incorporated into bacteriophage particles and transferred to another bacterium. Bacteriophages have a restricted host range and bacteria may mutate to become incapable of phage adsorption. Although transduction seems an unlikely candidate for gene transfer in the environment (Davison, 1999) there are some reports about transduction of chromosomal and plasmid markers in a freshwater (Saye *et al*., 1987, 1990) and in the marine environment (Jiang and Paul, 1998). Also, bacteriophage mediated HGT seems to play an important role in distribution of virulence genes (O'Shea and Boyd, 2002; Rabsch *et al*., 2002).

1.4. Studies of horizontal transfer of catabolic genes among bacteria using microcosm experiments

Since gene transfer experiments in natural environment are technically difficult, most experiments have been performed in microcosms designed to represent the natural environmental situation. Microcosm experiments enable manipulation of physicochemical variables (temperature, pH, humidity, carbon, nitrogen and phosphorous sources) that are impossible to manipulate in natural environment. However, microcosms are only an approximation of the natural environment and the results should be viewed within the limitations of their experimental design. In gene transfer experiments; it has been commonly observed that the frequency of transfer is lower in the presence of the native microbial population (Stewart and Sinigalliano, 1991; Top *et al*., 1990). Similarly, a newly introduced bacterial population usually declines upon introduction into the natural environment (van Veen *et al*., 1997). The reason for this may include predation, bacteriophages, growth inhibitors (heavy metals, antibiotics, bacteriocins), and competition with the indigenous microflora for nutrients or ecological niche (van Veen *et al*., 1997).

Examples of the microcosm experiments are given in Table 1.

Table 1. Microcosm studies assessing HGT of catabolic genes among bacteria.

Type of microcosm	Description and conclusions	Reference
Soil	The transfer of plasmids allowing 3-phenoxybenzoic acid (3-POB) degradation was observed only in sterile soil that received multiple additions of 3-POB. These conditions were important to avoid competition with indigenous microorganisms and allowing growth of the plasmid donor. Soil containing more organic matter and clay causes higher appearance rate of transconjugants than organic-poor soil.	Haldane *et al*., 1999
Soil	Transfer of the 80-kb, broad-host range, self-transmissible 2,4-dichlorophenoxyacetic acid (2,4-D) catabolic plasmid pJP4 into indigenous or introduced microorganisms was observed in variety of soils and at different contaminant level. The formation of transconjugants was shown to occur only in soil treated with high concentrations of the 2,4-D.	DiGiovanni *et al*., 1996; Neilson *et al*., 1994; Newby *et al*., 2000
Soil	The effect of conjugative transfer of a different 2,4-D degradative plasmids on 2,4-D degradation was examined. The transfer of plasmids was observed and correlated with an enhanced degradation of 2,4-D.	Top *et al*., 1998; Dejonghe *et al*., 2000

Soil	HGT of pJP4 into different indigenous soil bacteria was observed using earthworm-inoculated soil microcosms. The earthworms had positive influence to plasmid spreading by distributing donor and transconjugant bacteria throughout the soil sample.	Daane *et al*., 1996
Soil	Studies on genetic exchange of biphenyl and chlorobenzoate degradation genes between indigenous soil microbes and inoculated strain *Pseudomonas aeruginosa* in soil microcosm. The achieved recombinant strains were losing the ability to grow on biphenyl when cultured continuously on chlorobenzoate, and *vice versa*. As the investigated strains did not harbor any plasmids the authors suggested transfer of the catabolic genes by transposons.	Focht *et al*., 1996
Soil	Horizontal transfer of plasmid carrying *tfdA* (2,4-dichlorophenoxyacetic acid dioxygenase) gene from *E. coli* to *Ralstonia eutropha* and to indigenous phenol degraders was observed in soil microcosm experiment. However, the acquisition of the *tfdA* did not resulted in the ability to degrade PAA (phenoxyacetic acid) suggesting pour expression of the acquired genes.	De Lipthay *et al*., 2001
Soil	TOL plasmid harboring *P. fluorescens* was inoculated into soil microcosm with and without pine seedlings. After 3 months of regular treatment with *m*-toluate the catabolic plasmid was found to be transferred into indigenous bacteria.	Sarand *et al*., 2000
Activated sludge	Horizontal transfer of the 105-kb self-transmissible element carrying chlorocatechol degradative genes, from *Pseudomonas* sp. B13 into indigenous bacteria was observed. Although the genetic basis for the transfer of the element is unknown, the element is capable to integrate into the recipient genome.	Ravatn *et al*., 1998
Lake water	*Alcaligenes* sp. BR60 harboring a conjugative, broad-host range plasmid that carries a 17 kb transposon encoding 3-chlorobenzoate catabolism was introduced into the microcosm. Bacteria capable to utilize 3-chlorobenzoate were isolated after a 2-month period. 11 isolates were shown to be indigenous transconjugants.	Fulthorpe and Wyndham, 1991

In conclusion, the HGT of the catabolic genes carried by various mobile genetic elements in the microcosm conditions is frequently observed. Also, there are some general points influencing the frequency of transfer of genetic material in microcosm:

a) The selective pressure has positive effect to gene spread in microcosm (DiGiovanni *et al*., 1996; Haldane *et al*., 1999, Newby *et al*., 2000). This could not be caused from direct effect of the pollutant on the conjugation efficiency, but the contaminants seemed to support selective growth of the transconjugants. Low pollutant concentrations may be not sufficient to support growth of transconjugants, and the cells would need another selective advantage to successfully compete with co-existing microorganisms (Top *et al*., 2002).
b) The earthworms influence positively the HGT in soil (Daane *et al*., 1996).
c) The catabolic plasmids were most often transferred and expressed in strains belonging to the genera *Burkholderia*, *Ralstonia* and *Pseudomonas* (DiGiovanni *et al*., 1996; Newby *et al*., 2000, De Lipthay *et al*., 2001, Top *et al*., 1998; Dejonghe *et al*., 2000). This could be due to the enrichment and isolation methods that favor pseudomonads and due to the environment used in microcosm as pseudomonads are ubiquitous in soil.
d) The formation of transconjugants is in correlation with degradation of respective compounds added to microcosm (Top *et al*., 1998; Dejonghe *et al*., 2000).

