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JEKATERINA JUTKINA

The horizontal gene pool for aromatics degradation: bacterial catabolic plasmids of the Baltic Sea aquatic system

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the Baltic Sea aquatic system

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PRESS

Department of Genetics, Institute of Molecular and Cell Biology, University of Tartu, Estonia

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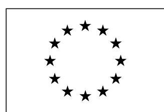

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original papers that will be referred in the text by Roman numerals I–III.

I Heinaru, E., Vedler, E., **Jutkina, J.**, Aava, M., Heinaru, A., (2009). Conjugal transfer and mobilization capacity of the completely sequenced naphthalene plasmid pNAH20 from multiplasmid strain Pseudomonas fluorescens PC20. *FEMS Microbiology Ecology*. 70, 563–574.

II **Jutkina, J.**, Heinaru, E., Vedler, E., Juhanson, J., Heinaru, A., (2011). Occurrence of Plasmids in the Aromatic Degrading Bacterioplankton of the Baltic Sea. *Genes*. 2, 853–868.

III **Jutkina, J.,** Hansen, L. H., Li, L., Heinaru, E., Vedler, E., Jõesaar, M., Heinaru, A., (2013). Complete nucleotide sequence of the self-transmissible TOL plasmid pD2RT provides new insight into arrangement of toluene catabolic plasmids. *Plasmid*. 70, 393–405.

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Author's contribution:
Ref. I – contributed to experiments and data analysis: sequenced and performed comparative analysis of the plasmids.
Ref. II – designed and performed experiments with isolated catabolic bacterial strains, wrote the paper.
Ref. III – designed and performed the experiments and wrote the paper.

The isolation of the bacterial strains used in this study has been undertaken by Eeva Heinaru.

ABBREVIATIONS

aa	amino acid(s)
Benz	benzoate
bp	base pair(s)
CP	coupling protein
DR	direct repeats
HGT	horizontal gene transfer
Inc	incompatibility
IR	terminal inverted repeat
IS	insertion sequence
kb	kilobase(s)
LGT	lateral gene transfer
MGE	mobile genetic element
MOB	mobility
MPF	membrane-associated mating pair formation
MRS	multimer resolution system
NAH/SAL/TOL plasmid	plasmid carrying genes for degradation of naphthalene/ salicylate/toluene
Nah	naphthalene
ORF	open reading frame
PAH	polycyclic aromatic hydrocarbons
PAR	active partitioning
PCB	polychlorinated biphenyl
Phe	phenol
PSK	postsegregational killing
Rep	replication initiation protein
Sal	salicylate
sRNA	small non-coding RNA
TA	toxin-antitoxin
TCA	citric acid or tricarboxylic acid
Tol	*m*-toluate
T4SS	type IV secretion system
Xyl	xylene/toluene

1. INTRODUCTION

Aromatic compounds are highly diverse and abundant components in the biosphere, serving as simple building blocks (aromatic amino acids) or more complex substances (lignin) for living organisms. Despite their essential role in the molecular architecture of life, different aromatic compounds nowadays constitute a remarkable part of environmental pollution and are considered hazardous due to their stability and persistence in the environment as well as their toxicity to the biota. Polychlorinated biphenyls (PCBs), different polycyclic aromatic hydrocarbons (PAHs) including fluorene, anthracene and phthalate, PAH-metabolites, and alkylphenolic compounds are among the most frequently found hazardous substances in the Baltic Sea (HELCOM, 2010; Kreitsberg et al., 2012; Potrykus et al., 2003). Oil is considered to represent the major threat to the Baltic Sea ecosystem due to constantly increasing shipping traffic with additional loads of numerous pollutants originating from the catchment areas of rivers running into the Baltic Sea. Estonian oil shale mining and processing industry is one of the active pollution hot spots in the Baltic Sea region (HELCOM, 2010). The extent of damage caused by oil pollution after it enters the marine environment is strongly dependent on undergoing modification processes as well as oil composition and the particular environmental conditions. Biodegradation, the utilisation and detoxification of contaminants by microorganisms, is considered to be the most important part in the oil breakdown process (ITOPF, 2002; Prince, 2010).

Isolates of numerous bacterial genera display the ability to use aromatic hydrocarbons as the sole or major carbon and energy source with strong dominance of proteobacterial representatives (Prince et al., 2010). Existence of a wide variety of hydrocarbon-degraders among bacteria can be partially explained by their high adaptability to constantly changing environmental conditions. Plasmids, along with other mobile genetic elements, have been elucidated to contribute significantly to the bacterial adaptation process (Frost et al., 2005). Catabolic plasmids deserved special attention as powerful tools for dissemination of catabolic genes in the bacterial community. Research on catabolic genes and their dissemination is important in terms of environmental oil-contamination threat (Top et al., 2002).

Despite comprehensive studies on catabolic plasmids worldwide, catabolic plasmids in bacteria from the Baltic Sea have remained poorly highlighted. Considering the special characteristics of the Baltic Sea such as low salinity level and slow rate of water exchange, the research on the local degradative inhabitants along with the relevant pool of mobile genetic elements are essential objects for research in order to understand the effective biodegradation mechanisms for this ecosystem.

The work presented in this thesis contributes to the knowledge of the Baltic Sea mobile genetic pool of degradative bacteria, with emphasis on large self-transmissible plasmids for spread of catabolic genes.

2. LITERATURE REVIEW

2.1. Catabolic pathways for aromatics degradation

Bacteria have evolved numerous mechanisms, which confer them with tolerance and survival under environmental pollution. A highly diverse range of bacteria, equipped with various catabolic genes necessary for degradation of aromatic compounds, have been isolated and characterized (Prince et al., 2010) and relevant new data is increasing rapidly due to the extended usage and availability of high-throughput technologies. Full genome sequencing of environmentally important bacterial strains such as *Burkholderia vietnamiensis* G4 (O'Sullivan and Mahenthiralingam, 2005), *Pseudomonas putida* KT2440 (Jiménez et al., 2002), *Cupriavidus necator* JMP134 (Pérez-Pantoja et al., 2008) and many others have contributed significantly to the understanding of catabolic processes and adaptation strategies of relevant degraders. In case of biodegradation studies, the term catabolism is frequently used in a very narrow meaning encompassing the processes of breakdown of different aromatic substances, which in the final stage end up as citric acid cycle (TCA) intermediates. Consequently, catabolic pathways are subdivided into central and peripheral routes (Figure 1). The degradation of aromatic compounds initially occurs through the action of peripheral pathway enzymes, resulting in the formation of central intermediates such as (chloro)catechol, gentisate and protocatechuate, which are subsequently channelled into TCA through central catabolic routes (Harwood and Parales, 1996). The aromatics degradation process begins with activation of a stable aromatic ring, which in aerobic conditions is conducted by oxygenases. These peripheral pathway enzymes prepare substrates for subsequent cleavage reactions by incorporating oxygen atoms into the aromatic ring (Harayama et al., 1992). Further transformation of obtained metabolites is conducted by aromatic ring cleavage dioxygenases, resulting in the formation of ring-opened intermediates which are directed to central catabolic routes. Which pathway is undertaken depends on the cleavage manner. If cleavage occurs between two hydroxyl groups it is the *ortho*-cleavage pathway, while cleavage adjacent to hydroxyl groups takes place in the *meta*-cleavage pathway (Vaillancourt et al., 2006). Frequently, catabolic pathways for complete or partial degradation of aromatic compounds are carried by extrachromosomal genetic elements, plasmids, enabling mobility of this genetic load within a genome and its lateral transfer to other bacteria. Lateral (horizontal) gene transfer (LGT, HGT) is considered a major force of rapid evolution of catabolic pathways (Dennis, 2005; Fulthorpe and Top, 2010; Springael and Top, 2004).

As discussed in reviews by van der Meer (van der Meer, 2006) and Williams (Williams, 2004), novel catabolic capacities may evolve through extended rearrangements of entire catabolic clusters or separate genes originating from different phylogenetic sources being introduced together. As a result, complex catabolic routes remind mosaics consisting of different separate modules assembled together into complete patterns. At present, several substantial

aspects of major plasmid-encoded catabolic routes (Figure 1) for degradation of aromatic pollutants have been outlined: (i) catechol is the most common central intermediate so far observed; (ii) degradation of toluene and naphthalene mostly occurs via the *meta*-catabolic pathway whereas chlorinated aromatic compounds are usually degraded via the *ortho*-catabolic route; (iii) the recent finding of naphthalene degradation via gentisate as central intermediate (Izmalkova et al., 2013) and degradation of chlorocatechols via the *meta*-catabolic pathway (Kunze et al., 2009) suggests the evolvement and spread of alternative catabolic pathways for degradation of the same aromatic compounds. Another good example of the plasmid-associated evolvement of the aromatic substrate utilization range is bacterial degradation of nitroaromatic compounds that is mediated by toluene catabolic (TOL) plasmids (Kulkarni and Chaudhari, 2007).

Our current knowledge about plasmid-associated catabolic genes is based on a modest number of well characterised completely sequenced catabolic plasmids and mostly on catabolic pathways which location is proven to be plasmid-born. New data derived from extensive sequencing projects contributes to our understanding of the evolution, the entire complexity and overall redundancy of plasmid-associated catabolic routes (Nojiri et al., 2009; Van der Meer, 2007; Williams, 2004).

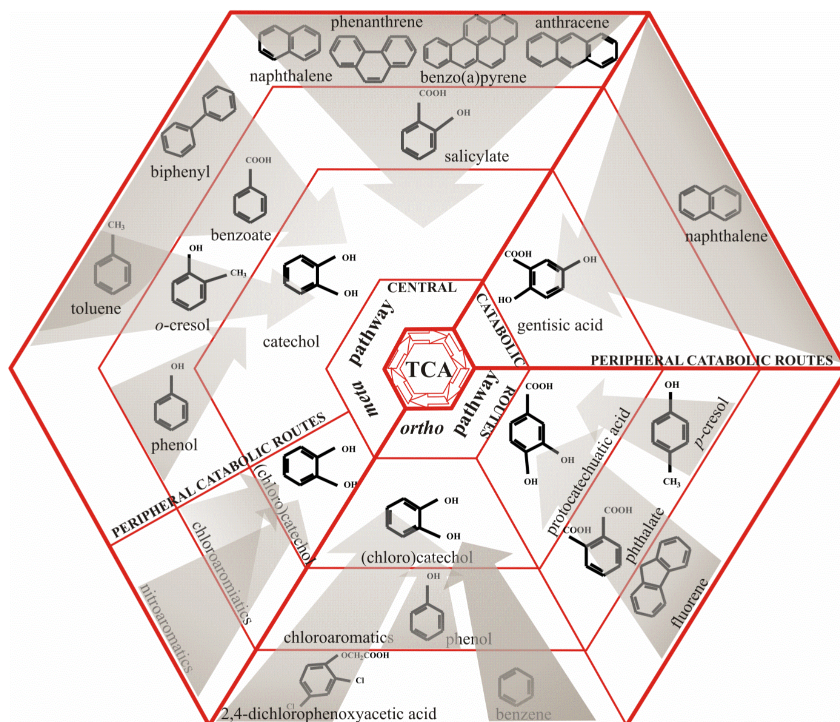

Figure 1. The major plasmid-encoded catabolic routes for degradation of aromatic compounds. Shaded arrows show the direction of catabolic routes from the peripheral to the central intermediates.

2.2. Dissemination of catabolic genes

Dissemination of catabolic genes by HGT is commonly associated with mobile genetic elements (MGEs), where plasmids play a significant role. Catabolic plasmids are considered well adapted vehicles of inter-genomic gene spread and main determinants of rapid evolution of catabolic pathways (Schmidt et al., 2001). It has been shown that catabolic genes are often located in close proximity to transposable elements, which may contribute to extensive intra-genomic rearrangements leading to expansion of catabolic properties and adaptation to novel pollutants (Tan, 1999).

Plasmid-encoded catabolic genes are commonly encompassed in class I (composite) and class II (Tn*3* family) transposons. Composite catabolic transposons consist of two copies of highly similar insertion sequence (IS) elements with catabolic genes in-between them. Commonly, class I catabolic transposons encode only a part of catabolic pathways, preferentially peripheral or so-called *upper* pathway enzymes. For example transposons Tn*5542* and Tn*5280* carry genes for degradation of benzene and chlorobenzene to catechol, respectively. The mobility of composite transposons is limited by size with bigger catabolic load diminishing the transposition frequency (Tsuda et al., 1999). Contrary to class I transposons, an entire catabolic pathway along with its regulatory genes is often encoded on large class II (Tn*3* family) transposons. Besides catabolic gene clusters the composition of class II transposons generally includes terminal inverted repeats (IRs), genes encoding transposase and resolvase, *tnpA* and *tnpR*, and a resolution site *res*. Effective transposition of large and structurally different catabolic transposons has been shown since it was initially proven through analysis of transposons Tn*4651* and Tn*4653*, which carry genes for toluene degradation (*xyl*), found on plasmid pWW0 of *Pseudomonas putida* strain mt-2 (Tsuda et al., 1989). Plasmid pWW0 is currently an object for intensive studies on the architecture of catabolic pathways with regard to their regulatory aspects (Silva-Rocha et al., 2011).

Evolution of catabolic pathways has been intensively studied on the basis of catabolic plasmids carrying genes for degradation of toluene (*xyl*) and naphthalene (*nah*), referred to as TOL and NAH plasmids (Yano et al., 2007). Although a number of studies indicates variation in organization of *xyl* genes clusters in different bacterial isolates, their formation remains unclear due to the lack of information regarding the corresponding MGEs (Chatfield and Williams, 1986; Sentchilo et al., 2000). Determination of complete nucleotide sequences of catabolic plasmids has accelerated the process of uncovering the probable mechanisms for assembly and dissemination of catabolic gene clusters. This can be illustrated by two examples. (i) The formation of the catabolic region of TOL plasmid pDK1, which shares a common ancestor with another TOL plasmid, pWW53, occurred through three subsequent recombination events, leading to a loss of *xyl meta* 1 operon, while pWW53 contains both *meta* 1 and *meta* 2 operons (Yano et al., 2010). (ii) A single recombination event between homologous *res* sites of transposons Tn*4660* and Tn*4658* of the plasmid pWW53 resulted in

formation of the new active transposon Tn*4656* which carries the entire set of *xyl* genes for toluene degradation (Tsuda and Genka, 2001; Yano et al., 2007).

