Molecular Analysis of Hydrocarbon-Degrading Bacteria and Alkane Hydroxylase (*alk*) genes in Association with Highly Tolerant Plant Species for Phytoremediation of Petroleum Oil Contamination in Soil

Dissertation

Submitted to obtain degree of Doctor at University of Natural Resources and Applied Life Sciences, Vienna

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Youth is not a time of life; it is a state of mind; it is not a matter of rosy cheeks, red lips and supple knees; it is a matter of the will, a quality of the imagination, a vigor of the emotions; it is the freshness of the deep springs of life.

Youth means a temperamental predominance of courage over timidity of the appetite, for adventure over the love of ease. This often exists in a man of sixty more than a boy of twenty. Nobody grows old merely by a number of years. We grow old by deserting our ideals.

Years may wrinkle the skin, but to give up enthusiasm wrinkles the soul. Worry, fear, self-distrust bows the heart and turns the spirit back to dust.

Whether sixty or sixteen, there is in every human being's heart the lure of wonder, the unfailing child-like appetite of what's next, and the joy of the game of living. In the center of your heart and my heart there is a wireless station; so long as it receives messages of beauty, hope, cheer, courage and power from men and from the infinite, so long are you young.

When the aerials are down, and your spirit is covered with snows of cynicism and the ice of pessimism, then you are grown old, even at twenty, but as long as your aerials are up, to catch the waves of optimism, there is hope you may die young at eighty.

[***Youth*** by Samuel Ullman 1840-1924]

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Finally, I dedicate this work for my beloved two families; Andria and Hödl families for their love, support and encouragement throughout my endeavor. My sincere grateful is for Martin Hödl, who will always be an important person in my life.

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Molecular Analysis of Hydrocarbon-Degrading Bacteria and Alkane Hydroxylase (*alk*) genes in Association with Highly Tolerant Plant Species for Phytoremediation of Petroleum Oil Contamination in Soil

SUMMARY

Phytoremediation is a cost-effective and environmentally sound method for the degradation of organic and inorganic contaminants. For successful phytoremediation, diversity of pollutant degrading bacteria and rapid spread of the corresponding functional genes are essential for efficient break-down of pollutants. This Ph.D. research attempted to enhance the effect of phytoremediation of petroleum-oil contaminated soil through an understanding of the diversity of hydrocarbon-degrading bacteria associated with tolerant plant species and the expression of alkane monooxygenase genes (*alkB*), encoding enzymes responsible for degrading environmentally toxic hydrocarbons.

To this end, we analysed the performance of 26 different plant species in soil contaminated with petroleum oil. Among others, Italian ryegrass (*Lolium multiflorum* var. Taurus), Birdsfoot trefoil (*Lotus corniculatus* var. Leo), and the combination of both plants demonstrated the highest tolerance. Subsequently, hydrocarbon degrading bacteria were isolated from the rhizosphere, root interior and shoot interior of these plants and were subjected to analysis of the 16S rRNA and alkane hydroxylase genes. The results showed higher numbers of culturable hydrocarbon-degrading bacteria in association with Italian ryegrass; endophytic bacteria mainly belonged to the *Gammaproteobacteria* (*Pseudomonas* and *Pantoea* species) whereas high G+C (*Rhodococcus, Microbacterium, Arthrobacter* and *Streptomyces*), low G+C Gram-positives (*Bacillus, Paenibacillus*) and *Gammaproteobacteria* colonised the rhizosphere. Fewer strains were obtained from Birdsfoot trefoil, mostly belonged to *Beta* (*Achromobacter, Alcaligenes*) and *Gammaproteobacteria*

(*Pseudomonas, Pantoea*) as well as to the low G+C Gram-positives (*Bacillus, Paenibacillus*). Known alkane hydroxylase genes (*alkB* and cytochrome P153-like) could be detected in only half of the isolates and were found to be located both on plasmids as well as chromosomally. Our results indicate that *alkB* genes may have spread through cross-phyla horizontal gene transfer events, most likely between Gram-positive and Gram-negative bacteria, particularly in the Italian ryegrass rhizosphere. For phytoremediation applications, our results suggest that Italian ryegrass appears more appropriate than Birdsfoot trefoil.

The second part of the project aimed to assess the role of endophytes in alkane degradation. Italian ryegrass was grown for 2 months in sterile soil with 0, 1 or 2% diesel and inoculated either with alkane degrading bacteria from the rhizosphere or with an endophyte. We studied plant colonization of these strains as well as the abundance and expression of alkane monooxygenase (*alkB*) genes in rhizosphere, shoot and root interior. Results indicated that the endophyte strains more efficiently colonised the plant, particularly the plant interior and showed higher expression of *alkB* genes, suggesting a more efficient degradation of the pollutant. The rhizosphere strains primarily colonised the rhizosphere and demonstrated lower expression of the *alkB* gene in the plant interior. Our study suggests that endophytes have significant potential for use in phytoremediation applications.

Keywords: *Petroleum-oil contamination, phytoremediation, hydrocarbon-tolerant plants, hydrocarbon-degrading bacteria, alkane monooxygenase (alkB) genes, horizontal gene transfer, gene expression.*

Molekulare Analyse des Kohlenwasserstoff-Entwürdigenden Bakteriums und der Gene der Alkan-Hydroxylase *(alk*) in Verbindung mit in hohem Grade toleranten Betriebssorten für Phytoremediation der Erdöl-Öl-Verschmutzung im Boden

ZUSAMMENFASSUNG

Phytoremediation ist eine kosten-effiziente und ökologisch nachhaltige Methode zur Stabilisierung bzw. zum Abbau organischer und anorganischer Schadstoffe. Erfolgreiche Phytoremdiation von Umweltgiften wie Erdöl erfordert hohe Diversität und Abundanz Schadstoff-metabolisierender Bakterien und einen schnellen Transfer von Abbaugenen. Diese Dissertation beschäftigte sich mit der Diversität von Kohlenwasserstoff-abbauenden Bakterien, die mit resistenten Pflanzen assoziiert leben, und mit der Expression des Alkan-Monooxygenase-Gens (*alkB*), welches für den Kohlenwasserstoffabbau verantwortlich ist. Ziel war es, einen Beitrag zur Entwicklung von Phyotoremediationsstragien für Erdölkontaminationen leisten.

Zunächst wurden 26 Pflanzenarten auf ihre Wachstumsleistung in erdölkontaminiertem Boden geprüft. Italienisches Raygras (*Lolium multiflorum* var. Taurus), gewöhnlicher Hornklee (*Lotus corniculatus* var. Leo), und eine Mischkultur aus diesen beiden Spezies zeigten die höchste Toleranz. Anschließend wurden Kohlenwasserstoff-abbauende Bakterien aus der Rhizosphäre, dem Wurzelinneren und dem Spross (Endophyten) dieser Pflanzen isoliert und 16S rRNA sowie Alkan-Hydroxylase Gene wurden analysiert. Die Endophyten von *L. multiflorum* waren hauptsächlich Gammaproteobakterien (*Pseudomonas* und *Pantoea* Arten), während die Rhizosphere von Actinobakterien (*Rhodococcus, Mikrobakterium, Arthrobakterium*, und *Streptomyces*) und *Firmicutes* (*Bacilus, Paenibacillus*) besiedelt war. *L.corniculatus* beherbergte eine gerigere Anzahl kultivierbarer Erdöl-abbauender Bakterien. Die Isolate aus dieser Pflanze gehörten den Beta- (*Achromobacter*, *Alcaligenes*) und Gammaproteobakterien (*Pseudomonas, Pantoea*) sowie den Firmicutes (*Bacillus, Paenibacillus*) an. Nur in 50% aller Isolate wurden bekannte Alkan-Hydroxylase Gene (*alk*B,

cytrochrome P153-artige) detektiert, und zwar sowohl auf Plasmiden als auch auf Chromosomen. Unsere Ergebnisse deuten darauf hin, dass sich *alk*B Gene, besonders in der Rhizosphäre von *L. multiflorum*, über horizontalen Gentrasfer im ganzen Bakterienreich verbreitet haben. *L.multiflorum* erscheint für den Einsatz in der Phytoremediation besser geeignet als *L.corniculatus*.

Im zweiten Teil des Projektes wurde die Rolle der Endophyten im Alkanabbau beleuchtet. *L.multiflorum* wurde zwei Monate lang in steriler Erde mit 0.1% und 2% Diesel vorgezüchtet und dann mit einem Alkan-abbauenden Isolat aus der Rhizosphere bzw. Endosphere inoculiert. Das bakterielle Besiedelungsverhalten sowie die Abundanz und Expression von *alk*B Genen in der Rhizosphere, im Wurzelinneren und im Spross wurde beobachtet. Die Ergebnisse zeigten, dass der Endophyt die Pflanze und vor allem das Pflanzeninnere effizienter besiedelte. Darüber hinaus wies dieser Stamm eine höhere Expression des *alk*B-Gen auf, was einen besseren Schadstoffabbau vermuten läßt. Das Rhizospärenbakterium kolonisierte vor allem die Rhizosphäre und zeigte nur eine schwache Expression des *alk*B Gens in der Pflanze. Unsere Ergebnisse deuten darauf hin, dass Endophyten ein großes Anwendungspotential für Phytoremediationstechnologien haben.

Stichwörter: *Erdölverschmutzung, Phytoremediation, Kohlenwasserstoff-tolerante Pflanzen, Kohlenwasserstoff-abbauende Bakterien, Alkan-Monooxygenase (alkB) Gene, horizontaler Gentransfer, Genexpression.*

CHAPTER 1

General Introduction

1.1. Background, Objectives and Scope of the Research

Increase of human needs towards petrochemical products such as natural gas, diesel, gasoline and asphalts has resulted in significant contamination of a number of terrestrial and marine sites with petroleum or petroleum by-products. Petroleum hydrocarbons are composed of various proportions of short, medium and long **aliphatics** (i.e. alkanes, alkenes), **aromatics** (i.e. benzene, toluene, ethylbenzene and xylene ; known as BTEX) as well as **polycyclic aromatic hydrocarbons** (known as PAHs; such as naphthalene, phenanthrene, anthracene) (Frick, *et al*., 1999). Alkanes constitute about 20-50% of crude oil and in addition, alkanes are produced throughout the biosphere by living organisms as waste product, structural elements, as chemoattractants or are part of defence mechanisms (van Beilen, *et al*., 2003).

Petroleum hydrocarbons in the environment are a concern due to:

1) their volatility, which poses a fire/explosion hazard.
2) their toxicity to living organisms; There are evidences of mutagenic effects to bacteria cells and carcinogenic effects to animal cells. PAHs and alkanes (n-C_9 to n-C_{14}) have been found to be more toxic to plants than heavier hydrocarbon compounds above n-C_{15} (Frick, *et al*., 1999).
3) Their mobility. Lighter hydrocarbons are mobile and can be a problem at considerable distances from their point of release due to transport in groundwater or air.
4) their persistence in the environment,

5) their potential interference with water retention and transmission, and with nutrient supplies in soils (O'Connor Associates Environmental Inc., 2008).

The maximum allowed total organic carbon in soil according to the Austrian environmental law is 10 mg/kg soil and 0.1 mg/kg soil for PAHs (http://www.umweltbundesamt.at/).

According to Huang *et al*., 2004, a range of *in situ* and *ex situ* remediation methods including natural attenuation, chemical, physical and mechanical engineering approaches as well as the application of microorganisms have been implemented to reduce the petroleum hydrocarbons contaminations. Each of these methods has advantages and disadvantages in regard to its costs and capacity to remediate the contaminant, for example, physical removal of contaminated soil and washing with solvents are expensive and also need facilities that transfer the contaminated soil to the clean-up location. In the past 15 years, phytoremediation has gained attention as an alternative to deal with petroleum oil contamination due to its low cost compared to engineering-approaches, easiness of implementation and environmentally sound way of application (Pilon-Smits, 2004). **Phytoremediation** is defined as the use of plants and their associated microbes for environmental cleanup from organic or inorganic contaminants (Salt, *et al*., 1995). Due to high hydrophobicity, hydrocarbons tend to interact with non-aqueous phases, strongly adsorbed in soil organic matter and small pores and, as a consequence, become potentially unavailable for microbial degradation. Therefore, it has been suggested that phytoremediation is a promising alternative because plant roots can penetrate small soil pores and access contaminants (Hutchinson, *et al*., 2003). Root exudates comprising nutrients and co-metabolites support the growth of contaminant-degrading microorganisms in the rhizosphere (Frick, *et al*., 1999; Lee, *et al*., 2008), while microbes through its degrading capacity and additional plant growth promoting activities reduce the contaminant's phytotoxicity.

For phytoremediation of petroleum hydrocarbons the following requirements have to be met: a) ability of the plant to produce sufficient biomass in the presence of the contaminant and b) to host degrading and plant growth-promoting bacteria (Huang, *et al*., 2004). The capability of plants to grow in contaminated soil is a plant-species and –genotype dependent trait (Siciliano, *et al*., 2001), whereas the ability of bacteria to degrade petroleum hydrocarbons is due to the containment of degradation genes. For the break-down of alkanes or PAHs hydroxylases (or known as well as oxygenases) are necessary such as alkane hydroxylase encoded by for n-alkane degradation, naphthalene dioxygenase encoded by *ndoB*/*nah* for the degradation of naphthalene, and cathecol 2,3-dioxygenase encoded by *xylE* genes for toluene degradation (Whyte, *et al*., 1997). Therefore, finding the appropriate plant species, which also hosts a microflora containing functional hydrocarbon degradation genes, is of importance for effective phytoremediation.

As petroleum hydrocarbons consist of almost 50% of alkanes, in this Ph.D. study the focus was on analyzing genes responsible for alkane degradation (alkane hydroxylase (*alk*) genes). Particularly, the aim was to understand the functional diversity of hydrocarbon degrading bacteria as well as the alkane degradation genes (*alk* genes) in association with highly tolerant plant species. Understanding the ecology of degrading bacteria will improve phytoremediation strategies.

This thesis is structured in five chapters. **Chapter 1** describes the background and objectives of the research and provides a general introduction on phytoremediation processes, hydrocarbon degrading bacteria, alkane hydroxylase genes and horizontal gene transfer among bacteria communities. **Chapter 2** presents results from experiments addressing the capability of different plant species to grow on soil contaminated with petroleum oil and the analysis of hydrocarbon degrading, plant-associated bacteria. We found that plant species showed different tolerance levels towards petroleum oil contamination and Italian ryegrass (*Lolium multiflorum* L.) and Birdsfoot trefoil (*Lotus corniculatus* L.) showed the best performance towards the contaminant.

A high diversity of hydrocarbon degrading bacteria was isolated from the rhizo- and endosphere of these plants, however, only in half of the isolates alkane hydroxylase genes could be detected. Alkane degrading genes were located either on plasmids or in chromosome of the bacterial strains, and identical alkane monooxygenase genes were identified in different strains, particularly in strains obtained from the Italian ryegrass rhizosphere. These findings suggest the rhizosphere of Italian ryegrass as hot spot for horizontal transfer of *alkB* genes. In **chapter 3** the colonization behaviour and *alkB* gene expression of two strains, *Pseudomonas sp*. ITRI53 (an endophyte) and *Rhodococcus sp*. ITRH43 (a rhizosphere strain), were investigated after applying these strains to Italian ryegrass. We aimed to assess whether endophytes could colonize and express *alkB* genes *in planta*, as most studies so far published have addressed only the rhizosphere. The study demonstrated that the endophyte promoted the growth of Italian ryegrass plantlets during experiment and they colonized the plant better as compared to the rhizosphere strain. Moreover, we could quantitatively measure the *alkB* genes abundance and expression in rhizosphere, shoot and root interior of Italian ryegrass. This result proved the potential role of endophyte in hydrocarbons degradation. **Chapter 4** is intended to review recent phytoremediation technologies which include application of simultaneous remediation methods and the use of genetically modified plants or bacteria to improve phytoremediation processes. Finally, general conclusions on the research performed are summarized in **Chapter 5**.

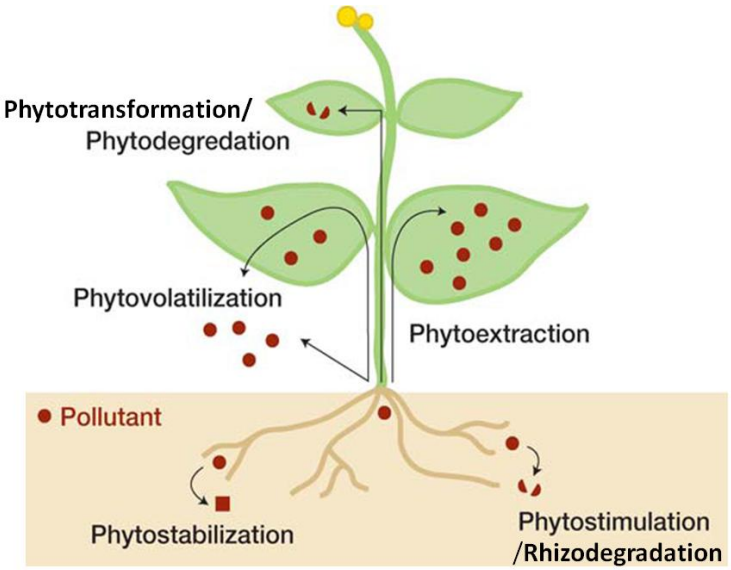
1.2. Phytoremediation Technologies

Phytoremediation is defined as the use of plants and their associated microbes for environmental cleanup from organic or inorganic contaminants (Salt, *et al*., 1995). Although plant and microbes can degrade various contaminants independently, the combined action of both organisms greatly increases degradation efficiency. The role of plants is to support the growth of contaminant-

degrading microbes through the improvement of soil physical and chemical soil conditions due to root activity and the release of root exudates (containing nutrients, co-metabolites or specific enzyme for contaminant degradation). Plants benefit from the ability of those microbes to degrade contaminants thus reducing phytotoxicity and allow the plants to grow in adverse conditions (Günther, *et al*., 1996, Schnoor, *et al*., 1995, McCutcheon and Schnoor, 2003). However, root exudates may also reduce bacterial activity in degrading the contaminant. For example, phenanthrene break-down by *Pseudomonas putida* was repressed after incubation with root extracts of oat (*Avena sativa*), osage orange (*Maclura pomifera*) and hybrid willow (*Salix alba* x *matsudana*) (Rentz, *et al*., 2004). Organic compounds contained in root extracts were apparently responsible for the observed repression as the type of carbon source can regulate the expression of catabolic genes (e.g. by catabolite repression regulation) (Rentz, *et al*., 2004). Overall, besides the importance of bacterial communities, plant species is of concern in phytoremediation, not only because the plant selects its preferred microbial communities (Siciliano, *et al*., 2001) but also due to the type of root exudates that select microbial communities and influence their activity.

The simplest form of phytoremediation is called natural attenuation, because in the presence of low contaminant concentrations the natural vegetation and its associated microflora remediate the soil. cleans the environment without human interference. In this case, only monitoring is needed. However, if contaminants in higher concentrations have to be removed, other approaches are needed. Various available techniques suitable for various types of pollutants are described in **Figure 1** (Pilon-Smits, 2004). **Phytostabilization** refers to stabilizing the pollutant and thereby immobilizing a toxic contaminant in soil, avoiding leaching, runoff or the conversion into more toxic or more bioavailable forms (reviewed by McCutcheon and Schnoor, 2003; Pilon-Smits, 2004). **Phytoextraction** is used to remediate mostly inorganic pollutants such as heavy metals by accumulating these compounds in their tissues. The plant material can be subsequently used for

non-food purposes, ashed, or in case of valuable metals, recycling of the accumulated elements can also be done. The latter technique is called **phytomining**. After uptake in plant tissues, certain pollutants can be released from the plant in volatile form, this mechanism is called **phytovolatilization**. Plants can also degrade or transform organic pollutants directly via their own enzymatic activities after taking up the contaminants through a process called **phytodegradation or phytotransformation**. In **phytostimulation** or **rhizodegradation**, plants facilitate biodegradation of organic pollutants in interaction with microbes in their rhizosphere by releasing nutrients, co-metabolites or specific enzyme that supports the microbial degradation process.



Source: Pilon-Smits, 2004

Fig.1. Mechanisms in phytoremediation of organic and inorganic pollutants

The behaviour of hydrocarbons in soil depends on physical and chemical properties such as the molecular structure, composition, weight, solubility and affinity for lipids or oils and are generally not very soluble in water (Paterson, *et al*., 1994). Furthermore, a large fraction of petroleum hydrocarbons can be strongly adsorbed on soil organic matter, trapped in soil small pores (Hutchinson, *et al*., 2003) and long chain alkanes can contribute to the formation of oil films and

slicks resulting in the blockage of water, soluble nutrients and gas exchange (Leahy and Colwell, 1990). These properties decrease the bioavailability of hydrocarbons to be degraded by soil bacteria and thus limit the process of bioremediation. Taking the phytotoxicity and low bioavailability of hydrocarbons into account, phytoremediation is a promising method to deal with petroleum hydrocarbon contaminants in comparison to bioremediation based solely on microorganisms. Considering the nature of hydrocarbons as mentioned above, rhizodegradation might be the most efficient mechanism for the phytoremediation of hydrocarbons as compared to other phytoremediation techniques (Fig. 1). However, Frick *et al*., (1999) showed that rice seedlings, alfalfa and soybean are able to extract hydrocarbons (^{14}C-methane, 14C-benzene, ^{14}C-anthracene) via their roots or leaves. Alfalfa was also observed of being able to volatize unidentified hydrocarbon compounds when grown on crude-oil contaminated soil (Frick *et al*., 1999). The uptake of hydrocarbons by plants has been found to be related to the plant lipid contents, plants with higher lipid content generally contained higher concentrations of PAHs than plant tissues with lower lipid contents, i.e. alfalfa roots (10 g lipid kg^{-1} dry roots) adsorbed more naphthalene than fescue roots (4.5 g lipid kg^{-1} dry roots) (Frick *et al*., 1999).

Additionally, plants were reported to be able to metabolize hydrophobic organic contaminants such as polychlorinated biphenyl through phytodegradation. Plant metabolism of organic carbons other than photosynthates is not aimed to provide source of energy like in the case of microbial metabolism of carbons but rather to avoid the build-up of toxicity. The plant metabolism of organic compounds is following a mechanism called the *Green-Liver* model (Burken, 2003) (Fig. 2), which shares similarity with mammalian liver function, including the capability to detoxify contaminants and to remove the compounds from the susceptible organelle.

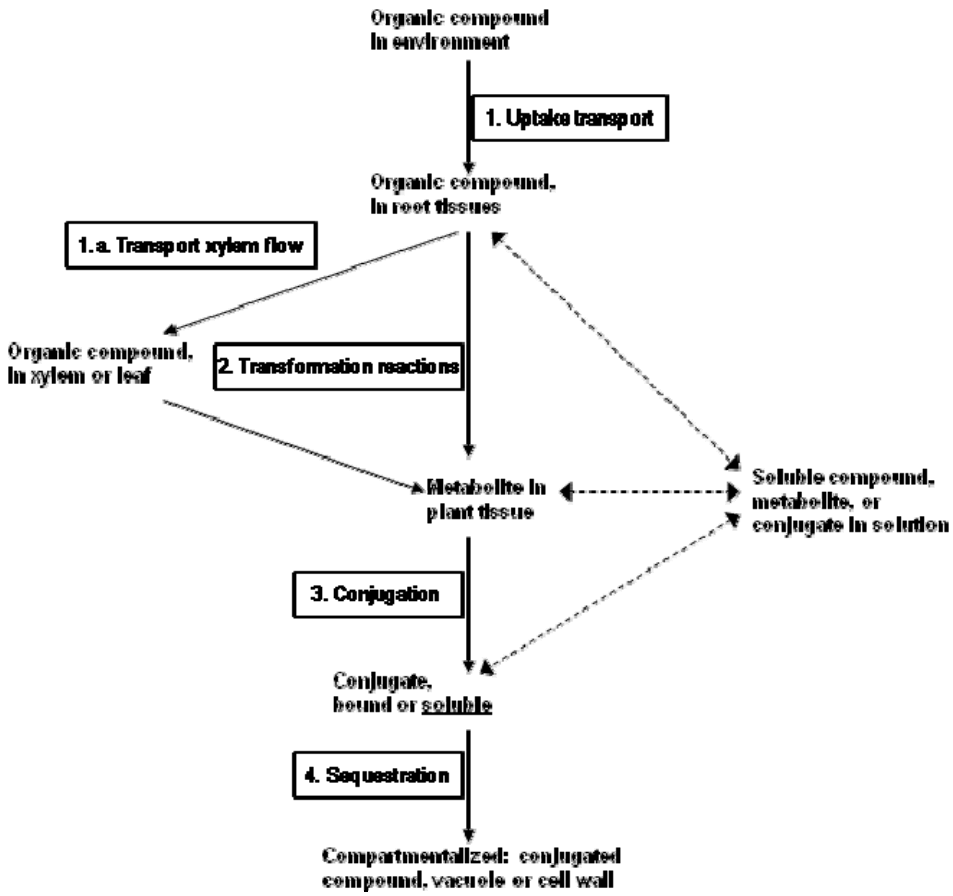


Fig. 2. Schematic diagram of the Green-Liver model of organic contaminant uptake and metabolism by plants in phytoremediation applications. Dashed lines represent pathways that are possible in aquatic phytoremediation (Burken, 2003).