1.5. Studies of horizontal transfer of catabolic genes *in situ*

Genes encoding enzymes involved in biodegradation processes are carried by a wide diversity of mobile elements. Obviously, these elements have a role in spreading of catabolic determinants in the natural ecosystems using different transfer and integration mechanisms. Nevertheless, there is a little direct information and only few examples available about where and when bacterial adaption through catabolic gene exchange takes place in natural environments (Top *et al*., 2002).

There are several studies about natural horizontal transfer of the genes encoding naphthalene degradation enzymes. Herrick *et al*. (1997) have reported *in situ* horizontal transfer of a naphthalene-catabolic gene (*nahAc*) between naturally occurring bacteria isolated from a coal tar waste-contaminated field. Identity of the *nahAc* gene among seven taxonomically diverse hosts was confirmed by the sequencing. The mechanism of HGT was uncertain since *nahAc* was located on the chromosome or on plasmids of different size. Most of the plasmids were still the one type of conjugative plasmid persisting in this coal tar-contaminated field site more than 4 years and probably spreading by conjugation. All naphthalene-catabolic plasmids investigated to date from *Pseudomonas*-strains are self-transmissible. This suggests that coal tar contamination has applied selective pressure for the proliferation of this plasmid (Stuart-Keil *et al*., 1998).

Also, Hohnstock *et al*. (2000) have performed several laboratory and field experiments to examine HGT of the naphthalene-catabolic plasmid. They conclude the possibility of HGT under favorable laboratory and field conditions and that recipient survival is necessary but not sufficient for conjugation to occur. Conjugation was triggered by naphthalene supplement, likely by stimulating growth of potential donors in environments. It is also possible that naphthalene stimulated growth of newly formed transconjugants to the detectable rate (Hohnstock *et al*., 2000). Recently, evidence for natural horizontal transfer of a naphthalene dioxygenase gene in a polycyclic aromatic hydrocarbon-contaminated site was observed (Wilson *et al*., 2003).

Thiem *et al*. (1994) were able to detect 3-chlorobenzoate-degrading bacterium *Pseudomonas* sp. strain B13 14 months after it had been injected into the aquifer. They also suggest possible transfer of 3-chlorobenzoate catabolic pathway genes to other natural microbes, as they detected no 3-chlorobenzoate-degrading bacterium from study site before introducing *Pseudomonas* sp. B13.

The occurrence of probable HGT is well documented in the case of genes involved in 2,4-D metabolism. There are several data about nearly identical 2,4-D degradation genes from diverse bacteria in different field spots (Ka *et al*., 1994; Matheson *et al*., 1996).

Wand *et al*. (1997) have demonstrated that laboratory-born *P. putida* strains capable degrade phenol and toluene were not resistant in natural conditions despite of continuous selective phenolic pressure in release area (detection of the released strain was possible only 32 h after release). It was supposed due to competition with indigenous microbes growing faster at low temperatures. They also did not detect transfer of the TOL-plasmid derivative harbored on the released strains to indigenous microflora (Wand *et al*., 1997).

1.6. Eukaryotic genes acquired by prokaryotes

Numerous genes (e.g. ATP/ADP translocase of *Chlamydia* and *Rickettsia*; arylsulfatase and cation transport system component of *E. coli*) are believed to be horizontally transferred from eukaryotes to bacteria and archea (Koonin *et al*., 2001). In most cases the selective advantage for bacteria is not clear. One of the clearest examples is the chloroplast-type ATP/ADP translocase detected in *Chlamydia* and *Rickettsia*. The advantage of having this enzyme for these intracellular parasites is obvious allowing them to scavenge ATP from the host (Winkler and Neuhaus, 1999). Some eukaryotic enzymes acquired by bacteria are useful for interactions with their eukaryotic hosts or as virulence factors that degrades host proteins (Koonin *et al*., 2001). As demonstrated by Kay *et al.* (2002), *in situ* transfer of the antibiotic resistance genes from transgenic plant to the cocolonizing bacteria is also possible. However, the transfer was observed only in case of the homologous sequences present in plant and bacteria.

1.7. How to identify transferred genes?

As an evidence of horizontal gene transfer event could be finding of identical copies of a gene in bacteria that are not closely related. That is, if a gene has been transferred recently from one bacterium to another and therefore has had no time to mutate appreciably. Secondly, if the %G+C content of the investigated gene is different from other genes in this bacterium, it could be acquired through horizontal transfer. Also, if the codon usage of the investigated gene deviates significantly from the codon usage characteristic for the host, this can indicate HGT (Lawrence and Ochman 1997, 1998).

Based on the abovementioned criteria Jeltsch and Pingoud (1996) have reported that HGT had a considerable influence on the distribution and evolution of restriction-modification (RM) systems of bacteria. The results of their study showed that in 6 of 29 RM systems investigated, HGT has occurred. In all six cases in which codon usage deviation was detected, the methyltransferase gene was supposed to be acquired before restriction endonuclease gene because deviation of the gene coding for the endonuclease is larger than that of the gene coding for the methyltransferase. They conclude that in general, genes for bacterial defense systems, like restriction enzymes, toxins, antibiotic resistance genes and other genes that provide an immediate adaptive advantage to host are likely to spread horizontally.

1.7.1. Tools for identifying transferred catabolic genes

Traditional studies on gene transfer in the environment have mainly based on cultivation, selecting for donor, recipient and transconjugant cells using a combination of appropriate markers. These approaches have complemented with molecular methods such as PCR-typing, reverse transcription-PCR analysis of environmental mRNA, DNA-DNA hybridization and sequencing. Also, restriction fragment length polymorphism analyses and subsequent hybridizations are favorable methods used to determine the structure and localization of transferred genes. All these methods need prior extraction of the material (DNA, RNA or bacterial cells) from the environment (reviewed in van Elsas and Bailey, 2002). By contrast, the use of the fluorescent markers, such as *gfp* (green fluorescent protein) gene, allows direct *in situ* observation of the mobilization of plasmids (Christensen *et al*., 1996; Dahlberg *et al*., 1998; Molbak *et al*., 2003).

1.8. Phenol degradation by the bacteria

Soil microorganisms, particularly pseudomonads, are capable of degrading a wide variety of aromatic compounds, e.g. phenols. Although these bacteria have a wide variety of enzymes for the initial attack on the different aromatic compounds, the catabolic pathways converge on a few central intermediates such as catechol and substituted catechols. Next step in the cleavage of the aromatic ring occurs by the ring-fission dioxygenases and the degradation is continued either by meta-or ortho pathway (Dagley, 1986).