(Meta)genome-based studies facilitate the detection of HGT events, and they are advancing attempts to estimate the probable extent and relevance of HGT for bacterial communities in general (Tamames and Moya, 2008). Nevertheless, detection of HGT beyond the test tube and (meta)genome sequencing analysis directly in natural environments still remain a challenge (Fulthorpe and Top, 2010).

2.3. Classification and bacterial host range of catabolic plasmids

Initially the research on dissemination of catabolic genes was focused on bacteria of the genus *Pseudomonas*, gram-negative aerobic *Gammaproteobacteria* that are ubiquitous in the environment and catabolically versatile, and their plasmids (Schmidt et al., 2001). The majority of catabolic plasmids isolated so far that carry genes for degradation of naturally occurring polluting compounds, such as toluene and naphthalene, have been isolated from different pseudomonads. Many of them belong to the plasmid groups IncP-2, IncP-7 and IncP-9 (Dennis, 2005). IncP-7 plasmids are considered to have a narrow host range compared to IncP-9 family members because their existence and transferability is found to be likely restricted to the genus *Pseudomonas* (Shintani et al., 2005). However, a conjugation experiment conducted in river water samples by Shintani and colleagues (Shintani et al., 2008) have demonstrated the transfer of IncP-7 plasmid pCAR1 to a *Stenotrophomonas* strain. Considerably wider hosts range is found for broad-host-range catabolic plasmids of the IncP-1 group, which are commonly associated with degradation of man-made (xenobiotic) chemicals (Top et al., 2000). Along with pseudomonads, the IncP-1 plasmid bearing strains represent phylogenetically distant bacteria, belonging to different proteobacterial genera (Norberg et al., 2011; Suzuki et al., 2010).

Members of each Inc family have a similar backbone region, in particular the region encoding replication functions, while the additional catabolic load they carry may vary. Assignment of plasmids to incompatibility (Inc) groups is the basic classification of plasmids currently used, and grew out of the originally conducted incompatibility studies. Based on this classification two closely related plasmids, belonging to the same Inc group, display the inability to stably coexist in the same bacterial cell (Cook et al., 2001; Novick, 1987). The current classification is convenient for *Pseudomonas* plasmids, while a growing number of sequenced catabolic plasmids from other taxonomic groups have remained unclassified. Recent genome-wide studies brought to light the significant role of catabolic plasmids in such biodegraders as members of genera *Burkholderia* (*Betaproteobacterium*) and *Sphingomonas* (*Alphaproteobacterium*) (Nojiri et al., 2009; O'Sullivan and Mahenthiralingam, 2005).

It was found that catabolic plasmids share common features within the particular bacterial taxonomic groups. Completely sequenced catabolic plasmids pCAR3 (Shintani et al., 2007) and pNL1 (Romine et al., 1999) of *Novosphingobium* sp. KA1 and *Sphingomonas aromaticivorans* F199, respectively, are phylogenetically closely related to each other and distantly related to catabolic plasmids of pseudomonads (Nojiri et al., 2009). General plasmid classification and their host range remain an important topic of debates and intensive studies. Along with Inc-specific replicon typing, alternative methods, for example classification of conjugative transfer systems (Garcillan-Barcia et al., 2009), have been proposed to improve plasmid taxonomy and monitoring in the environment. Also, a range of hosts for several Inc groups has recently been revised by undertaking thorough genome-based computational analysis (Suzuki et al., 2010). Suzuki and colleagues predicted that the evolutionary host range of the IncP-9 plasmids is likely broader than estimated so far and may resemble that of members of the IncP-1 family. The ability of plasmids to expand their host range through adaptation to new, even unfavourable, hosts has also been experimentally proven (Garcillan-Barcia et al., 2009).

2.4. General organisation of catabolic plasmids

Catabolic plasmids are usually large (up to several hundreds of kilobases) circular DNA molecules. Linear forms are common for catabolic plasmids residing in gram-positive bacteria, particularly in *Rhodococcus* spp. (Fetzner et al., 2007). The large size of catabolic plasmids is associated not only with the catabolic load they carry, but also with the ability to self-transfer by establishing cell-to-cell contact between a resident donor cell and a recipient cell, so-called conjugation. These plasmids can also serve as helpers for co-resident non-conjugative mobilizable plasmids to disperse laterally among bacteria. Although self-transmissibility is an optional function and is not necessarily required for plasmid existence and stable inheritance, the absence of genes encoding conjugation apparatus is a rather unusual trait among the catabolic plasmids described so far (Li et al., 2013). Being large molecules, catabolic plasmids have a low copy number in the host cell. Their persistence and stability over many bacterial generations is firmly ensured by several different mechanisms: active partitioning, that direct plasmid copies to dividing bacterial cells; multimer resolution, that enables separation of plasmids from each other; and post-segregational killing, which favours proliferation of plasmid-bearing bacteria by causing death of plasmid-free daughter cells after cell division (Thomas, 2000). The autonomous replication and maintenance at a stable copy number are the basic features of a plasmid replicon in general (Nordström, 1985). Control of plasmid copy number is an important aspect of prosperous co-existence of plasmids and their hosts.

All aforementioned functions comprise the plasmid backbone while other incorporated genetic determinants, like genes encoding catabolic pathways, are

termed as accessorial. Similarly to catabolic routes, the backbone regions of plasmids are frequently found to display mosaics, which have arisen by fusion of separate blocks of different origin. It has been proposed that a restricted amount of different plasmid backbones is likely to exist in nature and novel plasmids emerge on their basis (Haines et al., 2005).

2.4.1. Replication

2.4.1.1. Basic features of plasmid replication

The origin of replication (*ori*) is a crucial feature for each replicon. *ori* is a minimal plasmid region, which encompasses essential *cis*-acting elements required for plasmid autonomous replication (Figure 2). It can also be defined as the site where the replication process begins (del Solar et al., 1998). Well studied catabolic plasmids of the IncP group contain the distinct origin of replication *oriV* and replicate by the theta (θ) mechanism (Adamczyk and Jagura-Burdzy, 2003; Dennis and Zylstra, 2004). Basic elements of *oriV* are iterons, directly repeated sequences, to which plasmid-encoded replication initiator protein (Rep) binds. Iterons are essential for initiation of replication process and its control (Chattoraj, 2000). In addition to Rep protein, the important role in replication initiation process belongs to chromosomally-encoded protein DnaA. DnaA binds specifically to DnaA-box sequence and facilitates DNA melting within the AT-rich region of *oriV* and subsequent recruitment of host cell replication factors, helicase and primase (del Solar et al., 1998). The AT-rich region of θ-replicating plasmids usually consists of 13 nucleotide clusters, which are critical for proper *oriV* functioning (Rajewska et al., 2012). Replication initiation may also require several additional host cell factors, IHF (integration host factor), HU (heat-unstable nucleoid protein) and Fis (factor for inversion stimulation), which play structural and stabilising roles (del Solar et al., 1998; Fekete et al., 2006). Formation of a nucleoprotein complex is followed by uni- or bidirectional plasmid DNA replication. In case of unidirectional replication a single replicating fork proceeds after priming of the replication origin takes place and the primer extension process begins. The replicating intermediates resemble the Greek letter theta „θ“.

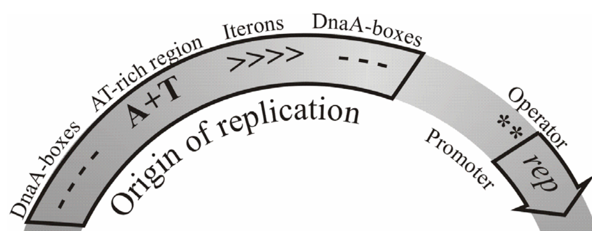

Figure 2. General structure of the *oriV* of a θ-replicating plasmid that is located in close proximity to the replication initiator encoding *rep* gene. The main components and regulatory elements of the *oriV* are outlined.

The described organisation of the replication system is common for the majority of θ-replicating plasmids, though absence of either a Rep-encoding gene or some structural elements within *oriV* region of different plasmids has also been outlined (Bruand et al., 1993; Osborn et al., 2000).

Although theta is the most common replication type, plasmids can also replicate via strand displacement and rolling-circle mechanisms (del Solar et al., 1998). Strand displacement is less abundant, nevertheless broad-host range plasmids of the IncQ family replicate by this model (Sakai and Komano, 1996). Strand displacement does not require any additional host cell factors like DnaA, whose function is accomplished by plasmid encoded replication proteins. Three replication proteins, RepA (helicase), RepB (primase) and RepC (initiator) constitute the replication machinery of plasmids replicating by the strand displacement model. DNA melting occurs within the AT-rich region stimulated by RepA protein. Helicase is recruited by initiator protein, which binds to the iterons of the replication origin. Subsequent priming of *ori* sites followed by bidirectional plasmid DNA replication results in the displacement of the non-template strand (del Solar et al., 1998).

Plasmids replicating via rolling-circle mechanism are usually small, less than 10 kb, cryptic or carrying antibiotic resistance determinants. The replication process begins with introducing a site-specific nick to double-stranded plasmid DNA by the Rep protein. Extension of the released 3`OH end leads to displacement of the nicked DNA strand resulting in formation of one double-stranded DNA molecule and a single-stranded intermediate that is subsequently converted to a double-stranded form (del Solar et al., 1998).

2.4.1.2. Iteron-based plasmid copy number control

Replication initiation requires binding of Rep monomers to *oriV*. Additionally, Rep can form dimers that interact with inverted repeats in the *rep* promoter region, called operator, thereby repressing transcription of its own gene (Giraldo and Fernandez-Tresguerres, 2004). Both monomeric and dimeric Rep forms are also thought to be involved in paring of iterons, originating from separate plasmid molecules, leading to inactivation of respective replication origins (Figure 3). This process is termed handcuffing. The handcuffing system is considered as the main mechanism of plasmid copy number control in iteron-based plasmids (Paulsson and Chattoraj, 2006). Although the importance of handcuffing in control of plasmid replication is indisputable, the precise mechanism and nucleoprotein complex structure still remains unsolved (Rakowski and Filutowicz, 2013).

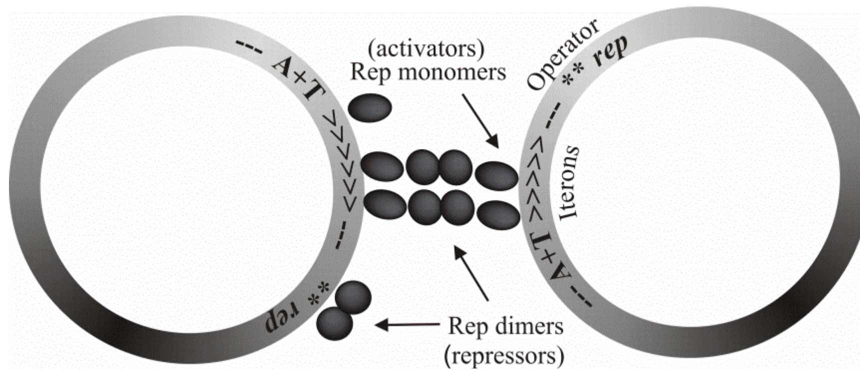

Figure 3. The elements involved in iteron-based plasmid copy number control: Rep dimers and monomers, iterons in *oriV*, operator in the *rep* gene promoter region.

Initiator proteins play a dual role in replication control. The increased concentration of Rep molecules leads to replication abolishment via mechanisms mentioned above, while saturation of iterons by Rep monomers is critical for the replication start. Upon increases in plasmid copy number, redistribution of initiators between separate origins results in their insufficient saturation, which in turn prevents replication start (Chattoraj, 2000). This is called initiator titration. Along with handcuffing, initiator titration is thought to accomplish plasmid incompatibility phenomenon (Rakowski and Filutowicz, 2013).

2.4.1.3. Plasmid replicon type and range of hosts

A lot of plasmids carrying more than one different replicon type have been isolated and characterised, many of them belong to the IncF plasmid family (Szczepanowski et al., 2005). Although the necessity of multiple replicons for plasmid existence is still unclear, it has been suggested that they either broaden host range and help to overcome the incompatibility barrier within the cell or are just a product of plasmid genome reshuffling (Summers, 2009).

Many broad-host-range plasmids of incompatibility group P-1 encode two Rep proteins, TrfA1 and TrfA2, which production is ensured by two different in-frame translational start sites. The shorter protein, TrfA2, is believed to confer plasmid replication of IncP-1 in a broad range of hosts, while TrfA1 is essential for replication in *Pseudomonas aeruginosa* only and can also be important for establishment of efficient plasmid replication in a new bacterial hosts after horizontal transfer (Smith and Thomas, 1984; Yano et al., 2012). Differently to the general model of replication initiation control for iteron containing plasmids, transcription of the *trfA* gene is under control of global regulatory proteins (KorA, KorB, TrbA). It has been suggested that mutations in both, the regulators encoding genes and non-coding plasmid regions likely

influence the plasmid host range leading to their spatial separation and further sequence divergence (Yano et al., 2013).

The ability of plasmids to replicate in a wide range of hosts is not always conferred solely by their minimal replicon features (*oriV* and Rep). For example, thorough studies of IncP-9 plasmid replication revealed the involvement of additional plasmid backbone elements in this process. The necessity of an active partitioning system for autonomous replication of IncP-9 plasmids in *Escherichia coli* was recognized. The partitioning system is encoded by *par* genes located closely to the replicon region (Sevastsyanovich et al., 2005). Recently, the genetic determinants that influence a range of hosts and are not related to replication and partition functions were determined: the *traDEF* genes were found crucial for transfer of naphthalene-catabolic plasmid NAH7 from *E. coli* to *Pseudomonas* (Miyazaki et al., 2008).

2.4.2. Maintenance and stability

Large plasmids imply a heavy metabolic load on the host cell, since their existence is largely dependent on host-encoded products. Crucial processes, such as transcription and translation of plasmid genes, and plasmid replication, are mediated by chromosomally-encoded determinants. Therefore, the copy number of large plasmids is generally very low, restricted only to a few copies per cell. To avoid being lost, large plasmids secure their stable maintenance within a cell culture by applying several different mechanisms: active partitioning (PAR), resolution of plasmid multimers to separate units and post-segregational killing (PSK). All three systems are proposed to be present at each replicon at least for large virulence plasmids of this type (Sengupta and Austin, 2011). Moreover many plasmids carry more than one module of each system. This finding is not restricted to large virulence plasmids only. For example, three different PSK systems have been outlined for the large low copy number plasmid pGRT1 that enables its host, *Pseudomonas putida* strain DOT-T1E, to tolerate high concentration of toluene (Molina et al., 2011).