The first step in the Green-Liver model is the uptake and transport of initial organic substrate into plant tissues. Once the initial substrates reside inside plant tissue, the transformation process will take place which involves many different reactions including oxidation and reductions resulted in metabolites. Oxidation of lipophilic compounds is very important for increasing the solubility and this step engages specific enzymes such as cytochrome P450 type enzymes, which belong to the key enzyme families in plant detoxification mechanism through oxidation processes. Following oxidation some compounds are conjugated and the resulting conjugates are generally more water-

soluble and have reduced toxicity compared to their parental substrates. The last step of Green-Liver model is the sequestration of conjugated compounds, which is analogous to mammalian excretion. Unlike mammalians, plants have no active excretion mechanism to remove the conjugated compounds, instead the plants insulate or sequester the conjugated compounds from the catabolic and anabolic metabolism of the plant. This process may also be called as compartimentalization that involves three terminal locations within plant tissues; storage in the vacuole, apoplast (region of plant cell which is outside the plasma membrane), or covalent binding to the cell wall (Burken, 2003).

Not all plant species are suited for phytoremediation of petroleum hydrocarbons. The potential plants shall attain properties such as tolerance towards hydrocarbons, a fibrous root system and the release root exudates that support the degradation of hydrocarbons (Pilon-Smits, 2004). Various plant species that have demonstrated tolerance towards petroleum hydrocarbons are listed in **Table 1**. The fibrous roots of plants can penetrate soil aggregates and small pores, increase exposed surface areas and enhance biodegradation of entrapped hydrophobic contaminants. Similarly, not all plant-associated bacteria are able to degrade hydrocarbons. The appropriate associated bacteria for phytoremediation are those which are tolerant to hydrocarbons and are capable to metabolize the hydrocarbons as their carbon sources for their own growth by specific catabolic genes encoding for hydrocarbon degradating genes.

Table 1. Plant species that showed tolerance[1] to petroleum hydrocarbons
(reviewed by Frick *et al*, 1999; (Kaimi, *et al*., 2007)

Alfalfa (*Medicago sativa* L.)	Meyer zoysiagrass (*Zoysia japonica* var. Meyer)
Alpine bluegrass (*Poa alpina*)	Oat *(Avena sativa)*
Arctic willow (*Salix arctica*)	Prairie buffalograss (*Buchloe dactyloides*)
Alsike clover (*Trifolium hybridum*)	Poplar trees (*Populus deltoides x nigra*)
Arctared red fescue (*Festuca rubra*)	Reed canary grass (*Phalaris arundinacea*)
Annual ryegrass (*Lolium multiflorum*)	Reed grass (*Phragmites australis*)
Barley (*Hordeum vulgare*)	Round sedge (*Carex rotundata*)
Birdsfoot trefoil (*Lotus corniculatus*)	Rock sedge (*Carex rupestris*)
Black medick (*Medicago lupulina*)	Red clover (*Trifolium pratense*)
Bering hairgrass (*Deschampsia beringensis*)	Ryegrass or perennial ryegrass (*Lolium perenne* L.)
Big bluestem (*Andropogon gerardi*)	Sorghum (*Sorghum bicolor*)
Blue grama (*Bouteloua gracilis*)	Soybean (*Glycine max*)
Bell rhodesgrass (*Chloris gayana*)	Sunflower (*Helianthus annuus*)
Bermuda grass (*Cynodon dactylon* L.)	Sudangrass (*Sorghum vulgare* L.)
Bush bean (*Phaseolus vulgaris* L.)	Snow willow (*Salix reticulata*)
Canola (*Brassica rapa*)	Side oats grama (*Bouteloua curtipendula*)
Canada wild-rye (*Elymus canadensis*)	Switchgrass (*Panicum virgatum*)
Cattails (*Typha latifolia*)	Tall cotton-grass (*Eriophorum angustifolium*)
Carrot (*Daucus carota*)	Tilesy sage (*Artemisia tilesii*)
Crested wheatgrass (*Agropyron desertorum*)	Three-square bulrush (*Scirpus pungens*)
Common buffalograss (*Buchloe dactyloides*)	Tall fescue (*Festuca arundinacea* Schreb.)
Duckweed (*Lemna gibba*)	Quackgrass *(Elytrigia repens)*
Fababean (*Vicia faba*)	Verde kleingrass (*Panicum coloratum* var. Verde)
Field pea (*Pisum arvense*)	Water sedge (*Carex aquatilis*)
Indiangrass (*Sorghastrum nutans*)	White clover (*Trifolium repens*)
Jack pine (*Pinus banksiana*)	Wheat (*Triticum aestivum*)
Little bluestem (*Schizachyrium scoparius*)	Western wheatgrass (*Agropyron smithii*)
Maize (*Zea mays* L.)	Winter rye (*Secale cereale* L.)

[1] Tolerance is defined here as the ability of a plant to grow in hydrocarbon contaminated soil; it does not necessarily mean the plant is healthy.

1.3. Hydrocarbon Degrading Bacteria: diversity, responsible enzymes and biodegradation pathway

Hydrocarbon degrading bacteria can be defined as bacteria with the capability to degrade (leading to the formation of less complex intermediate compounds) and/or to mineralize (leading to the formation of water and carbon dioxide) hydrocarbons. Heterotrophic bacteria depend on carbon and electron sources from environment to be used as their energy resource. Microbial

degradation of organic contaminants therefore occurs as result of microorganisms using the contaminants as carbon, energy or nutrient source for their own growth and reproduction. Besides environmental factors such as oxygen, temperature, soil physical-chemical conditions, bioavailability of the contaminant and available nutrients (Romantschuk, *et al*., 2000), the capability to degrade hydrocarbons in soil is also influenced by other factors such as a) bacterial species-dependent capability; that makes every species differ in their capability to metabolize hydrocarbons (Siciliano, *et al*., 1998), b) the bacterial ability to quickly distribute genetic information within a population and thereby to adapt to environmental changes (van Elsas, *et al*., 2003), c) the presence of the contaminant as selective pressure to maintain their degrading capability (van der Lelie, *et al*., 2005) and d) catabolic genes encoding degradation enzymes (Romantschuk, *et al*., 2000). In regard to the catabolic genes, it has been demonstrated that of the presence of petroleum hydrocarbons in soil increased the abundance of specific catabolic genes, however, this stimulation of catabolic activity was not directly related to the increase of total bacterial numbers. This might be due to horizontal gene transfer of degradation genes, which are frequently located on mobile elements (Siciliano, *et al*., 2003).

Various bacteria living in the rhizosphere or endosphere of plants have been reported to be involved in the degradation of petroleum hydrocarbons (**Table 2**). Although capability in degrading hydrocarbons spread across wide range of bacterial species, but in general, bacterial genera *Pseudomonas*, *Arthrobacter*, *Alcaligenes*, *Corynebacterium*, *Flavobacterium*, *Achromobacter*, *Micrococcus*, *Mycobacterium*, and *Nocardia* have reported as the most active bacteria in the degradation of hydrocarbons in soil (Frick *et al*., 1999), whereas *Cellulomonas, Clavibacter, Curtobacterium, Pseudomonas* and *Microbacterium* have been suggested as the most promising

endophytic bacteria (Ryan, *et al*., 2008). Endophytic bacteria are defined as those bacteria that colonize the internal tissue of the plant showing no negative effects on their host (Ryan, 2008).

Table 2. Genera of hydrocarbon degrading bacteria, which grow in the presence of PAHs, BTEX or alkanes

Soil and Rhizosphere Bacteria[1]		Endophytic Bacteria (colonize root, shoot and leaf interior of plant)[2]
Acidovorax	*Methylococcus (only CH_4)*	*Acinetobacter*
Alcaligenes	*Micrococcus*	*Arthrobacter*
Arthrobacter	*Mycobacterium*	*Bacillus*
Achromobacter	*Norcadia*	*Cellulomonas*
Acinetobacter	*Paenibacillus*	*Clavibacter*
Actinomyces	*Proteus*	*Curtobacterium*
Aeromonas	*Pseudomonas*	*Enterobacter*
Alcaligenes	*Rhodococcus*	*Herbaspirillum*
Aquaspirillum	*Rhizobium*	*Micrococcus*
Bacillus	*Sarcina*	*Microbacterium*
Burkholderia	*Serratia*	*Pseudomonas*
Brevibacterium	*Spirilum*	*Paenibacillus*
Bosea	*Sinorhizobium*	*Sphingomonas*
Chromobacterium	*Streptomyces*	*Variovorax*
Corynebacterium	*Staphylococcus*	*Xanthomonas*
Cytophaga	*Sphingomonas*	
Erwinia	*Variovorax*	
Flavobacterium	*Vibrio*	
Methylobacter (only CH_4)	*Xanthomonas*	
Methylobacterium (only CH_4)		

[1] *Source*: (Cookson, 1995; Frick, *et al*., 1999; Van Hamme, *et al*., 2003)

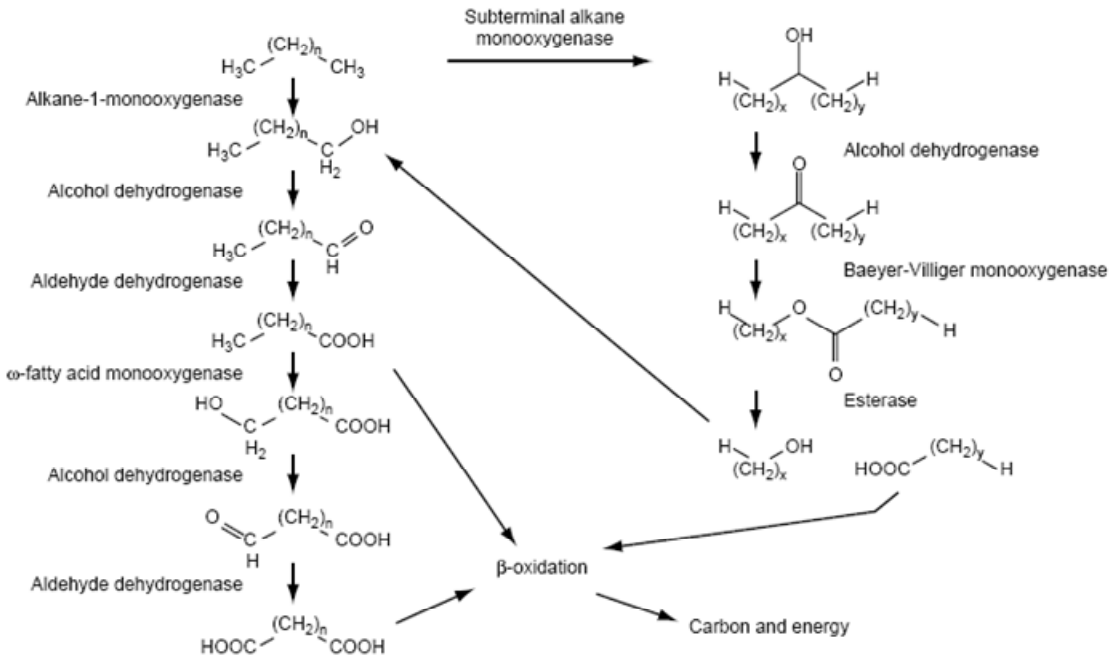
[2] *Source*: (Moore, *et al*., 2006, Ryan, 2008)

Bacterial pathways for the degradation of hydrocarbons contaminants have been the subject of intense study and suggest several important physiological events as key factors that lead to the efficient catabolism of these compounds, which are: **bioavailability**, or the amount of a substance that is physiochemically accessible to microorganisms; **chemotaxis**, or the directed movement of motile organisms towards or away from chemicals in the environment; and **transport mechanisms** for the intracellular accumulation of aromatic molecules (Parales, *et al*., 2008). Degradation of hydrocarbon by bacteria takes place through complex sequence of reduction-oxidation reactions,

which are catalyzed by enzymes. Aliphatic hydrocarbons are oxidized by several alkane hydroxylase enzyme systems including cytochrome P450 enzymes, an integral membrane mono- or di-iron alkane hydroxylase (i.e. alkane monooxygenase), a soluble di-iron methane monooxygenase (sMMO) and a membrane-bound copper-containing (possibly iron-containing) methane monooxygenase (pMMO) (van Beilen and Funhoff, 2005). For the reaction to take place, the compound must pass through the bacteria's cell membrane so the organism's electron transport system can be used for energy storage. Frequently, the bacteria are attached to alkane droplets, which make it more available to bacterial attack, however, the enhanced bioavailability can cause a toxicity problem for the bacteria. In addition, soil moisture lower than 50% and pH above 8.5 appear to inhibit hydrocarbon degradation (Cookson, 1995).

The degradation of hydrocarbons can be divided into aerobic and anaerobic metabolism modes. The **aerobic** alkane degradation pathway is performed by oxidation or incorporating molecular oxygen in the hydrocarbon by a membrane-bound alkane monooxygenase and two soluble enzymes, rubredoxin and rubredoxin reductase, which act as electrons carriers between NADH and the hydroxylase for conversion of alkane to alcohol. Commonly, the oxidation takes place on one or both terminal methyl group or at a sub-terminal location **(Fig. 3)**. The alcohol can be further oxidized to an aldehyde and acid prior to proceeding into the β-oxidation and tricarboxylic acid cycle (TCA cycle) to produce energy (van Beilen, *et al*., 2003). This pathway is best describing the alkane degradation by *Pseudomonas putida* GPO1, in which the genes coding for alkane monooxygenase are located on a plasmid (Marín, *et al*., 2001). Short chain alkanes except methane are more difficult to degrade and may require co-metabolism- defined as the degradation of a compound only in the presence of other organic material that serves as the

primary energy source. Branched alkanes and cyclic alkanes are much less susceptible to degradation.



Source: van Beilen *et al*., 2003

Fig. 3. Metabolic pathway for degradation of alkanes by terminal and sub-terminal oxidation.

Anaerobic degradation of hydrocarbons is restricted to anaerobic photoheterotrophic bacteria *(*i.e. *Blastochloris sulfoviridis),* Fe(III)-reducing bacteria (i.e. *Geobacter*), denitrifying bacteria (i.e. *Azoarcus, Dechloromonas, Pseudomonas and Thauera*), and sulfate-reducing bacteria (i.e. *Desulfobacterium, Desulfobacula*) or to proton-reducing and methanogenic bacteria living in syntrophic consortia (phenomenon that one species lives off the products of another species). It has been reported that hydrocarbons such as toluene, alkylbenzenes, benzene, naphthalene, phenanthrene, $>C_6$ n-alkanes, branched alkanes and hydrocarbon mixtures can be degraded under anaerobic conditions.

1.4. Diversity of Alkane Hydroxylase Systems

As described above, the first step for alkane degradation under aerobic conditions is oxidation by an alkane hydroxylase enzyme. Hydroxylases catalyze the addition of hydroxyl groups (-OH) by attaching oxygen atoms (oxidation) during hydroxylation reactions. The terms of hydroxylase and oxygenase is commonly used interchangeably. However, oxygenases belong to the calss of oxidoreductases that catalyze the incorporation of oxygen to the substrate (http://medical-dictionary.thefreedictionary.com/). In prokaryotes and eukaryotes several enzyme systems have evolved to mediate hydroxylation or oxygenation of aliphatic hydrocarbons. Depending on the chain-length of the alkane substrate, different enzyme systems are required to oxidate their substrate and thereby initiate the biodegradation process. In summary, the responsible alkane hydroxylase enzymes, their encoding genes, substrate range and examples of host organisms are as follows:

1. Bacterial **soluble di-iron methane monooxygenase (sMMO)** encoded by the *mmo* gene cluster (McDonald, *et al*., 1997); substrate range C_1-C_{10} (*Methylisinus trichosporium, Methylococcus capsulatus*), C_2-C_8 for butane monooxygenase (*Pseudomonas butanovora*) (van Beilen and Funhoff, 2005).
2. Bacterial integral membrane copper or iron-containing **particulate methane monooxygenase (pMMO)** encoded by *pmoA, pmoB* and *pmoC* genes (Hoffmann, *et al*., 2002); substrate range for short alkanes C_1 – C_5 (found in all known methanothrops) (van Beilen and Funhoff, 2005).
3. **Cytochrome P450 alkane hydroxylase** enzyme family; in bacteria these enzymes are encoded by CYP153 genes (encoding class I P450s), in eukaryotic yeast and fungi are encoded by CYP52 genes, in mammals are CYP2E and CYP4B (encoding class II P450s);

substrate range for bacteria C_4 – C_{16} (e.g. *Sphingomonas* sp., *Mycobacterium* sp., *Acinetobacter* sp.), substrate range for eukaryote C_{10} – C_{16} (e.g. *Candida maltose, Yarrowia lipolytica*), substrate range for mammals C_6 – C_{10} (humans and rabbits) (van Beilen and Funhoff, 2005).

4. Bacterial integral membrane non-heme iron **AlkB-related alkane hydroxylase** or **alkane monooxygenase** (alkB) encoded by a *alk* gene cluster; substrate range between medium and long chain alkanes C_5 – C_{16} (e.g. *Acinetobacter, Burkholderia, Mycobacterium, Pseudomonas*) and for *Rhodococcus* C_6 – C_{36} (van Beilen, *et al*., 2001, 2003, van Beilen and Funhoff, 2005).

In recent reviews by van Beilen *et al*. (2003, 2007) it has been suggested that in most bacterial strains, alkane hydroxylase genes seem to be distributed over the genome, they can be located on chromosomes, plasmids, or transposons. These genotypes are widespread in the environment since alkanes are ubiquitous in surroundings due to biogenic and anthropogenic activities. Alkane hydroxylase gene homologs (defined as *similarity in gene nucleotide sequence that is perceived come from similar ancestor* (Webber and Ponting, 2004)), particularly for gene hemologs encoding *alkB*-related alkane hydroxylases and bacterial CYP450, have been found in phylogenetically, different bacteria indicating that the horizontal gene transfer has played an important role in spreading these genes. However, understanding the evolution of such diverse families of alkane hydroxylases with essentially the same function still remains a challenge. Moreover, very frequent multiple alkane hydroxylase genes are present in one alkane degrading bacterium rendering them the capactity to utilize a wide range of alkanes. To date, the best *alkB* gene system is the integral membrane *alkB*-related alkane hydroxylase found in *Pseudomonas putida* strain GPo1.

Soluble and particulate methane monooxygenase (sMMO and pMMO) are two types of enzyme systems that are known to oxidize methane, propane and butane. Besides the oxidation of methane, sMMO is able to oxidize saturated and unsaturated alkanes as well as halogenated, aromatic and heterocyclic compounds, whilst pMMO has a much narrower substrate range, which appears to be restricted to alkanes and alkenes up to C_5 (van Beilen and Funhoff, 2005). Butane monooxygenase (BMO) found in *P. butanovora* is not up-regulated by butane, but is induced by the products of monooxygenase activity, butyraldehyde and 1-butanol (Doughty, *et al*., 2006). This gene regulation system is different to the alkane monooxygenase (*alkB*) genes in *P. putida* GPo1, which is induced by presence of alkanes and repressed during growth on medium containing simple organic acids (known as catabolite repression system) (Doughty, *et al*., 2006).

The **cytochrome P450 alkane hydroxylase** enzyme system comprises up to date more than 4000 different enzymes, of which 10-15% are found in prokaryotes. So far only few P450s enzymes have been identified and characterized. The most researched are CYP101 or better known as P450 camphor, an enzyme found in *P. putida* ATCC 29609 that oxidizes camphor, and Class II P450 (soluble) enzyme from *Bacillus megaterium* ATCC 14581 thought to be involved in fatty acid metabolism. The CYP450 enzyme families are divided into two classes. Class I P450 enzymes are soluble enzymes located in the cytoplasm, and consists of 3-component systems comprising cytochrome P450, ferredoxin and ferredoxin reductase subunits. These enzymes need heme (conjugated protein) as well as iron-sulfur as cofactor during catalysis. This enzyme system is found among bacteria that oxidize C_5-C_{10} alkanes, alicyclic compounds and limonene, encoded by CYP153 gene family (van Beilen and Funhoff, 2005) and is commonly found in alkane degrading bacteria that lack the integral membrane alkane hydroxylase (van Beilen *et al*., 2006). Class II P450s enzymes are contained in the microsome, consist of two-component systems comprising a

membrane-bound cytochrome P450 and a reductase, and need heme as cofactor. The enzymes are encoded by genes belonging to the CYP52 family and are usually found in multiple copies in various yeast strains. In mammals, the CYP2E1 gene is involved in the metabolism of endogenous compounds and xenobiotics and is the key enzyme in the microsomal pathway for ethanol oxidation.

AlkB-related integral membrane alkane hydroxylase or alkane monooxygenase (*alkB*) is the most common enzyme found in alkane degrading bacteria, first discovered in a hexane-degrading fluorescent *Pseudomonas* strain now known as *P. putida* strain GPo1. This enzyme system is a cytoplasmic integral membrane protein comprising alkane monooxygenase (AlkB), one or two rubredoxins and electron providing rubredoxin reductase, and needs iron as cofactor (Fig.4).

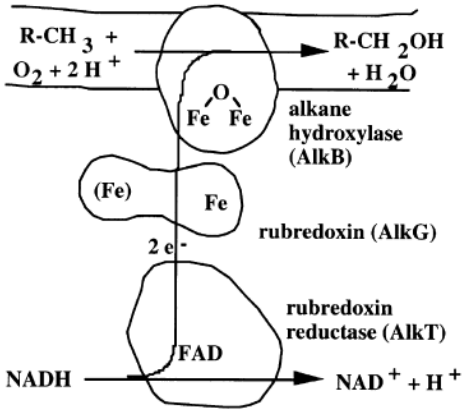


Fig.4. Alkane monooxygenase system of *P. putida* GPo1. The AlkB is an integral cytoplasmic membrane protein containing di-iron. Rubredoxin (AlkG) transfers electrons from the flavoprotein rubredoxin reductase (AlkT) to AlkB (Staijen *et al*., 2000).

Functional *alkB* homologs are present in many alkane degrading α-,β- and γ-Proteobacteria and high G+C content Gram positive bacteria. Moreover, many bacterial strains contain multiple, quite divergent *alkB* genes, which might have the following characteristics: 1) the enzymes have different substrate ranges, 2) different enzymes might be active during different growth phases, 3)

AlkBs could have different affinity or preference towards different alkanes, or 4) it is possible that some *alkB* genes are pseudogenes or no longer expressed (van Beilen and Funhoff, 2005). In *P. putida* GPo1 and *Alcanivorax burkomensis*, the substrate range of AlkB is determined by an amino acid position within the enzyme (van Beilen *et al*., 2005).

The alkane monooxygenase encoding gene in *P. putida* GPo1 is present in two *alk* gene clusters or operons located on OCT plasmid and is part of a 55 kb mobile element that integrated in the OCT plasmid. According to van Beilet *et al*. (2001), the *alk* clusters consist of a 18 kb fragment containing the *alkBFGHJKL* operon and 16.9 kb fragments containing the *alk*ST operon. The *alkBFGHJKL* operon encodes different enzymes required for the oxidation of alkanes to acetyl-CoA, namely; alkane hydroxylase (*alkB*), two rubredoxins (*alkF* and *alkG*), an alcohol and aldehyde dehydrogenase (*alkJ* and *alkH*), an acyl-CoA synthetase (*alkK*) and an outer-membrane protein of unknown function (*alkL*), whereas the *alkST* operon encoding rubredoxin reductase (alkT) and alkS that positively regulates the expression of *alkBFGHJKL* operon and also the alk*ST* genes. The two clusters are separated by a 9.7 kb fragment, in which *alkN* is located, a gene coding for a methyl-accepting transducer protein that may be involved in alkane chemotaxis (**Fig. 5**).

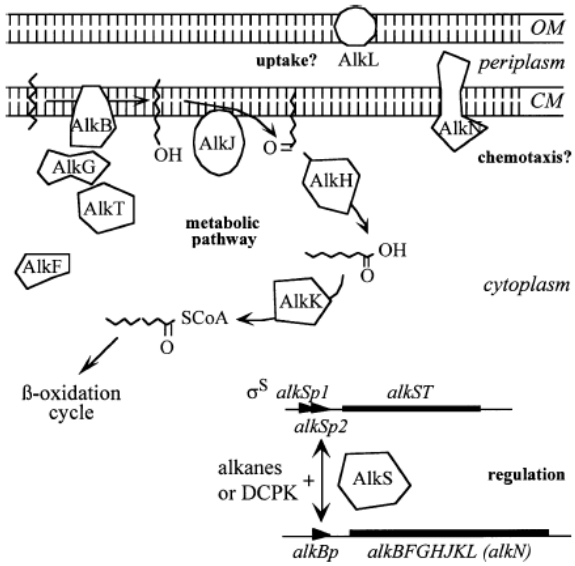


Fig. 5. Location and function of the *alk* gene operon and products during alkane degradation. The products are alkane hydroxylase (*alkB*), two rubredoxins (*alkF* and *alkG*), alcohol dehydrogenase (*alkJ*), aldehyde dehydrogenase (*alkH*), acyl-CoA synthetase (*alkK*) and an outer-membrane protein of unknown function maybe involved in uptake (*alkL*), methyl-accepting transducer protein that may be involved in alkane chemotaxis (*alkN*), rubredoxin reductase (*alkT*) and positive regulator of the expression of *alkBFGHJKL* operon and the alkST genes (*alkS*) (van Beilen *et al*., 2001, Van Hamme *et al*., 2003).