Two different types of phenol hydroxylases have been identified in bacteria: single-chain flavoproteins and multicomponent hydroxylases. The later seem to be more widely represented and distributed among different bacteria.

1.8.1. Multicomponenent phenol hydroxylases

Bacterial multicomponent monooxygenases are family of nonheme, di-iron enzymes capable of using molecular oxygen to hydroxylate a variety of organic compounds. All known multicomponent monooxygenases are transcribed from single operons that code for four to six polypeptides (reviewed in Notomista *et al*., 2003).

Pseudomonas sp. strain CF600 (isolated from activated sludge in England) can catabolize phenol and some of its methylated derivatives as the sole carbon and energy source using meta-cleavage pathway encoded on a large IncP2 plasmid (Shingler *et al*., 1989). The phenol hydroxylase of CF600 was first multicomponent bacterial hydroxylase of aromatic compounds described. The coding region of this enzyme consists of six ORFs (designated *dmpKLMNOP*) arrayed in an operon structure (Nordlund *et al*., 1990). Up to date a variety of phenol-degrading strains harboring the Dmp family of enzymes have been isolated from different habitats. These include *P. putida* P35X from river mud in England (Ng *et al*., 1994), *P. putida* H (*phl* genes located in large plasmid) from river water in Germany (Janke *et al*., 1981), and *P. putida* BH from activated sludge in Japan (Hashimoto and Fujita, 1987). Nordlund *et al*. (1993) have detected *dmp* gene analogs with high frequency also in marine phenol degrading bacterial isolates (collected from sea water of the coast of Norway). Examination of the size and order of the hybridizing DNA fragments of these strains suggests that not only structural genes for phenol catabolism are conserved but they are also encoded in the same order as *dmp* genes in CF600 (Nordlund *at al*., 1993).

Watanabe *et al*., (1998) have investigated phenol-degradating bacteria from activated sludge in Japan. They obtained two groups of multicomponent phenol hydroxylases, sharing similarity with *dmp* genes. The 16S rDNA sequences of these strains were also assessed and noticed that all strains closely related to the

genera *Acinetobacter* and *Pseudomonas* in the γ subclass of the *Proteobacteria* harbored phenol hydroxylase of one type while bacteria harboring multi-component phenol hydroxylase with different sequence were members of the β subclass of the *Proteobacteria*. Therefore they suggest that horizontal transfer of the multicomponent phenol hydroxylase genes between members of the different subclasses of *Proteobacteria* does not occur often (Watanabe *et al*., 1998).

Degradation of phenol by *Acinetobacter calcoaceticus* NCBI8250 involves σ^{54} –dependent expression of a chromosomally encoded multicomponent phenol hydroxylase and catechol 1,2-dioxygenase encoded by the *mop* operon (Ehrt *et al*., 1994; Ehrt *et al*., 1995). Also, phenol-degrading *A. calcoaceticus* PHEA-2, isolated from wastewater of an oil refinery, has phenol hydroxylase-encoding genes similar to *dmp* genes of *Pseudomonas* sp. CF600 (Xu *et al*., 2003).

Ralstonia eutropha strain E2 (previously *Alcaligenes* sp.) is able to transform phenol to catechol using a chromosomally encoded multicomponent (PoxRABCDEFG) phenol hydroxylase. The *poxRABCDEFG* products are homologous to those of *dmpRKLMNOPQ* of *Pseudomonas* sp. CF600, sharing 30–65% identity (Hino *et al*., 1998).

Comomonas testosteroni TA441 has a catabolic gene cluster (*aph* genes) similar to phenol- and dimethylphenol degradation gene clusters encoding multicomponent phenol hydroxylase from *Pseudomonas* sp. CF600 (*dmp* genes) and *P. putida* P35X (*phh* genes) (Ng *et al*., 1994; Arai *et al*., 1998).

1.8.2. Single component phenol hydroxylases

Phenol hydroxylase (encoded chromosomally by *tbuD* gene) from *P. pickettii* PKO1 is a simple flavoprotein which substrate range is phenol and three isomeric methylphenols. The genes encoding enzymes for meta-cleavage of catechol or 3-methylcatechol are located downstream of the *tbuD* and degradation of phenol occurs via meta-pathway (Kukor and Olsen, 1991, 1992).

The second example for single component phenol monooxygenase (*pheA*) is characterized from *Pseudomonas* sp. EST1001 plasmid DNA (Nurk *et al*., 1991). *pheA* is cotranscribed with *pheB* encoding catechol 1,2 dioxygenase gene in the order *pheB* to *pheA* (Kivisaar *et al*., 1991). The *pheBA* cluster is flanked by two IS elements (IS*1472* and IS*1411*) (Figure 1). The promoter of the operon is located upstream of the IS*1472* and shows homology to the chromosomal *catBC* promoter region which is recognized by CatR (Kasak *et al*., 1993). The regulatory protein CatR activates also transcription from the *pheBA* promoter (Kasak *et al*., 1993; Parsek *et al*., 1995).

Figure 1. Organization of the *pheBA* operon. Open reading frames are indicated with solid boxes, non-coding regions with solid lines. Transcription directions are shown with arrows.

The 2,4-dichlorophenol hydroxylases of *Acinetobacter* and *Alcaligenes* species have also been shown to be flavoproteins, although these enzymes show no activity with unsubstituted phenol (Beadle and Smith, 1982; Liu and Chapman, 1984).

The first two enzymes for phenol degradation via meta pathway of *B. stearothermophilus* BR219 are encoded by plasmid-located genes *pheA* and *pheB*. The single component phenol hydroxylase (PheA) of this organism resembles any of the described phenol hydroxylase from gram-negative origin and is also able to catalyze formation of indigo (Kim and Oriel, 1995).

AIMS OF THE STUDY

1. The first goal of the present study was to investigate the persistence and possible horizontal transfer of the released *phe*-operon in phenol-polluted river water microflora.
2. The second goal was to characterize thoroughly one of the new biodegradation plasmid, generated through acquisition of the *phe*-genes.
3. The third goal was to determine flanking sequences of the acquired *phe*-operon with the purpose to find possible mechanisms of the *pheBA* distribution among indigenous microbial community.

