The ecology of alkane-degrading bacteria in phytoremediation of diesel fuel

Dissertation

Submitted to obtain the Doctoral degree at the University of Natural Resources and Life Sciences, Vienna

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Vienna, March 2011

To my Mother.....

Acknowledgements

First of all, I would like to thank Allah Almighty for all He has done to show me this day.

I am greatly thankful to Higher Education Commission of Pakistan and Dr. Angela Sessitschfor providing me an opportunity to work at AITAustrian Institute of Technology, Seibersdorf, Austria. Without them I wouldn't have been writing this acknowledgment.

I would like to express my gratitude to Dr. Angela Sessitschfor being an outstanding, encouraging, and patient supervisor. Her constant encouragement, support, and invaluable suggestions made this work successful. My sincere thanks go to Dr. Thomas G. Reichenauer for his support during this work and for reviewing this work. I would like to thank Verania Andria and Muhammad Afzal for their extensive help during this research.

Special thanks goes to my lovely container mates and friends, Katrin Glaser, SouzanSanoussi, Johanna Pultar, and Muhammad Naveed for lot of fun and help at work and also for hard times that we had in common.

I am grateful to all my colleaguesfor their kind supports, help, valuable advice, and for being patient answering my questions. My sincere thanks go to my Ph.D. fellows, and close friends -Melanie,Yao, Stefan, Naveed, Katharina, Claudia, Michaela,Ratri, Joy, Dhairyasheel,Edward, Parvez, Patrick,Alex, Branislav, Birgit,Marlies, Tanja, Evelyn,

Marianne, Elena, Milica, Iris, Günter, Frank, Levente, StephaneCompant, Aatif, Ihsan, Kamran, and Mohsinfor their feedbacks, moral supports and for being great friends throughout. I am also thankful to Anton Grahsl, who assisted in performing the greenhouse experiments. You people made my work and stay in Vienna a lot easier than it would ever have been.

Finally, I dedicate this work for my beloved mother for her love, support, and encouragement throughout my endeavor. I would specially thank my elder brothers Shafique and Khalique, who kept faith in me and who inspired all my professional ambitions. I am thankful to my brothers, sister, and their families for their support during my PhD study.My sincere thanks to Mariam for making my life much happier.

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Chapter 1

Abstract

Phytoremediation is highly versatile, solar-driven *in situ* pollutant extraction system for removal of ecosystem trembling contaminants from soil, water, sediments and air. In phytoremediation plants and their associated microbes work in symbiotic relationship. Root exudates comprising nutrients and co-metabolites support the growth of contaminant-degrading microorganisms in the rhizosphere while microbes through their degrading capacity and additional plant growth-promoting activities reduce the contaminants'phytotoxicity. Successful phytoremediation requires appropriate plant species and inoculant strains that, besides degrading pollutants are also able to survive in natural soil for long periods of time.

We analyzed the hydrocarbon degradation potential and colonization of four alkane degrading strains (ITSI10, ITRI15, ITRH76, and BTRH79)in the rhizosphere and endosphere of Italian ryegrass and birdsfoot trefoil,using cultivation-and cultivation-independent approaches as well as the diversity of alkane degrading bacteria.Contaminated soil was prepared by spiking agricultural soil with 10 g diesel fuel kg^{-1} soil. Italian ryegrass (*Loliummultiflorum*var. Taurus) and birdsfoot trefoil (*Lotus corniculatus* var. Leo) were inoculated with four alkane degrading strains. Hydrocarbon degradation (up to 57 %) was observed in all inoculated treatments of vegetated and unvegetated samples. Italian ryegrass in combination with compost and BTRH79 showed highest degradation, while birdsfoot trefoil performed best with compost and strainITSI10. Cultivation-based as well as cultivation-independent analysis showed that both strains were competitive colonizers.The combination between vegetation, inoculation with well performing degrading bacteria and compost amendment was an

efficient approach to reduce hydrocarbon contamination. Two *Pantoea* sp. strains, ITSI10 and BTRH79, established well in the plant environment despite the presence of a variety of other, indigenous alkane-degrading bacteria**.**The present study suggests that the application of degrading bacterial strains, which are able to compete with the native microflora and to tightly associate with plants, are promising candidates to be used for phytoremediation applications.