2.4.2.1. Active partitioning

Large low copy number plasmids have evolved active partitioning (PAR) systems, which secure appropriate segregation of plasmid copies along with dividing bacterial cells (Ebersbach and Gerdes, 2005).

Proper functioning of plasmid-segregating machinery requires three components: a *cis*-acting DNA site, a centromere, and two plasmid-encoded partition proteins. One of these proteins is a motor protein, which is either ATPase or GTPase, and another is a centromere-binding protein (CBP). Formation of the partition complex by means of cooperative binding of centromere-binding proteins to the centromeric site initiates the process of plasmid segregation. Further assembly of a large nucleoprotein complex, segrosome, followed by

subsequent recruitment of motor proteins enables active separation of the plasmid copies (Schumacher, 2008). Based on the properties of known motor proteins (the Walker-type cytoskeletal P loop ATPases, actin-like ATPases and tubulin-like GTPases) three main types of PAR systems have been outlined (Gerdes et al., 2010). The most abundant is the type I system containing ATPase proteins with a variant Walker A box motif, usually referred to as ParA, and their partners, the CBP, called ParB. ParA proteins can be further sub-divided into types Ia and Ib on the basis of size and nucleotide sequence. Type Ia ATPases are larger molecules compared to type Ib representatives. They contain a DNA-binding helix-turn-helix (HTH) motif at their N-terminus, enabling transcriptional autoregulation of their own *par* operons (Gerdes et al., 2000). The size of ParB and corresponding ParA originating from the same operon are positively related. Where large *parA* and *parB* genes of type Ia are found exclusively on plasmids, type Ib *par* loci are found on both plasmids and bacterial chromosomes. ParB proteins of both subtypes contain HTH motifs and N-terminal domains for binding to the centromere and interaction with ParA proteins, respectively (Gerdes et al., 2010).

Plasmid centromeres are usually positioned upstream or downstream of the partition loci and their organization varies significantly. However, well-characterised plasmid centromeres share common elements which are multiple DNA repeat sequences that differ in number, orientation and sequence composition among different plasmid types. The *parS* centromere of plasmid P1, in addition to repeat sequences, contains a binding site for IHF, which mediates the formation of segrosome (Hayes and Barilla, 2006). The centromeric sites of IncP-7 catabolic plasmids are located between the *par* genes in the partition region, that resemble the type Ia partition system (Yano et al., 2010). *parS* regions of these plasmids contain 2 to 4 conserved 17 bp palindromic repeat sequences, which are crucial for the proper functioning of the plasmid-segregating system (Miyakoshi et al., 2012; Yano et al., 2010).

The molecular mechanism of type I partitioning is not clearly determined yet. Two different models have been proposed: (i) the filament pulling model, in which ParA proteins polymerize into filaments (Ringgaard et al., 2009) and (ii) the diffusion-ratchet model, according to which ParB follows the high ParA-ATP concentration on the nucleoid (Vecchiarelli et al., 2012). Comparison of the two models is reviewed in (Szardenings et al., 2011) and briefly introduced on Figure 3.

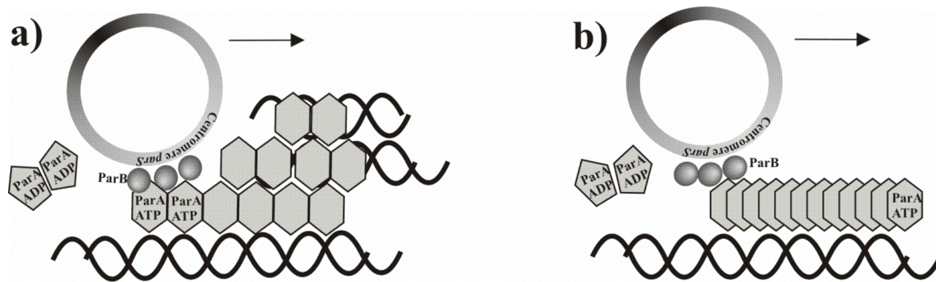

Figure 3. Schematic representation of proposed type I plasmid active partitioning systems: a) the diffusion-ratchet model, b) the filament pulling model. The arrow shows the moving direction of a plasmid-protein complex on the chromosome. Basic elements of the plasmid-segregating machinery are outlined. Brief description of the models: (a) Diffusion-ratchet model. ATP binding causes conformational changes of ParA enabling its nonspecific attachment to the nucleoid and subsequent association with the ParB-plasmid complex. The nucleoprotein complex diffuses randomly until it contacts to the ParA-ATP molecules. ParB stimulates hydrolysis of the bound ParA-ATP releasing ParA and ADP molecules as well as the plasmid-protein complex itself from the nucleoid. Disconnected plasmid diffuses towards new available ATP-bound ParA proteins and the circle repeats. Thus, plasmid driven by centromere-bound ParB proteins follows the ParA-ATP gradient on the nucleoid enabling segregation. (b) The filament pulling model. ATP-bound ParA proteins polymerize into filaments attaching to the nucleoid. A randomly diffusing plasmid becomes connected to the filament by means of interactions with *parS*-bound ParB proteins. The filament begins to disassemble resulting in release of ParA-ADPs and plasmid consistent movement on the nucleoid. The circle repeats when the filament disassembles completely.

2.4.2.2. Multimer resolution

Upon replication, and before plasmids are segregated to daughter cells, the involvement of multimer resolution system (MRS) is usually required to ensure plasmid stability within the bacterial population. Replication and homologous recombination processes lead to formation of plasmid multimers, which in turn results in asymmetric partitioning and subsequent plasmid loss if joined plasmid copies are not separated from each other (Austin et al., 1981). The prevalence and rapid accumulation of dimeric forms known as "dimer catastrophe" induces plasmid curing, i.e. its complete elimination from the host cell progeny (Summers et al., 1993). MRS is also known as site-specific recombination system and consists of a specific recombinase and a *cis*-acting site, generally termed *res*, where multimer resolution occurs. Recombinase encoded by either plasmid or host cell mediates recombination process between two *res* sites yielding two separate plasmid copies (Zielenkiewicz and Ceglowski, 2001).

Recruitment of host-encoded proteins is common for small, high-copy-number plasmids. Colicin plasmid ColE1 has been a model for understanding the mechanism of particular MRS (Blaby and Summers, 2009). Large plasmids generally encode their own site-specific recombinases. It is believed that re-

solvases of these large plasmids are derived from transposon resolvases referring to their dual role: stabilising plasmid maintenance and facilitating horizontal gene transfer (Sengupta and Austin, 2011).

2.4.2.3. Postsegregational killing

An efficient mechanism for stable persistence of large, low-copy-number plasmids in bacterial lineage that leads to elimination of plasmid-free cells in bacterial population was discovered in the 1980s in the *Escherichia coli* plasmids F and R1 (Gerdes et al., 1986; Ogura and Hiraga, 1983). This phenomenon is termed postsegregational killing (PSK). Genetic determinants ensuring PSK of plasmid-free host cells (Gerdes et al., 1986) have later been found in abundance on both plasmids and chromosomes (Leplae et al., 2011). Although, functions of chromosomally-encoded elements have not been completely understood yet, they are thought to play an important role in host protection against bacteriophages, response to different stress conditions and regulation of other essential physiological processes within the host, like cell motility (Bukowski et al., 2011; Magnuson, 2007).

The basis of a PSK mechanism is cell killing by a toxic protein component, called toxin, which is metabolically more stable than its inhibitor, called antitoxin or antidote. Antitoxin can be either protein or small non-coding RNA (sRNA). Constant production of antitoxin is required to prevent the detrimental function of the toxin by assembling toxin into a stable complex or to inhibit its translation by antisense mechanism. Since plasmid loss after cell division results in fast degradation of antitoxin and subsequent killing of plasmid-free cells mediated by free toxic components, toxin-antitoxin (TA) systems are also frequently referred to as addiction modules in plasmids (Hayes, 2003; Yarmolinsky, 1995).

All known TA systems are currently divided into five groups, type I-V, based on the antitoxin features and its control mechanism as recently reviewed by Schuster and Bertram (2013). Type I antitoxin is a sRNA that acts on post-transcriptional regulatory level. It intercepts the toxin's mRNA abolishing translation of the protein by hiding the ribosome binding site and/or causing degradation of the mRNA-sRNA complex. Type II antitoxin is a protein that in addition to its major function (toxin inactivation) serves as a transcriptional autorepressor. As a result of antitoxin binding, toxin action sites become blocked or the toxin undergoes a change that inactivates its detrimental function. The antitoxin of type III TA systems is a RNA molecule that undergoes processing prior to interacting directly with the toxic protein. Special characteristics of type IV TA systems are the mode of action of the antitoxin. The antitoxin (a protein) does not interact with the toxin directly but rather opposes and mitigates the effects of it action. Type V antitoxin is an endoribonuclease that regulates activity of the toxin on post-transcriptional level. TA systems of type III–V have been described recently and are currently represented by single case studies (Schuster and Bertram, 2013). Only type I

and II TA systems are found on plasmids, and the majority of plasmid-encoded TA's belong to type II. (Hayes, 2003).

Genes encoding TA systems of type II are clustered together in an operon, whereas the antitoxic component is commonly positioned before its partner. Toxin-antitoxin complexes serve as main regulators of the entire TA module (Yamaguchi and Inouye, 2011). While toxins of type I TA systems mainly disrupt the cell membrane, those of type II inhibit replication, translation or ATP synthesis (Schuster and Bertram, 2013). Until recently type II TA modules have been classified into ten groups on the basis of similarity between amino acid sequences of toxic proteins. This was based on the assumption that toxin and antitoxin components were tightly linked in that each toxin group corresponded to a certain antitoxin group. Recent findings derived mostly from bioinformatics studies considerably extended the range of both toxin and anti-toxin families. Moreover, the tight linkage between particular toxin-antitoxin families has been abolished (Leplae et al., 2011).

Type II addiction modules are also believed to proliferate via horizontal gene transfer (Van Melderen, 2010). In addition to plasmid maintenance, these elements can be advantageous in competition between different plasmids. TA containing plasmids were shown to outcompete none-TA plasmids with the same replicons, due to applying PSK strategy (Cooper and Heinemann, 2000).

2.4.3. Mobility

2.4.3.1. Genetic determinants of plasmid mobility

The ability to transfer between cells is probably the most important feature of plasmids in respect to propagation of antibiotics resistance and different metabolic properties. Therefore, the process of conjugation have been intensively studied for decades, revealing a high variety and great complexity of protein machineries that have been evolved by plasmids for horizontal gene transfer (Guglielmini et al., 2013; Nagai and Kubori, 2011). In a generalized form the mechanism of plasmid transfer can be characterised by cooperative work of the MOB (derived from mobility) and MPF (derived from membrane-associated mating pair formation) systems. Basic elements involved in the conjugation process are shown in Figure 4. MOB module contains a minimal set of genetic elements, including origin of transfer (*oriT*), a *cis*-acting DNA element, and several genes that encode a MOB protein complex also called DTR (derived from DNA transfer and replication). Plasmids that encode the entire translocation machinery including both MOB and MPF modules are called conjugative or self-transmissible (Smillie et al., 2010). A MOB region is crucial for plasmid transmissibility. However, for establishing cell-to-cell contact and subsequent translocation mobilisable plasmids (i.e. encoding only MOB system) require assistance of a co-resident helper plasmid which possesses genes encoding intact MPF apparatus.

The key protein component of MOB modules is relaxase. Together with one or several accessorial proteins it binds to *oriT* resulting in the formation of a nucleoprotein complex called relaxosome. The essential biochemical reactions aiming the preparation of plasmid for transfer that take place prior to translocation of a plasmid copy to a recipient cell are known as DNA processing (Alvarez-Martinez and Christie, 2009). Since plasmid is transferred in a single stranded DNA form, a requirement of successful conjugation is synthesis of a second plasmid DNA strand in both the donor and the recipient. Plasmid transfer is found to be normally associated with conjugal replication that is also considered as a part of plasmid DNA processing (Willetts and Wilkins, 1984). The relaxase supported by auxiliary proteins cleaves the transferring DNA strand (T-strand) in a specific site (*nic*) within *oriT*, after which it generally remains bound to the 5`end of the T-strand and likely guides it through further transportation events (de la Cruz et al., 2010). The relaxase-T-strand intermediate is subsequently recruited by "coupling protein" (CP), which exhibits ATPase activity and pumped through the translocation channel into the target cell. The plasmid translocation apparatus, the CP-MPF complex, belongs to the type IV secretion system (T4SS), and is often called T4CP-T4SS. T4SSs represent a group of bacterial macromolecular transport systems, which either serve as plasmid translocation facilities or secretion complexes for intercellular transfer of proteins and DNA molecules that are commonly used by bacterial pathogens (Alvarez-Martinez and Christie, 2009). Although type IV coupling proteins perform their function in a complex with translocation channels, phylogenetic analysis of T4CPs undertaken by Smillie and colleagues (2010) have shown their close relatedness to relaxases, therefore placing T4CP as part of the MOB module. Further extensive analysis on evolution of conjugation and T4SSs revealed a tendency of T4CPs to co-evolve with both relaxases and T4SSs (Guglielmini et al., 2013).

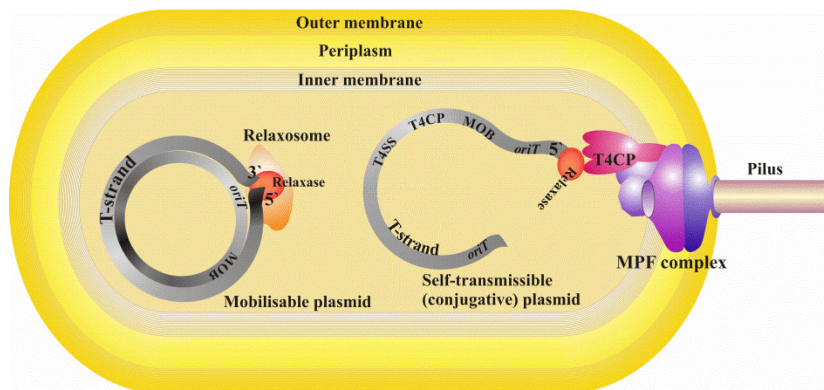

Figure 4. Basic steps and elements of the conjugation process taking place prior to a plasmid translocation to a recipient cell: plasmid DNA processing and its recruitment by translocation apparatus.