2. RESULTS AND DISCUSSION

2.1. Horizontal transfer of the *pheBA* operon among indigenous *Pseudomonas* strains

2.1.1. The historical view of the study site (Reference I)

A subterranean fire took place in an oil shale mine "Estonia" in 1988. Thousands of tonnes of phenols polluted extinguishing water was pumped out of the mine. To reduce the produced phenolic pollution *in situ*, nonpathogenic phenol-degradative derivatives of *Pseudomonas putida* PaW85 were released into accident area. These bacterial strains harbored plasmids encoding first enzymes involved in phenol degradation: catechol 1,2-dioxygenase (Kivisaar *et al*., 1991) and phenol-monooxygenase (PMO) (Nurk *et al*., 1991). The respective genes *pheB* and *pheA* are forming an operon and are cotranscribed under the same promoter (Kasak *et al*., 1993). The *pheBA* genes are boarded by two IS elements, IS*1472* up- and IS*1411* downstream, respectively. IS*1411* is shown to encode an active transposase (Kallastu *et al*., 1998). One of the strains harbored in addition to the *pheBA* operon also the TOL plasmid pWW0 (Williams and Murray, 1974). After bioaugmentation, the treated underground water with phenol-degrading bacteria was pumped out via a reservoir in the enrichment plant to the watershed in a northerly direction (Fig. 1, reference I).

One area with potential selective value for phenol degradation genes is located 15 km from the enrichment plant reservoir, where the bacterial biomass used for bioaugmentation was produced. This area contains ash-dumps, where the solid by-products formed in the semi-coking processing of oil shale are disposed. The leachate from the ash-dumps contains aromatic compounds, e.g., phenol, dimethylphenols, cresols and resorcinols (Kettunen and Rintala, 1995). This leachate, a continuous source of phenols, is discharged via rivers into the Baltic Sea (Fig. 1, reference I). The additional source of the unsubstituted phenol in this district was resorcinol adhesives plant near the ash dumps.

Water samples relevant for this study were collected during 1994 from several sampling points (Fig. 1, reference I). The colony forming bacteria with biodegradative properties were plated out from different water samples onto phenol-minimal plates. The isolation, purification and identification of the bacteria used in this study were performed by Eeva Heinaru and Ene Talpsep. For possible re-detection of the *pheBA* operon 41 strains that grew well on phenol were picked out.

2.1.2. Diversity of phenol hydroxylases (Reference I)

The origin of phenol degradation genes in 41 selected strains was investigated by colony hybridization. Three probes were used: the multicomponent phenol hydroxylase specific gene probe and two single component phenol hydroxylase gene specific probes, [*dmpKLMNOP* (Shingler *et al*., 1992), *tbuD* (Kukor and Olsen, 1992) and *pheA* (Nurk *et al*., 1991), respectively]. Of the 41 isolates, 10 revealed no hybridization with the probes used. Isolated DNA from 18 investigated strains exhibited homology to the *dmp* genes. This is in accordance with results of the other works demonstrating wide distribution of the multicomponent phenol hydroxylases among phenol-degrading bacteria. By using gene-probing techniques, homologous genes to *dmpKLMNOP* have been identified from several pseudomonads (Ng *et al*., 1994, Watanabe *et al*., 1998, Nordlund *et al*., 1993) and also from distinct bacteria e.g. *Acinetobacter* (Ehrt *et al*., 1994; Xu *et al*., 2003) and *Ralstonia* (Hino *et al*., 1998). All these genes encoding multicomponent phenol hydroxylases are linked with the catechol 2,3-dioxygenase gene and the aromatic substrates are metabolized via meta-cleavage pathway.

Four of these *dmpKLMNOP*-hybridization positive isolates also hybridized with *tbuD* at a low level. 13 isolates hybridized strongly with the *pheA* specific probe but not with the other two probes. The strains that hybridized with the *pheA* probe were isolated only from the water samples which were contaminated with ash dump leachate.

2.1.3. Proof of the *phe* operon derivates (Reference I)

All *pheA* positive isolates were gram-negative bacterial strains belonging to the genus *Pseudomonas* as identified by using the BIOLOG GN system. Nine distinct *pheA* positive strains that revealed unique genomic repPCR patterns (Fig. 2, reference I) were selected for further investigation: *P. corrugata* MB2/12, *P. fragi* B1/25, *P. fluorescens* biotype B MT4/4, *P. fluorescens* biotype B 2.67, *P. fluorescens* biotype B MB3/26, *P. fluorescens* biotype F Cb36, *P. fluorescens* biotype F MB3/29, *P. fluorescens* biotype C 5F6 and *P. fluorescens* biotype G JP1/5 (previously *P. stutzeri* JP1/5, overestimated by E. Heinaru). None of the strains characterized in this study were established as a derivative of the released *P. putida* strains, and we do not have data that this laboratory strain has survived in the environment. Also, Top *et al*. (2002) have concluded that even if the donor dies off quickly, a selective pressure (an organic compound that can be degraded by plasmid encoded enzymes) can strongly affect the extent of gene spread in a soil habitat. In situ transfer of catabolic genes to the indigenous bacteria is beneficial as these organisms (as compared to laboratory strains) are well adapted to the given habitat and to the constantly changing natural conditions (de Lipthay *et al.*, 2001).

Seven of these strains belonged to *P. fluorescens* biotypes what is not surprising as *P. fluorescens* strains capable of degrading various phenolic compounds are predominant in current polluted watershed (Heinaru *et al*., 1997). The studied strains harbored plasmid replicons of different size (Fig. 3A, reference I), and the *pheA* gene was localized in plasmid DNA in all the strains, except MB3/29 (Fig. 3B, reference I). This is in accordance with data referring that catabolic genes are often found in plasmids. Also an increase of plasmid frequency within microbial communities from polluted habitats has been observed (reviewed in Sayler *et al*., 1990).