Closely related genes to *P. putida alk* genes were found in *P. putida* strain P1 originally isolated from a pentane enrichment culture, however, in this strain the *alk* genes are located on chromosome. Comparing *alk* gene sequences and the cluster arrangement of strains *GPo1* and strain P1 (see **Fig. 6**) revealed the presence of insertion sequences (IS) in both strains. The segment between *alkL* and *alkN* and up-and downstream of *alkST* are entirely composed of insertion sequences, and encode proteins like transposase which is involved in DNA transposition events. These IS elements may have played a role in the recruitment of these genes, and also increase the potential of further exchange of the genes between different hosts and replicons (Top and Springael, 2003). This may explain the widespread ocurrence of *alkB* homologs in different bacterial genera.

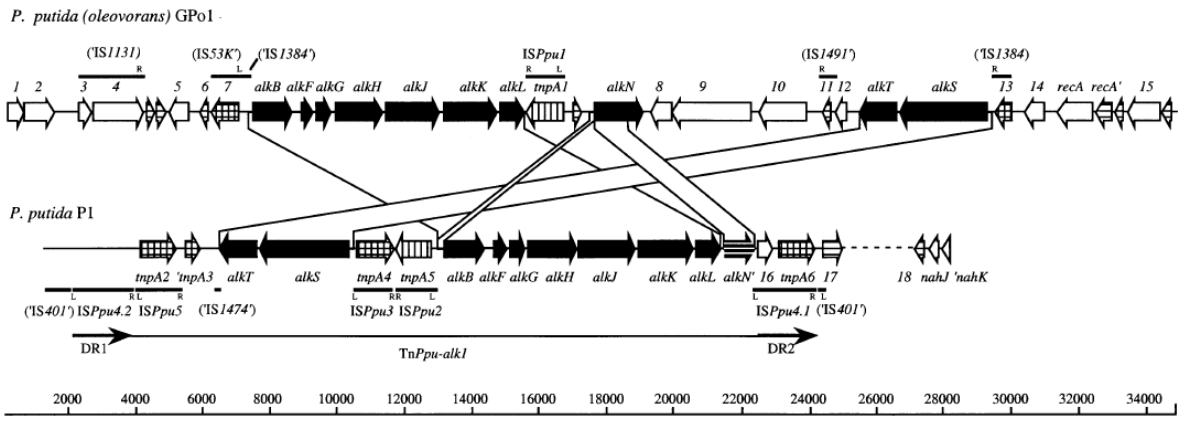


Fig. 6. Schematic arrangement of *P. putida* GPo1 and *P. putida* P1 *alk* genes and flanking DNA. The scale is in bp. Arrows represent potential coding regions. Black arrows represent genes involved, or presumably involved, in alkane degradation and vertically hatched arrows represent complete or partial transposase genes. Horizontally hatched arrows (also in combination with vertically hatched) indicate that the ORFs are incomplete or interrupted by stop codons or frameshifts. Black bars correspond to (incomplete) insertion sequences. IS*Ppu1–5* are new IS elements named in this study. The other black bars represent incomplete IS elements, with the name of the most closely related IS element shown between parentheses. The orientation of the IS elements is marked by L and R for left and right end. Bars linking the GPo1 and P1 DNA segments indicate homologous regions. DR means direct repeats formed by IS*Ppu4.1* and IS*Ppu4.2* (van Beilen *et al*., 2001).

In a single operon, regulation of gene expression can be regulated by controlling the amount of *alk* mRNA (known as *transcriptional regulation*), or the transcription terminates soon after the transcription begins unless certain conditions are met (so called *attenuation*), or alternatively, the translation of the gene may be inhibited even after the mRNA has been made (so called *translational regulation*). In the case of bacteria adjusting to major changes in the environment, they require regulatory systems that simultaneously regulate numerous operons. These systems are called *global regulatory mechanisms*, which involve catabolite respression (mechanism that ensures the availability of the best carbon and energy source), available alternative sigma transcription factors (σ^{54}), global regulatory proteins, or available components of the electron transport chain that are used to monitor the physiological status, which can have substantial impact on a metabolic pathway (Snyder and Champness, 2003). The alkane degradation pathway

encoded by the *alk* operons located on the OCT plasmid of *P. putida* GPo1 follows the catabolite repression mechanism as explained in **Fig. 7**. It is a useful model to study global regulation responses that affect the induction of catabolic pathways.

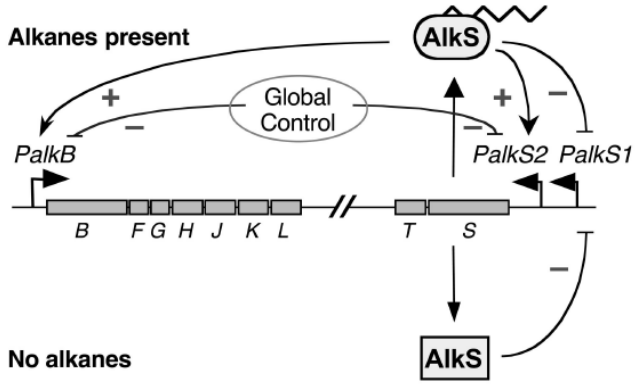


Fig. 7. Regulation of the genes encoding the *P. putida* GPo1 alkane degradation pathway. The genes are grouped into two clusters, *alkBFGHJKL* and *alkST*, both of which are regulated by the AlkS protein. When no alkanes are present, *alkS* is expressed at low levels from promoter *PalkS1*; AlkS negatively modulates the expression of this promoter, resulting in low constant expression of the gene. When alkanes become available, AlkS activates transcription from the *PalkB* and *PalkS2* promoters, generating a positive amplification loop for *alkS* expression. Induction of these two promoters by alkanes is negatively modulated by a dominant global control when cells are grown in the presence of alternative carbon sources, such as some organic acids (succinate or pyruvate) or amino acids (Dinamarca *et al*., 2003)

1.5. Horizontal Gene Transfer

Horizontal gene transfer (HGT) between bacteria is the exchange of genetic material between donor and recipient cells, and stable persistence in the latter either by integration or autonomous replication. HGT is one of mechanisms, by which bacteria acquire genetic information from either related or phylogenetically distinct populations in the community. It plays an important role in prokaryotic evolution and adaptation. Genes of prokaryotes can be transferred from one cell to

another by three distinct processes, namely: 1) transformation, an uptake of free DNA by bacterial cells; 2) transduction, a process whereby the gene is transferred via a bacterial virus as vector; and 3) conjugation, a gene transfer process that takes place during cell contact, and which involves specific structures to enable DNA transfer (Lorenz, 1992, van Elsas, 2003). Hot spots for HGT are sites / habitats, which support high microbial density and metabolic activity (van Elsas *et al*., 2003). These include environments such as the rhizosphere, the phyllosphere or the animal/human gut . Abiotic (e.g. temperature, pH, moisture content, micro and macro-nutrient availability, transfer-stimulating compounds availability, presence of selective pressure such as a pollutant, presence of surface and oxygen) and biotic (presence of plants or grazing, antagonistic, competing or syntropic organisms) parameters may influence HGT (van Elsas *et al*., 2003; Johnsen and Kroer, 2007). However, it is not known to which extent these factors affect natural HGT as they have different effects under different conditions (van Elsas *et al*., 2003). The detection of gene transfer can be conducted through selection of specific genetic traits contained in the transferred gene as selective marker, such as genes encoded resistance to antibiotics or pollutants. Recently, utilization of green fluorescent protein gene (*gfp*) tagged on plasmid carrying catabolic gene has removed some constraints imposed by the need to select the transconjugants. The donor cell contains a conjugative plasmid tagged with the green fluorescent protein (GFP) gene (*gfp*) downstream from a LacI repressible promoter. The donor chromosome encodes LacI, which represses the expression of GFP. During conjugation, the plasmid is transferred from the donor cells to the recipients, which become transconjugants. Expression of *gfp* is not repressed in the transconjugant cells, and these cells consequently fluoresce green. In contrast to previous detection methods, which rely on successful cultivation of transconjugants on selective media, this approach only requires that recipient cells can express the *gfp* gene cassette. This approach allows *in situ* HGT detection (Davison, 1999; Sørensen *et al*., 2005). Indirectly, HGT of functional genes

can be detected through nucleotide sequence analysis, by which identical DNA sequences of specific genes found in different bacterial genera are considered as an indication of such genes being transferred horizontally (Wilson *et al*., 2003).

Transformation processes take place in natural habitats probably as a means to deal with environmental changes, to protect cells, to improve nutrient acquisition and for transformational repair. The main problem of transformation is the exposure of free DNA to DNase that is ubiquitous in the environment. According to work by Lorenz & Wackernagel, 1994, free DNAs have been suggested to mostly derive from bacterial excretion, lysis or the release of membrane-derived vesicles containing chromosomal and plasmid DNA. These continuous production and release of DNA by organisms may be one reason for the presence of free DNA in environment despite of potential cleavage by DNases . Moreover, the particulate constituents of soil, such as clay and quartz, play a role in protecting DNA from DNase by forming complexes of DNA and clay minerals. DNA can be also bound to solid material or remain in dead cells (**Fig. 8**). Experiments, in which purified plasmid DNA was inoculated into different non-sterile soils, showed that the plasmids remained intact for 2-5 days before degradation commenced. Transformation requires competent recipient cells in the environment, cells that possess the ability to take up free DNA from surroundings. This property is widely distributed among the taxonomic and trophic groups of bacteria, which infers a long evolutionary history of natural competence (Lorenz & Wackernagel, 1994). The transformation frequency among them is variable and depends on the growth state, cell type specificity and nutritional availability. Competent bacterial cells tend to take up DNA from their own species, while others take up any DNA (Lorenz & Wackernagel, 1994). In general, the presence of homology between the taken up DNA and the recipient genome is a prerequisite for successful acquisition of DNA into chromosome (van Elsas *et al*., 2003).

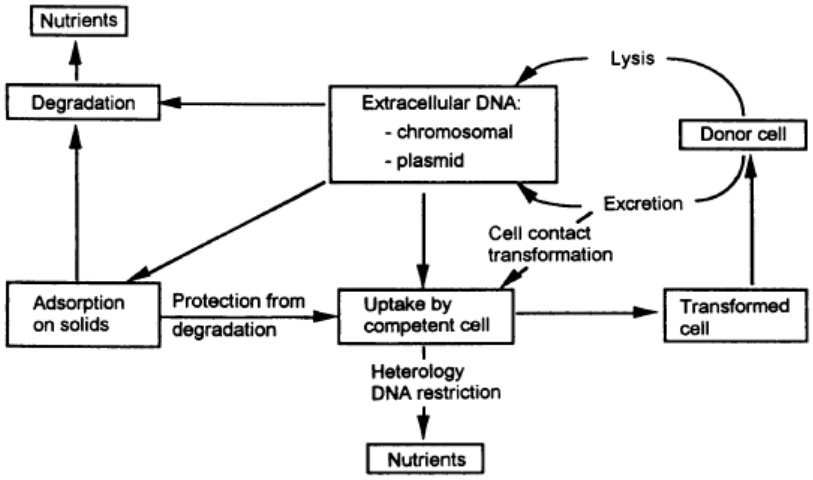


Fig. 8 Schematic overview of gene transfer of free DNA in the aquatic and terrestrial environment (Lorenz & Wackernagel, 1994)

Transduction is the transfer of genetic material to bacteria by bacteriophages and can be categorized into two types: 1) *generalized transduction*, in which essentially any region of the bacterial DNA can be transferred from one bacterium to another through phage infection, and 2) *specialized transduction*, in which only certain genes close to the attachment site of lysogenic phage in the chromosome can be transferred. In generalized transduction, while phages are packaging their own DNA, they mistakenly package some DNA of bacterial host. These phages are still capable of infecting other cells, but progeny phages will not be produced. The packaged, bacterial DNA may recombine with the host chromosome if the tranferred and the recipient DNAs have sequences in common. If the injected DNA is a plasmid, it may replicate after it enters the cell and thus being maintained. If the incoming DNA contains a transposon, it may insert itself into a plasmid or the chromosome of the recipient (Snyder and Champness, 2003).

Conjugation is the most important mechanism of HGTG. Genes that encode degradation pathways or antibiotic resistance determinants are often located on plasmids, transposons or other mobile

genetic elements. Plasmids are independently replicating, circular or linear DNA molecules, while transposons are mobile elements, which are transferred to other genetic locations with the help of transposase enzymes. (Snyder and Champness, 2003).

The following scenarios of conjugation among bacteria exist:

a) transfer of self-transmissible plasmids and conjugative transposons. In this mechanism, plasmids and transposons are able to transfer themselves to the host cell during conjugation. Genes that encode transfer functions (*tra*) are required (Snyder and Champness, 2003).
b) mobilization of a non-self-transmissible plasmid, which contains an origin of conjugal transfer (*oriT*) and mobilization genes (*mob*) genes, can be transferred by the presence of a conjugative plasmid (the latter is not usually transferred in the same time)(Snyder and Champness, 2003).
c) cointegration, where two different circular plasmids may fuse. Thus, a non-self-transmissible, non-mobile plasmid may combine with a transmissible, conjugative plasmid and thereby both plasmids can be subject of HGT. Such a plasmid fusion is often facilitated by the presence of insertion elements or conjugative transposons on one of the plasmids (Davison, 1999)
d) retro-transfer, where a donor strain harbors a conjugative plasmid which is transferred to a recipient cell, where it may mobilize an already existing non-conjugative plasmid, which will then be transferred back to the donor (Haagensen *et al*., 2002).

Besides the biotic and abiotic factors, the transfer of plasmids depends on plasmid-specific characteristics that affect the host metabolism. The transfer and replication systems of plasmids define their host range (some plasmids have a broad-host range, and some are very specific),

whereas the regulation of transfer genes determines the extent of transfer amongst potential hosts (Johnsen and Kroer, 2007). Another categorization of plasmid is based on the ability to stably coexist in the same host cell for many generations with other plasmid. This categorization is called incompatibility group. Two plasmids that cannot stably coexist in the same cell are said to be incompatible and are in the same incompatibility (Inc) group. Incompatibility can be due to plasmids having the same mechanism of replication or partitioning system. Examples of Inc group of plasmids are IncP-1, IncP-2, and IncP-9, which are also identified as self-transmissible and broad-host range plasmids (Snyder and Champness, 2003). Plasmids harboring catabolic genes are commonly called catabolic plasmids. Among these, the IncP-2 and InP-9 plasmids often encode degradation of naturally occurring compounds, whereas genes that encode degradation of mostly xenobiotic compounds are often encoded by IncP-1 plasmids (Top and Springael, 2003).

Transposons are classified as class I and class II transposons, which are often located on catabolic plasmids. Class I transposons are called retrotransposons, which work by copying themselves (initially to RNA) and pasting copies back into genome in multiple places, examples are Tn5280 found in *Pseudomonas* sp. P51 and contain chlorobenzene degradation genes (Top and Springael, 2003). Class II transposons do not involve RNA intermediates in their transposition mechanism, instead they move by a cut and paste mechanism based on homologous recombination by using transposase. Examples of class II transposons are Tn4651 found in *Pseudomonas putida* mt-2 which is involved in toluene and xylene degradation (Top and Springael, 2003; Snyder and Champness, 2003).

REFERENCES

Burken, J. G., (2003) Uptake and Metabolism of Organic Compounds: Green-Liver Model. In: Phytoremediation: Transformation and Control of Contaminants. S. C. M. a. J. L. Schnoor (ed). New Jersey: John Wiley & Sons, Inc., pp. 355-386.

Cookson, J. T. J., (1995) *Bioremediation Engineering: Design and Application*. McGraw-Hill, Inc., New York.

Davison, J., (1999) Genetic exchange between bacteria in the environment *Plasmid* **42**: 73-91.

Doughty, D. M., Sayavedra-Soto, L.A., Arp, D.J., Bottomley, P.J., (2006) Product repression of alkane monooxygenase expression in *Pseudomonas butanovora Journal of Bacteriology* **188**: 2586-2592.

Frick, C. M., Farrel, R.E., Germida, J.J., (1999) Assessment of phytoremediation as an in-situ technique for cleaning oil-contaminated sites. In. Saskatoon, Canada: Department of Soil Science, Univ. of Saskatchewan

Günther, T., Dornberger, U. and Fritsche, W., (1996) Effects of ryegrass on biodegradation of hydrocarbons in soil. *Chemosphere* **33**: 203-215.

Haagensen, J. A. J., Hansen, S.K., Johansen, T., Molin, S., (2002) In situ detection of horizontal gene transfer of mobile genetic elements. *FEMS Microbiology Ecology* **42**: 261-268.

Hoffmann, T., Horza, H-P., Kemnitza, D., Conrad, R., (2002) Diversity of the particulate methane monooxygenase gene in methanotrophic samples from different rice field soils in China and the Philippines. *Systematic and Applied Microbiology* **25**: 267-274

Huang, X.-D., El-Alawi, Y., Penrose, D.M., Glick, B.R., Greenberg, B.M., (2004) A multi-process phytoremediation system for removal of polycyclic aromatic hydrocarbons from contaminated soils. *Environmental pollution* **130**: 465-476.

Hutchinson, S. L., Schwab, A.P., Banks, M.K., (2003) Biodegradation of petroleum hydrocarbons in the rhizosphere. In: Phytoremediation: Transformation and Control of Contaminants. S. C. M. a. J. L. Schnoor (ed). New Jersey: John Wiley & Sons, Inc., pp. 355-386.

Johnsen, A.R., Kroer, N., (2007) Effects of stress and other environmental factors on horizontal plasmid transfer assessed by direct quantification of discrete transfer events. *FEMS Microbiology Ecology* **59**: 718-728.

Kaimi, E., Mukaidani, T., Tamaki, M., (2007) Screening of twelve plant species for phytoremediation of petroleum hydrocarbon-contaminated soil. *Plant Production Science* **10**: 211-218.

Leahy, J. G., Colwell, R.R., (1990) Microbial degradation of hydrocarbons in the environment. *Microbiological Reviews* **54**: 305-315.

Lee, S.-H., Lee, W-S., Lee, C-H., Kim, J.G., (2008) Degradation of phenanthrene and pyrene in rhizosphere of grasses and legumes. *Journal of Hazardous Materials* **153**: 892–898.

Lorenz, M. G., (1992) Gene transfer via transformation in soil/sediment environments. In: Gene Transfer and Environment. M. J. Gauthier (ed). Berlin: Springer-Verlag, pp. 95-101.

Lorenz, M. G., Wackernagel, W., (1994) Bacterial gene transfer by natural genetic transformation in the environment. *Microbiological Reviews* **58**: 563-602.

Marín, M., Smits, THM., van Beilen, JB., Rojo, F., (2001) The alkane hydroxylase gene of *Burkholderia cepacia* RR10 is under catabolite repression control. *Journal of Bacteriology* **183**: 4202–4209.

McCutcheon, S.C., Schnoor, J. L., (2003) Overview of phytotransformation and control of waste. In: Phytoremediation: Transformation and Control of Contaminants. S. C. M. a. J. L. Schnoor (ed). New Jersey: John Wiley & Sons, Inc., pp. 355-386.

McDonald, I. R., Uchiyama, H., Kambe, S., Yagi, O., Murrel, J.C., (1997) The soluble methane monooxygenase gene cluster of the trichloroethylene-degrading methanotroph *Methylocystis* sp. strain M. *Applied and Environmental Microbiology* **63**: 1898-1904.

Moore, F. P., Barac, T., Borremans, B., Oeyen, L., Vangronsveld, J., van der Lelie, D., Campbell, C.D., Moore, E.R.B. , (2006) Endophytic bacterial diversity in poplar trees growing on a BTEX-contaminated site: The characterisation of isolates with potential to enhance phytoremediation. *Systematic and Applied Microbiology* **29**: 539–556.

O'Connor Associates Environmental Inc., M. E. I., (2008) Canada-wide standard for petroleum hydrocarbpns (PHC) in soil In. C. C. o. M. o. t. Environment (ed). Canada, pp.

Paterson, S., Mackay, D., McFarlane, C., (1994) A model of organic chemical uptake by plants from soil and the atmosphere. *Environmental Science and Technology* **28**: 2259-2266.

Pilon-Smits, E., (2004) Phytoremediation. *Annual Review of Plant Biology* **56**: 15-39.

Parales, R. E., Ju, K-S., Rollefson, J.B., Ditty, J.L., (2008) Bioavailability, Chemotaxis, and Transport of Organic Pollutants In: Microbial Biodegradation: Genomics and Molecular Biology. E. Díaz (ed). Norfolk, UK: Caister Academic Press, pp. 145-188.

Rentz, J. A., Alvarez, P.J., Schnoor, J.L., (2004) Repression of Pseudomonas putida phenanthrene-degrading activity by plant root extracts and exudates. *Environmental Microbiology* **6**: 574-583.

Romantschuk, M., Sarand, I., Petänen, T., Peltola, R., Johnsson-Vihanne, M., Koivula, T., Yrjälä, K., Haahtela, K., (2000) Means to improve the effect of in situ bioremediation of contaminated soil: an overview of novel approaches. *Environmental Pollution* **107**: 179-185.

Ryan, R. P., Germaine, K., Franks, A., Ryan, D.J., Dowling, D.N., (2008) Bacterial endophytes: recent developments and applications. *FEMS Microbiology Letters* **278**: 1-9.

Salt, D., Blaylock, M., Kumar, N.P.B.A., Dushenkov, V., Ensley, BD., (1995) Phytoremediation: a novel strategy for the removal of toxic metals from the environment using plants. *Biotechnology* **13**: 468–474.

Schnoor, J. L., Licht, L.A., McCutcheon, S.C., Wolfe, N.L. and Carreira, L.H., (1995) Phytoremediation of organic and nutrient contaminants. *Environmental Science and Technology* **29**: 318-323.

Siciliano, S. D., Fortin,N., Mihoc,A., Wisse,G., Labelle,S., Beaumie,D., Ouellette,D., Roy,R., Whyte,L.G., Banks,M.K., Schwab, P., Lee,K., Greer,C.W., (2001) Selection of specific endophytic bacterial genotypes by plants in response to soil contamination. *Applied and Environmental Microbiology* **67**: 2469–2475.

Siciliano, S. D., Germida, J. J., (1998) Mechanisms of phytoremediation: biochemical and ecological interactions between plants and bacteria. *Environmental Review* **6**: 65-79.

Siciliano, S. D., Germida, J.J., Banks, M.K., Gree, C.W., (2003) Changes in microbial community composition and function during a polyaromatic hydrocarbon phytoremediation field trial. *Applied and Environmental Microbiology* **69**: 483-489.

Snyder, L., Champness, W., (2003) *Molecular Genetics of Bacteria*. ASM Press, Washington, USA.

Sørensen, S.J., Bailey, M., Hansen, L.H., Kroer, N., Wuertz, S., (2005) Studying plasmid horizontal transfer *in situ*: a critical review. *Nature Reviews Microbiology* **3**:700-710.

Staijen, I. V., van Beilen, J.B., Witholt, B., (2000) Expression, stability and performance of the three-component alkane mono-oxygenase of *Pseudomonas oleovorans* in *Escherichia coli*. *European Journal of Biochemistry* **267**: 1957-1965.

Top, E. M., Springael, D., (2003) The role of mobile genetic elements in bacterial adaptation to xenobiotic organic compounds. *Current Opinion in Biotechnology* **14**: 262-269.

van Beilen, J. B., Panke, S., Lucchini, S., Franchini, A.G., Röthlisberger, M., Witholt, B. , (2001) Analysis of *Pseudomonas putida* alkane-degradation gene clusters and flanking insertion sequences: evolution and regulation of the *alk* genes. *Microbiology* **147**: 1621-1630.

van Beilen, J. B., Li, Z., Duetz, W.A., Smits, T.H.M., Witholt, B., (2003) Diversity of alkane hydroxylase systems in the environment. *Oil & Gas Science and Technology – Rev. IFP* **58**: 427-440.

van Beilen, J. B., Smits, T.H.M., Roos, F.F., Brunner, T., Balada, S.B., Röthlisberger, M., Witholt, B., (2005) Identification of an amino acid position that determines the substrate range of integral membrane alkane hydroxylases *Journal of Bacteriology* **187**: 85-91.

van Beilen, J. B., Funhoff, E.G., (2005) Expanding the alkane oxygenase toolbox: new enzymes and applications. *Current Opinion in Biotechnology* **16**: 308-314.

van Beilen, J. B., Funhoff, E.G., van Loon, A., Kaysse, L., Bouza, M., Holtackers, R., Röthlisberger, M., Li, Z., Witholt, B. , (2006) Cytochrome P450 alkane hydroxylases of the CYP153 family are common in alkane-degrading eubacteria lacking integral membrane alkane hydroxylases. *Applied and Environmental Microbiology* **72**: 59-65.

van der Lelie, D., Barac, T., Taghavi, S., Vangronsveld, J., (2005) Response to Newman: New uses for endophytic bacteria in plants. *Trends in Biotechnology* **23**: 8-9.

van Elsas, J. D., Turner, S., Bailey, M.J., (2003) Horizontal gene transfer in the phytosphere. *New Phytologist* **157**: 525-537.