2.4.3.2. Mobility-based plasmid classification

Considering the importance of the transfer-associated region of the plasmid backbone with regard to dissemination of accessorial genes, a mobility-based plasmid classification has been recently developed on the basis of relaxase protein sequences available in databases and promptly applied along with replicon typing in classification of newly sequenced mobile genetic elements. MOB typing allows to distinguish between six phylogenetic groups of relaxases: MOB_C, MOB_F, MOB_H, MOB_P, MOB_Q, and MOB_V, with deeper branching within each group (Francia et al., 2004; Garcillan-Barcia et al., 2011; Garcillan-Barcia et al., 2009).

According to this classification system catabolic plasmids of the IncP-9 plasmid family belong to MOB_{F11} subclade of the MOB_{F1} phylogenetic clade/ group, which also contains plasmids of IncW and IncN families (Garcillan-Barcia et al., 2011). MOB_{F12} is represented by catabolic plasmids pNL1 and pCAR3 of yet unknown incompatibility features. Encompassing also IncF plasmids, the MOB_{F1} phylogenetic group is considered of great importance especially for *Gammaproteobacteria* as outlined by Garcillan-Barcia (Garcillan-Barcia et al., 2009).

IncP-7 catabolic plasmids are assigned to the MOB_{H1} clade of the MOB_H relaxase family. All plasmids affiliated to this group are found to be large and conjugative (Garcillan-Barcia et al., 2011; Garcillan-Barcia et al., 2009).

The most abundant group of relaxases is MOB_P. Consisting of deeply branched clades, it includes plasmids of great importance in respect to possession and spread of catabolic genes. It includes relaxases of the thoroughly studied IncP-1 plasmids, which are well resolved into the MOB_{P11} subclade (Garcillan-Barcia et al., 2011; Garcillan-Barcia et al., 2009). MOB_{P12} and MOB_{P13} subclades are remarkable because they contain well-studied plasmids of yet non-determined Inc families, which enable their host to tolerate toluene. These are catabolic plasmid pBVIE04 (known as pTOM) representing MOB_{P13} (Garcillan-Barcia et al., 2009), and efflux pump encoding plasmid pGRT1 affiliated to MOB_{P12} (Molina et al., 2011).

Taking into consideration the limitations of Inc typing such as presence of multiple replicons and growing number of distinct plasmids which are not classified to any incompatibility family, for example numerous plasmids of phytopathogenic bacteria (Sundin, 2007) and the above mentioned catabolic plasmids, the mobility-based grouping offers a relevant alternative solution (Garcillan-Barcia et al., 2011).

2.4.3.3. Diversity of plasmid encoded mating pair formation systems

The mating pair formation system of conjugative plasmids consists of a protein complex spanning the inner and outer membranes and periplasm and an extracellular pilus. Both the protein complex and pilus are required for establishing contact with a recipient bacterial cell. Being a part of T4SSs, MPF systems of conjugative plasmids have been shown to resemble either DNA or protein translocation systems of bacterial pathogens like VirB/VirD4 of *Agrobacterium*

tumefaciens (Schröder and Lanka, 2005) and Dot/Icm of *Legionella pneumophila* (Nagai et al., 2002), respectively. These two thoroughly investigated systems served as prototypes for the general grouping of all T4SSs into type IVA (VirB/VirD4 like) and type IVB (Dot/Icm like) on the basis of their architecture and composition. The recognition of close relatedness between *dot/icm* genes and *tra* genes of IncI plasmids like ColIb-P9 played a significant role in developing of this classification. Compared to type IVA systems (T4ASS), type IVB (T4BSS) representatives are more abundant among bacteria and generally located on plasmids. The type IVB translocation apparatus consisting of around twice as many different components also display a more complicated structure than T4ASS. An advantage of this complexity is the ability to mate equally well in both liquid and solid media (Nagai and Kubori, 2011).

Classifying plasmids on the basis of presence of either type IVA or type IVB conjugation genes is of great importance for phytopathogenic bacteria, in particular for *Pseudomonas syringae* species. The genes encoding virulence factors are often located on a certain type of plasmids, which are shown to be universally distributed among *Pseudomonas syringae* pathovars. These plasmids have been allocated to a separate pPT23A plasmid family since their *repA* gene sequences, that are the only genetic determinants commonly present in all known PFPs (pPT23A-family plasmids), does not share homology with any incompatibility group members (Sundin, 2007; Zhao et al., 2005).

A more profound classification of T4SSs enables grouping of conjugative plasmid transfer systems into three types. Plasmids of IncH1, -H2, -J, -T, -V and -X groups commonly possess F-like T4SS also called MPF_F. I-like multiprotein complex (MPF_I) is commonly encoded by members of IncI, -B and -K plasmid groups. The third known type of conjugative plasmid transfer systems, P-like T4SS (MPF_T), is associated with Ti plasmids of *Agrobacterium tumefaciens* and plasmids commonly belonging to incompatibility groups P, M, N and W. This classification is based on the differences in genetic composition of transfer apparatus (Lawley et al., 2003) supported by sequence similarities of its single components, basically ATPases (Smillie et al., 2010). General trends seen from morphology of conjugative pili are found to be congruent to the sequence-based analysis. Thin and thick long flexible pili encoded by respective I-like and F-like T4SSs mediate formation of mating aggregates in liquid as successfully as on solid media. P-like T4SSs that encode short (less than 1 μm in lengths) rigid pili accomplish surface-dependant cell-to-cell contact and thereby display absence of genetic determinants for mating pair stabilisation (Mps). F-pilus (up to 20 μm in lengths) has been the main model for understanding the mechanism of conjugation, specifically pilus architecture, assembly and retraction, but also mating pair formation and relevant environmental conditions related to this process (Arutyunov and Frost, 2013; Lawley et al., 2003). Recent bioinformatic analysis undertaken by Guglielmini and colleagues (Guglielmini et al., 2013) shed light on evolution of conjugative systems beyond well-characterized T4SS types, revealing a general tendency of all T4SSs to specialize and adapt to cell envelope structure.

3. AIM OF THE STUDY

The collection of non-medical environmental and laboratory microbial strains, CELMS, which is a member of ECCO (European Culture Collections' Organisation) is situated at the Institute of Molecular and Cell Biology, University of Tartu, and was principally established on the basis of bacterial isolates originating from contaminated sites affected by oil shale mining and chemical industry. A large number of bacterial strains with versatile catabolic capabilities were isolated and added to the collection in 2008 when an extensive research project on biodegradative microbial communities of the Baltic Sea was undertaken at the Department of Genetics, Institute of Molecular and Cell Biology, University of Tartu. Thorough characterisation of isolated bacteria in the context of their catabolic potential is the major aspect of our research group. The previous studies brought out a number of effective catabolic strains which can be applicable in development of bioremediation technologies for cleaning up oil-derived pollution. Since catabolic genes are often located on plasmids that also facilitate lateral transfer of the catabolic traits in the bacterial community, characterisation of plasmid content of effective bacterial degraders is of great importance. Therefore, my work focused on unravelling and characterising the plasmid pool of catabolic isolates. The objectives of my study can be summarized as follows:

1. To facilitate identification of catabolic bacterial strains isolated from Baltic Sea surface water and their screening for the presence of plasmids.
2. To screen plasmid-bearing isolates for the presence of IncP plasmids with subsequent analysis of the identified plasmid pool.
3. To characterise the plasmid-derived catabolic and backbone genes in light of plasmid evolution and horizontal gene transfer.
4. To determine the complete nucleotide sequences of catabolic plasmids pNAH20 and pD2RT of respective effective biodegraders *Pseudomonas fluorescens* strain PC20 and *Pseudomonas migulae* strain D2RT followed by thorough analysis of their genetic structure.

4. RESULTS AND DISCUSSION

4.1. Insight into catabolic bacteria inhabiting Baltic Sea surface water (Ref. II)

Little is known about plasmids in the marine ecosystem of the Baltic Sea. Leitet and colleagues (Leitet et al., 2006) have shown that 19% of the 130 different Baltic Sea isolates of *Alphaproteobacteria*, *Gammaproteobacteria*, *Actinobacteria* and *Bacteroidetes* phylogenetic groups contained small plasmids of unknown function with plasmid sizes ranging between 1.5 and 15 kb. A number of studies have focused on isolation and characterisation of single plasmids from Baltic Sea water. For example, plasmids belonging to the IncP-1 plasmid family have been investigated (Norberg et al., 2011).

This study revealed the presence of bacteria in Baltic Sea surface water near Estonia containing both large and small plasmid(s) that enable bacteria to degrade different aromatic compounds i.e. phenol, benzoate, *m*-toluate, salicylate and naphthalene. The regions from where water samples were collected and isolated catabolic strains originate are shown in Figure 5. 29% of 209 isolated biodegraders carry plasmid(s). Large plasmids were detected in more than half of all isolated plasmid-containing strains (34 out of 61) (Table 1, modified from Table 2, Ref. II). A high proportion of the strains bearing large plasmids could be associated with the degradative capabilities of these isolates and, consequently, with the potential presence of various catabolic plasmids. Catabolic IncP-9 plasmids were detected. However, screening for presence of replicons belonging to IncP-1 and IncP-7 plasmid families yielded no positive results (Ref. II).

Analysis of 16S rRNA gene sequences has shown that all 61 plasmid-containing isolates belong to the bacterial phylum *Proteobacteria*. The majority of them are affiliated to the genera *Pseudomonas* (31) *and Acinetobacter* (26) (Table 1, modified from Table 2, Ref. II). Numerous studies on bacterial biodegradation have been focusing attention on single isolates of these two genera. Bacteria of these phylogenetic groups are found to be highly represented among cultivable fraction of oil degraders (Kostka et al., 2011). It has been shown that *Acinetobacter* species that inhabit aquatic ecosystems are more prevalent in freshwater as they are strongly influenced by salinity (Jurelevicius et al., 2013).

In this study the overwhelming dominance of *Gammaproteobacteria* (95%) is additionally supported by strain AP3 which is affiliated to the genus *Aeromonas* (Table 1, modified from Table 2, Ref. II). *Aeromonas* spp. are ubiquitous aquatic bacteria and have gained attention as carriers of resistance plasmids (Gordon et al., 2008) and oil degrading biosurfactant producers (Ilori et al., 2005).

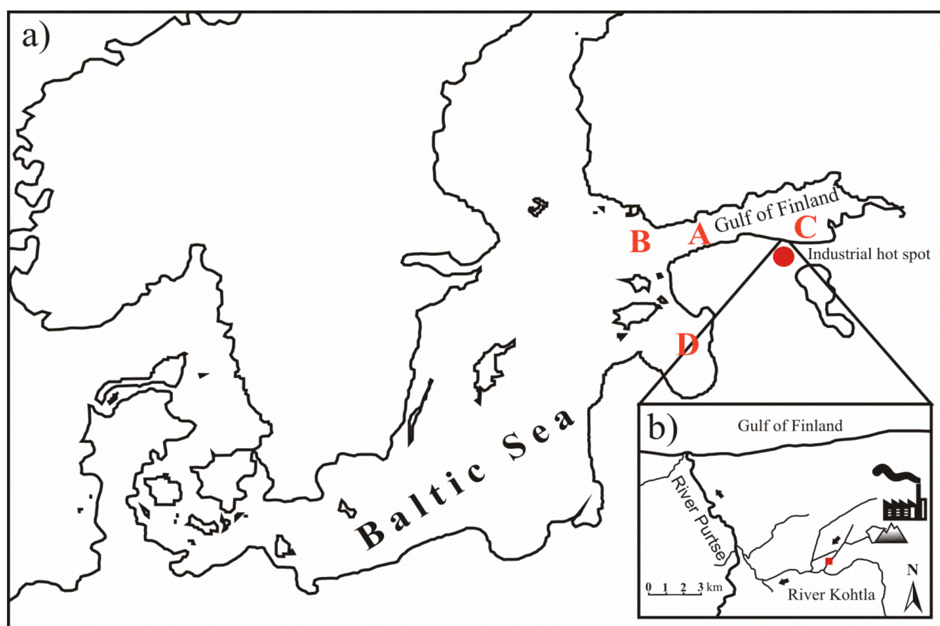

Figure 5. Water samples collection sites from a) the Baltic Sea regions A, B, C, D near Estonia; b) the map of the sample collection site in the northeastern Estonia. The red rectangle indicates the river water sampling site from where the bacterial strain *Pseudomonas fluorescens* PC20 was isolated, the arrows show the route of phenolic leachate from the ash dump area to the Gulf of Finland. The map (b) has been modified from the paper published by Heinaru and colleagues (Heinaru et al., 2000).

Table 1. Catabolic properties of plasmid-bearing bacterial strains isolated from Baltic Sea surface water samples A, B, C and D (modified from Table 2, Ref. II).