Total DNA isolated from the investigated strains was cleaved with the restriction endonuclease *Hin*dIII, which cleaves several times into coding and noncoding areas of the *pheBA* operon (Fig. 4, reference I) and therefore gives a specific pattern of restriction fragments. Data from serial hybridizations with the specific gene probes from the original *pheBA* operon demonstrated that all nine strains hybridized with *pheA* and *pheB* specific gene probes and had the same structural organization as the original *phe* genes carried in the control plasmid pEST1412 (Fig. 4, reference I). Moreover, the DNA of all 9 strains gave hybridization with original promoter probe, and in the 8 cases also IS*1472* was present (Fig. 4, reference I). In contrast, the sequence specific to IS*1411* was absent in 6 cases (Fig. 4, reference I). To summarize, eight operons include one or both IS-elements in the same order (IS*1472* upstream and IS*1411* downstream of *phe* genes) as in the original operon.

Based on the data obtained, we suggest that horizontal transfer of the released *pheBA* operon into the characterized *Pseudomonas* strains had occurred in natural conditions. Unfortunately, we did not have data about distribution of different phenol hydroxylases in this watershed before the release of the laboratory bacteria into the environment. Nevertheless, the primary source of the *phe*-genes, *Pseudomonas* sp. S13 was isolated in 1976 by P. A. Williams and was therefore not of local origin (Kivisaar *et al*., 1989) and hereby the natural formation of the second identical *pheBA* operon with the same promoter sequence and flanking IS elements is very unlikely. Nordlund *et al*. (1993) have tested the presence of the *pheA* gene counterparts among phenol-degradative marine isolates and did not detect hybridization signal with this gene. Up to date, there is no data about detection of similar *phe*-genes from geographically distinct localities. On the contrary, sequences belonging to nearly identical counterparts of the IS*1472* and IS*1411* from various sources are available in databases. Therefore, the presence of three or more genes in the same order in distant genomes is very unlikely unless these genes form an operon. Furthermore, it is suggested that each operon emerges only once during evolution and when the same operon is presented in distantly related genomes, horizontal transfer seems to be happened (Koonin *et al*., 2001).

2.1.4. Study of expression of the phenol monooxygenase in *pheA*-positive strains (Reference I)

In *P. putida* PaW85, syntheses of plasmid-encoded phenol-monooxygenase (encoded by *pheA*) and catechol 1,2-dioxygenase (encoded by *pheB*) was shown to be inducible by cis, cis-muconate in the presence of the LysR type positive transcription factor CatR. The expression level of the *pheA* gene in the presence of phenol and benzoate as inducers has been described as equal (Kasak *et al*., 1993). We tested PMO activities of the investigated nine strains and control strain *P. putida* EST1026 (Kivisaar *et al*., 1990), grown on minimal M9 media supplemented with phenol or benzoate in the presence or absence of glucose. As the results showed (Table 1, reference I), the expression of the *pheA* in seven of nine strains was still inducible with both substrates as in control strain. Therefore, the involvement of the CatR-like regulatory protein in the regulation of the *phe* operons in these strains was suggested. One strain (2.67) was not able to utilize benzoate as the sole carbon source and strain 5F6 revealed constitutive expression of the PMO. The maintenance of the inducible expression of the *phe*-genes seems to be beneficial as the type of regulation of gene expression on the plasmid could affect plasmid stability in changing natural conditions (Ensley, 1985). Boronin *et al*. (1985) have demonstrated plasmid instability under the conditions when the constitutive expression of the plasmid-carried catabolic genes is not necessary, whereas plasmids with inducible catabolic genes that were not expressed in nonselective conditions were stably maintained. Also, LysR type regulatory proteins are considered to be the most common type of positive transcription regulators in bacteria (Rothmel *et al*., 1990). Therefore, it is probable that new hosts of the *phe* genes, belonging to the genus *Pseudomonas*, already had CatR-like protein allowing direct usage of the acquired *phe* genes without preceding rearrangements in the regulatory region of the operon.

2.2. Structure of the pAM10.6

The hybridization data of the investigated nine strains revealed that the *pheBA* operon of the *P. fluorescens* Cb36 locates in a relatively small (about 10 kb) plasmid replicon (Fig. 3, ref. I). Transformation of the *P. putida* PaW85 with total plasmid DNA purified from the Cb36 resulted phenol growing transformants that harbored small *pheBA* carrying plasmid. This plasmid was named pAM10.6.

Most of the catabolic plasmids reported up to now are large plasmids over 50 kb in size that often belong to IncP2 and IncP9 incompatibility group (Top *et al*., 2002). Therefore, the pAM10.6 as 10-kb plasmid is exceptional among biodegradation plasmids and sequencing of the whole plasmid seemed

convenient to verify the authenticity of the *phe*-operon and to investigate the method of integration of the *pheBA* operon into a novel plasmid replicon.

2.2.1. *pheBA* operon in the pAM10.6 (Reference I)

We did not detect any differences between the sequences of the original *pheBA* operon and the *phe*-genes present in pAM10.6. However, in the case of pAM10.6 the *tnpA* of the IS*1411* (ORF2) was truncated 80 nucleotides from its start (Fig. 5, reference I) by the 240-bp DNA segment, designated ARMphe. The second, identical copy of the ARMphe was found at the beginning of the *phe* operon (Fig. 5, reference I). There is 60 nt between the –35 sequence of the *pheBA* promoter and the 3´end of the upstream repeat of ARMphe. ARMphe has an IS-like structure with 39-nt inverted repeats (IR) at both ends. IRs of ARMphe reveal remarkable sequence homology to the left hand IR of the class II transposon Tn*4654* (Tsuda *et al*., 1989; Fig. 5, reference I). Thus, the *phe* operon on pAM10.6 flanked by IS-like DNA elements looks like a composite transposon. However, in the sequence of the ARMphe no open reading frames (ORF) encoding transposase characteristic to transposons or insertional elements is included but it is known that efficient *recA*-independent DNA rearrangements could be mediated by inverted repeats alone (Bi and Liu, 1996). Therefore, the ARMphe element could play the same role in formation of plasmid pAM10.6.