Van Hamme, J. D., Singh,A., Ward, O.P., (2003) Recent advances in petroleum microbiology. *Microbiology and Molecular Biology Reviews* **67**: 503-549.

Webber, C., Ponting, C.P., (2004) Genes and homology. *Current Biology* **14**: 332-333.

Whyte, L. G., L Bourbonniere, L., Greer, C.W., (1997) Biodegradation of petroleum hydrocarbons by psychrotrophic Pseudomonas strains possessing both alkane (*alk*) and naphthalene (*nah*) catabolic pathways. *Applied and Environmental Microbiology* **63**: 3719-3723.

Wilson, M. S., Herrick, J.B., Jeon, C.O., Hinman, D.E., Madsen, E.L., (2003) Horizontal transfer of *phnAc* dioxygenase genes within one of two phenotypically and genotypically distinctive naphthalene-degrading guilds from adjacent soil environments. *Applied and Environmental Microbiology* **69**: 2172–2181.

CHAPTER 2

Phylogenetic and functional diversity of alkane degrading bacteria associated with Italian ryegrass (*Lolium multiflorum*) and Birdsfoot trefoil (*Lotus corniculatus*) in a petroleum oil-contaminated environment*

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Summary

Twenty-six different plant species were analysed regarding their performance in soil contaminated with petroleum oil. Two well-performing species, Italian ryegrass (*Lolium multiflorum* var. Taurus), Birdsfoot trefoil (*Lotus corniculatus* var. Leo), as well as the combination of both plants were selected to study the ecology of plant-associated, culturable alkane-degrading bacteria. Hydrocarbon degrading bacteria were isolated from the rhizosphere, root interior and shoot interior and subjected to the analysis of 16S rRNA, the 16S and 23S rRNA intergenic spacer region and alkane hydroxylase genes. Furthermore, it was investigated whether alkane hydroxylase genes are plasmid located. Higher numbers of culturable, degrading bacteria were associated with Italian ryegrass, which were also characterized by a higher diversity, particularly in the plant interior. Only in half of the isolated bacteria known alkane hydroxylase genes (*alkB* and cytochrome P153-like) could be detected. Degradation genes were found both on plasmids as well as in the chromosome. Our results indicate that *alkB* genes have spread through horizontal gene transfer, particularly in the Italian ryegrass rhizosphere, and that horizontal gene transfer was not restricted to only plasmid transfer. Furthermore, results indicate mobility of catabolic genes between Gram-negative and Gram-positive bacteria. In regard to phytoremediation applications, where support of numerous degrading bacteria and rapid spread of degrading genes is essential for efficient break-down of pollutants, Italian ryegrass seems to be more appropriate than Birdsfoot trefoil.

1. Introduction

Phytoremediation is a low-cost method, which employs plants and their associated microbes to treat contaminated sites. Plants and microorganisms interact to absorb, degrade, or remove toxic pollutants from soil, ground- and surface–water (Pilon-Smits, 2005; Lafferty Doty, 2008). In respect to phytoremediation of organic contaminants, which are slowly transferred from the soil to the plant, rhizodegradation (i.e. degradation by microorganisms in the rhizosphere of plants) is the major mechanism of detoxification (Reichenauer and Germida, 2008). Degrading bacteria are supported through the release of root exudates. Several plant species have demonstrated the ability to tolerate petroleum hydrocarbons. Among others, prairie grasses and legumes have been shown to be suitable for phytoremediation of petroleum hydrocarbon contaminants (Reilley *et al*., 1996; Kaimi *et al*., 2007, Hutchinson *et al*., 2003). Grasses have a fibrous root system which creates a high root surface area and which may penetrate to a soil depth of up to 3 m (Aprill and Sims, 1990), whereas legumes have the advantage to fix atmospheric nitrogen (Vavrek, 2000) and thus can potentially improve the N:C-ratio in a soil contaminated by hydrocarbons.

Apart from mineralizing organic contaminants, bacteria can also reduce the phytotoxicity of contaminants to a level where plants can grow in unfavourable soil conditions (Siciliano and Germida, 1998). The ability of bacteria to degrade aliphatic compounds found in petroleum is primarily conferred by enzymes such as the alkane monooxygenase encoded by *alkB.* Bacterial oxidation of n-alkanes is a very common phenomenon in soil and water as alkanes constitute about 20-50% of crude oil and in addition, alkanes are produced throughout the biosphere by living organisms as waste product, structural element, as chemoattractants or are part of defence mechanisms (van Beilen *et al*., 2003). Although very common among various bacterial domains (reviewed by van Beilen and Funhoff, 2007), only the *Pseudomonas putida* strain GPo1 alkane

hydroxylase system (*alk* gene cluster) has been studied in detail with respect to enzymology and genetics (van Beilen *et al*., 2002, Whyte *et al*., 2002). The *alkB* gene encodes an integral-membrane non-heme di-iron monooxygenase and is located in strain GPo1 on the OCT plasmid (Chakrabarty *et al*., 1973, Dinamarca, 2003), a broad-host range IncP-2 plasmid. PCR and genome sequencing studies have shown that many strains contain homologues of the GPO1 *alkB* genes, mainly located on the chromosome (van Beilen *et al*., 2001) Closely related *alkB* genes were found in *Pseudomonas putida* strain P1, placed on a chromosomally located class I transposon, although this strain harbours a 114 kb plasmid (van Beilen *et al*., 2001). Quite divergent *alkB* homologues to GPO1 were found mainly in Gram-positives (van Beilen *et al*., 2002). Alkane-degrading yeasts and fungi mainly posses enzymes related to cytochrome P450 class II (CYP52) alkane hydroxylase (van Beilen and Funfhoff, 2005). Recent studies showed that also a range of bacteria contain CYP153 genes encoding cytochorome P450 alkane hydroxylase (van Beilen *et al*., 2006). The *alkB* and CYP153 hydroxylase genes allow bacteria to grow in media containing long chain length alkanes (van Beilen and Funhoff, 2007).

Other studies showed that different plant species host distinct microbial populations in their rhizo- and endospheres which is most likely due to different root exudates and substances produced by the plant (Siciliano *et al*., 2001, Smalla *et al*., 2001; Benizri and Amiaud, 2005, Nunan *et al*., 2005) and different compartments of the plant might harbour different bacterial assemblages (Idris *et al*., 2004; Moore *et al*., 2006). In regard to phytoremediation of petroleum oil, less information is available on degrading bacteria and their degradation genes hosted by different plants. Therefore, the aim of this study was to select plants being highly tolerant to petroleum oil contamination and to analyse the diversity of alkane-degrading bacteria and their alkane hydroxylase (*alk*) genes isolated from the rhizo- and endosphere of two selected plants, namely Italian ryegrass

(subsequently termed IT) and Birdsfoot trefoil (subsequently termed BT). The two plant species were associated with highly distinct hydrocarbon degrading communities carrying different types of alkane hydroxylase genes, which were located either on plasmids or in the chromosome. Our results suggest that different alkane hydroxylase genes were subjected to horizontal gene transfer in different magnitudes as well as that the plant species greatly influenced the abundance and diversity of alkane degrading bacteria.

2. Materials & Methods

2.1. Plant screening

The petroleum oil-contaminated soil (17.2 g total hydrocarbon kg^{-1} soil as determined by FTIR) was sampled from a landfill for deposition of crude oil contaminated soil from oil pumping sites in Zistersdorf, Lower Austria. The soil was taken randomly from 10 sampling points at the landfill each from 0-25 cm soil depth. Subsequently, all soil samples were mixed, homogenized and sieved by a 2 mm sieve. The soil was filled into pots (10 cm x 10 cm x 11 cm) and irrigated from the bottom. In each pot 200 mg seeds of each plant were sown, the plants selected are shown in Table 1. In the first screening experiment (without compost amendment) 26 plant species were tested. Plants were cultivated in contaminated soil as well as in agricultural soil mixed with 40% sand and each treatment was done in triplicates. In the second screening experiment (with 10% (v/v) compost amendment), the 12 best performing plant species as well as mixture of Italian ryegrass and Birdsfoot trefoil were tested. Plants were grown for 3 months until being harvested. The tolerance of each plant species toward petroleum contamination was measured based on comparing above-ground biomass with the uncontaminated control. The biomass was harvested, heat-dried and weighted. The Least Significant Difference statistical test (P<0.05) was applied to

compare the mean values of the treatment and the control for all pots containing the same plant species.

2.2. Isolation of alkane-degrading bacteria

Alkane-degrading bacteria were isolated from rhizosphere, shoot interior and root interior of Italian ryegrass (IT), Birdsfoot trefoil (BT) and the mix of Italian ryegrass and Birdsfoot trefoil (IT+BT), which were cultivated for 3 months in petroleum oil-contaminated soil amended by 10% (v/v) compost. The plants were pulled and shaken to dislodge the bulk soil attached to the roots and the shoots were cut (2 cm above soil). The rhizosphere soil was obtained by putting the roots with its attached soil into 3 ml of 0.9% (w/v) NaCl and agitated 180 rpm for half an hour. Subsequently agitated roots and cut shoots were washed for 2 minutes in sterile distilled water before surface sterilization with 70% ethanol for 5 min (shoots) or 10 min (roots), followed by a 1 min wash in 1% NaOCl added by 0.01% Tween 20 solution, and then a final rinse in sterile distilled water (3 times, 1 min each time). The surface sterility was checked on 10% TSA medium. The plate was incubated at 30^0C for 48 hrs, no growth was observed.

The isolation method applied steps with and without enrichment and used two different isolation solid media which were previously mixed well with 1% (v/v) filter-sterilized diesel. The enrichment was conducted by putting 1 ml of rhizosphere slurry or 1 g of surface sterilized and macerated shoots and roots in Falcon tubes containing 10 ml liquid minimal basal medium amended with 1% (v/v) filter-sterilized diesel and incubated for 5 days in 30^oC with agitation at 180 rpm. The isolation media used were 10% Tryptic Soy Agar (TSA, Merck) and Minimal Salt Basal Medium (Alef, 1994) containing 1.83 g/L Na_2HPO_4, 0.9 g/L KH_2PO_4, 2 g/L NH_4Cl, 1 g/L Na_2SO_4 , 0.67 g/L $CaCl_2.2H_2O$, 0.01 g/L $MgCl_2.6H_2O$, 0.001 g/L $FeSO_4.7H_2O$, 0.001 g/L $MnSO_4.4H_2O$, and 0.01 g/L

yeast extract. Both media were amended with cycloheximide (100 mg/L) to avoid fungal growth and 15 g agar (Difco,USA).

For bacterial isolation without enrichment 1 g of surface sterilized roots and shoots was homogenized with 2 mL 0.9% (w/v) NaCl solution. Aliquots (100 µL) were then spread onto isolation medium. Plant surfaces were checked for their sterility by blotting them tightly on 10% TSA and incubating plates for 2 days at 30^{o}C. No growth was observed. For the isolation from rhizosphere soil, the soil slurry from previous agitation of roots material was kept until the soil particles settled, then the aqueous phase (100 µL) of a 10^{-3} dilution was spread onto both isolation media. For the isolation of bacteria after enrichment, the aqueous phase (100 µL) of a 10^{-3} dilution of enriched rhizosphere, shoots or roots were plated on 10% TSA containing 1% filter-sterilized diesel. Isolation media were incubated at 30^{o}C for 2 days (TSA medium) and for 2 weeks (minimal basal medium). The isolation of bacteria from IT+BT was conducted by taking and proceeding separately shoots from IT and BT but roots were taken as mixture as roots could not be separated. Bacterial colonies on each plate were picked according to their distinguishable colony morphology. Three to five colonies of similar colony type were picked. Picked isolates were again grown on solid minimal basal medium without yeast and amended with 2% (v/v) filter-sterilized diesel followed by incubation in 30^{o}C for 2 weeks.

2.3. Identification of alkane-degrading isolates

All hydrocarbon degrading isolates were subjected to restriction fragment length polymorphism (RFLP) analysis of the 16S-23S rRNA intergenic spacer region (IGS) region to distinguish different strains. Genomic DNA was extracted from liquid cultures by using the Microbial DNA Isolation Kit (Mo Bio Laboratories, USA). The IGS region was amplified by PCR using the primers pHr (5'-

TGCGGCTGGATCACCTCCT-3') and P23SR01 (5'-GGCTGCTTCTAAGCCAAC-3') (Massol-Deya *et al*., 1995) as described by Rasche *et al*. (2006). Digestion of 10 µL of IGS PCR products was performed with 5U of endonuclease *Hha*I (Invitrogen) at 37^{0}C for 4 h. The resulting DNA fragments were analyzed by gel electrophoresis in 2.5% (w/v) agarose gels. The IGS characterization distinguished 81 bacterial strains which were further analyzed. A representative isolate of each IGS type was identified by partial 16S rRNA gene sequencing. 16S rRNA genes were amplified by applying PCR primer 8f (5'-AGAGTTTGATCCTGGCTCAG-3') (Weisburg *et al*., 1991) and 1520rev (5'-AAGGAGGTGATCCAGCCGGA-3') (Edwards *et al*., 1989) using the same PCR set up as above. PCR amplicons were purified with Sephadex™ G-50 (Amersham) and used as template for sequence analysis. Partial sequencing of 16S rRNA genes was performed by applying the BigDye V3.1 Terminator Kit (Applied Biosystem, Warrington,UK) and the reverse primer 518rev (5'-ATTACCGCGGCTGCTGG-3') (Liu *et al*., 1997), resulting in sequences of approximately 500 bp length. Sequences were subjected to BLASTn analysis with NCBI database (accession numbers FJ013273 to FJ013353).

2.4. Growth on different n-alkanes and PAHs

Strains were tested for their ability to utilize alkane and PAHs as sole carbon by growing them on plates containing solid minimal basal medium without yeast and amended with either 2% (v/v or w/v) of diesel, n-alkanes (C_6, C_8,C_{10},C_{12} and C_{16}), naphthalene or pyrene. Plates were prepared as described by Daane *et al*. (2001). For control, strains were grown on 10% TSA containing 0.2% (w/v) glucose. The hydrocarbon-containing plates were incubated for 7 days at 30^{o}C and the control was incubated for 2 days. All hydrocarbons used were at least 98% pure (Sigma-Aldrich).

2.5. Detection, sequencing and localization of alkane hydroxylase (alk) genes

The presence of known (*alk*) genes was tested by applying three sets of published PCR primers for detection of *alk* genes: 1) *P. putida alkB* genes derived primers: Pp alkB-for (5'-TGGCCGGCTACTCCGATGATCGGAATCTGG-3') and Pp alkB-rev (5'-CGCGTGGTGATCCGAGTGCCGCTGAAGGTG-3') resulting in a 870 bp fragment (Whyte *et al*., 2002); 2) primers based on *P. oleovorans* GPO1 *alkB* and *Acinetobacter sp*.ADP1 *alkM*: TS2S (5'-AAYAGAGCTCAYGARYTRGGTCAYAAG-3') and deg1RE (5'-GTGGAATTCGCRTGRTGRTCIGARTG-3') resulting in a 557 bp fragment (Smits. *et al*., 1999); and 3) cytochrome P153 *alk* genes: P450fw1 (5'-GTSGGCGGCAACGACACSAC-3') and P450rv3 (5'-GCASCGGTGGATGCCGAAGCCRAA-3') resulting in a 339 bp fragment (van Beilen *et al*., 2005). The *alk* PCR analysis was performed as described in the relevant references. Amplicons obtained from *alk* genes PCR were sequenced in two directions by using both forward and reverse primer accordingly. The sequencing procedure and BLASTN analysis were conducted as for partial 16S rRNA genes above. Alignments of resulted sequences with related sequences at NCBI database was done with the Multalin alignment (http://bioinfo.genotoul.fr/multalin/multalin.html). The TREECON software (van de Peer *et al*., 1994) was used to calculate distance matrices by the Jukes and Cantor algorithm and to generate phylogenetic trees using nearest-neighbor criteria.

All strains, in which known *alk* genes were detected (37 strains), were further tested in order to determine whether the *alk* genes are localized on a plasmid or on the chromosome. Due to lack of knowledge about plasmids presence and their size carried in 81 isolates, several plasmid isolation methods were applied as described by Crosa et al. (1994), Birnboim and Doly (1979), Olsen (1990; modified for Gram-positive strains by addition of 5mg mL^{-1} of lysozyme at lysis step), Stuart-Keil *et al*. (1998) and by using the Plasmid Midi Kit (Qiagen). Isolated plasmid DNA with various sizes were

checked by electrophoresis on 0.5% (w/v) or 2% (w/v) agarose gels. Contamination of chromosomal DNA in isolated plasmid DNA was examined by conducting 16S rRNA PCR analysis. Contamination of chromosomal DNA was removed by applying Plasmid Safe ATP-dependent DNase (Epicentre Biotechnologies), which efficiently digested chromosomal DNA. Furthermore, the localization of *alk* genes was tested by performing *alk* PCR as described above using pure plasmid DNA as template. Reference strains used as positive control in plasmid isolation were *Pseudomonas putida* PaW701 (DSMZ 3938, ATCC 12633) containing a 87 kb plasmid, *Bacillus subtilis* subsp. *subtilis* BD170 (DSMZ 10, ATCC 6051) carrying a 7 kb plasmid and *Escherichia coli* V517 carrying 9 different plasmids in range of 2.7 – 54 kb (obtained from L. Phillips, University of Saskatchewan, Canada).

2.6. PCR-based screening for IncP-1 and IncP-9 plasmids

Screening was performed with Gram-negative strains containing an *alkB* gene. Plasmid DNA obtained from these strains was analyzed by PCR with primers targeting the backbone of IncP-1 (Götz *et al*., 1996) and IncP-9 plasmids (Krasowiak *et al*., 2002). PCR amplicons obtained by IncP-1 specific primers (*trfA2*) were confirmed by Southern blot hybridization with a mixed probe generated from IncP-1 (RP4) and IncP-1 (R751).

2.7. Plasmid curing

Plasmid curing was performed with strains ITRH43, ITRI53 and MixRI75. Plasmid curing was performed by using acridine orange as curing agent with incubation at sub-lethal temperature and daily sub-culturing. The method was a modification from Trevors (1986) and Mesas *et al*. (2004). Ten percent of a dense plasmid-harbouring bacterial culture in Luria Bertani (LB) broth was inoculated into LB agar medium supplemented with 200 µg ml^{-1} acridine orange, and grown at 40^{o}C. The culture was sub-cultured every day into fresh LB agar medium containing 200 µg/mL

acridine orange. On the 5th and 10th day, the sub-culture was diluted and spread onto LB plates as well as on minimal basal medium containing 1% filter-sterilized diesel. As expected, CFUs were reduced on minimal basal medium. Single colonies from LB plates were randomly picked and grown overnight in LB medium. The presence of *alk* genes was checked by colony PCR with *alk*-targeting PCR primers. The native strain was used as positive control and the IGS PCR- RFLP was conducted to assure the chromosomal identity of the cured strain with its parental strain. The presence of plasmid after curing was checked by using Eckhardt in- well lysis method (Eckhardt, 1978).

2.8. Nucleotide sequence accession numbers

The partial 16S rRNA gene sequences determined in this study were deposited in the GenBank database with the accession numbers FJ013273 to FJ013353. The nucleotide sequences of partial *alk* genes determined in this study were deposited as well with the accession numbers FJ014891 to FJ014897 for CYP153 genes, FJ014898 to FJ014906 for Pp-*alkB* genes and FJ014916 to FJ014920 for TS2S-*alkB* genes.

3. Results

3.1. Screening of petroleum oil-tolerant plant species

Twenty-six different legume and grass species and varieties were screened for growth on soil contaminated with crude petroleum oil (Table 1). Most plant species showed significant growth reduction, particularly when grown without compost amendment, and few plants (grasspea (*Lathyrus sativus*), cereal rye (*Secale multicaule*), false flax (*Carnelina sativa*), kidney bean (*Phaseolus vulgaris*) and livid amaranth (*Amaranthus lividus*) were not able to grow at all. Biomass production of few plant species (Birdsfoot trefoil (*Lolium corniculatus*), alfalfa (*Medicago sativa*))

were not affected by the contamination when grown with compost. Italian ryegrass (IT, *Lolium multiflorum* var. Taurus), Birdsfoot trefoil (BT, *Lotus corniculatus* var. Leo) as well as these two plant species growing together were selected for analysis of plant-associated microbial communities as they demonstrated similar or better growth polluted compared to unpolluted soil.

Table 1. List of plant species tested in a two months pot experiment. Control pots contained agricultural soil whereas treatment pots contained crude-oil contaminated soil with and without 10% (v/v) compost amendment. Italian ryegrass, Birdsfoot trefoil and a mixture of IT and BT amended with 10% compost (v/v) were selected for the isolation of hydrocarbon-degrading bacteria.

			Biomass (mean (g) ± SD)		
Common Name	**Acr.**	**Scientific Name**	**Control**	**Without compost**	**With compost**
Italian ryegrass	IT	*Lolium multiflorum* var. Taurus	$4.62 \pm 0.42^{a*}$	1.51 ± 0.18^{b}	1.66 ± 0.09^{b}
Birdsfoot trefoil	BT	*Lotus corniculatus* var. Leo	1.76 ± 0.54^{a}	0.66 ± 0.10^{b}	1.61 ± 0.04^{a}
Mix Italian ryegrass and Birds-foot trefoil	IT+BT	Mix *Lolium multiflorum* var.Taurus and *Lotus corniculatus* var.Leo	3.19 ± 0.23^{a}	1.01 ± 0.14^{c}	1.71 ± 0.19^{b}
Perennial ryegrass, English ryegrass	EG	*Lolium perenne* var.Prana	1.81 ± 0.49^{a}	0.40 ± 0.06^{b}	0.23 ± 0.16^{b}
Birds-foot trefoil	HR	*Lotus corniculatus* var. Rocco	1.24 ± 0.15^{a}	0.64 ± 0.11^{b}	1.19 ± 0.13^{a}
Alfalfa	LH	*Medicago sativa* var.Harpe	1.77 ± 0.37^{a}	0.94 ± 0.12^{b}	2.07 ± 0.33^{a}
Red clover	RG	*Trifolium pratense* var. Gumpensteiner	2.02 ± 0.48^{a}	0.10 ± 0.09^{b}	0.03 ± 0.16^{b}
Red fescue	RR	*Festuca rubra* var. Reverent	2.92 ± 0.74^{a}	0.40 ± 0.15^{b}	0.31 ± 0.02^{b}
Whiteclover	WH	*Trifolium repens* var. Gr.Huta	1.96 ± 0.51^{a}	0.32 ± 0.08^{b}	0.47 ± 0.15^{b}
Sainfoin	EI	*Onobrychis viciifolia*	0.75 ± 0.33^{a}	0.10 ± 0.09^{b}	0.38 ± 019^{ab}
Kidney-vetch	WU/1	*Anthyllis vulneraria*	1.40 ± 0.1^{a}	0.12 ± 0.16^{b}	1.04 ± 0.25^{c}
Spelt	ZS	*Triticum spelta* var. Album	1.75 ± 0.44^{a}	0.08 ± 0.10^{b}	0.40 ± 0.12^{b}
Emmer	SW	*Triticum dicoccon*	0.79 ± 0.34^{a}	0.24 ± 0.08^{b}	0.56 ± 0.25^{ab}
Perennial ryegrass, English ryegrass	EP	*Lolium perenne* var. Prana	3.59 ± 0.45^{a}	0.17 ± 0.07^{b}	
Hybrid ryegrass	BG	*Lolium boucheanum* var. Gumpensteiner	3.05 ± 0.29^{a}	0.05 ± 0.02^{b}	
Black medic	GV	*Medicago lupulina* var.Virgo	1.71 ± 0.12^{a}	0.02 ± 0.01^{b}	
Orchardgrass	KT	*Dactylis glomerata* var.Tandem	1.80 ± 0.07^{a}	0.04 ± 0.01^{b}	
Timothy	TT	*Phleum pratense* var. Tiller	2.59 ± 0.47^{a}	0.10 ± 0.08^{b}	
Kentucky bluegrass	WC	*Poa pratensis* var. Compact	1.28 ± 0.62^{a}	0.03 ± 0.04^{b}	
Kentucky bluegrass	WO	*Poa pratensis* var. Oxford	1.38 ± 0.37^{a}	0.01 ± 0.01^{b}	
Kidney bean	BW/1	*Phaseolus vulgaris* var. vulgaris	3.13 ± 0.01^{a}	$1{,}392 \pm 0.0^{b}$	
Grasspea	PM	*Lathyrus sativus* var. Colorolus Merkur	1.03 ± 0.72	Not growing	
Cereal rye	JO	*Secale multicaule*	0.61 ± 0.54	Not growing	
Falseflax	LE	*Camelina sativa*	0.98 ± 0.67	Not growing	
Kidney bean	BA/1	*Phaseolus vulgaris* var. Vulgaris	1.70 ± 0.01	Not growing	
Livid amaranth	RM	*Amaranthus lividus*	1.44 ± 0.56	Not growing	

*The same letter following mean ± SD shows that the mean value is not significantly different with a level of confidence 95% (Least Significant Difference test, $P<0.05$).