Strain	The most similar homologue from the GenBank database, acc. nr.	16s rRNA gene identity	Nr. of plasmid DNA bands*	Ability to degrade aromatic compounds				
				Phe	Benz	Tol	Sal	Nah
Gammaproteobacteria								
AB1	*Acinetobacter tjernbergiae*, FR822986	99%	1L	+	+	+	-	+
AP3	*Aeromonas rivuli*, FJ976900	100%	1L + 4S	+	+	-	-	+
A2	*Acinetobacter* sp., FJ192980	99%	1S	+	+	+	-	-
A8	*Pseudomonas putida*, AM411059	99%	1L	+	+	-	+	-
A12	*Acinetobacter haemolyticus*, AY047216	99%	3S	+	+	+	-	-
A17	*Pseudomonas fluorescens*, GU198122	99%	1L	-	+	-	+	-
A19	*Acinetobacter calcoaceticus*, AY800383	99%	1L	+	-	+	-	-
A30	*Acinetobacter* sp., FJ192980	99%	2S	+	+	+	-	-
A34	*Acinetobacter calcoaceticus*, AY800383	99%	1L	+	+	+	+	-
A52	*Acinetobacter haemolyticus*, AY047216	99%	1S	+	+	+	-	-
A56	*Acinetobacter calcoaceticus*, AY800383	99%	1L	-	-	-	+	-
A67	*Acinetobacter* sp., FJ192980	99%	2S	+	+	+	-	-
A71	*Acinetobacter* sp., FJ192980	99%	3S	+	+	+	-	-
2Aphe4	*Pseudomonas stutzeri*, FJ859914	100%	1L	+	-	-	-	-
2A7	*Pseudomonas stutzeri*, FJ859914	100%	1L	+	-	-	-	-
2A20	*Pseudomonas stutzeri*, EU652047	99%	1L	+	+	+	-	-
2A54 **	***Pseudomonas stutzeri*, EU652047**	**100%**	**1L + 1S**	+	+	+	-	-

Strain	The most similar homologue from the GenBank database, acc. nr.	16s rRNA gene identity	Nr. of plasmid DNA bands*	Ability to degrade aromatic compounds				
				Phe	Benz	Tol	Sal	Nah
B10v	*Pseudomonas stutzeri*, JF727659	99%	4S	-	-	+	-	-
B17	*Pseudomonas putida*, GU186116	99%	1L + 1S	+	+	+	-	+
B37	*Pseudomonas putida*, GU186116	99%	1S	+	+	-	-	+
B43	*Acinetobacter johnsonii*,DQ911549	99%	6S	+	+	+	-	+
2Bsal	*Pseudomonas migulae*, EU111725	99%	1S	+	+	-	+	+
2B31	*Pseudomonas veronii*, AB494445	99%	1S	-	+	+	-	+
2B49	*Pseudomonas guinae*, AM491811	99%	3S	-	-	+	-	-
C14 **	***Pseudomonas putida*, DQ178233**	**99%**	**2L + 3S**	**+**	**+**	**-**	**+**	**-**
C70	*Pseudomonas pseudoalcaligenes*, NR_037000	99%	1L	+	+	-	+	+
CN1b	*Acinetobacter junii*, AM184300	100%	2S	+	+	+	+	+
CN3b	*Acinetobacter junii*, AM184300	100%	1S	+	+	+	-	+
CS2	*Acinetobacter junii*, AM184300	100%	1L	+	+	-	-	-
CP1	*Acinetobacter junii*, AM184300	100%	1L + 1S	+	+	+	-	+
2C20v	*Pseudomonas stutzeri*, AJ244724	99%	1L	-	+	-	-	-
2C31 **	***Pseudomonas putida*, AF094746**	**100%**	**1L**	**-**	**+**	**+**	**+**	**-**
2C43 **	***Pseudomonas putida*, AF094746**	**100%**	**1L**	**+**	**+**	**-**	**+**	**-**
2C48	*Acinetobacter johnsonii*, DQ911549	99%	5S	-	+	-	-	-
2C52	*Acinetobacter johnsonii*, DQ911549	99%	5S	+	+	+	-	+
2C56	*Acinetobacter johnsonii*, EU337121	99%	3S	+	+	+	-	-
2C68	*Pseudomonas putida*, AF094746	100%	1L	-	+	-	-	-
2Cphe2	*Acinetobacter johnsonii*, DQ911549	99%	5S	+	+	+	-	+
2Ctol2 **	***Pseudomonas putida*, AF094746**	**100%**	**1L**	**+**	**+**	**+**	**+**	**+**
2Ctol3	*Pseudomonas stutzeri*, FJ859914	99%	1L	+	-	-	-	-
D2RT	***Pseudomonas migulae*, EU111725**	**99%**	**1L**	**-**	**+**	**+**	**+**	**-**
D10	*Pseudomonas aeruginosa*, FJ620575	99%	1L	-	+	-	+	-
D14	*Acinetobacter johnsonii*, DQ911549	98%	4L + 6S	+	+	-	-	-
D19	*Acinetobacter johnsonii*, EU337121	99%	5S	+	+	-	-	-
D25	*Pseudomonas lini*, AY035996	99%	1L + 1S	-	+	-	+	-
D65lp	*Pseudomonas anguilliseptica*, AF439803	99%	1S	-	+	-	-	-
D65v	*Acinetobacter schindleri*, GU339299	99%	1S	+	+	-	-	+
D67 **	***Pseudomonas migulae*, EU111725**	**99%**	**1L**	**-**	**+**	**+**	**+**	**-**
D69v	*Pseudomonas lini*, AY035996	99%	2S	+	+	+	+	+
DN1	*Acinetobacter lwoffii*, FJ860882	99%	1L + 3S	+	+	-	-	+
DP1	*Acinetobacter lwoffii*, FJ860882	99%	3S	+	+	-	-	+
DR2A1	*Acinetobacter lwoffii*, FJ860882	99%	2S	+	+	+	-	+
2Dphe2	*Pseudomonas stutzeri*, AJ244724	100%	2L	+	+	-	-	-
2D47 **	***Pseudomonas stutzeri*, EU652047**	**100%**	**2S**	**+**	**+**	**+**	**+**	**-**
2D49 **	***Pseudomonas stutzeri*, EU652047**	**100%**	**2S**	**+**	**+**	**+**	**+**	**-**
2D61 **	***Pseudomonas putida*, CP002290**	**100%**	**1L**	**+**	**+**	**+**	**+**	**-**
2D67	*Acinetobacter johnsonii,* EU337121	99%	1S	+	+	+	-	+
2D68 **	***Pseudomonas putida*, CP002290**	**100%**	**1L**	**+**	**+**	**-**	**+**	**-**
Betaproteobacteria								
2A10	*Acidovorax radicis*, HM027578	99%	1L	-	+	-	-	-
Alphaproteobacteria								
D69k	*Novosphingobium sp.,* AB360760	100%	1L	-	+	+	+	-
2D23	*Sphingomonas xenophaga,* AY611716	99%	2L + 1S	-	-	+	-	-

* Plasmids were separated according to size by agarose gel electrophoresis. Each band of plasmid DNA located above or below the chromosomal DNA band was defined as individual large plasmids (L) or small plasmids (S), respectively. + denotes positive growth on minimal media containing phenol (Phe), benzoate (Benz), *m*-toluate (Tol), salicylate (Sal), naphthalene (Nah). ** IncP-9 positive isolates. Strains belonging to the genus *Acinetobacter* and the strain AP3 of the genus *Aeromonas* are outlined by shading. Bacterial strains that have been analysed in more detail are shown bold.

Class *Betaproteobacteria* is represented only by a single bacterial strain; 2A10 of genus *Acidovorax* (Table 1, modified from Table 2, Ref. II). Members of this genus have been frequently isolated and found in abundance in PAH-contaminated sites (Singleton et al., 2009).

The ability to degrade different aromatic compounds including naphthalene and toluene is also an important feature of plasmid-bearing catabolic stains of the genera *Novosphingobium* (Romine et al., 1999) and *Sphingomonas* (Basta et al., 2004), both belonging to class *Alphaproteobacteria*. In this study these genera are both represented by a single isolate, D69k and 2D23, respectively (Table 1, modified from Table 2, Ref. II).

A number of other studies have also examined the bacterial community in Baltic Sea water and sediments using both cultivation- and molecular-based methods (Edlund et al., 2006; Langenheder et al., 2003; Riemann et al., 2008). Data derived from applying next-generation sequencing techniques has in recent years considerably extended our knowledge about the abundance and community composition of the bacteria inhabiting the Baltic Sea. Recent analysis also brought out inconsistency in the results originating from different studies which could be due to the use of different techniques and influence of altering environmental conditions (Koskinen et al., 2011). However, the impact of riverine bacteria on the Baltic Sea bacterial community and high abundance of *Gammaproteobacteria* representatives have been outlined as a common trend which is consistent with the results of this study.

4.2. Evidence of a wide distribution of IncP-9 plasmids in the Baltic Sea bacterial community (Ref. I, II and III)

Ten bacterial strains out of the 61 plasmid-bearing Baltic Sea surface water isolates were found to carry IncP-9 plasmids based on *repA* gene amplification by PCR with IncP-9 replicon specific primers (Table 1, modified from Table 2, Ref. II; Table 2, modified from Table 3, Ref. II). All ten strains belong to the genus *Pseudomonas*. Self-transmissible TOL plasmids were detected in strains *Pseudomonas migulae* D67 and *Pseudomonas putida* 2D61 (Table 2, modified from Table 3, Ref. II). Three *P. putida* isolates (2C43, 2Ctol2, 2D68) were found to carry self-transmissible SAL plasmids (enabling the host to degrade salicylate) (Table 2, modified from Table 3, Ref. II). For all of the IncP-9 *repA* gene containing *Pseudomonas stutzeri* strains (2A54, 2D47, 2D49) and two *P. putida* strains (2C31 and C14) the location of *repA* on plasmids remains to be experimentally proven (Table 2, modified from Table 3, Ref. II). Nevertheless, this study is the first to detect IncP-9 plasmid features in *P. stutzeri* strains (Ref. II). Also, *P. migulae* strain D67 carrying plasmid pD67 appears to be the first natural IncP-9 plasmid bearing isolate of this species (Ref. II). However, the ability of *P. migulae* representative to harbor IncP-9 plasmid pWW0 was demonstrated previously in microcosms with oil-contaminated soil (Jussila et

al., 2006). Although no bacterial strains carrying IncP-9 plasmids were isolated from sampling site B, the presence of IncP-9 plasmids at all four sampling sites in the Baltic Sea (Figure 5) was determined from extracted total community DNA (Table 4 of Ref. II).

Table 2. IncP-9 plasmid-bearing isolates and plasmid transferability testing (modified from Table 3, Ref. II).

Strain	Catabolic phenotype	Identified species	Sampling point	Transconjugant / Catabolic phenotype / Transfer frequency
2A54	Phe, Benz, Tol	*P. stutzeri*	A	No transfer
C14	Phe, Benz, Sal	*P. putida*	C	No transfer
2C31	Benz, Tol, Sal	*P. putida*	C	No transfer
2C43	Phe, Benz, Sal	*P. putida*	C	PaW340 (p2C43) / **Sal** / < 1.0×10^{-8}
2Ctol2	Phe, Benz, Tol, Sal, Nah	*P. putida*	C	PaW340 (p2Ctol2) / **Sal** / $3.89(\pm0.05) \times 10^{-6}$
D67	Benz, Tol, Sal	*P. migulae*	D	PaW340 (pD67) / **Tol** / $2.12(\pm0.53) \times 10^{-4}$
2D47	Phe, Benz, Tol, Sal	*P. stutzeri*	D	No transfer
2D49	Phe, Benz, Tol, Sal	*P. stutzeri*	D	No transfer
2D61	Phe, Benz, Tol, Sal	*P. putida*	D	PaW340 (p2D61) / **Tol** / $4.37(\pm0.11) \times 10^{-5}$
2D68	Phe, Benz, Sal	*P. putida*	D	PaW340 (p2D68) / **Sal** / $1.14(\pm0.31) \times 10^{-4}$

Phe, Benz, Sal, Tol, Nah, the ability to degrade phenol, benzoate, salicylate, *m*-toluate and naphthalene, respectively.

4.2.1. IncP-9 plasmids in the catchment area of a river flowing into the Baltic Sea (Ref. I)

Considering the high inflow of river water to the Baltic Sea, the plasmid pool of the sea is presumably tightly linked to its catchment area. Plasmid-mediated horizontal gene transfer could serve as a binding bridge between these two different bacterial communities. In the Gulf of Finland in the northeastern part of the Baltic Sea the river Purtse empties into the sea. Inland, Purtse is joined by the river Kohtla that flows through an oil shale mining region in northeastern Estonia (Figure 5). Leachate from the oil shale mining has resulted in a continuous pollution of the river with phenolic compounds. The bacterial strain *Pseudomonas fluorescens* PC20 carrying IncP-9 plasmid pNAH20 has been previously isolated in our laboratory from water of the river Kohtla and thoroughly characterized as an effective degrader of aromatic compounds (Heinaru et al., 2005; Heinaru et al., 2000). *P. fluorescens* PC20 with pNAH20 was later used in a rhizoremediation/bioaugmentation experiment close to the river Kohtla in an area near the semi-coke (oil shale chemical industry solid waste) depository. It was found to be persistent in natural environment for more than a year (Juhanson et al., 2009). Approximately one year and four month after the start of the rhizoremediation/bioaugmentation experiment, indigenous

representative of *P. fluorescens* was isolated from the soil and found to carry NAH plasmid pNS8 (Ref. I). The isolate was different from PC20 (Figure 6 and Figure 7 of Ref. I) and its residing plasmid pNS8 appeared to be a derivative of pNAH20, thereby demonstrating horizontal transfer of an IncP-9 plasmid in soil under natural circumstances (Ref. I). Only one difference in the genetic structure of these plasmids was observed: a double insertion of an IS*Pre2*-like element in pNS8 and only one copy of it in pNAH20 (Figure 5 of Ref. I).

To summarize, the present study provides the first insight into the genetic pool of IncP-9 plasmids in both the Baltic Sea bacterioplankton community and its catchment area in an Estonian oil shale mining region revealing their wide occurrence and horizontal transfer ability under natural environmental conditions. These findings point to a plausible significant role of the IncP-9 plasmid family for degradation of oil compounds in the Baltic Sea, and make IncP-9 plasmids reasonable candidates for the development of an effective bioremediation technology of the Baltic Sea region.

4.2.2. Diversity of catabolic IncP-9 plasmids (Ref. I and II)

Analysis of the *repA* gene sequences affiliated to IncP-9 plasmids demonstrated significant diversity among these plasmids. The majority of sequences (five) derived from *P. putida* strains 2Ctol2, 2C43, 2C31, 2D61 and 2D68 are affiliated to the ε subgroup of IncP-9 plasmids with 99–100% nucleotide sequence identity of the *repA* gene within the subgroup (Figure 6, modified from Figure 1, Ref. II). The *repA* gene sequence of TOL plasmid pD67 from *P. migulae* strain D67 was affiliated to subgroup θ and shared 100% identity with the IncP-9 TOL plasmid pSVS15 (Figure 6, modified from Figure 1, Ref. II). Subgroup θ is currently represented only by these two plasmids. The *repA* gene sequence of NAH plasmid pNAH20 was 100% identical to the corresponding sequence of IncP-9 NAH plasmid pDTG1 representing subgroup δ (Figure 6, modified from Figure 1, Ref. II). Along with the β subgroup, δ encompasses the biggest number of IncP-9 isolates (Sevastsyanovich et al., 2008).