2.2.2. pAM10.6 encodes σ^S regulated catalase (Reference II)

Sequencing of the pAM10.6 revealed an ORF 1,518-bp in length with direction of the transcription opposite to *phe* genes (Fig. 1A, reference II). This open reading frame encodes 506 amino acids, corresponding to the protein with a M_r of 57 kDa. The putative ribosome-binding site was found 6-bp upstream of the putative ATG start codon (Fig. 2, reference II). Further upstream, at position –212 relative to this ATG, a sequence CTATACT, that matches exactly the proposed consensus for –10 sequence of promoters recognized by the alternative sigma factor, σ^s (Espinosa-Urgel *et al*., 1996) was detected. Searches in databases with deduced amino acid sequence derived from this ORF revealed similarity to the catalase sequences from *Neisseria meningitidis* (Parkhill *et al*., 2000) and *Haemophilus influenzae* (gene *hktE*) (Bishai *et al*., 1994), showing respectively 83% and 81% identity over 500 aa. The gene was thereby expected to encode a monofunctional catalase and was designated *katA*. The functionality of *katA* was verified by measuring enzyme activity (Fig. 3, reference II) and also by demonstrating the ability of the *katA* gene product to detoxify H_2O_2 (Fig. 4, reference II).

2.2.2.1 The regulation and function of KatA (Reference II)

The expression of catalase is tightly regulated in most bacterial species studied. The catalase synthesis increases in response to oxidative challenge or when the cells enter the stationary phase of growth (Demple, 1991). In the majority of cases, maximum levels of these enzymes are observed when the cells enter and remain in the stationary phase of growth (Loprasert *et al*., 1996). In the case of pAM10.6, significant catalase activity was detected during all phases of growth but the amount of catalase was increased over 4- fold when cells entered the stationary growth phase (Fig. 6, reference II) suggesting that the KatA is a stationary phase catalase. Stationary-phase inducible catalases are usually regulated by σ^S (Loewen and Hengge-Aronis, 1994; Schellhorn and Hassan, 1988). We controlled σ^S dependent regulation of the *katA* expression by measuring the catalase activity also in σ^S-deficient strains. The catalase activities in transformants were measured in stationary phase cultures. As expected, the KatA activity in *P. putida* σ^S-deficient strain PKS54 was 3–4-times lower than in the wild-type PaW85, whereas the difference with *E. coli* was 2–3 fold (Fig. 3, reference II), suggesting σ^S dependent regulation of KatA.

Catalases are not essential for growth and survival of bacteria under common laboratory conditions but are often unavoidable for successful manage in environment. According to the literature, enhanced catalase level in bacteria is associated with defense of pathogens against oxidative agents of the host (Haas and Goebel, 1992; Chamnongpol *et al*., 1995; Rocha and Smith, 1997). So far, there are no data about the relation between the catabolism of aromatic compounds and catalase activity. There are no other genes beside the *katA* and the *pheBA* operon outside the pAM10.6 basic replicon, and we can speculate that the *katA* might have played a significant role for the host before the insertion of the *pheBA* operon into the pAM10.6. Due to this DNA-acquisition the strain *P. fluorescens* Cb36 should have evolved from the plant-associating strain to the strain with biodegradative abilities. Phylogenetic analysis of the amino acid sequences of catalases indicated non-pseudomonad origin, suggesting horizontal transfer of the *katA* gene. Also, the GC ratio of *katA* nucleotide sequence diverges significantly from the GC ratio of its host genome, supporting the assumption of horizontal transfer. Similarly, the phylogenetic comparision of catalase-peroxidases revealed that plasmid-encoded KatP from *E. coli* O157:H7 could be transferred from other organism (Klotz *et al*., 1997).

Therefore, pAM10.6 could be viewed as the plasmid consisting of blocks of genes acquired by horizontal gene transfer.

2.2.3. Structure of the pAM10.6 basic replicon

2.2.3.1. Maintenance functions (Reference III)

One of the important factors influencing the maintenance of catabolic plasmid in the natural environment is the stability of the plasmid in the host organism also under nonselective conditions (Sayler *et al*., 1990). Several mechanisms that reduce the plasmid loss have been discovered (Gerdes *et al*., 1986; Nordström and Austin, 1989; Rawlings, 1999). The comparison of the nucleotide sequence of the residual region (excluding the *pheBA* operon and *katA*) of pAM10.6 on BLAST server showed the presence of the two putative ORFs (Fig. 1A and Fig. 2, reference III) on the complementary strand to the leader region of the *phe*-genes mRNA. The predicted amino acid sequences of these open reading frames showed significant similarities with poison-antidote plasmid addiction system proteins PasA and PasB of pTF-FC2 (Smith and Rawlings, 1997) (76 and 77% overall amino acid identity, respectively). The *pasA* gene encodes an antidote, *pasB* encodes a toxin, and *pasC* encodes a protein that enhances the neutralizing effect of the antidote (Dorrington and Rawlings, 1990; Smith and Rawlings, 1997). Like in the case of pTF-FC2, the beginning of the pAM10.6 *pasB* gene locates inside the coding region of *pasA* (Fig. 2, reference III) but the *pasC* gene analog is missing in pAM10.6. We examined the role of pAM10.6 *pas* genes on plasmid stability and found out that the *pas*-system of the plasmid pAM10.6 stabilizes plasmid maintenance in *P. putida* cells under non-selective conditions for at least 200 generations (Fig. 3, reference III).

2.2.3.2. Replication functions (Reference III)

The search for open reading frames of pAM10.6 showed that there were two putative ORFs upstream to the *pas*-operon. The sequence alignment of the deduced amino acid sequence of the first ORF showed some identity with Rep proteins of several plasmids. Therefore this gene was named as *repA* (Fig. 1A, reference III). The plasmid with deletion in this gene was not able to replicate in *P. putida* PaW85 (see pMPKΔEB in Fig. 1B, reference III). This suggests that *repA* encodes a replication protein. *repA* encodes 204 amino acids and has a putative Shine-Dalgarno sequence GAA four nucleotides upstream of the initiation codon (Fig. 2, reference III).

The short (399 bp) ORF downstream of *repA* showed no considerable similarity with the known protein sequences and proved to be not necessary for plasmid maintenance.

2.2.3.3. Host range of the pAM10.6 (Reference III)

A Km-resistant minireplicon of pAM10.6 (*pheBA* operon and *katA* gene replaced with Km resistance gene) was used to determine host range of this plasmid. According to our results, host range of the pAM10.6 is limited with pseudomonads. It was also determined that pAM10.6 is not conjugative plasmid and even not mobilizable with the help of IncP plasmids. Phe+ conjugants were obtained only with Cb36 as the donor strain where pAM10.6 was transferred together with the large plasmid of this strain. Therefore, it is most likely that pAM10.6, as it is in present, has formed in the current host due to the sequential linking of the beneficial genes acquired through horizontal gene transfer.