3.2. Diversity of alkane degrading isolates

Generally, enrichment for 5 days in minimal basal medium resulted in few culturable bacterial isolates. More strains were isolated by using TSA medium containing diesel oil without enrichment. However, strains, in which *alk* genes were detected, were primarily isolated by using minimal basal medium containing diesel oil without enrichment. From isolation using TSA and minimal basal medium without enrichment, on average isolation of plant-associated bacteria from Italian ryegrass (IT) resulted in 2.9 x 10^2 CFU g^{-1} fresh shoot, 4.4 x 10^2 CFU g^{-1} fresh root and in 10^6 CFU ml^{-1} rhizosphere soil slurry. Birdsfoot trefoil (BT) hosted 1.2 x 10^2 CFU g^{-1} fresh shoot, 3.3 x 10^2 CFU g^{-1} fresh root and 6.3 x 10^4 CFU ml^{-1} rhizosphere soil slurry. From IT+BT 87 CFU g^{-1} were isolated from IT fresh shoot and 1.2 x 10^2 CFU g^{-1} from BT fresh shoot, whereas 3.1 x 10^2 CFU g^{-1} were isolated from fresh roots and 2.2 x 10^4 CFU ml^{-1} rhizosphere soil slurry. In total 266 colonies were obtained, which after being tested for their growth capability on minimal basal medium with 2% (v/v) diesel as sole carbon source, decreased to 164 alkane-degrading isolates. Twenty-two, 7 and 13 isolates were obtained from IT, BT and IT+BT shoots, respectively, whereas 18, 9 and 18 were isolates were isolated from sterilized roots from IT, BT and IT+BT, respectively. The rhizosphere yielded 18, 9 and 18 isolated from IT, BT and IT+BT, respectively. By RFLP analysis of the 16S-23S IGS region 81 types could be differentiated. From each IGS type one representative isolate was chosen for further analysis (Table 2, 3 and 4). Only one strain, MixRH13 (*Pseudomonas anguiliseptica*) (SI Table 1), was found in all three treatments (IT, BT and IT+BT). Only few strains, which were found in IT or BT were found in the IT+BT treatment.

The majority of alkane–degrading isolates were obtained from IT. Most strains isolated from IT, particularly the endophytes, belonged to the *Gammaproteobacteria* comprising mostly *Pseudomonas* and *Pantoea* species (Table 2, SI Table 1, Fig. 1). Besides few *Alpha*- and

Betaproteobacteria and a *Bacteroidetes* strain, several high G+C (*Rhodococcus, Microbacterium, Arthrobacter*, *Dietzia, Cellulosimicrobium* and *Streptomyces*) and low G+C Gram-positives (*Bacillus, Paenibacillus*) were isolated from the IT rhizosphere. Most strains showed 97-100% similarity to known 16S rRNA genes, whereas several endophyte strains (ITSI32, ITSI67, ITRI66, ITRI59, ITSI21) were only distantly related to known bacteria (93-96% similarity) and probably represent novel genera within the *Bacteroidetes* group as well as within the *Beta*- and *Gammaproteobacteria* (Table 2, SI Table 1). Only few Gram-positive strains were isolated from the root or shoot interior of IT plants (Fig. 1).

Compared to Italian ryegrass, fewer strains were obtained from BT. Isolates belonged mostly to the *Beta*- (*Achromobacter, Alcaligenes*) and *Gammaproteobacteria* (*Pseudomonas, Pantoea*) as well as to the low G+C Gram-positives (*Bacillus, Paenibacillus*) (Table 3, SI Table 1, Fig. 1). In addition, one *Flavobacterium* strain and one high G+C Gram-positive strain were obtained. Most strains showed 97-100% similarity to known bacteria, only three strains (BTSI16, BTSI34, BTRH72) had lower similarity values (91-96%).

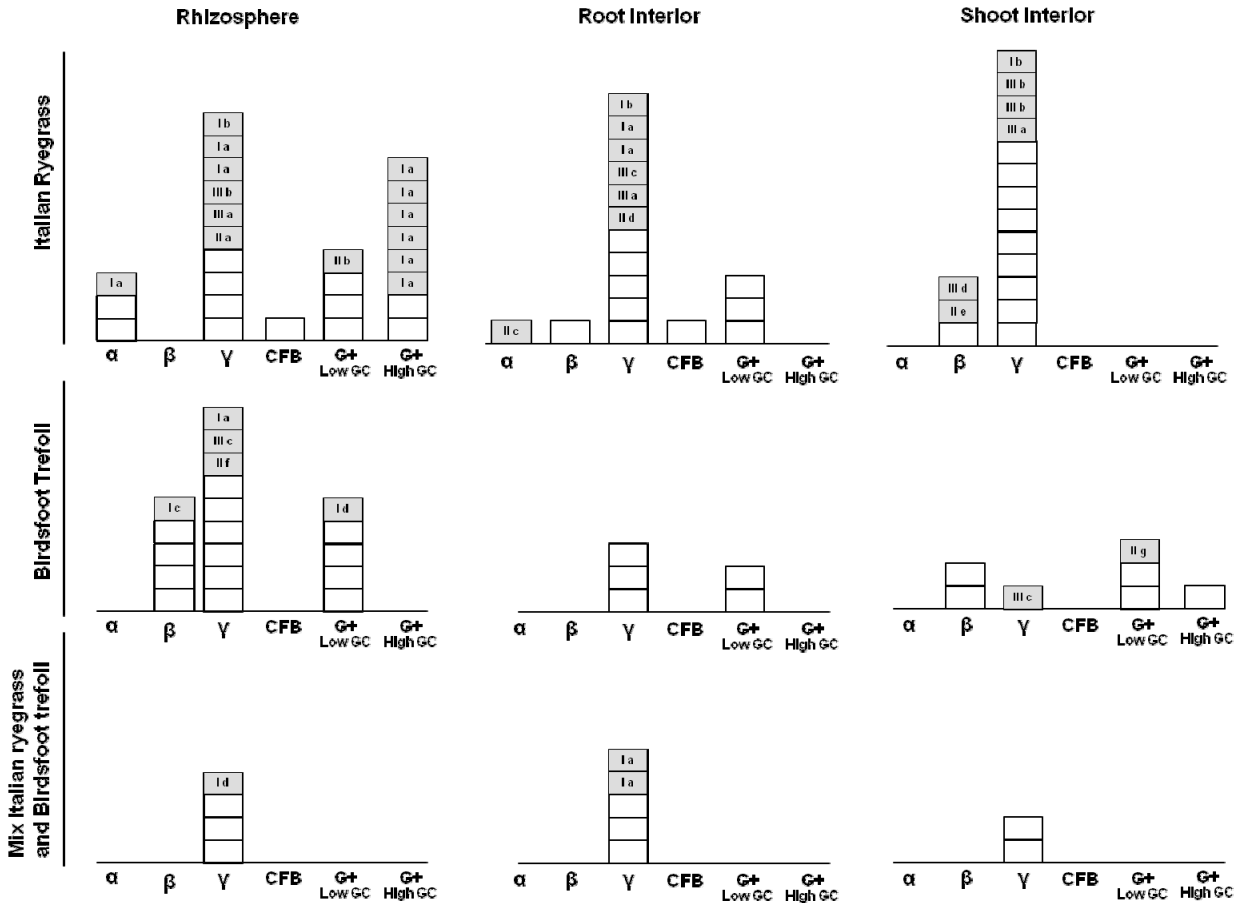

Fig. 1. Distribution of alkane-degrading isolates in relation to the host plant, site of isolation, detectable alkane hydroxylase (*alk* genes) and diversity of *alk* gene homologues. Each box represents one bacterial strain. The empty box means no *alk* genes were detected, whereas shadowed box indicate that *alk* genes were detected. The initial (I,II,III) shows the type of *alk* genes detected using: I (PpalkB primer set), II (CYP450 primer set), and III (TS2S-deg1RE primer set). The same letter following the initial means identical nucleotide sequences of *alk* genes.

Table 2. Bacterial strains isolated from IT in which alkane hydroxylase encoding genes (*alk* genes) were detected by PCR. Rhizosphere strains are termed ITRH, root endophytes ITRI and shoot endophytes ITSI.

IGS Type	16S rRNA gene similarity (NCBI accession number /%)	Phylogenetic Group	PCR primers for *alk* genes detection	*alk* seq. type	Tentative location of detected *alk* genes	Isolation medium
Rhizosphere						
Proteobacteria						
ITRH1	*Ochrobactrum anthropi* (AB120120 / 99)	*Alphaproteobacteria*	PpalkB[a]	Ia	Chromosome	Basal
ITRH31	*Pseudoxanthomonas sp*. E16 (AY488509 / 99)	*Gammaroteobacteria*	CYP450 [b]	IIa	Plasmid	Basal
ITRH16	*Pseudomonas sp*.MACL12A (EF198249 / 99)	*Gammaproteobacteria*	TS2S-degRe1[c]	IIIa	Plasmid	10% TSA
ITRH17	*Pseudomonas sp*.MACL12A (EF198249 / 99)	*Gammaproteobacteria*	TS2S-degRe1	IIIa	Chromosome	10% TSA
ITRH25	*Pseudomonas sp*.Rs87 (AM905943 / 99)	*Gammaproteobacteria*	PpalkB	Ia	Plasmid	Basal
ITRH76	*Pseudomonas sp*.Ri71(AM110075 / 99)	*Gammaproteobacteria*	PpalkB	Ib	Chromosome	Basal
ITRI22	*Pseudomonas sp*.BWO (EU006700/ 99)	*Gammaproteobacteria*	PpalkB	Ie	Chromosome	10% TSA
Gram Positives						
ITRH39	*Bacillus sp.* G2DM-19 (DQ416802 / 99)	Low GC, G+	CYP450	IIb	Chromosome	10% TSA
ITRH43	*Rhodococcus rhodochrous* (AB183422 / 99)	High GC, G+	PpalkB	Ia	Plasmid	Basal
ITRH47	*Microbacterium sp.* YT0620 (AB376082 / 99)	High GC, G+	PpalkB	Ia	Chromosome	Basal
ITRH49	*Arthrobacter oxydans* str.1662 (EU086791 / 99)	High GC, G+	PpalkB	Ia	Plasmid	Basal
ITRH56	*Dietzia sp*. BBDP47 (DQ337507 / 99)	High GC, G+	PpalkB	Ia	Plasmid	Basal
ITRH48	*Arthrobacter sp.* 19503 (AJ315071 / 99)	High GC, G+	PpalkB	Ic	Chromosome	Basal
ITRH51	*Streptomyces sp*. CNQ-023 (EU214930 / 99)	High GC, G+	PpalkB	Ic	Plasmid	Basal
Root Interior						
Proteobacteria						
ITRI4	*Sphingopyxis macrogoltabida* (AB372255 / 100)	*Alphaproteobacteria*	CYP450	IIc	Plasmid	Basal
ITRI24	Pseudomonas boreopolis (AJ864722 / 100)	*Gammaproteobacteria*	CYP450	IId	Plasmid	Basal
ITRH16	*Pseudomonas sp*.MACL12A (EF198249 / 99)	*Gammaproteobacteria*	TS2S-degRe1	IIIa	Plasmid	10% TSA
ITRI19	*Pseudomonas sp*. SMCC B0205 (AF500277 / 100)	*Gammaproteobacteria*	TS2S-degRe1	IIIb	Plasmid	10% TSA
ITRI22	*Pseudomonas sp*.BWO (EU006700/ 99)	*Gammaproteobacteria*	PpalkB	Id	Chromosome	10% TSA
ITRI53	*Pseudomonas anguiliseptica* (AM902193 / 99)	*Gammaproteobacteria*	PpalkB	Ia	Plasmid	Basal
ITRI73	*Pseudomonas anguiliseptica* (AM902193 / 99)	*Gammaproteobacteria*	PpalkB	Ia	Plasmid	Basal
Shoot Interior						
Proteobacteria						
ITSI70	*Alcaligenes sp.*T12RB (EU304280 / 99)	*Betaproteobacteria*	CYP450	IIe	Chromosome	10% TSA Enriched
ITSI67	*Alcaligenes sp*.T12RB (EU304280 / 94)	*Betaproteobacteria*	TS2S-degRe1	IIId	Plasmid	Basal
ITRH16	*Pseudomonas sp*.MACL12A (EF198249 / 99)	*Gammaproteobacteria*	TS2S-degRe1	IIIa	Plasmid	10% TSA
ITRI19	*Pseudomonas sp*. SMCC B0205 (AF500277 / 100)	*Gammaproteobacteria*	TS2S-degRe1	IIIb	Plasmid	10% TSA
ITRI22	*Pseudomonas sp*.BWO (EU006700/ 99)	*Gammaproteobacteria*	PpalkB	IIId	Chromosome	10% TSA
ITSI32	*Enterobacter sakazakii* (AB274298 / 94)	*Gammaproteobacteria*	TS2S-degRe1	IIIc	Plasmid	Basal

Table 3. Bacterial strains isolated from BT in which alkane hydroxylase encoding genes (*alk* genes) were detected by PCR. Rhizosphere strains are termed BTRH, root endophytes BTRI and shoot endophytes BTSI

IGS Type	16S rRNA gene similarity (NCBI accession number /%)	Phylogenetic Group	PCR primers for *alk* genes detection	*alk* seq. type	Tentative location of detected *alk* genes	Isolation medium
Rhizosphere						
Proteobacteria						
BTRH5	*Alcaligenes faecalis* (EU075145 / 99)	*Betaproteobacteria*	PpalkB[a]	Ie	Plasmid	Basal Enriched
ITRI19	*Pseudomonas sp*. SMCC B0205 (AF500277 / 100)	*Gammaproteobacteria*	TS2S-degRe1[c]	IIIb	Plasmid	10% TSA
BTRH79	*Pantoea agglomerans* str. HK 14-1 (AY335552 / 100)	*Gammaproteobacteria*	CYP450[b]	IIf	Plasmid	10% TSA
BTRH11	*Pantoea sp*. iCTE592 (DQ122350 / 99)	*Gammaproteobacteria*	PpalkB	Ia	Plasmid	Basal Enriched
Gram Positives						
BTRH40	*Bacillus sp*. SK83 (EU417673 / 100)	Low GC, G+	PpalkB	If	Plasmid	10% TSA
Shoot Interior						
Proteobacteria						
ITRI19	*Pseudomonas sp*. SMCC B0205 (AF500277 / 100)	*Gammaproteobacteria*	TS2S-degRe1	IIIb	Plasmid	10% TSA
Gram Positives						
BTSI33	*Bacillus licheniformis* str.RPk (EU445292 / 99)	Low GC, G+	CYP450	IIg	Plasmid	Basal Enriched

Table 4. Bacterial strains isolated from IT+BT in which alkane hydroxylase encoding genes (*alk* genes) were detected by PCR. Rhizosphere strains are termed MixRH and root endophytes MixRI.

IGS Type	16S rRNA gene similarity (NCBI accession number /%)	Phylogenetic Group	PCR primers for *alk* genes detection	*alk* seq. type	Tentative location of detected *alk* genes	Isolation medium
Rhizosphere						
MixRH30	*Enterobacteriaceae bacterium* 58 (AY579163 / 99)	*Gammaproteobacteria*	PpalkB[a]	If	Plasmid	10% TSA
Root Interior						
MixRI74	*Pseudomonas sp.* BWDY-40 (DQ200853 / 99)	*Gammaproteobacteria*	PpalkB	Ia	Plasmid	Basal
MixRI75	*Pseudomonas sp.* BWDY-40 (DQ200853 / 99)	*Gammaproteobacteria*	PpalkB	Ia	Plasmid	Basal

[a]Alkane hydroxylase genes were detected by using PCR primers derived from published *Pseudomonas putida* GPo1 *alkB* genes sequence, commonly known as *Pseudomonas oleovorans* ATCC 29347 (Whyte *et a*l., 2002).
[b]Alkane hydroxylase genes were detected by using PCR degenerate primer CYP153 amplifyng cytochrome P450 alkane hydroxylase (van Beilen *et a*l*.,* 2006)
[c]Alkane hydroxylase genes were detected by using degenerate PCR primers amplifying alkane hydroxylase genes in Gram-negative and Gram-positive strains (Smits *et a*l., 1999).

3.3. Growth on hydrocarbons and diversity of alkane hydroxylase genes

The growth capability test performed with all (81) degrading isolates on different n-alkanes (C6, C8, C10, C12 and C16) and PAHs (naphthalene and pyrene) demonstrated that most of the isolates which could grow utilized all tested n-alkanes and PAHs. Exceptions were ITRI24 (*Pseudomonas*), which could grow on PAHs but was only able to grow on short n-alkanes C_6 and C_8, whereas ITRI53 and MixRI75 (both *Pseudomonas*) were able to grow on all n-alkanes but not on PAHs.

Although all of isolates were able to grow on n-alkanes, only less than the half of all isolates carried detectable *alk* genes. Mostly strains isolated from the IT rhizosphere showed *alk* genes, which could be amplified by PCR (Fig. 1, Table 1-3). Also, besides hosting a large amount and various alkane degraders, in strains isolated from IT various *alk* gene types were detected. The prevailing detectable *alk* gene type was highly homologous to *alkB* encoding alkane-monooxygenase commonly found in *Pseudomonas* strains (detected by using PpalkB and TS2S-deg1RE PCR primer sets). The identical partial *alkB* DNA sequence and closely related homologues were found within and across genera of *Alpha-*, *Beta-* and *Gammaproteobacteria*, high and low G+C Gram-positives (Fig. 2, Fig. 3). The degenerated TS2S-deg1RE primer was designed to amplify Gram-positive and Gram-negative strains (Smits *et al*., 1999), but we experienced that the primer only amplified *alkB* genes from Gram-negative strains (Fig. 3). For some *Pseudomonas* strains, DNA sequences obtained from amplicons produced with the PpalkB and TS2S-deg1RE primer sets were identical at one end of sequence, suggesting both primer targeted the same *alk* gene (data not shown), but for the strains described in Fig.3, the *alkB* sequences amplified with the TS2S-deg1RE primers were not similar. We conclude that these strains probably contain different *alkB* homologues. Few cytochrome P153 (CYP153)-like *alk* genes were detected in *Alpha-*, and *Gammaproteobacteria* and by low G+C Gram-positive strains isolated from IT and BT. Only

distantly related DNA sequences of CYP153 were found among the isolates (Fig. 4). Due to the low DNA sequence similarity, the detected CYP153 gene DNA sequences could not be aligned with CYP153 gene sequences published in the NCBI database.

3.4. Plasmid analysis

As degradation genes are frequently located on plasmids, we tested whether the *alk* genes detected by PCR are located on a plasmid or on the chromosome. Both, plasmid as well as chromosome-located genes was found (Table 2, 3 and 4). Even identical *alk* gene fragments were both, located on a plasmid as well as on the chromosome. No correlation could be detected between the location of the alk gene and the phylogeny of the strain or the host plant. PCR analysis with primers targeting IncP-1 or IncP-9 plasmids revealed that only ITRH1 (*Ochrobactrum*), MixRI74 and MixRI75 (both *Pseudomonas*) and ITSI67 (*Alcaligenes*) carried plasmids belonging of the incompatibility group IncP-1. For none of the plasmids amplified with IncP-9 specific primers PCR products were obtained.

To further confirm that *alk* genes are indeed located on plasmids, plasmid curing was performed with few strains that had identical *alkB* gene fragments (ITRH43, ITRI53 and MixRI75). Although the *alkB* genes were not detected anymore after plasmid curing, the capacity of cured strains to grow on n-alkanes did not vanish totally, however the range of degraded alkanes differed (Table 5). This indicates the presence of more than one *alk* gene, which all together are responsible for the degradation capacity. The cured strain of ITRH43 (*Rhodococcus*) lost its ability to grow on naphthalene and pyrene as well as on medium chain alkanes ($C_{10,}$ C_{12}, C_{16}) but still could grow on short chain alkanes (C_6 and C_8). In contrast, the cured strain of ITRI53 (*Pseudomonas*) was unable

to grow anymore on short alkanes but was able to grow on medium chain alkanes (C_{12} and C_{16}) (Table 5).

Table 5. Hydrocarbon utilization performance of the isolates after plasmid curing, grown on minimal basal medium containing 2 % hydrocarbon (Glu = Glucose (as control), Die = diesel, Naph = naphthalene, Pyr = pyrene). The isolates were detected carrying *alkB* genes on plasmid

IGS	16S rRNA gene homology (NCBI accession number /%)	Plasmid Curing	Glu (0.2%)	Die	Naph	Pyr	n-C_6	n-C_8	n-C_{10}	n-C_{12}	n-C_{16}
ITRH43	*Rhodococcus rhodochrous* (AB183422 / 99)	**Before**	+	+	+	+	+	+	+	+	+
		After	+	--	--	--	+	+	--	--	--
ITRI53	*Pseudomonas anguiliseptica* (AM902193 / 99)	**Before**	+	+	--	--	+	+	+	+	+
		After	+	+	--	--	--	--	--	+	+
MIXRI75	*Pseudomonas sp.* BWDY-40 (DQ200853 / 99)	**Before**	+	+	--	--	+	+	+	--	--
		After	+	--	--	--	+	--	--	--	--

4. Discussion

Different plant species are not equally suited for phytoremdiation applications, in which they preferably should produce substantial biomass and support the appropriate, degrading microflora. Frequently, plants are fertilized with compost to optimize nutrient availability under unfavorable conditions. The plant screening performed in this study revealed that different plant species respond differently to petroleum oil as well as to compost amendment. Italian ryegrass and Birdsfoot trefoil belonged to the best performers in regard to tolerance towards petroleum oil contamination, however, they responded differently to compost amendment suggesting that even under unfavorable conditions the need of fertilization depends (in addition to the nutrient status of the soil) on the plant species.

The investigated plant species, Italian ryegrass and Birdsfoot trefoil, did not only respond differently to petroleum contamination and compost treatment, but showed also highly different

degrading microbial communities in the rhizosphere and plant endosphere. Italian ryegrass, which tolerated petroleum oil also without compost amendment, but produced less biomass than control plants, hosted higher numbers of degrading bacteria, which were also characterized by a higher diversity. This suggests that Italian ryegrass supports a microflora, which more efficiently degrades alkanes or is better able to degrade a broad range of alkanes. Consequently, different plant species, tolerating a certain contaminant, might be differently suited to host degrading microbial communities. In the case of Italian ryegrass, an efficiently degrading microbial community might have supported plant growth in the presence of the pollutant, whereas in the case of Birdsfoot trefoil, which was not able to grow in contaminated soil, compost providing nutrients and additional microbes was required to support growth. Similarly, Siciliano *et al*. (2001) reported that the enrichment of hydrocarbon-degrading bacteria by plants depends, in addition to the type and amount of contaminant, on the plant species. It is well known that plants host specific associated microbial communities, both in the rhizosphere as well as inside the plant. The structure of these communities is mostly shaped by root exudates and plant physiological responses (Sessitsch *et al*., 2002; Costa *et al*., 2006; Weisskopf *et al*., 2008). We can only speculate on the kind of metabolites produced by Italian ryegrass, but they allowed a better interaction with bacteria degrading alkanes. Grasses have been reported to contain alkanes *in planta* (Marseille *et al*., 1999), which might result in the enrichment of alkane-degrading bacteria.

Overall, we observed that only few strains were found in both plants investigated indicating that distinct plant species acted as reservoirs of different degrading populations. Interestingly, in the treatment, in which Italian ryegrass and Birdsfoot trefoil were planted together, in total only few strains were obtained, and only three strains were detected carrying *alk* genes. The very dense root system of Italian ryegrass over that of Birdsfoot trefoil made it difficult to separate the two

roots systems to compare the root interior and rhizosphere strains to those of the individual plant, but few shoot interior strains were found as well within individual plants. Lower numbers and diversities of degrading isolates in the mixed treatment may be due to competition between plants for space and nutrients, which might have impacted the production of root exudates (Jose *et al*., 2006; Siemens and Bossley, 2007). The fact that Birdsfoot trefoil was outcompeted by Italian ryegrass again suggests that the latter plant has a better tolerance towards petroleum hydrocarbons, which might be partly due to a more efficient, degrading associated microflora.