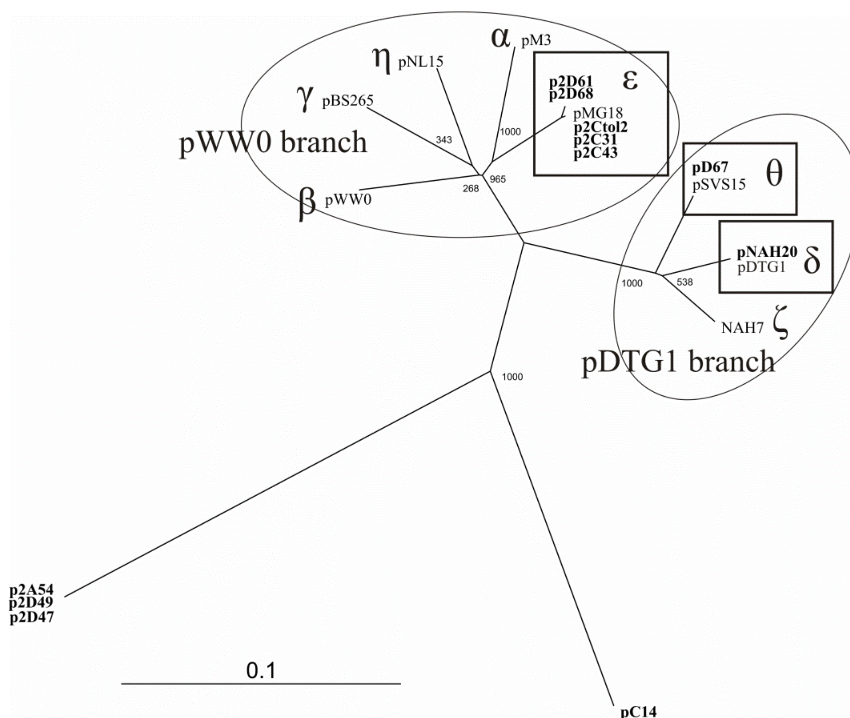

Figure 6. Phylogenetic analysis of *repA* gene sequences amplified with IncP-9 replicon-targeted primers (modified from Figure 1, Ref. II). pNL15, pBS265, NAH7, pM3, pWW0, pMG18, pSVS15, pDTG1 sequences represent the previously known IncP-9 subgroups named with the letters of the Greek alphabet. A neighbor-joining unrooted phylogenetic tree of the IncP-9 plasmid family was constructed based on 355 bp partial *repA* gene sequence analysis. The plasmids related to this study are shown in bold and related plasmid subgroups marked by rectangles. Ovals define the pWW0 and pDTG1 plasmid branches. Bootstrap values (out of 1,000) are shown adjacent to branch nodes.

Analysis of the complete nucleotide sequence of pNAH20 determined in this study (Ref. I) revealed its common origin with the NAH plasmid pDTG1 isolated from a coal-tar-contaminated site in Wales (Dennis and Zylstra, 2004) (Figure 7).

Both plasmids share the same plasmid size (83,042 bp) and almost identical nucleotide sequence, with two differences: (i) one nucleotide mismatch in the catabolic gene *nahQ*, (ii) the type and insertion site of an IS element. In the case of pNAH20, an IS*Pre2*-like element inserted into the end of *orf93* that putatively encodes a transcriptional regulator for the nearby located *mpf* genes (Figure 3 of Ref. I). In pDTG1 the insertion of the IS*Pre1*-like element into the catabolic region of this plasmid disturbed the expression of the *meta* catabolic pathway (Dennis and Zylstra, 2004). A high degree of identity between pNAH20 and pDTG1 (Ref. I) together with the finding that all other plasmids belonging to the subgroup δ (originated from Japan, Russia and Belarus) encode

catabolic pathways (summarized by Sevastsyanovich *et al.,* 2008), might imply an important role of IncP-9 δ plasmids for dissemination of catabolic genes worldwide. Catabolic IncP-9 plasmids which complete nucleotide sequence has been determined reveal a high conservation level of their structure. Based on comparative analysis of plasmids pDTG1, pWW0 and NAH7 the 39-kb region has been defined as the general backbone region for this plasmid group (Sota et al., 2006). In this work the newly sequenced NAH plasmid pNAH20 endorses this finding (Figure 7, Figure 3 of Ref. I).

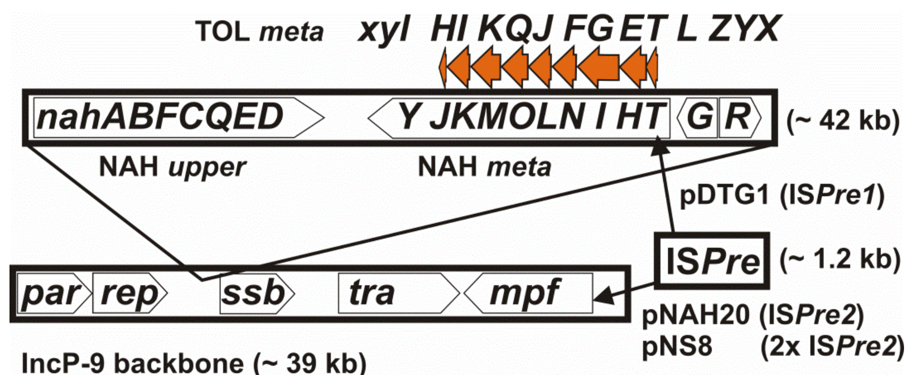

Figure 7. Common structure of IncP-9 plasmids in light of formation of NAH plasmids pDTG1, pNAH20 and its derivative pNS8. Colored arrows indicate corresponding genes common for both TOL and NAH plasmids. Black arrows show the insertion positions of IS elements. The location of catabolic region in the backbone is also outlined. General structure of the 39 kb backbone region of IncP-9 plasmids has been previously defined by Sota and colleagues (Sota et al., 2006).

In addition to the above-mentioned plasmids shown to be affiliated to previously known IncP-9 subgroups, the IncP-9 *repA* gene sequences derived from *P. stutzeri* strains 2A54, 2D47 and 2D49 formed a new phylogenetic lineage showing 54–64% identity with the other subgroups (Figure 6, modified from Figure 1, Ref. II). Furthermore, the amplified product originating from *P. putida* strain C14 formed a second new lineage in the phylogenetic tree of the IncP-9 plasmids shearing 56–64% identity with the other *repA* gene sequences (Figure 6, modified from Figure 1, Ref. II).

Amplification of new phylogenetically distant replicons has extended the knowledge about the diversity of the IncP-9 plasmid family. The obtained results are consistent with data published by Sevastsyanovich and colleagues (Sevastsyanovich et al., 2008), who determined atypical IncP-9 plasmids using an incompatibility testing technique. As a result of our work, two new lineages of IncP-9 plasmids have been added to the two previously known major clusters of pDTG1 and pWW0. The work thus points out the yet unascertained diversity of IncP-9 plasmids.

4.3. The common gene pool of plasmid-encoded catabolic traits (Ref. I, II and III)

The evolution of catabolic traits and bacterial adaptation to environmental changes by means of horizontal gene transfer has been intensively studied on the basis of catabolic plasmids, in particular NAH and TOL plasmids (Yano et al., 2007). While the first studies mostly focused on the genetic composition of catabolic pathways, the phylogeny and enzymatic functions of catabolic genes, recent works address questions regarding dissemination and formation of catabolic traits in the context of their location on mobile genetic elements.

The results of the analysis of *nah* and *xyl* catabolic genes of the respective IncP-9 plasmids pNAH20 (Ref. I) and pD67 (Ref. II and III), along with the *xyl* genes of the novel TOL plasmid pD2RT (Ref. III) from Baltic Sea water isolate *Pseudomonas migulae* strain D2RT (Ref. II and III), are consistent with previous findings and assumptions pointed out in a number of excellent reviews (Nojiri et al., 2009; van der Meer, 2006; Williams, 2004). Briefly these are: (i) degradation of both naphthalene (*nah)* and xylene/toluene (*xyl*) follows a common *meta* catabolic route, (ii) catabolic operons show modular structure, i.e. single genes and gene blocks apparently have been recruited from different origins during the process of pathway formation, and (iii) transposition-related genes are likely responsible for proliferation of catabolic genes and shaping the structure of catabolic modules.

4.3.1 Insight into evolutionary history of the studied catabolic plasmids (Ref. III)

An extensive study on the diversity of TOL plasmids conducted by Sentchilo and co-workers (Sentchilo et al., 2000) revealed the presence of *xyl* operons organized in a common manner on different plasmid types. Based on this finding, it was suggested that entire *xyl* pathways spread between different plasmid vehicles as stable entities, although no transposition genes have yet been determined on the plasmid types studied. The affiliation of the studied plasmids has also remained unascertained (except IncP-9 plasmid pSVS15) (Sentchilo et al., 2000). In the present work it was determined that a highly conserved catabolic region consisting of single *upper* and lower *xyl* operons is incorporated into dissimilar plasmid backbones resulting in the formation of TOL plasmids pD67 and pD2RT (Ref. III). Dissemination of these catabolic traits has most likely occurred by means of single insertion events conducted by Tn*4656*-like transposons (Ref. III).

The modular structure of catabolic operons is confirmed by several findings. First, comparative analysis of single genes of the *meta* operon revealed that the *xylJQKIH* genes of TOL plasmids pD2RT and pD67 are more homologous to *nahLOMKL* of NAH plasmids pNAH20 and pDTG1 than to corresponding sequences of TOL plasmid pWW53, although the remaining *xyl* genes (*xylTEGF*) resemble more those of pWW53. Second, the similarity between the cor-

responding gene products encoded by the *upper* operon of pD2RT/pD67 and those of other TOL plasmids vary significantly, strongly indicating a different origin of the xylene monooxygenase encoding *xylA* genes compared to other *xyl* genes located nearby (Ref. III).

The first two genes of the *upper xyl* operon of pD2RT/pD67, *xylU* and *xylW*, that are not required for degradation of toluene, have accumulated several mutations leading to significant changes on amino acid level compared to homologues of known TOL plasmids and presumable functionality loss (Ref. III). *xylU* has no assigned function yet and it shares high level of similarity mostly with its homologues of other TOL plasmids. The *xylW* gene encodes a benzyl alcohol dehydrogenase which presumably participates in degradation of some other, not yet identified, growth substrate (Williams et al., 1997). The occurrence of non-functional remnants of catabolic genes provides a good source for tracing evolutionary events and thereby helps to understand the process of formation of new catabolic traits (Williams, 2004).

4.4. Novel vehicles for catabolic pathways (Ref. II and III)

Only ten bacterial strains out of 61 isolated from Baltic Sea water were found to contain IncP plasmids. The type of the remaining plasmids is still unascertained (Ref. II). Considering the fame of IncP plasmid family in light of biodegradation, this finding emphasise our lack of knowledge about the true diversity of the vehicles carrying catabolic genes. Complete sequencing of plasmid pD2RT was undertaken to establish its plasmid type, its structure and the mobile genetic elements encompassing the catabolic genes (Ref. III).

4.4.1. General features of the plasmid pD2RT (Ref. III)

The complete sequence of the plasmid pD2RT extends the known range of *xyl* genes carriers by being the first completely sequenced TOL plasmid which is not related to the well-studied IncP plasmid groups. The plasmid pD2RT is 129,894 base pairs in size with an average G+C content of 53.75%. A total of 135 open reading frames (ORFs) were predicted to encode proteins, among them genes for catabolism of toluene, plasmid replication, maintenance and conjugative transfer. ORFs encoding proteins with putative functions in stress response, transposition and site-specific recombination were also predicted.

The overall genetic organization of plasmid pD2RT indicates two clearly distinguishable regions: one encompasses the plasmid backbone while the other so called accessory part of the plasmid, is responsible for enhancing host cell catabolic properties (74,363–129,853 bp) (Figure 8, Figure 1 of Ref. III). A number of mobile genetic elements and their remnants have been identified on plasmid pD2RT, and are all enclosed within the accessory region, that resembles the structure of a class II transposon, highly similar to Tn*1721*-related class II toluene catabolic transposon Tn*4656* of plasmid pWW53

(AB062597) (Tsuda and Genka, 2001). Similarly to Tn*4656* it contains two basic modules: the toluene-degrading *xyl* genes and the transposition-related segments, inverted repeats (IRs) and *res*-*tnpR*-*tnpA*. Deletion mutations in both *tnpR* and *tnpA* genes strongly affect the products they encode that in turn may impact the functional activity of the transposase-resolvase complex. Two identical inverted copies of IS*1492b* are inserted in the terminal IRs of the putative Tn*4656*-like transposon of pD2RT, resulting in the formation of a large composite transposon structure (Figure 9, Figure 5 of Ref. III).

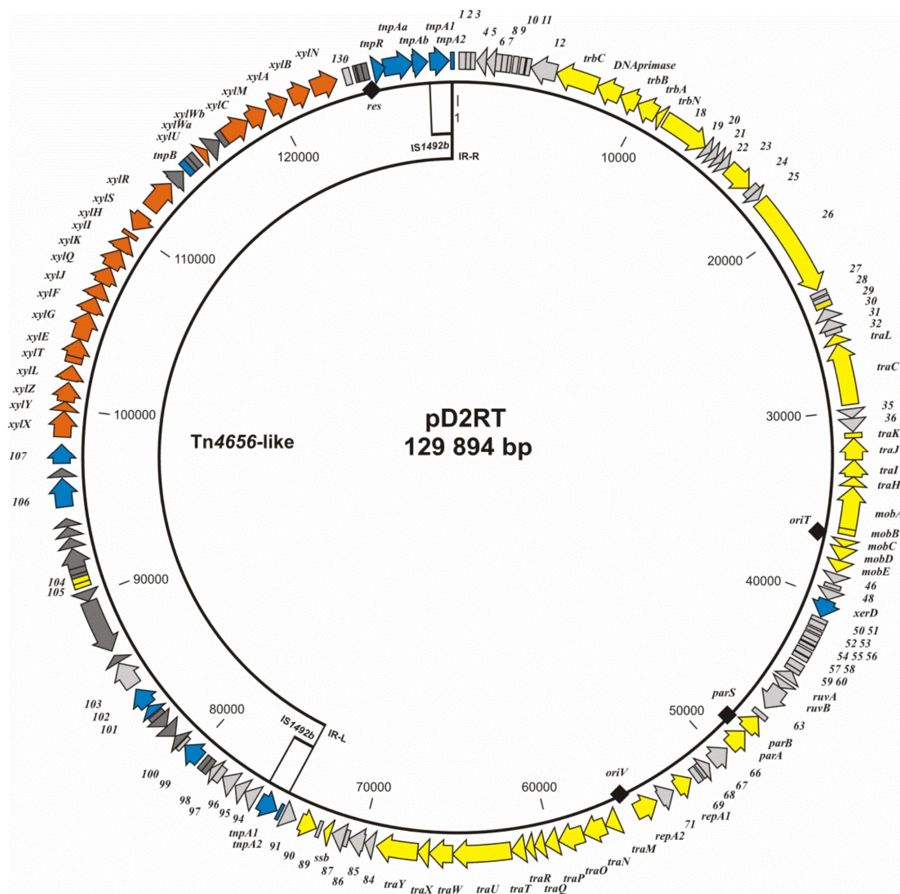

Figure 8. Genetic map of plasmid pD2RT (Figure 1, Ref. III). The predicted ORFs and gene remnants are shown as arrows indicating the direction of transcription, or as boxes if the product size is ≤ 120 aa. ORFs encoding putative plasmid backbone functions are coloured yellow, transposition and integration elements are blue, ORFs involved in the degradation of toluene are red, gene remnants are dark grey, and ORFs with no functional assignment are light grey. The region representing a proposed class II Tn*4656*-like transposon, flanked by IRs and two identical copies of IS*1492b* element, is also outlined. The black squares represent the predicted locations of *res*, *oriT*, *oriV* and *parS* sites.