2.3. The flanking sequences of the acquired *pheBA* operon in plasmids of indigenous strains other than Cb36 (pAM10.6) (Reference IV)

Up to date, there is a little information about the neighboring sequences of acquired genes transferred otherwise than in composition of conjugative plasmids. Restriction fragment length polymorphism analyses and subsequent hybridizations are favorable methods used to determine the structure and localization of transferred catabolic genes (Stuart-Keil *et al*., 1998; Peel and Wyndham, 1999; Hohnstock *et al.*, 2000). We have determined the up- and downstream sequences of the horizontally transferred *pheBA* operon. We also suggest possible DNA rearrangement mechanisms occurred during acquisition of a new genetic material.

2.3.1. Rearrangement of the *phe*-operon during acquisition (Reference IV)

Hybridization data showed, that in some cases during the acquisition the *phe*-DNA has undergone several deletions that lead to a loss of the one or both flanking IS elements (2.1.3.). The determination of the nucleotide sequence of the up- and downstream areas of the re-detected *phe*-operons adjusted the absence of these elements.

B1/25 is the only strain lacking upstream insertional sequence IS*1472*. In contrast to IS*1411*, that is demonstrated to be an active IS-element (Kallastu *et al*., 1993) there is no data about IS*1472* ability to excise from target DNA. Interestingly, the deletion of this IS element has occurred exactly between the cleavage sites of the restriction endonuclease *Afl*II (CTTAAG) locating just before and after the coding region of the IS*1472* (Fig. 1A, reference IV).

According to the sequence data, there are two distinct reasons for the absence of IS*1411* specific sequence among five investigated plasmids. As previously described (2.2.1.), in the case of the pAM10.6 of the strain Cb36, the *tnpA* of the IS*1411* was truncated by the IS-like element ARMphe. The same event has happened in the strains MB2.12 and 5F6 where ARMphe have inserted into IS*1411* at the very same site as in the pAM10.6 (Fig. 1B, reference IV). The possible role of the ARMphe in distribution of the *phe*-operon is discussed below (2.3.3.).

The second reason for the absence of the IS*1411* specific sequence was found to be excision of the whole IS-element. According to Kallastu *et al*. (1998), IS*1411* produces IS minicircles composed of the complete IS*1411* with additional 5-bp fragment separating two IS ends. This 5-bp sequence was derived from the 3'end of the *pheA* gene just flanking the left IR of IS*1411* (Kallastu *et al*., 1998). In the case of 2.67 and B1/25, the lost of IS*1411* has occurred in the same way. Hereby, we suppose that excision of IS*1411* has occurred before the acquisition of the *phe*-operon by B1/25 and 2.67 as the sequence following *pheA* in the PHE-plasmids is also identical in both cases.

2.3.2. The organization of the *phe*-operon in the PHE-plasmids of the *P. fluorescens* strains MT4/4, JP1/5 and MB3/26 is possibly the closest to the *phe*-operon released (Reference IV)

The genes encoding regulatory proteins of aromatic catabolic pathways are often found in close proximity to the catabolic genes. Frequently, these regulators are divergently transcribed from the genes they regulate (reviewed in McFall *et al*., 1998; Diaz and Prieto, 2000). The sequence of the *phe*-operon (GenBank accession number 57500) did not reveal gene for the regulatory protein and the *phe*-operon is showed to be regulated by the chromosomally encoded CatR (Kasak *et al*., 1993). However, during the laboratory experiments 17-kb transposable DNA element from the chromosome of the *P. putida* PaW85 had inserted directly upstream of the promoter sequence of the *pheBA* gene cluster resulting the plasmid pEST1026 (Kivisaar *et al*., 1990). Therefore it was not possible to detect presence of the possible upstream-located gene for regulator protein of the *pheBA* operon.

According to our data, only *P. fluorescens* MT4/4, JP1/5 and MB3/26 have the *pheBA* operon flanked by two original IS elements (IS*1411* and IS*1472*) in plasmids of different sizes (Reference I). Analyses of the flanking sequences of the *phe* operon of these strains exhibited complete identity. Upstream to IS*1472* on the opposite transcription direction is located an ORF which derived amino acid sequence has 50–52% identity (over 189 amino acids) with amino acid sequences of the transcriptional regulator proteins (CatR) from different pseudomonads (e.g. GenBank accession numbers AAG05898, AAK33065, BAB21461). Hereby, the promoter regions of the *pheBA* operon and putative

regulator gene must overlap (Fig. 2, reference IV). This is also in accordance with the data published about LysR type transcription regulators (Rothmel *et al*., 1990). Therefore, we suppose that the upstream located open reading frame coding for putative regulator protein is originating from the original PHE-plasmid once released. We conclude that the organization of the *phe*-operon in PHE-plasmids of the *P. fluorescens* strains MT4/4, JP1/5 and MB3/26 is possibly the closest to the *phe*-operon once released.

2.3.3. Possible transfer mechanism of the *pheBA* operon between different plasmid replicons (Reference IV)

Data obtained show that almost in all cases (except in Cb36 and MB2.12) the additional transposase genes are locating nearby to the acquired *phe*-genes (Fig. 1B, reference IV). Therefore we propose transposase mediated transfer as the major transfer mechanism of the *pheBA* operon between different plasmid replicons. Subsequently, ARMphe seems to play an important role in the distribution of the operon. Interestingly, in the cases where two copies of ARMphe were presented (Cb36 and MB2.12; Fig 1B, reference IV), there was no transposase gene observed around suggesting distribution of the operon possibly through these elements. Recently there are several reports describing small (100–200-bp) nonautonomous but still mobilizable insertion sequence derived elements from *Neisseria* ssp. (*nemis*) and *Streptococcus pneumoniae* (RUPs) (Mazzone *et al*., 2001; Oggioni and Claverys, 1999). ARMphe corresponds diversely to the characteristics described for these miniature insertional elements. *Nemis* and RUPs generate specific 2-bp target site duplication (TA) upon insertion (Mazzone *et al*., 2001; Oggioni and Claverys, 1999) also as ARMphe (Fig. 3, reference IV). Furthermore, ARMphe locates just adjacent to IS*1472* and inside of IS*1411* (Fig. 1B; reference IV). Mazzone *et al*. (2001) have shown that 30% of *nemis* found in *N. meningitidis* have inserted into or next to larger IS. A related type of nonautonomous transposon has also been described in *B. cereus*. This element, MIC231, is composed of two left ends of IS*231* flanking an endopeptidase gene and can be translocated by the IS*231* transposase supplied in trans (Chen *et al*., 1999).