From Italian ryegrass but not from Birdsfoot trefoil, a high number of different endophyte strains belonging to diverse phylogenetic groups were isolated. Production of alkanes by grasses or endophytic fungi as reported by Marseille *et al*. (1999) or uptake of alkanes from soil might explain the comparably high abundance and diversity of alkane degraders within the plant. Mostly, endophytes were different to rhizosphere strains and also roots and shoots hosted distinct culturable, degrading communities. Only few strains were found in the rhizosphere, root and shoot interior of selected plants, such as *Pseudomonas* strains ITRH16 and ITRI19. It has been reported that most endophytes originate from the rhizosphere (Sturz and Nowak, 2000; Sessitsch *et al*., 2002), however, the plant apoplast provides different growth conditions and therefore different strains efficiently colonize the plant interior (Idris *et al*., 2004). Moreover, only few strains and not necessarily those, which dominate the root interior, are transported via vascular tissues to the shoots. Endophytes were dominated by *Gammaproteobacteria*, which were also highly abundant in the rhizosphere in addition to high G+C-Gram-positives, which were not found in the endosphere. Almost all *Gammaproteobacteria* strains belonged to the genus *Pseudomonas*, a genus, which has been frequently reported to be involved in the degradation of aliphatic hydrocarbons (Holloway, 1996).

Only in half of the strains known alkane hydroxylases genes could be detected, although both enzyme types known for alkane degradation, namely *alkB* and CYP153 genes, have been targeted by using several published PCR primer pairs. This indicates that either only distantly related genes of the same enzyme classes or new enzyme classes might be responsible for the alkane degradation observed. Furthermore, plasmid curing indicated that several strains contain alkane degradation genes in addition to the genes detected by the PCR detection methods used in this study. According to van Beilen and Funhoff (2007) the occurrence of multiple alkane hydroxylases with overlapping substrate range is a common phenomenon among alkane degraders. Several degrading strains, particularly endophytes, in which alkane hydroxylase genes could not be identified, showed very low 16S rRNA gene similarity to known species indicating that new species or genera were isolated. Unknown taxa might also host novel alkane degradation genes.

The *alkB* genes which were detected by the PpalkB primer set, were originally derived from the sequence of the alkB gene of *Pseudomonas* GPo1 (Vomberg & Klinner, 2000). Our study demonstrated a much wider phylogenetic distribution. Identical or highly similar sequence of *alkB* genes of that type were found in *Alpha-*, *Beta-* and *Gammaproteobacteria* as well as in low and high G+C Gram-positives. This suggests that these genes have been spread by horizontal gene transfer, which is supported by the finding that many alkane hydroxylase genes were located on a plasmid. Frequently, related genes have been detected on plasmids (e.g. Dinamarca *et al*., 2003) or within mobile elements in the chromosome (e.g. van Beilen *et al*., 2001). Our results strongly suggest that *alkB* genes have been transferred from Gram-negative to Gram-positive bacteria (or from Gram-positives to Gram-negatives), which has been so far only demonstrated by Musovic *et al.* (2006). In that study a broad host range plasmid usually occurring in different types of *Proteobacteria* was found to be transferred also to *Arthrobacter*, however, maintenance of these

plasmids in Gram-positives was not confirmed. The high number of strains belonging to distinct phyla hosting identical *alkB* gene fragments together with the fact that the genes identified in our study were highly related to those found in *Pseudomonas* strains obtained from various other environments (Vomberg and Klinner, 2000; Daane *et al*., 2001; Whyte *et al*., 2002) indicate that this type of *alkB* gene has been prone to massive horizontal gene transfer. Interestingly, the most abundant *alkB* type (type Ia) was located in some strains on the chromosome (or possibly on a linear plasmid), whereas two thirds of the strains contained this gene on a (circular) plasmid. However, only in strains MixRI74 and MixRI75 *alkB* genes were located on IncP-1 plasmids. This findings suggest that the isolated strains may host yet unknown plasmids. Furthermore, horizontal gene tranfer was not restricted to the plasmid transfer. In contrast to the PpalkB-type *alkB* genes, those detected with the TS2S-deg1Re primer pair were only found in *Proteobacteria* and were only distantly related to other *alkB* genes deposited in the NCBI database as well as to each other. Only one strain (ITSI67) hosted an IncP-1 plasmid combined with a plasmid location of *alkB*. Similarly, CYP153-like genes were only distantly related to known alkane hydroxylase genes and showed a rather high heterogeneity. In regard to the occurrence of alkane degradation genes, CYP153-like genes were detected in association with both plants, whereas *alkB* genes amplified with the PpalkB primer pair was mostly found in the rhizosphere and endosphere of Italian ryegrass.

Horizontal gene transfer has been reported to support the biodegradation potential (Top *et al.*, 2002, 2003) by accelerating the development of an efficiently degrading microbial community upon contamination. Therefore, a plant, which selects a microflora hosting degradation genes on mobile genetic elements or which is able to promote horizontal gene tranfer is of advantage in a phytoremediation process. Efficient horizontal gene transfer of catabolic genes might affect the speed and efficiency of degradation as well as the range of substrates degraded. Our results

indicate that recent horizontal gene is responsible for the richness of degrading strains associated with Italian ryegrass. The fact that most strains containing identical *alkB* gene fragments were isolated from the rhizosphere further suggests that horizontal gene transfer occurred rather in the rhizosphere than in the endosphere. This might be due to higher bacterial numbers of donor and potential recipient strains in the rhizosphere as compared to the plant interior. Horizontal gene transfer is known to be determined by intrinsic as well as by environmental factors, such as nutrient availability (van Elsas *et al*., 2003). Furthermore, the rhizosphere has been described as a hot spot of horizontal gene transfer (van Elsas *et al*., 1988; Pukall *et al*., 1996; Kroer *et al*., 1998; Mølbak *et al*., 2003). It has been reported that the pea rhizosphere is approximately 10 times more conducive to plasmid transfer than barley (Schwaner and Kroer, 2001; Mølbak *et al*., 2007), which has been mostly attributed to the higher root exudation in pea leading to higher donor cell concentrations on roots (Mølbak *et al*., 2007). Similarly, Italian ryegrass may show different root exudation characteristics as compared to Birdsfoot trefoil, which promoted the growth of donor cells. Furthermore, potential exudation of aliphatic hydrocarbons in form of wax (Reynhardt and Riederer, 1994) and specific cometabolites for degradation of aliphatic hydrocarbons might have enhanced the selective pressure that induce abundance of genes responsible for the degradation (Olson *et.al*., 2003) and subsequently promoting spread of alkane hydroxylase genes (van Elsas *et al*., 2003). Alternatively, Italian ryegrass may have been the better host for microbial species such as *Pseudomonas* being highly competent for DNA transfer (Espinosa-Urgel, 2004) or microbial strains being highly susceptible to the acquisition of plasmids (DeGelder *et al*., 2007; Heuer *et al*., 2007). The finding of identical *alkB* gene fragments in plasmids and chromosomes indicates that not only plasmid transfer occurred but also transfers of other mobile elements.

In this study we only addressed culturable alkane degraders. Culturable bacteria often represent only a minority of the total microbial community, however, it can be assumed that the recovery rate is higher by using selective conditions as well as by combining isolation procedures. Nevertheless, we are aware that we might have missed some degraders. On the other hand, our study revealed that by targeting alkane hydroxylase genes directly the diversity of degraders is not fully addressed, as many strains contain novel or only distantly related degradation genes. Furthermore, by applying a cultivation approach we could observe that very distinct strains contain identical or highly similar alkane hydroxylase genes and thereby obtain information on horizontal gene transfer in different plant environments. Particularly in association with Italian ryegrass we detected a high diversity of alkane degrading bacteria and our results indicate that the extent of horizontal gene transfer of degradation genes by transfer of plasmids or other mobile elements is influenced by the plant species. Further analysis will reveal, whether the detected alkane hydroxylase genes are all involved in the degradation of hydrocarbons and to which extent endophytes contribute to the break-down of alkanes.

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References

Alef, K. (1994) *Biologische Bodensanierung-Methodenbuch.*VCH, Weinheim, Germany

Aprill W & Sims RC (1990) Evaluation of the use of prairie grasses for stimulating polycyclic aromatic hydrocarbon treatment in soil. *Chemosphere* **20**: 253-265.

Benizri E & Amiaud B (2005) Relationship between plants and soil microbial communities in fertilized grasslands. *Soil Biology & Biochemistry* **37**: 2055–2064.

Birnboim HC & Doly JA (1979) Rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research* **7:** 1513–1523.

Chakrabarty AM, Chou G & Gunsalus LC (1973) Genetic regulation of octane dissimilation plasmid in *Pseudomonas*. *Proceedings of the National Academy of Sciences USA* **70:** 1137-1140.

Costa R, Gotz M, Mrotzek N, Lottmann J, Berg G & Smalla K (2006) Effects of site and plant species on rhizosphere community structure as revealed by molecular analysis of microbial guilds. *FEMS Microbiology Ecology* **56**: 236–249.

Crosa JH, TolmaskyME, Actis LA& Falkow S (1994) Plasmids. *Methods for General and Molecular Bacteriology* (Gerhardt P, ed.), pp. 365-386. American Society for Microbiology, Washington.

Daane LL, Harjono I, Zylstra GJ & Häggblom MM (2001) Isolation and characterization of polycyclic aromatic hydrocarbon-degrading bacteria associated with the rhizosphere of salt marsh plants. *Applied Environmental Microbiology* **67**: 2683–2691.

DeGelder L, Ponciano JM, Joyce P & Top EM (2007) Stability of a promiscuous plasmid in different hosts: no guarantee for a long-term relationship. *Microbiology* **153**: 452–463.

Dinamarca MA, Aranda-Olmedo I, Puyet A & Rojo F (2003) Expression of the *Pseudomonas putida* OCT plasmid alkane degradation pathway is modulated by two different global control signals: Evidence from continuous cultures. *Journal of Bacteriology* **185:** 4772–4778.

Eckhardt E (1978) A rapid method for the identification of plasmid deoxyribonucleic acid in bacteria. *Plasmid* **1:** 584-588.

Edwards U, Rogall T, Blockerl H, Emde M & Bottger EC (1989) Isolation and direct complete nucleotide determination of entire genes: characterization of a gene coding for 16S ribosomal RNA. *Nucleic Acids Research* **17**: 7843-7853.

Espinosa-Urgel M (2004) Plant-associated *Pseudomonas* populations: molecular biology, DNA dynamics, and gene transfer. *Plasmid* **52**: 139–150.

Götz A, Pukall R, Smit E, Tietze E, Prager R, Tschäpe H, van Elsas JD & Smalla K (1996) Detection and characterization of broad-host range plasmids in environmental bacteria by PCR. *Applied Environmental Microbiology* **62**: 2621-2628.

Heuer H, Fox RE & Top EM (2007) Frequent conjugative transfer accelerates adaptation of a broad-host-range plasmid to an unfavourable *Pseudomonas putida* host. *FEMS Microbiology Ecology* **59**: 738–748.

Hutchinson SL, Schwab AP & Banks MK (2003) Biodegradation of petroleum hydrocarbons in the rhizosphere. *Phytoremediation: Transformation and Control of Contaminants* (McCutcheon SC & Schnoor JL, eds.), pp. 355-386. Wiley Interscience Series, John Wiley & Sons, New Jersey.

Holloway BW (1996) *Pseudomonas* genetics and taxonomy. *Molecular Biology of Pseudomonas* (Nakazawa T, Furukawa K, Haas D & Silver S, eds.), pp.22-32. ASM Press, Washington.

Idris R, Trifonova R, Puschenreiter M, Wenzel WW & Sessitsch A (2004) Bacterial communities associated with flowering plants of the Ni-hyperaccumulator *Thlaspi goesingense*. *Applied Environmental Microbiology* **70**:2667-2677.

Jose S, Williams R & Zamora D (2006) Belowground ecological interactions in mixed-species forest plantations. *Forest Ecology and Management* **233**: 231–239.

Kaimi E, Mukaidani T & Tamaki M 2007 Screening of twelve plant species for phytoremediation of petroleum hydrocarbon-contaminated soil. *Plant Production Science* **10**: 211-218.

Krasowiak R, Smalla K, Sokolov S, Kosheleva I, Sevastyanovich Y, Titok M & Thomas CM. 2002. PCR primers for detection and characterisation of IncP-9 plasmids. *FEMS Microbiology Ecology* **42:** 217-225.

Kroer N, Barkay T, Sørensen S & Weber D (1998) Effect of root exudates and bacterial metabolic activity on conjugal gene transfer in the rhizosphere of a marsh plant. *FEMS Microbiology Ecology* **25:** 375-384.

Lafferty Doty S (2008) Enhancing phytoremediation through the use of transgenics and endophytes. *New Phytologist* **179:** 318-333.

Liu W-T, Terence LM., Cheng H & Forney LJ (1997) Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. *Applied Environmental Microbiology* **63**: 4516–4522.

Marseille F, Disnar JR, Guillet B & Noack Y (1999) n-alkanes and free fatty acids in humus and A1 horizons of soils under beech, spruce and grass in the Massif-Central (Mont-Lozère), France. *European Journal of Soil Science* **50**: 433-441

Massol-Deya AA, Odelson DA, Hickey RF & Tiedje JM (1995) Bacterial community fingerprinting of amplified 16S and 16S-23S ribosomal DNA genes sequences and restriction endonuclease analysis (ARDRA). *Molecular Microbial Ecology Manual* (Akkermans ADL, van Elsas JD & de Bruijn FJ, eds.). Kluwer Academic Publisher, Dordrecht, The Netherlands.

Mesas JM, Rodriguez C & Alegre MT (2004) Plasmid curing of *Oenococcus oeni*. *Plasmid* **51**: 37–40.

Mølbak L, Licht TR, Kvist T, Kroer N & Andersen SR (2003) Plasmid transfer from *Pseudomonas putida* to the indigenous bacteria on alfalfa sprouts: characterization, direct quantification, and in situ location of transconjugant cells. *Applied Environmental Microbiology* **69**: 5536–5542.

Mølbak L, Molin S & Kroer N (2007) Root growth and exudate production define the frequency of horizontal plasmid transfer in the rhizosphere. *FEMS Microbiology Ecology* **59**: 167–176.

Moore FP, Barac T, Borremans B, Oeyen L, Vangronsveld J, van der Lelie D, Campbell CD & Moore ERB (2006) Endophytic bacterial diversity in poplar trees growing on a BTEX-contaminated site: The characterisation of isolates with potential to enhance phytoremediation. *Systematic and Applied Microbiology* **29**: 539–556

Musovic S, Oregaard G, Kroer N & Sørensen (2006) Cultivation-independent examination of horizontal transfer and host range of an IncP-1 plasmid among Gram-positive and Gram-negative bacteria indigenous to the barley rhizosphere. *Applied Environmental Microbiology* **72:** 6687-6692.

Nunan N, Daniell TJ, Singh BK, Papert A, McNicol JW & Prosser JI (2005) Links between plant and rhizoplane bacterial communities in grassland soils, characterized using molecular techniques. *Applied Environmental Microbiology* **71**: 6784–6792.

Olsen JE (1990). An improved method for rapid isolation of plasmid DNA from wild-type Gram-negative bacteria for plasmid restriction profile analysis. *Letters in Applied Microbiology* **10:** 209-212.

Olson PE, Reardon KF & Pilon-Smits AH (2003) Ecology of Rhizopshere Bioremediation. *Phytoremediation: Transformation and Control of Contaminants* (McCutcheon SC & Schnoor JL, eds.), pp. 317-353. Wiley Interscience Series, John Wiley & Sons, New Jersey.

Pilon-Smits E (2005) Phytoremediation. *Annual Review of Plant Biology* **56:** 15-39.

Pukall R, Tschäpe H & Smalla K (1996) Monitoring the spread of broad host and narrow host range plasmids in soil microcosms. *FEMS Microbiology Ecology* **20**: 53-66.

Rasche F, Velvis H, Zachow C, Berg G, van Elsas JD & Sessitsch A (2006) Impact of transgenic potatoes expressing anti-bacterial agents on bacterial endophytes is comparable with the effects of plant genotype, soil type and pathogen infection. *Journal of Applied Ecology* **43**:555-566.

Reichenauer TG & Germida JJ (2008) Phytoremediation of organic contaminants in soil and groundwater. *ChemSusChem* **1:** 708-719.

Reilley KA, Banks M K & Schwab A P (1996) Organic chemicals in the environment: dissipation of polycyclic aromatic hydrocarbons in the rhizosphere. *Journal of Environmental Quality* **25**: 212-219.

Reynhardt, EC & Riederer M (1994) Structures and molecular dynamics of plant waxes. *European Biophysics Journal* **23**:59–70.

Schwaner NE & Kroer N (2001) Effect of plant species on to the kinetics of conjugal transfer in the rhizosphere and relation to bacterial metabolic activity. *Microbial Ecology* **42:** 458-465.

Sessitsch A, Reiter B, Pfeifer U & Wilhelm E (2002) Cultivation-independent population analysis of bacterial endophytes in three potato varieties based on eubacterial and *Actinomyces*-specific PCR of 16S rRNA genes. *FEMS Microbiology Ecology* **39**:23–32.

Siciliano SD & Germida JJ (1998) Mechanisms of phytoremediation:biochemical and ecological interactions between plants and bacteria. *EnvironmentalReviews* **6:** 65–79.

Siciliano SD, Fortin N, Mihoc A, Wisse G, Labelle S, Beaumie D, Ouellette D, Roy R, Whyte LG, Banks MK, Schwab P, Lee K & Greer CW (2001) Selection of specific endophytic bacterial genotypes by plants in response to soil contamination. *Applied Environmental Microbiology* **67**: 2469–2475.

Siemens TJ & Blossey B (2007) An evaluation of mechanisms preventing growth and survival of two native species in invasive Bohemian knotweed (*Fallopia bohemica*, Polygonaceae). *American Journal of Botany* **94**: 776–783.

Smalla K, Wieland G, Buchner A, Zock A, Parzy J, Kaiser S, Roskot N, Heuer H & Berg G (2001) Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis: plant-dependent enrichment and seasonal shifts revealed. *Applied Environmental Microbiology* **67**: 4742-4750.

Smits THM, Röthlisberger M, Witholt B & van Beilen JB (1999) Molecular screening for alkane hydroxylase genes in Gram-negative and Gram-positive strains. *Environmental Microbiology* **1**: 307-317.

Stuart-Keil KG, Hohnstock AM., Drees KP, Herrick JB & Madsen EL (1998) Plasmids responsible for horizontal transfer of naphthalene catabolism genes between bacteria at a coal tar-contaminated site are homologous to pDTG1 from *Pseudomonas putida* NCIB 9816-4. *Applied Environmental Microbiology* **64:** 3633–3640.

Sturz AV & Nowak J (2000) Endophytic communities of rhizobacteria and the strategies required to create yield enhancing associations with crops. *Applied Soil Ecology* **15:** 83–190.

Top EM, Springael D & Boon N (2002) Catabolic mobile genetic elements and their potential use in bioaugmentation of polluted soil and waters. *FEMS Microbiology Ecology* **42**: 199-208.

Top EM & Springael D (2003) The role of mobile genetic elements in bacterial adaptation to xenobiotic organic compounds. *Current Opinion in Biotechnology* **14**: 262-269.

Trevors JT (1986) Plasmid curing in bacteria. *FEMS Microbiology Reviews* **32**: 149-157.

van Beilen JB, Panke S, Lucchini S, Franchini AG, Röthlisberger M & Witholt B (2001) Analysis of *Pseudomonas putida* alkane-degradation gene clusters and flanking insertion sequences: evolution and regulation of the *alk* genes. *Microbiology* **147**: 1621-1630.

van Beilen JB, Smits THM, Whyte LG, Schorcht S, Röthlisberger M, Plaggemeier T, Engesser KH & Witholt B (2002) Alkane hydroxylase homologues in Gram-positive strains. *Environmental Microbiology* **4**: 676-682.

van Beilen JB, Li Z, Duetz WA, Smits THM & Witholt B (2003) Diversity of alkane hydroxylase systems in the environment. *Oil & Gas Science and Technology* **58**: 427-440.

van Beilen JB, Holtackers R, Lüscher D, Bauer U, Witholt B & Duetz WA (2005) Biocatalytic production of perillyl alcohol from limonene by using a novel *Mycobacterium* sp. cytochrome P450 alkane hydroxylase expressed in *Pseudomonas putida*. *Applied Environmental Microbiology* **71**: 1737–1744.

van Beilen JB, Funhoff EG, van Loon A, Kaysse L, Bouza M, Holtackers R, Röthlisberger M, Li Z & Witholt B (2006) Cytochrome P450 alkane hydroxylases of the CYP153 family are common in alkane-degrading eubacteria lacking integral membrane alkane hydroxylases. *Applied Environmental Microbiology* **72:** 59-65.

van Beilen JB & Funhoff (2005) Expanding the alkane oxygenase toolbox: new enzymes and applications. *Current Opinion in Biotechnology* **16:** 308–314

van Beilen JB & Funhoff EG (2007) Alkane hydroxylases involved in alkane degradation. *Applied Microbiology and Biotechnology* **74**:13-21

van Elsas JD, Trevors JT & Starodub ME (1988) Bacterial conjugation between pseudomonads in the rhizosphere of wheat. *FEMS Microbiology Ecology* **53:** 299-306.

van Elsas JD, Turner S & Bailey MJ (2003) Horizontal gene transfer in the phytopshere. *New Phytologist* **157:** 525-537.

van de Peer Y & de Wachter R (1994) TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. *Computer Applications in the Biosciences* **10**: 569-570.

Vavrek MC & Campbell WJ (2000) Phytoremediation of petroleum.: Identification of plant traits that enhance degradation. *OSRADP Technical Report Series 00-012.* Louisiana Applied and Educational Oil Spill Research and Development Program, USA.

Vomberg A & Klinner U (2000) Distribution of *alkB* genes within n-alkane-degrading bacteria. *Journal of Applied Microbiology* **89**: 339-348.

Weisburg WG, Barns SM, Pelletier DA & Lane DJ (1991) 16S Ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology* **173**: 697-703.

Weisskopf L, Le Bayon R-C, Kohler F, Page V, Jossi M, Gobat J-M, Martinoia E & Aragno M (2008) Spatio-temporal dynamics of bacterial communities associated with two plant species differing in organic acid secretion: A one-year microcosm study on lupin and wheat. *Soil Biology & Biochemistry* **40**:1772–1780

Whyte LG, Schultz A, van Beilen JB, Luz AP, Pellizari V, Labbe D & Greer CW (2002) Prevalence of alkane monooxygenase genes in Arctic and Antarctic hydrocarbon-contaminated and pristine soils. *FEMS Microbiology Ecology* **41:** 141-150.

CHAPTER 3

Expression of alkane monooxygenase (*alkB*) genes by plant-associated bacteria in the rhizosphere and endosphere of Italian ryegrass (*Lolium multiflorum* L.) in diesel contaminated environment

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Abstract

For phytoremediation of organic contaminants, plants have to host a microflora efficiently expressing degradation genes. To assess the role of endophytes in alkane degradation, Italian ryegrass (*Lolium multiflorum* L.) was grown in sterile soil with 0, 1 or 2% diesel and inoculated either with alkane degrading bacteria originally derived from the rhizosphere of Italian ryegrass or with an endophyte. We studied plant colonization of these strains as well as the abundance and expression of alkane monooxygenase (*alkB*) genes in rhizosphere, shoot and root interior. Results showed that the endophyte strain better colonized the plant, particularly the plant interior, and also showed higher expression of *alkB* genes suggesting a more efficient degradation of the pollutant. The rhizosphere strain colonized primarily the rhizosphere and showed low *alkB* gene expression in the plant interior. Our study suggests that endophytes have a high potential to be used in phytoremediation applications.

Keywords: diesel contamination, phytoremediation, alkane monooxygenase genes, gene expression, endophyte.

1. **Introduction**

Phytoremediation combines the use of plants and their associated microorganisms to degrade toxic organic contaminants (Pilon-Smits, 2005). The efficiency of a phytoremediation process depends largely on the presence and activity of the plant-associated microflora carrying degradation genes required for enzymatic break-down of organic pollutants. The rhizosphere as well as the plant apoplast has been reported to host degrading bacteria (Siciliano *et al*., 2001), although comparably little is known about degradation activities of endophytes (Newman & Rreynolds, 2005). Endophytes are defined as bacteria that reside within plant tissue without conferring pathogenicity and frequently show plant growth-promoting activities (Sessitsch *et al*., 2004; Ryan, *et al*., 2008). There is increasing interest in the role of endophytes in phytoremediation applications due to their ability as vector delivering the biodegradative capacity inside the plant (Lodewyckx et al., 2002; Barac *et al*., 2004; Glick, 2004). However, it is not fully understood, whether potentially degrading endophytes are active once inside the plant and thereby contribute to the detoxification of the pollutant and better survival of the plant under toxic conditions.

Highly diverse bacteria containing alkane monooxygenase genes (*alkB*) have been isolated from the rhizosphere or endosphere from different plant species, including from Italian ryegrass (Siciliano *et al*., 2001; van Beilen *et al*., 2002; Hamamura, *et al*., 2005; Kaimi *et al*., 2007). The abundance of *alkB* genes has been assessed in the rhizosphere as well as in the root interior (Siciliano *et al.,* 2001; Whyte *et al*., 2002) but *alkB* gene expression under natural conditions has been rarely addressed (Powell *et al*., 2006).

The aim of this study was to analyze plant colonization and activities of two strains, the endophyte *Pseudomonas* sp. strain ITRI53 and the rhizosphere strain *Rhodococcus* sp. ITRH43, isolated from Italian ryegrass in a diesel-contaminated soil (Andria *et al*., submitted). Our study showed that the endophyte colonized the plant interior efficiently and showed high *alkB* gene expression inside the plant. Expression was particularly high in the presence of high diesel oil concentrations in soil and plants showed better growth when inoculated with strain ITRI53.