The second part of the study aimed to assess the role of *Enterobacte*rendophytes in hydrocarbon degradation,plant colonization, abundance and expression of CYP153 genes in different plant compartments, three plant species (Italian ryegrass, birdsfoot trefoil and alfalfa) were grown in sterilesoil spiked with 1% diesel and inoculated with three endophytic*Enterobacterludwigii* strains. We studies plant colonization of these strains as well as the abundance and expression of CYP153 genes in the rhizosphere, root and shoot interior. Results showed that all strains were capable of hydrocarbon degradation and efficiently colonizedthe rhizosphere and plant interior. Two strains, ISI10-3 and BRI10-9, showed highest degradation up to 68% and performed best in combination with Italian ryegrass and alfalfa. All strains principally expressed the CYP153 gene in all plant compartments, indicating an active role in degradation of diesel in various plant compartments.

Keywords: bioremediation, rhizosphere, degradation, *Enterobacterludwigii*, endophytes, gene abundance, gene expression

Zusammenfassung

Bei der Phytoremedation handelt es sich um eine höchst vielseitige, lichtgesteuerte *in situ*Methode, diezur Sanierung von mit Schadstoffen belasteten Böden verwendet wird. Dies geschieht durch eine Wechselwirkung von Pflanzen und den mit ihnen vergesellschafteten Bakterien. Die Wurzelexudate der Pflanze enthalten Nährstoffe und Co-Metaboliten, welche das Wachstum dieser schadstoff-reduzierenden Bakterien fördern. Durch die Fähigkeit Kontaminationen aus dem Pflanzengewebe zu beseitigenhelfen die Bakterien der Pflanze,und können gleichzeitig durch zusätzliche pflanzenwachstumsfördernde Aktivitäten die Phytotoxizität dieser Schadstoffe weiter herabsetzen. Erfolgreiche Phytoremediation benötigt sowohl eine geeignete Pflanzenart als auch geeignete Bakterienstämme, die, neben den erwähnten Fähigkeiten auch konkurrenzstark sind, d.h. im natürlichen Boden auch über lange Zeiträume überleben können.

In dieser Studie wurden das Potenzial zum Abbau von Kohlenwasserstoffen und die Kolonisierungseffizienz von vier Alkan-abbauenden Stämmen (ITSI10, ITRI15, ITRH76, and BTRH79) in der Rhizo- und Endosphäre von welschen Windelgras und gewöhnlichem Hornklee anhand von kultivierungsabhängigen und -unabhängigen Methodenevaluiert, sowie die Diversität der alkanabbauenden Bakterien bestimmt. Ein mit 10g Diesel pro kgkontaminierter Agrarboden wurde als Versuchsboden herangezogen. In diesem wurden welsches Windelgras (*Lolium multiflorum* var. Taurus) und gewöhnlicher Hornklee (*Lotus corniculatus* var. Leo) mit vier Alkan-abbauenden Bakterien inokuliert. Der Abbau von Kohlenwasserstoffen (bis zu 57%) wurde nach erfolgter Inokulation in allen bepflanzten und unbepflanzten Bodenproben nachgewiesen.

Der meiste Abbau wurde dabei bei der Kombination von welschem Windelgras mit dem Stamm BTRH79 gemessen, während gewöhnlicher Hornklee in Kombination mitzugeführtem Kompost und Stamm ITSI10 die beste Performance lieferte. Es zeigte sich, dassbeide Stämme sehr gut besiedelten. Die Kombination aus Vegetation, Inokulation mit Abbau-effizienten Bakterien unterder Verwendung von optimiertem Kompost bildet einen effizienten Zugang Böden zu reinigen, welche mit aliphatischen Kohlenwasserstoffen kontaminiert sind. Zwei *Pantoea* sp. Stämme, ITSI10 und BTRH79, konnten sich trotz dem Vorhandensein einer Vielzahl indigener alkanabbauender Bakterien im natürlichen Habitat der Pflanze durchsetzen. Die vorliegende Studie zeigt an, dass die Anwendung dieser Stämme, die mit der nativen Mikroflora in Konkurrenz treten können und im engen Kontakt zu der Pflanze stehen, vielverspechende Kandidaten zur Anwendung von Phytoremediation darstellen.

Der zweite Teil der Studie versuchte die Rolle von Endophyten der Gattung *Enterobacte*r im Abbau von aliphatischen Kohlenwasserstoffen, Pflanzenbesiedlung sowie die Häufigkeit und Expression von CYP153 Genen in verschiedenen Pflanzenkompartimenten in drei unterschiedlichen Pflanzenarten zu beleuchten. Welsches Windelgras, gewöhnlicher Hornklee und Alfalfa (*Medicago sativa*) wurden in sterilem Boden, welcher mit 1% Diesel versetzt wurde, angezogen und mit drei Endophytenstämmen von *Enterobacter ludwigii* inokuliert. Wir beobachteten die Besiedelungseffizienz dieser Stämme sowie die Häufigkeit und Expression der CYP153 Gene in der Rhizosphäre und dem Inneren von Stamm und Wurzel. Die Resultate zeigten an, dass alle drei Stämme die Fähigkeit zum Abbau von aliphatischen Kohlenwasserstoffen besitzen, und dass sie das Innere der Pflanze und die Rhizosphäre