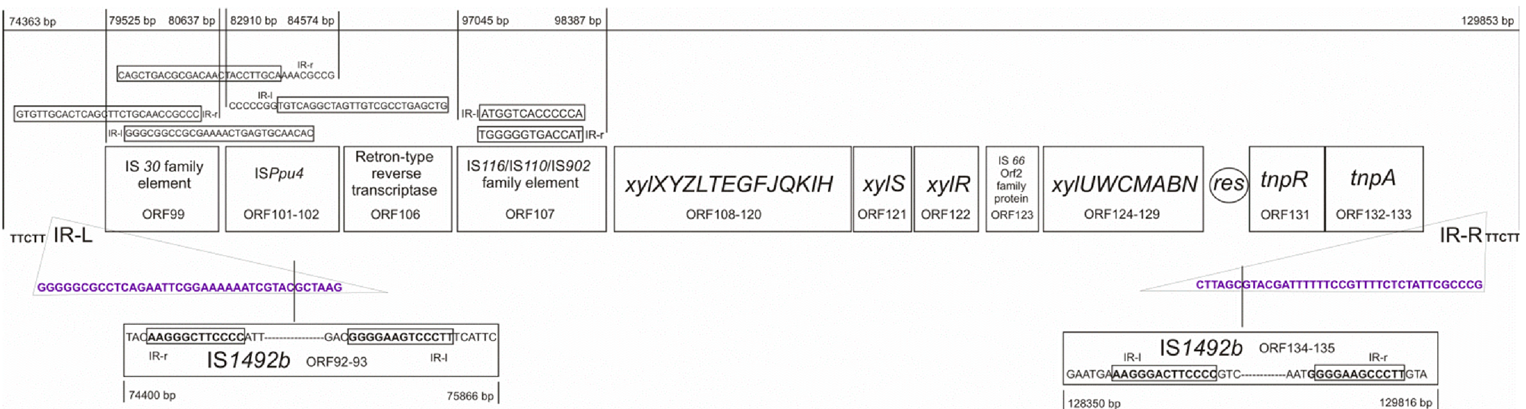

Figure 9. Genetic organization of the putative Tn*4656*-like class II transposon of pD2RT (Figure 5, Ref III). The order of major determined ORFs and genetic elements are outlined. Single ORFs and ORF clusters representing mobile genetic elements and catabolic genes are enclosed with rectangles. The *res* site is marked by a circle. Sequences of detected IRs of identified transposon and IS elements are triangled and boxed, respectively, and the location of these MGEs within the plasmid is also provided. The positions of insertions of both *IS1492b* elements into the left and right IRs (IR-L and IR-R) of the putative class II transposon are indicated with short vertical lines.

The main structure of the pD2RT backbone is represented by several gene clusters: *trbC-trbN-DNA topoisomerase* III (5,226–13,942), *traK-mobE* (31,178–38,546), *parBA* (47,152–49,321), *repA1-repA2* (51,932–55,315) and *traM-traY* (56,293–69,228). These are separated from each other by a range of ORFs with yet unknown functions, and single ORFs with functional assignment (Ref. III). For example, two accessory genes *ruvA* (ORF61) and *ruvB* (ORF62) that have been incorporated into the plasmid backbone (44,883–46,584) in close proximity to the partition-associated region (Figure 8) presumably confer *P. migulae* strain D2RT resistance to ultraviolet light (Ref. III). RuvAB proteins of pD2RT share around 70% identity with products of *rulAB* genes from plasmid pWW0 which have been determined to encode a DNA Polymerase V homologue that confers evolutionary fitness on its host *P. putida* under conditions of environmental stress (Tark et al., 2005).

The plasmid pD2RT contains two genes, *repA1* (ORF70) and *repA2* (ORF72), which are relevant to plasmid replication function. The *repA2* gene products play a crucial role for initiation of the plasmid pD2RT replication process in the strain *P. migulae* D2RT (Ref. III). Inactivation of the *repA1* gene affected neither the plasmid nor its hosts viability. Contrary, attempts to create the *P. migulae* D2RT derivative strain containing pD2RT with an inactivated *repA2* gene yielded no isolates (Ref. III). However, it cannot be entirely ruled out that RepA1 might also have some accessory role in the replication process and/or it could become essential at certain environmental conditions/host strains. The plasmid pD2RT replicase (RepA2) shows the highest degree of identity (99%) with the replicase of *Pseudomonas syringae* pv. *glycinea* strain B076 and around 62% with those of pPT23A family plasmids, originating from different *P. syringae* pathovars (Zhao et al., 2005). Plasmids belonging to the pPT23A family are shown to be universally distributed among *P. syringae* pathovars being important agents for virulence (Sundin, 2007). The putative origin of plasmid pD2RT replication (*oriV*) was found to be located upstream of *repA2* (Ref. III). It contains four almost identical 17 bp long direct repeats (DR) which could serve as iterons, i.e. multiple RepA binding sites (Ref. III). Iterons undertake an important regulatory function ensuring plasmid replication and replication control.

Two genes, *parA* (ORF65) and *parB* (ORF64), crucial for plasmid stability and maintenance, have been identified on pD2RT (Ref. III). *parB* and *parA* encode centromere-binding protein ParB and ParA ATPase, respectively, whose amino acid sequences are most closely related (98% and 99% identity) to the homologues of *P. syringae* pv. *glycinea* strain B076 and also show 73% and 49% identity with those of pPT23A family plasmid pDC3000A (Zhao et al., 2005) from *P. syringae* pv. *tomato* strain DC3000. The pD2RT partition system is related to the type Ia partitioning loci (Ref. III), assuming an active movement of replicated plasmid copies to dividing bacterial daughter cells (Gerdes et al., 2000). The putative centromere-like region *parS* containing two almost identical 19 bp palindrome sequences were detected between the predicted *parA* and *parB* genes (Ref. III). In addition to the *par* system, several genetic

determinants involved in plasmid stability were predicted: zeta toxin family protein (ORF23), RelE-RelB toxin-antitoxin system (ORF105 and ORF104), a putative DNA methylase (ORF26) and ORF90 that encodes bacteriophage abortive infection (Abi) protein (Ref. III).

The conjugation system of pD2RT belongs to the family of type IVB secretion systems (T4BSS), revealing the presence of three main gene clusters: *traKJIH*, *trbC-DNA primase-trbBAN* and *traMNOPQRTUWXY* (Ref. III) according to the criteria highlighted in (Sexton and Vogel, 2002). The genetic organization of the respective region of pD2RT and BLAST search analysis of the corresponding gene products showed the highest degree of similarity of this region to that of the plasmids pGRT1 and pBVIE04, and genomic DNA of *P. syringae* pv. *glycinea* strain B076. The conjugation system of pD2RT is also distantly related to the I-type conjugation system of IncI plasmids and the relevant elements of plasmids representing the pPT23A family (Ref. III).

The conjugal transfer in general encompasses: DNA processing, its recruitment to the translocation channel and subsequent translocation reaction. The translocation is mediated by the mating pair formation apparatus (Alvarez-Martinez and Christie, 2009). Plasmid pD2RT is self-transferable. Its transfer frequency to the recipient *P. putida* strain PaW340 was found to be around 2.6×10^{-5} transconjugants per donor cell (Ref. III).

The DNA processing of pD2RT is putatively conducted by the *mobABCDE* gene cluster with relaxase enzyme as a key player encoded by the *mobA* gene (ORF41) (Ref. III). Following the classification of conjugative transfer systems proposed by Garcillan-Barcia and colleagues (Garcillan-Barcia et al., 2009), which is based on phylogeny of relaxase proteins, the plasmid pD2RT belongs to the MOB_P family (Ref. III). MobA showed the closest homology to the plasmid pGRT1-encoded MobA (61% identity) of the subclade MOB_{P12} that originates from *Pseudomonas putida* strain DOT-T1E and to the plasmid pBVIE04-encoded homologous protein (48% identity) of the subclade MOB_{P13} of *Burkholderia vietnamiensis* strain G4. The putative *oriT* of pD2RT is located between the *mobB* (ORF42) and *mobC* (ORF43) genes encompassing inverted repeats followed by a nick site (Figure 4 of Ref. III).

The growth kinetics of the plasmid-bearing strains, wild type strains *P. migulae* D2RT (carrying pD2RT) and D67 (carrying pD67) along with transconjugants *P. putida* PaW340 (pD2RT) and *P. putida* PaW340 (pD67), were observed. Interestingly, differences were detected in the growth profiles of the wild type strains as well as of the transconjugant host, *P. putida* PaW340, influenced by particular TOL plasmid, pD2RT or pD67. The growth rate of the strains containing pD2RT was almost three times slower than that of the strains bearing pD67. The cultures required a remarkably longer time to reach the stationary phase. This observation may refer to the importance of plasmid and host compatibility in the biodegradative capacity of a catabolic plasmid-bearing strain (Figure 10, Figure 6 of Ref. III).

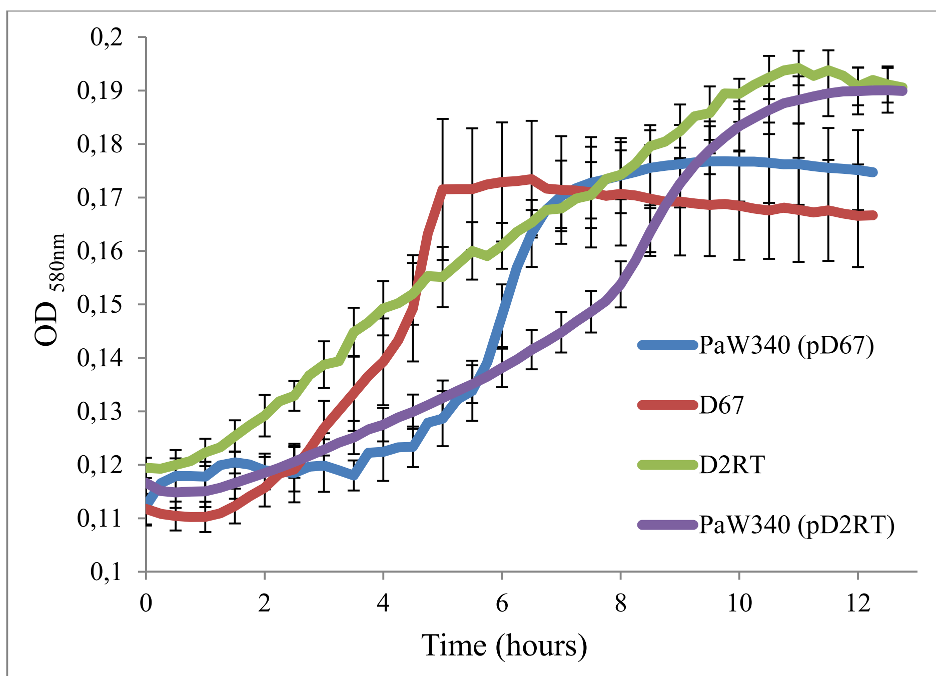

Figure 10. Growth curves of cultures grown with *m*-toluate as sole carbon and energy source (Figure 6, Ref. III). The standard error bars are presented.

4.4.2. The putative origin of the plasmid pD2RT backbone (Ref. III)

Analysis of the plasmid pD2RT architecture and gene content revealed the high degree of similarity to the Contig065 of plant-pathogenic multi-plasmid bacterial strain *P. syringae* pv. *glycinea* B076 (*Pseudomonas savastanoi* pv. *glycinea* B076) draft genome, which is presumably a plasmid-derived sequence (Qi et al., 2011) (Figure 11, Figure 2a and Figure 3 of Ref. III). The majority of ORFs in the backbone exhibit more than 90% identity between corresponding gene products and high conservation of their location and transcriptional orientation in relation to each other. From the performed comparative analysis of entire genomes as well as single backbone components it could be deduced that the new TOL plasmid pD2RT was likely formed by incorporation of the Tn*4656*-like transposon into the plasmid vehicle which shared the last common ancestor with a pPT23A family plasmid of a phytopathogenic pseudomonad.

The second closest homologue was found to be the backbone region of the plasmid pGRT1 of *Pseudomonas putida* strain DOT-T1E, although the extent of sequence similarity is considerably lower compared to that of *P. syringae* pv. *glycinea* B076 Contig065. Backbones of these two plasmids share the main structural components and also display similar organisation of particular gene clusters within the genome (Figure 11, Figure 2b and Figure 3 of Ref. III). However, the identity level between single gene products is mostly in the range of

50–80%. A remarkable difference in their backbone genetic content is the presence of *kfrA* gene associated with plasmid partitioning in pGRT1 and its absence in pD2RT. pD2RT is also distantly related to pBVIE04 of *Burkholderia vietnamiensis* strain G4 exhibiting the presence of common genes that are required for plasmid stability and replication as well as gene clusters crucial for conjugal transfer. Conversely, sequence similarity between corresponding proteins is low and generally restricted to conserved protein domains. Comparison of pD2RT and pBVIE04 genomes brings out certain plasticity of the common backbone structure, since the conserved gene clusters have different order and orientation within particular genomes (Figure 11, Figure 2c and Figure 3 of Ref. III).

Interestingly, all three plasmids enable their host bacteria to tolerate toluene; a crucial role in their survival. However, the mechanisms encoded on these plasmids are different: pD2RT is a TOL plasmid, pBVIE04 enables degradation of toluene via toluene-2-monoxygenase pathway, pGRT1 encodes a toluene efflux pump. Thus, by means of joining *xyl* genes with pGRT1 and pBVIE04 resembling carrier, pD2RT exhibits a novel combination of previously determined features, strongly associated with bacterial survival in the presence of toluene.