2. Materials and Methods

2.1. Bacterial strains and plant experiment

Pseudomonas sp. strain ITRI53 and *Rhodococcus sp*. strain ITRH43 were isolated from the root interior and the rhizosphere of Italian ryegrass, respectively. Both strains have the capacity to degrade alkanes and contain identical or highly similar alkane monooxygenase (*alkB*) genes, which are located on plasmids (Andria *et al.,* submitted). Strains were cultivated in 10% Luria Bertani broth amended with 1% (v/v) filter-sterilized, incubated 48 hr at 30^0C and 180 rpm agitation. Strains were harvested by centrifugation at 10,000 g for 1 min and resuspended in sterile 0.9% NaCl solution.

For the plant experiment Magenta boxes were filled with 72.5 g air-dried soil mixed with 40% (v/v) sand and sterilized by 30 kGy γ-radiation. Before sowing, the soil was amended with either0%, 1% or 2% (v/v) filter-sterilized diesel and then mixed with 25 ml inoculant suspension (app. 10^8 CFU/ml) containing either strain ITRI53 or strain ITRH43. Control treatments received 25 ml sterile 0.9% NaCl solution. Seeds of Italian ryegrass were surface-sterilized in a 20% (v/v) NaOCl for 10 min, washed 5 times with sterile water and placed on covered-sterile filters at 25^0C for 72 hours until seedlings started to grow. Twenty Italian ryegrass plantlets were placed in each box and each

treatment was triplicated. Plants were grown at 25^0C in a sterile environment by covering boxes with a sterilized lid and subjected to a cycle of 16 h light and 8 h dark for 2 months. Plants were watered with equal amounts of sterile water.

2.2. Sampling and extraction of DNA and RNA

After 2 months, shoots were cut from 2 cm above soil and the remaining plants were carefully harvested. Rhizosphere soil was collected by gently sampling the soil closely attached at the root surface. Subsequently, the roots were washed several times in sterile water. Roots and shoots (1 g) were surface-sterilized as described by Reiter *et al*. (2003), replacing distilled sterile water by DEPC-treated water. Sterility was checked by plating on Tryptic Soy Agar plates (TSA, Merck).

DNA from rhizosphere was extracted by using FastDNA Spin Kit for soil (Qbiogene), whereas for RNA isolation the FastRNA Pro Soil-Direct Kit (MP Biomedicals) was used. Roots and shoots were briefly ground in liquid N_2 and microbial cells were disrupted by bead-beating (Reiter *et al*., 2003). For isolation of DNA and RNA the DNeasy Plant Mini Kit and RNeasy Plant Mini Kit (Qiagen) were used. In RNA preparations genomic DNA was eliminated by DNase I enzyme (Ambion) digestion and potential presence of contaminating DNA was checked by PCR amplification of partial 16S rRNA genes (Rasche *et al*., 2006).

2.3. Detection of inoculants re-colonization and presence of alkB genes

Rhizosphere soil, surface-sterilized roots and shoots (1 g) were suspended in 2 ml of 0.9% (w/v) NaCl solution and shaken at 180 rpm for 30 min. After plant and soil particles settled, the aqueous phase (100 µL) of 10^{-3} dilutions was plated on 10% TSA in duplicates and incubated at 30^oC for 2 days to determine CFU/g dry soil or plant material. Ten colonies were randomly picked and the

identity of isolates with the inoculant strain was confirmed by restriction fragment length polymorphism (RFLP) analysis of the 16S-23S rRNA intergenic spacer region (IGS) as described by Rasche *et al*. (2006) as well as of *alkB* genes. The *alkB* genes were amplified as described by Whyte *et al.* (1996) and amplicons were digested with the restriction enzyme *Alu*I (Invitrogen), electrophoresed on 1.5% agarose gels and compared with the profiles obtained from the inoculant strains. Isolates and inoculant strains had identical restriction patterns.

2.4. Quantiative analysis of the abundance and expression of alkB genes

Reverse transcription was performed with 10-20 ng of extracted RNA, using the specific primer PpalkB-for (Bustin, 2000) applying Superscript II Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. Abundance and expression of *alkB* genes were quantified by real-time PCR using an iCycler IQ (Biorad) using DNA and cDNA as templates. Specific qRT-PCR primers were designed based on the conserved region within the *alkB* of the inoculant and closely related strains (Genbank accession number FJ014898 to FJ014915, AJ233397, AJ344083, AJ250560 and AY034587). The specificity for bacterial *alkB* genes of the designed primers RTalkB-f (5'-ATCCGCCTGAGGAAGTAGTG-3') and RTalkB-r (5'- CGGCCACTTCTTTATTGAGC-3') resulting in the amplification of a 300 bp fragment was checked by using Primer 3 and BLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Standards for qRT-PCR were generated by serial dilution of stocks containing purified PpalkB PCR products from *Pseudomonas sp.* ITRI53 DNA. Analyses were performed in triplicates and gene copy numbers were calculated as described by Powell *et al*. (2006). Reaction mixtures (25 µ) contained 12.5 µl of Q Mix (Biorad), 2 µl 10 mg/ml BSA, 0.8 µl DMSO, 0.5 µl 2 µM of each primer, 25-50 ng of DNA/cDNA template and RNase-free water. Thermal cycling conditions were as following: 4 min at 94^0C followed by 40 cycles of 94^0C for 20 s, 60^0C for 30 s, 72^0C for 45 s followed by a melt curve from 50^0C to 100^0C. Besides melt

curve analysis, PCR products were examined on 2% agarose gels. No primer-dimers were detected. Serial dilutions of DNA and cDNA were spiked with 10^6 copies of amplified *alkB* genes in to check for real-time PCR inhibition (López-Gutierrez *et al*., 2004). Highly linear standard curves (r^2 values > 0.95, PCR efficiency > 98%) over the dilution range and a detection limit of 10^1 copies were obtained indicating no PCR inhibition. The *alkB* gene copy numbers were quantified relative to a standard curve of positive control and were normalized to the copy number of control plants Statistical analysis was based on Least Significant Difference tests using SAS software (SAS Institute Inc., Cary, NC, USA) and regression analysis using Microsoft Excel.

3. Results and Discussions

Colonization of alkane degrading inoculant strains was determined by cultivation and a cultivation-independent approach. Both approaches clearly showed that strain ITRI53, originally isolated from the root interior of Italian ryegrass, better colonized all plant compartments than strain ITRH43, originally isolated from the Italian ryegrass rhizosphere (Table 1, Fig. 1). However, primarily the plant interior without or at low diesel concentrations was better colonized by strain ITRI53, whereas differences in the rhizosphere or in plants grown in 2% diesel were less pronounced. This is in agreement with Rosenblueth & Martínez-Romero (2006), who postulated that endophytic isolates are generally better able to colonize plant tissues than rhizosphere isolates. Strain ITRH43 entered the plant at certain conditions, however, endophyte strain ITRI53 was better adapted to grow in the plant interior. Interestingly, the diesel concentration influenced endophytic plant colonization of strain ITRI53, which was most efficient in the presence of high (2%) diesel concentrations. As the experiment was performed under sterile conditions, these observations cannot be explained by competition effects. Rhizosphere colonization of strain ITRI53 in the absence of a selective pressure was low, but endophytic colonization was better. Grasses have

been reported to contain alkanes *in planta* (Marseille *et al*., 1999), which might explain the higher abundance of alkane-degrading, endophytic bacteria in uncontaminated soil. The comparison of results obtained by cultivation and by quantifying *alkB* gene copies suggests that with increasing diesel concentration the number of culturable bacteria decreased. It might be that due to the stress conditions encountered the number of viable-but-non-culturable bacteria increased. Similarly, *Pseudomonas frederiksbergensis* cells entered a viable-but-non-culturable state in a mercury contaminated soil (Johnsen *et al*., 2003).

We observed that plants inoculated with strain ITRI53 showed better growth and survival in the presence of diesel. All (20) plants were able to grow in un-polluted soil, whereas less plantlets were able to grow in the presence of diesel (Table 2). Only 15 and 9 plants inoculated with strain ITRH43 survived in the presence of 1% and 2%, diesel, respectively, but 20 and 15 plants, respectively, survived when inoculated with strain ITRI53. Diesel oil is toxic to plants even at low levels (Adam & Duncan, 2003) and strain ITRI53 protected plants more efficiently than strain ITRH43. It might be that strain ITRI53, which belongs to the genus *Pseudomonas*, increases stress tolerance in the plant as many pseudomonads have been identified so far with plant growth-promoting properties (Compant *et al.,* 2005). Furthermore, strain ITRI53 colonized plants better and might have degraded alkanes more efficiently and thereby protected the plants. Both strains principally expressed *alkB* genes indicating an active role in the degradation of the pollutant and *alkB* gene expression was found in all compartments (Table 1, Fig. 1). As the abundance of a degrading strain in a particular environment is a pre-requisite for expressing degrading activities, the differences between strains ITRI53 and ITRH43 in regard to *alkB* gene expression followed essentially the same patterns as *alkB* abundance data. Strain ITRH43 only poorly colonized the plant interior in the presence of 1% diesel or in the absence of the pollutant, consequently we also

observed only low expression. Expression of the *alkB* gene of that strain was very high in the shoot interior, but only very low in the root interior.

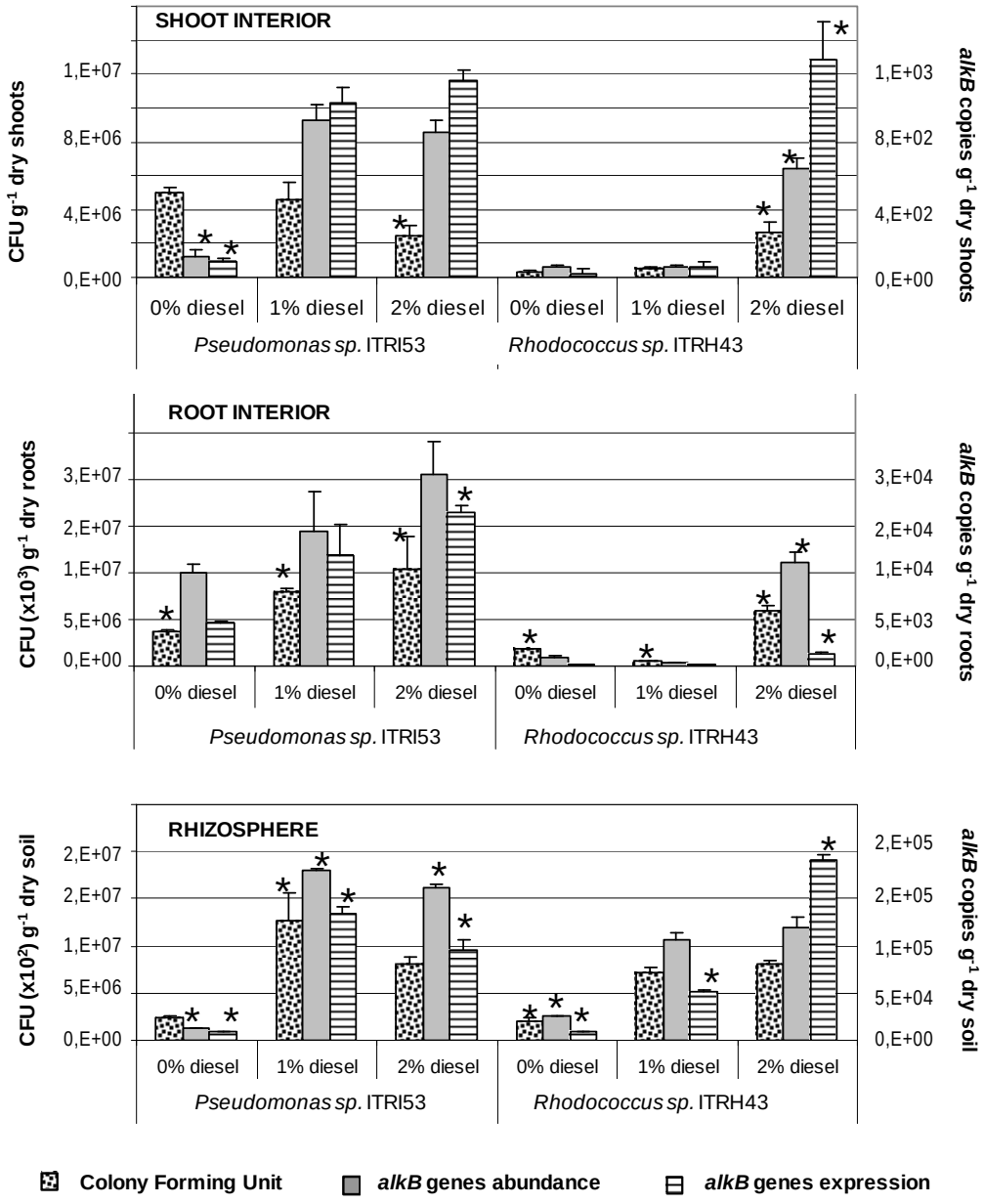

Fig. 1. Mean values of Colony Forming Unit (CFU), *alkB* genes abundance and *alkB* genes expression of endophyte *Pseudomonas sp.* ITRI53 and rhizosphere bacteria *Rhodococcus sp.*ITRH43 at rhizosphere, root and shoot interior of italian ryegrass, in presence of 0%, 1% and 2% of diesel. Error bars represent standard error. The asterix (*) shows significant difference between means for every parameter (CFU, *alkB* abundance and *alkB* expression) in different diesel concentration for the same inoculant. Means followed by the same letters are not significantly different according to Least Significant Difference test ($P < 0.05$).

Table 1. Mean values of Colony Forming Unit (CFU), *alkB* genes abundance and *alkB* genes expression in rhizosphere (coded RH), root interior (coded RI) and shoot interior (coded SI) of italian ryegrass inoculated either by endophytic bacteria *Pseudomonas sp.* ITRI53 or by rhizosphere bacteria *Rhodococcus sp*. ITRH43 in association with diesel amendment 0%, 1% and 2%. The standard error of the distribution is presented in parentheses.

Bacterial inoculant: ***Pseudomonas sp.* ITRI53**												
Diesel (v/v)	CFU/g dry weight[a]			*alkB* genes Abundance[b] (copies/g dry weight)			*alkB* genes Expression[c] (copies/g dry weight)			Ratio *alkB* expression/abundance[d]		
	RH	RI	SI	RH	RI	SI	RH	RI	SI	RH	RI	SI
0%	2.4E+05^{A} (1.0E+05)	3.8E+06^{B} (9.8E+04)	5.0E+07^{B} (3.5E+05)	1.2E+04^{AB} (6.8E+029	1.0E+04^{A} (9.8E+02)	1.3E+02^{B} (3.5E+01)	8.7E+03^{A} (6.0E+02)	4.7E+03^{B} (9.3E+01)	8.9E+01^{A} (2.7E+01)	0.7^{A}	0.5^{A}	0.5^{A}
1%	1.3E+06^{A} (2.9E+05)	8.0E+06^{B} (4.4E+05)	4.6E+07^{B} (9.8E+05)	1.8E+05^{A} (1.7E+03)	1.4E+04^{B} (4.4E+03)	9.3E+02^{C} (9.8E+01)	1.3E+05^{A} (9.0E+039	1.2E+04^{A} (3.4E+03)	1.0E+03^{B} (9.2E+01)	0.7^{A}	0.8^{A}	1.1^{B}
2%	8.1E+05^{A} (6.8E+05)	1.0E+07^{B} (3.5E+06)	2.4E+07^{C} (6.6E+05)	1.6E+05^{A} (3.8E+03)	2.1E+04^{B} (3.5E+03)	8.6E+02^{C} (6.6E+01)	9.6E+04^{A} (1.1E+04)	1.7E+04^{A} (6.4E+02)	1.2E+03^{B} (6.3E+01)	0.6^{A}	0.8^{A}	1.3^{B}

Bacterial inoculant: ***Rhodococcus sp.* ITRH43**												
Diesel (v/v)	CFU/g dry weight			*alkB* genes Abundance (copies/g dry weight)			*alkB* genes Expression (copies/g dry weight)			Ratio *alkB* expression/abundance		
	RH	RI	SI	RH	RI	SI	RH	RI	SI	RH	RI	SI
0%	2.0E+06^{A} (4.6E+05)	1.8E+06^{A} (1.2E+05)	3.2E+02^{B} (1.0E+01)	2.6E+04^{A} (9.8E+01)	2.92E+02^{B} (1.2E+02)	6.16E+01^{C} (1.2E+01)	9.2E+03^{A} (1.6E+02)	2.0E+02^{A} (5.2E+00)	6.5E+01^{B} (4.9E+01)	0.4^{A}	0.2^{A}	1.1^{B}
1%	7.1E+06^{A} (6.8E+05)	6.0E+04^{B} (0.1E+00)	4.6E+02^{C} (1.3E+01)	1.1E+05^{A} (6.8E+03)	9.0E+02^{B} (6.9E+01)	6.48E+01^{B} (7.29E+00)	5.2E+04^{A} (5.2E+02)	1.3E+02^{A} (3.9E+01)	6.2E+01^{B} (3.2E+01)	0.5^{A}	0.5^{A}	1.0^{B}
2%	8.0E+06^{A} (4.3E+05)	6.0E+06^{B} (4.8E+05)	2.6E+06^{C} (5.9E+05)	1.2E+05^{A} (1.1E+04)	1.1E+04^{A} (1.2E+03)	6.4E+02^{B} (5.9E+01)	1.9E+05^{A} (5.1E+03)	1.3E+03^{B} (9.9E+01)	1.3E+03^{B} (2.2E+02)	1.6^{A}	0.1^{B}	2.0^{A}

Significant difference test compared mean values for each parameter in different plant environments (RH, RI and SI) in certain diesel concentration (read within rows). Means followed by the same letters are not significantly different according to Least Significant Difference test ($P < 0.05$).

[a] Colony Forming Unit per gram dry weight of environmental samples.

[b] Gene copy number per gram dry weight of environmental samples resulted from q-RT PCR of extracted DNA. PCR efficiency 98% for both strains.

[c] Gene copy number per gram dry weight of environmental samples resulted from q-RT PCR of reverse transcribed extracted mRNA. PCR efficiency 95% for both strains.

[d] Ratio between reverse-transcribed mRNA and DNA gene copy number per gram weight of environmental samples.

Table 2. Average number of growing plantlets after 2 months (n=3)

Bacterial inoculant	No diesel	Diesel 1%	Diesel 2%
Pseudomonas sp. ITRI53	20	20	16
Rhodococcus sp. ITRH43	20	15	9

Also strain ITRI53 showed higher *alkB* gene expression in relation to *alkB* gene abundance in the shoot interior than in the root interior. This indicates that root and shoot metabolites influenced *alkB* gene expression. It appears that the endophyte strain maintained its degrading activity in the rhizosphere and in the plant interior independently from the diesel concentration, whereas the rhizosphere strain needed a higher diesel concentration as selective pressure to maintain its degradation activity within plant tissues, but not in the rhizosphere. However, for both inoculants, *alkB* gene abundance and expression were the highest in rhizosphere (Table 1) indicating that the rhizosphere provides a better supporting environment through root exudates providing nutrients for bacterial growth and co-metabolites for alkane degradation (Olson *et al.*, 2003).

For the endophyte as well as the rhizosphere strain, we did not find a general correlation pattern for colonization, *alkB* gene abundance or expression among plant environments in certain diesel concentration (Table 1). However, regression analysis revealed, that for the endopyhte strain ITRI53 only in the root interior the diesel concentration was linearly correlated with the number of colonies, *alkB* gene abundance and *alkB* gene expression (r^2= 0.90, P = 0.09 for CFU; r^2 = 0.99, P = 0.05 for *alkB* genes abundance; and r^2 = 0.98, P = 0.07 for *alkB* genes expression), whilst for the rhizosphere strain ITRH43 a similar correlation was observed only in the rhizosphere (r^2= 0.89, P = 0.04 for CFU; r^2 = 0.84, P = 0.05 for *alkB* genes abundance; and r^2 = 0.91, P = 0.09 for *alkB* genes

expression). This suggests that besides environmental factors, plant species and contaminant (Siciliano *et al*., 2001) the original habitat is important for the performance of an inoculant strain.

In conclusion, we showed that bacterial *alkB* genes can be expressed not only in the rhizosphere but also *in planta*. The endophyte strain tested was superior than the rhizosphere strain regarding colonization and *alkB* gene expression. Furthermore, inoculation of the endophyte strain resulted in better survival of plants due to plant growth-promoting and / or alkane degrading activities. Further studies will address the application potential of endophytes in phytoremediation applications.

References

Adam G & Duncan H (2003) The effect of diesel fuel on common vetch (*Vicia sativa* L.) plants. *Environmental Geochemistry and Health* **25**:123-130.

Andria V, Yousaf S, Reichenauer TG, Smalla K & Sessitsch A (2008) Phylogenetic and functional diversity of alkane degrading bacteria associated with Italian ryegrass (*Lolium multiflorum*) and Birdsfoot trefoil (*Lotus corniculatus*) in a petroleum oil-contaminated environment. *FEMS Microbiology Ecology (submitted)*.

Barac T, Taghavi S, Borremans B, Provoost A, Oeyen L, Colpaert, JV, Vangronsveld J & van der Lelie D (2004) Engineered endophytic bacteria improve phytoremediation of water-soluble, volatile, organic pollutants. *Nature Biotechnology***22**:583-588.

Bustin SA (2000) Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *Journal of Molecular Endocrinology* **25**:169-193.

Compant S, Duffy B, Nowak J, Clément C & Barka EA (2005) Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. *Applied Environmental Microbiology* **71**: 4951-4959.

Glick BR (2004) Teamwork in phytoremediation. *Nature Biotechnology*. **22**: 526-527.

Hamamura N, Olson SH, Ward DM & Inskeep WP (2005) Diversity and functional analysis of bacterial communities associated with natural hydrocarbon seeps in acidic soils at Rainbow Springs, Yellowstone National Park. *Applied Environmental Microbiology* **71**:5943-5950

Johnsen K, Ekelund F, Binnerup SJ & Rasmussen, LD (2003) Mercury decreases culturability of *Pseudomonas frederiksbergensis* JAJ 28 in soil microcosms. *Current Microbiology* **47**: 0125-0128.

Newman LA & Rreynolds CM (2005) Bacteria and phytoremediation: new uses for endophytic bacteria in plants. *Trends in Biotechnology* **23**: 6-8.

Kaimi E, Mukaidani T & Tamaki M (2007) Screening of twelve plant species for phytoremediation of petroleum hydrocarbon-contaminated soil. *Plant Production Science* **10**: 211-218.

Lodewyckx C, Vangronsveld J, Porteous, F Moore, ERB, Taghavi, S & van der Lelie, D (2002) Endophytic bacteria and their potential applications**.** *Critical Reviews in Plant Sciences* **21**:583-606

Marseille F, Disnar JR, Guillet B & Noack Y (1999) n-alkanes and free fatty acids in humus and A1 horizons of soils under beech, spruce and grass in the Massif-Central (Mont-Lozère), France. *European Journal of Soil Science* **50**: 433-441

Olson PE, Reardon KF & Pilon-Smits EAH (2003) Ecology of Rhizopshere Bioremediation. *Phytoremediation: Transformation and Control of Contaminants* (McCutcheon SC & Schnoor JL, eds.), pp. 317-353**.** Wiley Interscience Series, John Wiley & Sons, New Jersey.

Pilon-Smits EAH (2005) Phytoremediation. *Annual Review of Plant Biology* **56**: 15-39.

Powell SM, Ferguson SH, Bowman JP & Snape I (2006) Using real-time PCR to assess changes in the hydrocarbon-degrading microbial community in Antarctic soil during bioremediation. *Microbial Ecology* **52**:523-32

Rasche F, Velvis H, Zachow C, Berg G, van Elsas JD & Sessitsch A (2006) Impact of transgenic potatoes expressing anti-bacterial agents on bacterial endophytes is comparable with the effects of plant genotype, soil type and pathogen infection. *Journal of Applied Ecology* **43**:555-566.

Reiter B, Wermbter N, Gyamfi S, Schwab H & Sessitsch A (2003) Endophytic *Pseudomonas* spp. populations of pathogen-infected potato plants analysed by 16S rDNA- and 16S rRNA-based denaturating gradient gel electrophoresis. *Plant and Soil* **257:** 397–405

Rosenblueth M, Martínez-Romero E (2006) Bacterial endophytes and their interactions with hosts. *Molecular Plant-Microbe Interaction* **19**: 827-837.

Ryan RP, Germaine K, Franks A, Ryan DJ & Dowling DN (2008) Bacterial endophytes: recent developments and applications. *FEMS Microbiology Letters* **278**:1-9.