There is only evidence about possible involvement of a homologous recombination event in the linkage of the acquired *phe*-DNA into the composition of a host plasmid-DNA. The sequencing results of the *pheBA* upstream region of the strain 5F6 demonstrated that the identity of the determined sequence compared with the original *tnpA* of the IS*1472* was only 87%. *tnpA* of IS*1472* is sharing high similarity (99–86%) with transposase genes of many different sources (mainly from pseudomonads) indicating a wide spread of this type of elements. Therefore we suppose that in the case of 5F6 the homological recombination has occurred between transposase gene of acquired *pheBA* operon and pre-existing transposase gene of a host strain.

CONCLUSIONS

1. The genetic material (*pheBA* operon) released into the environment persisted and distributed through lateral gene transfer among indigenous microbial isolates under selective conditions during several years. Nine different *Pseudomonas* strains harboring horizontally transferred *pheBA* operons were investigated.
2. During the acquisition several rearrangements have occurred in the organization of the *pheBA* operon. Still, in the eight cases out of nine, re-detected *phe*-genes were connected to the same promoter as in the original *pheBA* operon and maintained the same regulation.
3. In most cases, additional insertional elements or genes encoding transposases were found nearby to the acquired *phe*-DNA. Therefore, transposase mediated transfer is the most probable distribution mechanism of the *pheBA* operon.
4. The characterization of the structure of one *de novo*-generated PHE plasmid suggests sequential acquirement of the genes allowing the host adaptation with environmental conditions. The genes encoding persistence functions (*pasAB*) are determining the plasmid maintenance under nonselective conditions when the catabolic genes are not expressed.

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SUMMARY IN ESTONIAN

Uurimus *pheBA* operoni horisontaalsest levikust looduslikes tingimustes

Horisontaalne geenitriiv on geneetilise info mittepärilik ülekandumine ühest organismist teise. See on prokarüootide hulgas laialt levinud protsess ning on mitmeid mehhanisme, mis võimaldavad bakteritel omandada võõrast ning levitada oma geneetilist materjali. Omandatud geenidel on siiski erinev potentsiaal populatsioonis kinnistumiseks ning tavaliselt on võõr-geenide edukaks püsimajäämiseks vajalik nende selektiivne väärtus antud tingimustes. Tööstuses ning põllumajanduses tekkinud toksiliste jäätmete sattumine looduslikku keskkonda loob tugeva selektiivse surve neid aineid lagundada võimaldavaid ensüüme kodeerivate geenide levikuks bakterite hulgas.

1988.a. "Estonia" kaevanduse tulekahju järgselt tekkinud fenoolse reostuse vähendamiseks kasutati fenooli lagundamist määravaid geene (*pheBA*) kandvaid laboratoorseid mikroorganisme. Kui fenoolide kontsentratsioon kaevanduse vees oli vähenenud lubatud tasemeni, lasti see vesi ümbruskonna jõgedesse. Seal lähedal asus ka pideva fenoolse reostuse allikas, tuhamäed, kust fenoolsete komponentidega reostatud tehnoloogiline vesi voolas Kohtla ja Purtse jõkke. Käesoleva töö eesmärgiks oli kontrollida, kas loodusesse vabastatud laboratoorsed *phe*-geenid on pideva fenoolse reostuse tingimustes püsima jäänud ning kas ja kuidas on toimunud nende geenide lülitumine kohaliku bakteriaalse mikrofloora geneetilise materjali koosseisu.

Me leidsime, *et al*gsed *phe*-geenid on fenoolse reostusega jõgedes säilinud ning omaks võetud kohaliku mikrofloora poolt. Me uurisime üheksat uut *phe*-operoni kandjat ning tegime kindlaks, et *pheBA* geenide lülitumisel peremeestüvede genoomi on toimunud mitmeid geneetilisi ümberkorraldusi operoni struktuuris, kuid vähemalt kaheksal juhul on säilinud algne regulatsioonitüüp. Kaheksal juhul on *phe*-geenid integreeritud peremeestüvedes sisalduvate plasmiidide koosseisu, ühel juhul on toimunud lülitumine kromosomaalse DNA koostisesse. Kuna uuritavate plasmiidide suurused ning restriktsioonanalüüs osutasid erinevatele replikonidele, tekkis küsimus: kuidas on *phe*-geenid levinud? Et kindlaks teha, milline võiks olla uuritavate geenide levikumehhanism, analüüsisime omandatud geenidega piirnevate alade nukleotiidset järjestust. Selgus, et kõigil vaadeldud juhtudel asuvad operoni läheduses insertsioonilised elemendid või transposaasi geenid. Nendest andmetest lähtudes eeldame, et *pheBA* operoni põhiliseks levikumehhanismiks kohaliku mikrofloora hulgas on olnud transposaaside vahendatud ülekanne.

Leidmaks kinnitust väitele, et tegemist on tõepoolest algse *phe*-DNA-ga, analüüsisime üht neid geene kandvat plasmiidi lähemalt. Selle plasmiidi täieliku nukleotiidse järjestuse määramine kinnitas *phe*-DNA autentsust. Sama plasmiid kannab monofunktsionaalset katalaasi kodeerivat geeni *katA*, mille järjestuse

analüüs lubab samuti oletada selle geeni omandamist horisontaalse geeni-ülekande teel. *katA* ekspressioon on indutseeritud rakkude statsionaarses kasvufaasis ning reguleeritud läbi statsionaarse faasi sigma faktori. *katA* geeni produkt on võimeline kaitsma bakteri rakke kõrge vesinikperoksiidi kontsentratsiooni eest. Seega võib öelda, et on toimunud bakteri jaoks kasulike geenide järjestikuline horisontaalne ülekanne ning lülitamine tema geneetilise info koosseisu.

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Teadustegevus

Alates 1994. aastast olen tegelenud biodegradatiivsete geenide horisontaalse geenitriivi uurimisega ning selle tagajärjel moodustunud uute geneetiliste üksuste struktuuri iseloomustamisega.