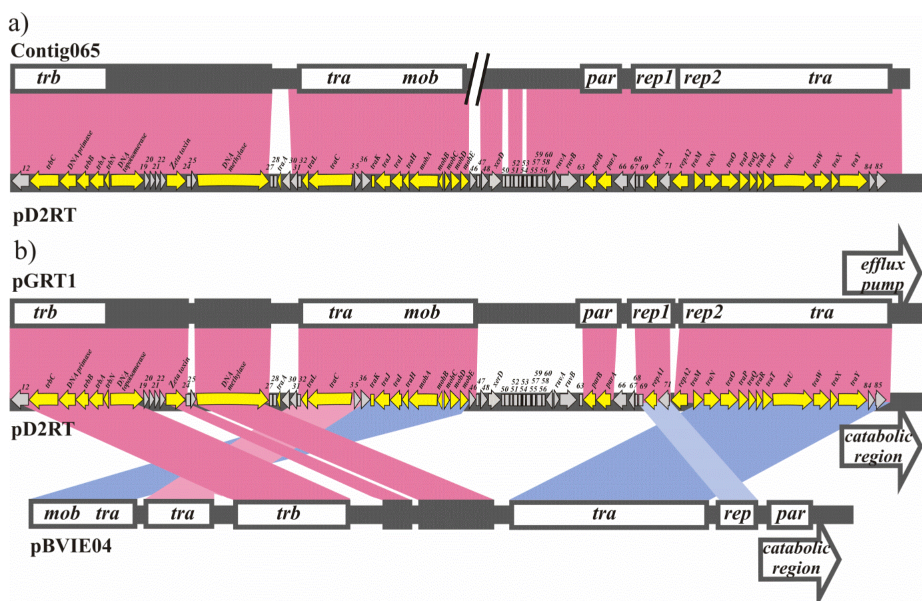

Figure 11. Comparison of the architecture of plasmid pD2RT backbone with corresponding regions of the most similar sequences in GenBank (Figure 3, Ref. III): a) Contig065 form the draft genome sequence data of *Pseudomonas syringae* pv. *glycinea* strain B076 (NZ_AEGG01000065); and b) plasmid pGRT1 of *Pseudomonas putida* strain DOT-T1E (NC_015855) and plasmid pBVIE04 of *Burkholderia vietnamiensis* strain G4 (NC_009228). Red bars indicate collinear homologous regions, while Blue bars indicate inverted regions of homology. Only the main backbone gene clusters and their corresponding location within the DNA molecule with respect to pD2RT are outlined for Contig065, pGRT1 and pBVIE04. Two slashes designate the break position in the linear sequence of Contig065.

5. CONCLUSIONS

Being exposed to intensive pollution from both land and directly at the sea the ecosystem of the Baltic Sea is endangered. Toxic aromatic compounds constitute one of the major threats to this vulnerable environment. Bioremediation, removal of pollutants with the help of microbes, is considered as an effective and perspective technology for coping with a contamination problems. Bacteria have evolved different catabolic pathways for the degradation of aromatics, and the respective catabolic genes can be located on the chromosome or on the extrochromosomal genetic elements like plasmids. Plasmids are also found to contribute significantly to the horizontal (lateral) transfer of catabolic genes, thereby facilitating dissemination of biodegradative capabilities among bacterial communities. The important role of catabolic plasmids for bioremediation processes has been assured. Nevertheless, the plasmid pool of the Baltic Sea is poorly understood. The present study was focused on unravelling the genetic pool of catabolic plasmids of the Baltic Sea aquatic system.

The following conclusions can be made on the basis of the conducted research work:

1. Analysis of the aromatics-degrading bacterial strains isolated from Baltic Sea surface water revealed the high percentage (29%) of plasmid-bearing strains. More than half of catabolic plasmid-bearing isolates carried at least one large plasmid. This finding indicates that the plasmids are likely associated with the degradative capabilities of the cultivable bacteria, the vast majority of which was found to belong to bacterial phylum *Proteobacteria,* in particular to the genera *Pseudomonas* and *Acinetobacter*.
2. Screening of the plasmid-bearing strains for the presence of different types of IncP plasmid families resulted in detection of representatives belonging to the P-9 incompatibility family. This finding underlines the important role of IncP-9 plasmids for biodegradation and also reveals the yet unascertained genetic pool of catabolic gene carriers. The prevalence of IncP-9 plasmids in the Baltic Sea aquatic system was confirmed by detection of particular replicons in total bacterial community DNA extracted from all tested surface water samples and in bacterial strains isolated from the polluted river water and its catchment area that empties into the Baltic Sea. Evidence of horizontal gene transfer of the IncP-9 catabolic NAH plasmid pNAH20 under natural conditions during a bioaugmentation/rhizoremediation experiment in a polluted site provides another confirmation for their relevant contribution to the biodegradation of pollutants.
3. Twelve plasmid-bearing pseudomonads highlighted in this study (ten Baltic Sea water isolates, the pNAH20 plasmid bearing strain *P. fluorescens* PC20 isolated from the polluted river water and pNAH20 derivative containing strain *P. fluorescens* NS8 originated from the polluted site nearby the river) were found to carry IncP-9 plasmids belonging to different phylogenetic subgroups. These were the already known subgroups ε, θ and δ as well as two novel phylogenetic lineages. This finding points to a high diversity of

IncP-9 plasmids in the Baltic Sea region along with bringing to light previously unknown phylogenetic branches. The range of bacteria carrying IncP-9 specific genetic determinants was also enriched with a new species, *P. stutzeri*. The high conservation of the backbone of IncP-9 plasmids, specifically for the plasmids belonging to the same subgroup, was confirmed by determining the complete nucleotide sequence of pNAH20. Plasmid pNAH20 was found to be almost identical to plasmid pDTG1 originating from a coal-tar-contaminated site in Wales.

4. The complete nucleotide sequence of the self-transmissible TOL plasmid pD2RT of *P. migulae* strain D2RT provides insight into the yet unascertained genetic pool of catabolic plasmids. The interesting finding is that the backbone of pD2RT shares common features with the plasmids of phytopathogenic pseudomonads and the closest homologue in sequence databases is a genomic fragment (assumed to be plasmid-born) of the plant-pathogenic multi-plasmid bacterial strain *Pseudomonas syringae* pv. *glycinea* B076 (*Pseudomonas savastanoi* pv. *glycinea* B076). The general backbone structure of pD2RT is also found to resemble those of well characterised plasmids such as catabolic plasmid pBVIE04 (pTOM) of *Burkholderia vietnamiensis* strain G4 and efflux pump-encoding plasmid pGRT1 of *Pseudomonas putida* strain DOT-T1E. Both plasmids are also associated with host tolerance to toluene.
5. The analysis of the pD2RT plasmid region containing catabolic genes lead to the following main conclusions. First, catabolic genes for toluene degradation have been encompassed within a Tn*4656*-like transposon and have disseminated as an entire unit among different plasmid types as the highly similar catabolic region was found on the IncP-9 TOL plasmid pD67 of the strain *P. migulae* D67. Second, the genes ensuring degradation of naphthalene and toluene of the Baltic Sea aquatic system have a common nature, since the comparative analysis of the *meta* operon revealed that the *xylJQKIH* genes of TOL plasmids pD2RT and pD67 are more homologous to *nahLOMKL* of NAH plasmids pNAH20 and pDTG1 than to corresponding sequences of other known catabolic plasmids.

To summarize, the present research work provides valuable information on the degradative bacterial strains isolated in order to use them in the development of effective bioremediation technologies and extends our general knowledge regarding the nature of TOL and NAH plasmids.

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SUMMARY IN ESTONIAN

Aromaatsete ühendite lagundajate mobiilne geenifond:
Läänemere bakterite kataboolsed plasmiidid

Olles eksponeeritud inimese poolt põhjustatud intensiivsele reostusele nii otseselt kui ka maismaa kaudu, on Läänemere ökosüsteem ohus. Toksilised aromaatsed ühendid on ühed ohtlikematest saasteainetest, mis ohustavad Läänemere tundlikku keskkonda. Bioremediatsiooni ehk reoainete kõrvaldamist mikroobide tegevuse abil peetakse efektiivseks ja paljulubavaks tehnoloogiaks saastusega seotud probleemide lahendamisel. Bakterid on kohastunud aromaatsete ühendite lagundamiseks erinevate kataboolsete radade abil. Neid radu kodeerivad geenid võivad paikneda nii kromosoomis kui ka ekstrakromosomaalselt – plasmiidides. Plasmiidid soodustavad kataboolsete geenide horisontaalset (lateraalset) ülekannet ning sellega kaasnevat biodegradatiivsete omaduste levitamist bakteriaalses koosluses. Vaatamata sellele, et kataboolsete plasmiidide oluline roll on bioremediatsiooni protsessis kinnituse leidnud, on Läänemere plasmiidne kooslus praktiliselt tundmatu ning jäänud uurijate tähelepanuta. Antud töö keskendub Läänemere kataboolsetele plasmiididele, täpsemalt nende plasmiidide tuvastamisele, taksonoomiale ning geneetilise struktuuri ja koostise määramisele.

Antud uurimistöö tulemused ja nende põhjal tehtud järeldused on järgmised:

1. Plasmiide sisaldavate bakteritüvede osakaal kõikide isoleeritud kataboolsete omadustega bakteritüvede seas oli suur, moodustades 29%. Suuri plasmiide leiti rohkem kui pooltel plasmiide sisaldavatel tüvedel, mis vihjab nende tõenäolisele seotusele isoleeritud tüvede kataboolsete tunnustega. Enamus plasmiide omavatest isolaatidest kuuluvad proteobakterite hulka, täpsemalt bakteriperekondadesse *Pseudomonas* ja *Acinetobacter*.
2. Plasmiide kandvate tüvede iseloomustamine erinevate plasmiidsete IncP rühmade esindajate olemasolu suhtes näitas IncP-9 plasmiidide esinemist Läänemere kultiveeritavatel bakteritel, mis osutab selle plasmiidide perekonda esindajate olulisele rollile biodegradatsiooni protsessis. IncP-9 plasmiidide laia levikut Läänemeres tõestas lisaks nende tuvastamine kõikide Läänemere veeproovide totaalses DNA-s ning, nende isoleerimine reostatud jõeveest ja jõe valgala saastatud piirkonnast, mis suubub Läänemerre. IncP-9 gruppi kuuluva kataboolse NAH plasmiidi pNAH20 horisontaalne ülekanne looduskeskkonnas bioaugmentatsiooni/risoremediatsiooni eksperimendi käigus kinnitab selle plasmiidide perekonna esindajate olulist rolli ja kaasatust reoainete lagundamise protsessi.
3. Kuna enamus isoleeritud plasmiide jäi antud töö käigus identifitseerimata ja klassifitseerimata, tõestab see, et Läänemere plasmiidikooslus sisaldab peamiselt senitundmatuid kataboolsete geenide kandjaid.
4. Kaksteist (12) selles töös käsitletud bakteriperekonda *Pseudomonas* kuuluvat tüve (kümme tüve isoleeritud Läänemere pinnase veest, saastatud jõeveest pärinev ning NAH plasmiidi pNAH20 kandev tüvi *P. fluorescens*

PC20 ning saastatud jõe valgala piirkonnast isoleeritud tüvi *P. fluorescens* NS8, mis sisaldab plasmiidi pNAH20 derivaati) kannavad IncP-9 plasmiide, mis kuuluvad erinevatesse fülogeneetilistesse alamgruppidesse: juba teadaolevad alamgrupid ε, θ ja δ ja kaks uut fülogeneetilist haru. Need tulemused osutavad IncP-9 plasmiidide suurele mitmekesisusele Läänemere regioonis ning uute, senini tundmatute fülogeneetiliste harude olemasolule. IncP-9 plasmiidide peremeestering on samuti rikastunud uue pseudomonase liigiga, milleks on *P. stutzeri*. IncP-9 plasmiidide selgroogude kõrge konserveerumine, eriti samasse alamgruppi kuuluvate plasmiidide puhul, on kinnitust leidnud plasmiidi pNAH20 nukleotiidse järjestuse määramisel. Plasmiid pNAH20 genoom on peaaegu identne Walesist kontamineeritud piirkonnast pärineva plasmiidi pDTG1 nukleotiidse järjestusega.

5. Konjugatiivse plasmiidi pD2RT, mille peremeestüveks on *P. migulae* D2RT, nukleotiidne järjestus on esimene täielikult sekveneeritud TOL plasmiid, mis ei kuulu tuntud IncP plasmiidide gruppi. Huvitav on see, et pD2RT selgroog sarnaneb taimpatogeensetes bakterites leiduvate plasmiidide selgroogudega ning sellele kõige lähedasem teadaolev järjestus on genoomne fragment (eeldatavasti plasmiidse päritoluga) plasmiide sisaldavast taimepatogeenist, tüvest *P. syringae* pv. *glycinea* B076 (*Pseudomonas savastanoi* pv. *glycinea* B076). Plasmiidi pD2RT selgroo üldine struktuur sarnaneb samuti selgroo järjestustega varem põhjalikult kirjeldatud plasmiididest – kataboolne plasmiid pBVIE04 (pTOM) bakteritüvest *Burkholderia vietnamiensis* G4 ja tolueeni transportsüsteemi kodeeriv plasmiid pGRT1 bakteritüvest *Pseudomonas putida* DOT-T1E, kusjuures mõlemad plasmiidid tagavad peremeestüvele tolerantsuse tolueeni suhtes.
6. Plasmiidi pD2RT kataboolse piirkonna analüüs võimaldas teha kaks peamist järeldust. Esiteks, kataboolsed geenid tolueeni lagundamiseks paiknevad Tn*4656* taolises transposoonis ja on levinud erinevatesse plasmiididesse terviküksustena. Sellele viitab kõrgelt konserveerunud kataboolse ala esinemine nii pD2RT plasmiidis kui ka IncP-9 TOL plasmiidis pD67 bakteritüvest *P. migulae* D67. Teiseks, geenid mis tagavad tolueeni ja naftaleeni lagundamist Läänemeres, on ühise päritoluga, kuna *meta* operoni järjestuste võrdlev analüüs näitas, et TOL plasmiididest pD2RT ja pD67 pärinevad *xylJQKIH* geenid on sarnasemad *nahLOMKL* geenidega NAH plasmiididest pNAH20 ja pDTG1 võrreldes teistest plasmiididest pärinevate kataboolsete geenide järjestustega.

Kokkuvõtlikult, käesolev uurimistöö annab väärtuslikku informatsiooni kataboolsete omadustega bakteritüvedest, kes olid isoleeritud eesmärgiga kasutada neid efektiivse bioremediatsiooni tehnoloogia arendamisel ning rikastab üldteadmisi TOL ja NAH plasmiidide kohta.

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