Sessitsch A, Reiter B & Berg G (2004) Endophytic bacterial communities of field-grown potato plants and their plant-growth-promoting and antagonistic abilities. *Canadian Journal of Microbiology* **50**:239-249

Siciliano SD, Fortin N, Mihoc A, Wisse G, Labelle S, Beaumie D, Ouellette D, Roy R, Whyte LG, Banks MK, Schwab P, Lee K & Greer CW (2001) Selection of specific endophytic bacterial genotypes by plants in response to soil contamination. *Applied Environmental Microbiology* **67**: 2469–2475.

Siciliano SD, Germida JJ, Banks K & Greer CW (2003) Changes in microbial community composition and function during a polyaromatic hydrocarbon phytoremediation field trial. *Applied Environmental Microbiology* **69**: 483-489

van Beilen JB, Smits THM, Whyte LG, Schorcht S, Röthlisberger M, Plaggemeier T, Engesser KH & Witholt B (2002) Alkane hydroxylase homologues in Gram-positive strains. *Environmental Microbiology* **4**: 676-682.

Whyte LG & Greer CW (1996) Assessment of the biodegradation potential of psychotropic microorganisms. *Canadian Journal of Microbiology* **42**: 99 – 106.

Whyte LG, Schultz A, van Beilen JB, Luz AP, Pellizari V, Labbe D & Greer CW (2002) Prevalence of alkane monooxygenase genes in Arctic and Antarctic hydrocarbon- contaminated and pristine soils. *FEMS Microbiology Ecology* **41**: 141-150.

CHAPTER 4

NEW APPROACHES IN PHYTOREMEDIATION RESEARCH

4.1. Introduction

Phytoremediation technologies have been used to clean up metals, pesticides, explosives and petroleum hydrocarbons contamination in streams, groundwater and soil. Phytoremediation has merit in comparison to other *in situ* or *ex situ* remediation technologies particularly due to its cost effectiveness and environmental sustainability. Nevertheless, the use of phytoremediation techniques is limited by some factors, namely:

- the ability of the plant to grow. Plants can only grow in contaminant concentrations where phytotoxicity does not occur and under environmental conditions, such as soil texture, pH, salinity, oxygen availability, temperature which are within the limits tolerated by plants. Phytoremediation of the target contaminant will not be successful unless the soil is pretreated to reduce phytotoxicity or a resistant plant species is selected Frick *et al*., 1999; Pilon-Smits, 2004).(Pilon-Smits, 2004; Frick *et al*., 1999)
- the root depth, because plants have to be able to reach the pollutant. Therefore, usually phytoremediation is used for contamination in shallow soils. Root depth is typically 50 cm for herbaceous species or 3 m for trees (Pilon-Smits, 2004), although some trees may have root systems that can extend to a depth of 60 m, but in most plant root density generally decreases with depth (Frick *et al*., 1999).
- phytoremediation is slower than *ex situ* remediation methods (such as the use of excavation, incineration, soil washing and other engineering methods), typically requiring

several seasons for in situ clean-up. Consequently, phytoremediation is not an appropriate solution in case of the contaminant pose an immediate danger to human or environment (Frick *et al*., 1999).

- bioavailability of contaminant for plants and microbial degradation. Particularly for hydrophobic pollutants that are tightly bound to soil particles. Hydrocarbon bioavailability in soil may be enhanced to some extent by adding surfactants (Pierzynski *et al*., 2005, Pilon-Smits, 2004).
- The chemical nature of the contaminant also affects the effectiveness of phytoremediation. The water-soluble contaminant may leach before phytoremediation can take place, similarly to volatile petroleum hydrocarbons that evaporate into the air directly from soil or through the plant (Frick *et al*., 1999).

This chapter discusses new approaches in phytoremediation technology, which try to overcome the limitations as described above by using combination of several remediation techniques and the application of genetically modified organisms to combine or express several beneficial traits in a single organism.

4.2. Enhancing Petroleum Oil Degradation by Using the Combination of Remediation Techniques

Mechanical remediation technologies and biological techniques, such as bioremediation or phytoremediation, are not mutually exclusive because pollutant distribution and concentration are heterogeneous for many contaminated sites, and the most efficient and cost-effective remediation solution may be a combination of different technologies (Pilon-Smits, 2004). Examples of combining various remediation techniques are as follows:

1. Bioremediation of refinery sludge containing hydrocarbon was conducted by applying landfarming techniques. Landfarming, also known as land treatment or land application, is an above-ground remediation technology for soils that reduces concentrations of petroleum constituents through biodegradation. This technology usually involves spreading excavated contaminated soils in a thin layer on the ground and stimulating aerobic microbial activity within the soils through aeration and/or the addition of minerals, nutrients, and moisture (*http://www.epa.gov/oust/pubs/tum_ch5.pdf*). In this experiment, the landfarming consisted of depositing the refinery sludge on the soil surface and mixing it with the top 1 m of the soil 1 week later and subsequently aerating the top 1 m of soil once a month with a tractor. This procedure resulted in degradation of 80% of total hydrocarbons in 11 months (Marin *et al*., 2005).

2. Combination of two biological remediation techniques, phytoremediation and bioaugmentation, has been conducted to remediate semi-coke solid waste (containing PAHs, oil products, sulfuric compounds). The field experiment used grass species and three *Pseudomonas* strains inoculated to the soil in mixture. The results showed that the plants produced double biomass and that the number of degrading bacteria were increased, whereas the concentrations of oil and phenolic compounds were decreased (Truu *et al*., 2003). However, bioaugmentation of diesel-degrading bacteria resulted in varying biodegradation activities in different soil properties (Bento *et al*., 2005). Consequently, understanding site characteristics including microbial parameters is needed prior to deciding on the proper bioremediation method. Similar effort through bioaugmentation of degradation capacity in the contaminated soil was conducted by introducing *Pseudomonas putida* UWC3 as donor strain carrying plasmids harbouring genes for degradation of the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D). This

resulted in increased degradation activity, whereas the indigenous community could only very slowly degrade the herbicide. It was suggested that horizontal transfer of the catabolic plasmid from donor strain to indigenous bacteria was responsible for that effect (Dejonghe *et al*., 2000). This bioaugmentation approach is taking advantage of horizontal transfer of catabolic genes carried on mobile genetic elements such as plasmids or transposons in various microbial soil community members.

3. Petroleum hydrocarbons consist of a mixture of compounds such as alkanes, cycloalkanes, polycyclic aromatic hydrocarbons (PAHs) and inorganic compounds. Long chain alkanes $>C_{15}$ and PAHs are recalcitrant in the environment and are tightly adsorbed in soil pores (Cookson, 1995). To overcome the limitations for phytoremediation of such compounds, the combination of several remediation techniques, which are carried on sequentially one step after another, might be of solution. Reviewing the study by Huang *et al*., 2004, the first step is the application of landfarming (regular soil mixing) to increase soil aeration and photo-oxidation, an oxidation process under influence of light. Following two weeks of landfarming, soil augmentation was conducted by spraying cultures of three PAHs-degrading strains, *Pseudomonas putida, Pseudomonas aeruginosa* and *Flavobacterium* sp. The last step involved sowing the tall fescue (*Festuca arundinacea*), a common grass species. Before sowing seeds were inoculated (by incubation) with the plant-growth promoting rhizobacteria (PGPR) belonging to *Pseudomonas putida*, *Azospirillum brasilense* and *Enterobacter cloacae*. The three bacterial strains were known to produce indole-acetic acid, siderosphores and 1-aminocyclopropane-1-carboxylate (ACC) deaminase, all of which contribute to the ability of bacteria to promote plant growth. The results of this sequential combination of remediation techniques showed that after landfarming the compounds with less than four benzene rings and volatiles were successfully removed, then the

bioaugmentation process resulted in reduced the concentrations of higher PAHs such as pyrene, benzo(*a*)anthracene, chrysene. The phytoremediation step supported by PGPR was able to remove recalcitrant PAHs with six ring compounds after 90 days.

4.3. Enhancing Phytoremediation by Using Genetically Modified Organisms

The application of genetically modified (transgenic) plants and/or microorganisms has gained importance in phytoremediation applications. Transgenic organisms are intended to exhibit higher tolerance, accumulation, transport and/or degradation capacity of targeted organisms for various pollutants. Although some plants have the inherent ability to detoxify some xenobiotic pollutants, they generally lack the catabolic pathway for complete degradation or mineralization of these compounds as compared to microorganisms. Hence, transfer of genes involved in xenobiotic degradation from microbes or other eukaryotes to plants will further enhance their potential for remediation of these dangerous groups of compounds (Eapen and D'Souza, 2007). For the development of transgenic plants, a direct method is to overexpress and/or to introduce genes from other organisms, such as bacterial genes involved in contaminant degradation. A common method for the introduction of these genes is *Agrobacterium tumefaciens*-mediated plant transformation. Targeted species for transgenic plants usually are large, fast-growing plants such as poplar (*Populus* sp.) and willow (*Salix* sp.) trees. According to recent reviews (Doty, 2008; van Aken, 2008), the aims of using transgenic plants to remediate organic pollutants can be summarized as follows:

1. *To increase the tolerance towards the pollutant*: transgenic tobacco (*Nicotiana tabacum*) was used in phytoremediation of xenobiotic organic pollutant 2,4,6-trinitrotoluene (TNT) by the introduction of bacterial genes encoded for PETN reductase, the enzyme

responsible to remove nitrate from TNT. Nitroaromatic compounds are highly phytotoxic, phytoremediation of these pollutants using non-transgenic plants is severely hindered. When bacterial genes mediating the break-down of nitroaromatics were expressed in plants, the plants became more tolerant of the pollutant and reduced the concentration of TNT surroundings the roots, allowing also microbial communities to survive. The transgenic tobacco in this study was able to tolerate higher TNT concentrations than non-transgenic tobacco (Doty, 2008; van Aken, 2008).

2. *To increase removal rates of a variety of small organic compounds*: It was attempted to achieve this aim by overexpressing the mammalian cytochrome P450 enzyme (encoded by *CYP2E*), *which* is involved in the metabolism of xenobiotic substances. This approach was tested in tobacco and poplar (Doty, 2008, van Aken, 2008) and could significantly remove small organic pollutants such as trichloroethylene (TCE), carbon tetrachloride, chloroform, benzene and vinyl chloride. These mammalian enzymes functioned well in plants without any need to modify the gene or to include other enzymes. When the *CYP2E1* gene was overexpressed in tobacco plants, transgenic plants produced 100 times more TCE degradation metabolites than wildtype plants (van Aken, 2008). Accordingly, expression of the P450 enzyme was 30,000 times higher than the native P450 gene. The expression of a P450 enzyme encoded by *CYP2B6* and involved in the degradation of a wide range of herbicides in rice resulted in transgenic plants being able to grow in the presence of herbicides. Similarly, the expression of glutathione S-transferases (GST) in transgenic tobacco has resulted in increased tolerance to herbicides (Doty, 2008).
3. *To increase ex planta secretion of pollutant-degrading enzymes into the environment*: this approach is advantageous as it does not require the plant to take up the pollutants, it rather helps rhizosphere or endosphere bacteria to degrade the pollutants which are too

phytotoxic or less bioavailable. Degradation of polychlorinated phenolic pollutants was achieved by expressing cotton root-specific laccase gene (*LAC1*) in *Arabidopsis* or fungal laccase genes in tobacco plants. Resulting transgenic plants secreted the laccase into the rhizosphere, and removed the pollutants bisphenol and pentachloro-phenol with high efficiency (Wang *et al*., 2004). A similar approach was used in *Arabidopsis* and tobacco that act on aromatic pollutants (Uchida *et al*., 2005). Resulting transgenic *Arabidopsis* plants expressed the aromatic-cleaving extradiol dioxygenase (DbfB) and transgenic tobacco plants expressed haloalkane dehalogenase (DhaA). These plants secreted these enzymes resulting in a higher tolerance to higher concentration of pollutants.

Phytoremediation of toxic metals has been successfully improved with transgenic plants (Pilon-Smits, 2004, Doty, 2008, Rugh, 2004). Transgenic plants were developed to extract the metals from the soil and transfer them to plant tissues for further harvest and disposal. For metals such as mercury and selenium, an alternative strategy is to convert the metal to volatile forms. *Arabidopsis* and poplar plants were engineered to express the bacterial mercuric ion reductase enzyme (*merA*) and organomercurial lyase (*merB*) and the transgenic plants showed higher tolerance towards methyl mercury at normally toxic concentration, to accumulate mercury and transform it into a less toxic form (Doty, 2008).

The rhizosphere is the environment, which is most important in frame of a phytoremediation application, however many bacteria are only able to degrade one or few contaminating compound(s). Combining several catabolic abilities in one bacterial strain was considered to be promising because the metabolic routes for the complete degradation of xenobiotic compounds are usually mediated by different organisms hosting different genes. By applying organisms being able to completely degrade a pollutant, the formation of toxic intermediates can be avoided

(Pieper and Reineke, 2000). This strategy of combining complementary metabolic activities has been used to engineer transgenic bacteria capable of mineralizing PCBs by combining an oxidative pathway for chlorobiphenyl transformation (encoded by *bph* genes) into chlorobenzoate with a chlorobenzoate degradation pathway. Several transgenic bacteria have been constructed by introducing *bph* genes into chlorobenzoate degraders through conjugation, which resulted in transgenic bacteria capable of growing and completely dechlorinating 2- and 4-chlorobiphenyl (Pieper and Reineke, 2000). An additional, challenging problem in the application of degrading bacteria is that they are out-competed by indigenous communities. Therefore, selecting the highly competitive bacterial strains is crucial, both for selecting non-transformed bacterial degraders as well as for the development of transgenic bacteria. Following this criterion, it has to be considered that 1) an engineered bacterial strain should be stable after cloning and the target gene should show high expression, 2) this strain should tolerate or degrade the contaminant, and 3) strains should persist in the plant environment (Zhuang, 2007). Poplar rhizobacteria were constructed with the aim to enhance trichloroethylene (TCE) by introducing genes encoding the toluene monooxygenase (TOM) of *Burkholderia cepacia* strain G4. This transgenic approach was important because strain G4 did not compete well with the native poplar rhizospheric bacteria (Doty, 2008).

Since attention has focused on the potential role of endophytes in mediating the degradation during phytoremediation, the use of endophyte inoculants known to degrade pollutants has been tested. As not every bacterium with necessary pollutant-degrading capacity can grow within the plant species, where the contamination is present, research has been carried out to provide microbes that can live in the required plants with the ability to degrade the pollutant (Romantschuk *et al*., 2000). Transgenic *Burkholderia cepacia* strain L.S.2,4; a natural endophyte of yellow lupine, was able to enhance phytoremediation of toluene *in planta* after the strain received

pTOM, the toluene-degradation plasmid of Burkholderia cepacia G4, a relative of the yellow lupine endophyte via conjugation. After yellow lupine plants were inoculated with this altered endophyte, the plants were more tolerant towards toluene, and showed 50-70% reduction of toluene evapotranspiration through the leaves (Barac *et al*., 2004). Furthermore, it was found that the introduced pTOM plasmid was horizontally transferred *in planta* from transgenic bacteria to the endogenous endophytic communities living in poplar tree in presence or absence of toluene. Such a transfer could be used to change natural endophytic microbial communities to improve the phytoremediation (Taghavi *et al*., 2005).

Genetic engineering of organisms to enhance phytoremediation has obvious environmental benefits, although potential risks and national regulations have to be considered as well. The potential risk of transgenic trees due to their long life cycle is more challenging to assess. Therefore, the risk assessment on ecological impact and close monitoring during field trials is suggested (Eapen and D'Souza, 2007). Some precautionary actions can be taken for example by avoiding the cultivation of the transgenic plants used for phytoextraction in agricultural areas as the transgenic plants are able to accumulate metal in higher concentration than natural hyperaccumulator plants and thus pose a higher risk of contaminating of food chains. Furthermore, crops used for phytoextraction shall be harvested before the seeds set, thus reducing threat of crossing with other crops intended for food or entering food supply. Careful selection of the species to be transformed can avoid routes of transgene release, for example the use of trees that will not resprout from wind-blown branches should be avoided. However, if the transgene did 'escape' into native populations, the gene involved in pollutant degradation would be unlikely to confer any selective advantage or negative environmental impact (Doty, 2008)

REFERENCES

Barac, T., Taghavi, S., Borremans, B., Provoost, A., Oeyen, L., Colpaert, J.V., Vangronsveld, J., van der Lelie, D., (2004) Engineered endophytic bacteria improve phytoremediation of water-soluble, volatile, organic pollutants. *Nature Biotechnology* **22**: 583-588.

Bento, F. M., Camargo, F.A.O., Okeke, B.C., Frankenberger, W.T., (2005) Comparative bioremediation of soils contaminated with diesel oil by natural attenuation, biostimulation and bioaugmentation. *Bioresource Technology* **96**: 1049-1055.

Cookson, J. T. J., (1995) *Bioremediation Engineering: Design and Application*. McGraw-Hill, Inc., New York.

Dejonghe, W., Goris,J., El Fantroussi, S., Höfte, M., De Vos, P., Verstraete,W., Top, E.M., (2000) Effect of dissemination of 2,4-dichlorophenoxyacetic acid (2,4-D) degradation plasmids on 2,4-D degradation and on bacterial community structure in two different soil horizons *Applied and Environmental Microbiology* **06**: 3297-3304.

Doty, S. L., (2008) Enhancing phytoremediation through the use of transgenic and endophytes. *New Phytologist* **179**: 318-333.

Eapen, S., Singh, S., D'Souza, S.F., (2007) Advances in development of transgenic plants for remediation of xenobiotic pollutants. *Biotechnology Advances* **25**: 442-451.

Frick, C. M., Farrel, R.E., Germida, J.J., (1999) Assessment of phytoremediation as an in-situ technique for cleaning oil-contaminated sites. In. Saskatoon, Canada: Department of Soil Science, Univ. of Saskatchewan

Huang, X.-D., El-alawi, Y., Penrose, D.M., Glick, B.R., Greenberg, B.M., (2004) A multi-process phytoremediation system for removal of polycyclic aromatic hydrocarbons from contaminated soils. *Environmental pollution* **130**: 465-476.

Marin, J. A., Hernandez, T., Garcia, C., (2005) Bioremediation of oil refinery sludge by landfarming in semiarid conditions: Influence on soil microbial activity. *Environmental Research* **98**: 185-195.

Pieper, D. H., Reineke, W., (2000) Engineering bacteria for bioremediation. *Current Opinion in Biotechnology* **11**: 262-270.

Pierzynski, G. M., J.T. Sims, J.T., Vance, G.V., (2005) Remediation of soil and groundwater. In: Soils and Environmental Quality. Taylor & Francis, pp. 464-467.

Pilon-Smits, E., (2004) Phytoremediation. *Annual Review of Plant Biology* **56**: 15-39.

Romantschuk, M., Sarand, I., Petänen, T., Peltola, R., Johnsson-Vihanne, M., Koivula, T., Yrjälä, K., Haahtela, K., (2000) Means to improve the effect of in situ bioremediation of contaminated soil: an overview of novel approaches. *Environmental Pollution* **107**: 179-185.

Rugh, C. L., (2004) Genetically engineered phytoremediation: one man's trash is another man's transgene. *Trends in Biotechnology* **22**: 496-498.

Taghavi, S., Barac, T., Greenberg, B., Borremans, B., Vangronsveld, J., van der Lelie, D., (2005) Horizontal gene transfer to endogenous endophytic bacteria from poplar improves phytoremediation of toluene. *Applied and Environmental Microbiology* **71**: 8500-8505.

Truu, J., Talpsep, E., Vedler, E., Heinaru, E., Heinaru, A., (2003) Enhanced biodegradation of oil shale chemical industry solid wastes by phytormediation and bioaugmentation. *Oil Shale* **20**: 421-428.

Uchida, E., Ouchi, T., Suzuki, Y., Yoshida, T., Habe, H., Yamaguchi, I., Omori, T., Nojiri, H., (2005) Secretion of bacterial xenobiotic-degrading enzymes from transgenic plants by an apoplastic expressional system: an applicability for phytoremediation. *Environmental Science and Technology* **39**: 7671-7677.

van Aken, B., (2008) Transgenic plants for phytoremediation: helping nature to clean up environmental pollution. *Trends in Biotechnology* **26**: 225-227.

Wang, G.-D., Li, Q-J., Luo, B., Chen, X-Y., (2004) *Ex planta* phytoremediation of trichlorophenol and phenolic allelochemicals via an engineered secretory laccase. *Nature Biotechnology* **22**: 893-897.

Zhuang, X., Chen, J., Shim, H., Bai, Z., (2007) New advances in plant growth-promoting rhizobacteria for bioremediation. *Environment International* **33**: 406-413.

CHAPTER 5

GENERAL CONCLUSIONS

For an effective phytoremediation, selection of highly tolerant plant species and their associated microflora expressing the desired degrading activity is of importance. The first work phase within this Ph.D was aimed to select plants being highly tolerant to petroleum oil contamination and to analyse the diversity of alkane-degrading bacteria and their alkane hydroxylase (*alk*) genes isolated from the rhizo- and endosphere of two selected plants. The results showed that not all plant species were able to grow in soil contaminated with petroleum oil. Italian ryegrass (*Lolium multiflorum* var. Taurus) and the legume Birdsfoot trefoil (*Lotus corniculatus* var. Leo) were among the most tolerant ones and can therefore be considered as potential plant species to be applied in phytoremediation applications of petroleum oil. Studying the diversity of associated hydrocarbon-degrading bacteria and *alk* genes from the two plant species revealed high numbers and diversities of culturable, hydrocarbon degraders carrying *alk* genes in association with italian ryegrass, particularly in the plant interior. However, the two plant species were associated with highly distinct hydrocarbon degrading communities, indicating that the plant species greatly influenced the abundance and diversity of alkane degrading bacteria. Furthermore, we detected that the hydrocarbon degraders carried different types of known *alk* genes, mainly *alkB* genes encoding alkane monooxygenase and *CYP153*-like genes encoding cytochrome P450 type oxygenases, both are enzymes responsible for catalyzing alkane degradation. These detected *alk* gene were located either on plasmids or in the chromosome of the bacterial strain. As we found highly similar nucleotide sequence of *alkB* genes among bacterial isolates, it appeared that *alkB* genes have spread through horizontal gene transfer (HGT) among bacterial communities in

different magnitudes, and that horizontal gene transfer was not restricted to only plasmid transfer. Results also revealed HGT between Gram-positive and Gram-negative bacteria. The hot spot for *alkB* horizontal gene transfer was particularly in the Italian ryegrass rhizosphere. Finally, in regard to phytoremediation applications, where support of numerous degrading bacteria and rapid spread of degrading genes is essential for efficient break-down of pollutants, Italian ryegrass seems to be more appropriate than Birdsfoot trefoil.

Successful bacterial colonization in the plant environment and the presence of catabolic genes are not necessarily indicating that the genes are active in degrading the contaminant. Analysis of gene expression through quantifying the mRNA transcripts of degrading genes could be a sign of activity of the targeted genes. Therefore, the second work phase was intended to assess colonization and alkane monooxygenase (*alkB*) gene expression of selected inoculant strains in different compartments of Italian ryegrass and under influence of different diesel concentrations. Particular objective was to assess the role of endophyte in alkane degradation and to compare endophytic colonization and activity with activities in the rhizosphere. We therefore compared two inoculants strains, an endophyte originally isolated from Italian ryegrass, *Pseudomonas* sp. strain ITRI53, and a rhizosphere strain, *Rhodococcus* sp. strain ITRH43. Generally they were able to colonize all compartments of Italian ryegrass and also to express their *alkB* genes, but the endophyte strain colonized the plant more efficiently, especially the plant interior, and this strain also showed high expression of *alkB* genes. Whilst, the rhizosphere strain primarily colonized the rhizosphere and showed low *alkB* gene expression in plant interior. The expression of *alk* genes was particularly high in the presence of high diesel oil concentrations in soil and plants showed better growth when inoculated with endophytic bacteria, suggesting that endophytes have a high potential to be used in phytoremediation applications.

Finally, in this study we were able to reveal the phylogenetic and functional diversity of alkane degrading bacteria in association with highly tolerant plant species as well as the *alkB* gene expression in different plant compartments. Specifically, we demonstrated the functional diversity and capability of alkane-degrading endophytes to express *alkB* gene inside the plant. However, in this Ph.D work we only addressed the culturable alkane degraders and applied sterile environment while assessing *alkB* gene expression. Culturable bacteria often represent only a minority of total bacteria community whereas applying sterile environment may underestimate the effect of competition among bacterial communities to colonization capability and the regulation of gene expression of inoculated strain. To anticipate the novel but unculturable alkane-degraders, non-cultivation study may be useful to know whether the culturable alkane-degraders are representing the total bacterial communities. Furthermore, with the intention to utilize the high potential of endophytes in phytoremediation application, non-sterile study on endophytic colonization, *alkB* gene expression and its horizontal transfer in rhizosphere and inside the plant tissue is needed. The endophytic colonization and HGT of alkane-catabolic genes can be assessed *in situ* by applying reporter-gene technology, through introduction of reporter genes, such as genes encoding Green fluorescent protein (Gfp) or Red fluorescent protein (Rfp) which is tagged on catabolic plasmid carried by the endophyte inoculants. Lastly, measurement of total petroleum oil reduction and degradation in soil due to the endophyte inoculation may reassure the potential of endophyte in phytoremediation.