effizient besiedelten. Zwei Stämme, ISI10-3 und BRI10-9, zeigten höchste Abbauraten von bis zu 68% der aliphatischen Kohlenwasserstoffe und funktionierten am Besten in Kombination mit welschem Windelgras und Alfalfa. Das CYP153Gen wurde von den getesteten Stämmen haben in allen Pflanzenteilen exprimiert, welches eine aktive Rolle dieses Gens im Abbau von Diesel in verschiedenen Planzenkompartimenten andeutet.

Keywords: bioremediation, rhizosphere, degradation, *Enterobacterludwigii*, endophytes, gene abundance, gene expression

Chapter 1

General Introduction

1.1 Background, objectives and scope of the research

Industrialization and modern human activities have lead towards increased use of petroleum and its products such as natural gas, diesel, gasoline, and asphalts. This has resulted in significant contamination of a number of terrestrial and marine sites. Petroleum hydrocarbons are composed of various proportions of short, medium, and long aliphatics (i.e. alkanes, alkenes), aromatics (e.g. benzene, toluene, ethyl benzene and xylene) as well as polycyclic aromatic hydrocarbons (known as PAHs; such as naphthalene, phenantherene, and pyrene) (Frick et al., 1999). Among hydrocarbon pollutants, diesel fuel is a common contaminant in soil and water, which contains a complex mixture of alkanes and aromatic compounds leaking from storage tanks and pipelines or released from accidental spills (Gallego et al., 2001). Alkanes constitute about 20-50% of crude oil and in addition, alkanes are produced throughout the biosphere by living organisms as waste products, structural elements, as chemoattractants or are part of defense mechanisms (van Beilen et al., 2003).

Hydrocarbons in sediments originate from diverse sources, which can be summarized in the following categories (Kim et al., 1999; Readman et al., 2002):

- anthropogenic (industrial chemicals)
- petroleum inputs
- incomplete combustion of fuels
- forest and grass fires
- biosynthesis of hydrocarbons by aquatic or terrestrial organisms

- post-depositional transformation of biogenic precursors
- diffusion from the mantle, petroleum source rocks or reservoirs

Hydrocarbons can become dangerous especially if they enter the food-chain, since several of the more persistent compounds, as PAHs and PCBs are carcinogenic (Perelo, 2010). These contaminants are of concern because of:

- their toxicity to living organisms, many of them cause carcinogenic and mutagenic diseases in living organism (Frick et al., 1999).
- their persistence in the environment.
- their potential interference with water retention and transmission and with nutrient supplies in soils (Turle et al., 2007).
- their volatility, which causes explosion hazard.

The first step of any contaminated site management strategy, before applying any of the available remediation techniques, is the contaminant source control or elimination. The two main objectives of sediment management strategies are on the one hand to minimize contaminant risk to human health and the environment, and on the other hand to minimize cost (Zeller and Cushing, 2006). A range of *in situ* and *ex situ* remediation methods have been implemented including natural attenuation, chemical physical and engineering approaches, dredging, dry excavation, capping and related technologies as well as the application of microorganisms, to reduce the petroleum hydrocarbons contaminations (Huang et al., 2004). Each of these methods has advantages and disadvantages in regard to its costs and capacity to remediate the contaminant. For example dredging consist in the physical removal of contaminated soil also layers. Typically, it is the most expensive technique, which results in the greatest mass removal from the aquatic environment (Zeller and Cushing, 2006). Washing with solvents is expensive

and also need facilities that transfer the contaminated soil to clean up site. Capping consists of covering of contaminated site with clean material, thus isolating the sediment. Capping requires a long term monitoring and poses a certain risk because contaminants are left in the place.

In the past two decades, phytoremediation has gained attention as an alternative to clean up petroleum oil contaminated sites due to its low cost compared to engineering approaches, easy to implement and environment friendly way of application (Pilon-Smits, 2005). Phytoremediation is defined as the use of plants and their associated microbes for environmental clean-up from organic or inorganic contaminants (Salt et al., 1995). Phytoremediation is a highly versatile, solar-driven, *in situ* pollutant extraction system for the removal of ecosystem trembling contaminants from soil, water, sediments, and air. It signifies a highly perceptive and promising field of biotechnology. It is considerably less expensive and effective against metals, pesticides, solvents, explosives, and crude oil contaminations. 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, phytoremediation is suggested as 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. Plant endophytes, bacteria which colonize the endosphere of plants without exhibiting pathogenicity to their hosts, are also important during phytoremediation. They may either degrade organic contaminants after plant uptake or improve plant stress tolerance (Weyens et al., 2009a).

For successful phytoremediation of petroleum hydrocarbon contaminated soils, the following requirements have to be met; a) ability of the plant to grow on contaminated soil and produce sufficient biomass and b) presence and activity of degrading and plant growth-promoting bacteria (Huang et al., 2004). The capacity of plants to grow in contaminated soil is a plant-species- and genotype-dependent trait (Siciliano et al., 2001a), whereas the ability of bacteria to degrade petroleum hydrocarbons is due to their degradation enzymes. For the break-down of alkanes or PAHs hydroxylases (also known as oxygenases) are necessary such as alkane hydroxylases 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).

Inoculation of soils or plants with bacteria showing appropriate degradation abilities is not always successful due to the poor survival of degrading microorganisms in a usually highly competitive environment (Miethling and Karlson, 1996; Rousseaux et al., 2003). Inoculant strains can be poor survivors or may lose catabolic activity when inoculated into complex microbial ecosystems (McClure et al., 1991; Watanabe et al., 1998). Consequently phytoremediation requires appropriate plant species and inoculant strains that, besides degrading pollutants, are also able to survive in natural soil for long periods of time (Schwartz, 2000). In this PhD study the aim was to identify efficient hydrocarbon degrading strains and plants. A specific aim was to study the colonization of selected inoculant strains in the rhizosphere and endosphere of plants using cultivation-dependent and -independent approaches as well as to analyse the diversity of alkane degrading bacteria.

This thesis is structured in four chapters. **Chapter 1** describes the background and objectives of the research and provides a general introduction on phytoremediation process,

hydrocarbon degrading bacteria and colonization of inoculated bacteria. **Chapter 2** presents results from experiments addressing the capability of hydrocarbon degradation and plant colonization by selected bacterial strains isolated from Italian ryegrass and birdsfoot trefoil. We found that the inoculant strains were able to degrade hydrocarbon concentrations in a contaminated soil. In particular, the combination between vegetation, inoculation with well performing strains and compost amendment was the most efficient approach to reduce hydrocarbon levels. Two strains, *Pantoea sp.* ITSI10 and BTRH79, established well in the plant environment despite the presence of a various other indigenous alkane-degrading bacteria. These findings suggest that the application of degrading bacterial strains, which are able to compete with the native microflora and to tightly associate with plants, is a promising strategy for the phytoremediation of hydrocarbon polluted soils. In **chapter 3** we analyzed hydrocarbon degradation and plant colonization potential of three endophytic *Enterobacter ludwigii* strains (ISI10-3, IRI10-4 and BRI10-9) in the rhizosphere and plant interior of three different plant species (Italian ryegrass, birdsfoot trefoil and alfalfa) in sterilized, diesel-spiked soil. The gene abundance and expression of CYP153 genes of *E. ludwigii* strains during phytoremediation in the rhizosphere and endosphere was also investigated. Our results showed that all the *E. ludwigii* strains were capable of hydrocarbon degradation. The endophyte strains were capable of colonizing efficiently the rhizosphere and plant interior, which lead to efficient hydrocarbon degradation. The strains ISI10-3 and BRI10-9 showed highest degradation up to 68% and performed best in combination with Italian ryegrass and alfalfa. The bacterial CYP153 genes were expressed not only in rhizosphere but also in the plant interior. The inoculation of endophyte strains resulted in better survival of plants, which might be due to plant growth-promoting or alkane-degrading activities. We suggest that the species *E. ludwigii*, which is

mostly known for its clinical relevance as isolates have been mostly isolated from clinical specimens can be used for the remediation of contaminated soils. Finally, general conclusions on the research performed are summarized in **Chapter 4.**

1.2. Phytoremediation technologies

Phytoremediation is a biological technology process that utilizes natural plant processes to enhance degradation and removal of contaminants in contaminated soil or ground water. Phytoremediation is defined as the use of plants and their associated microbes for environmental cleanup from organic or inorganic contaminants (Salt et al., 1995). Generally, phytoremediation can be cost-effective for a) large sites with shallow residual –levels of contamination by organic, nutrient or metal pollutants, where contamination does not pose an imminent danger and only polishing treatment is required and b) where vegetation is used as a final cap and closure of the site (Schnoor et al., 1995). Although plants 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 physical and chemical conditions of soil due to root activity and release of root exudates. 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; McCutcheon and Schnoor, 2003). Besides the importance of bacterial communities, the plant species or genotype is important, not only because the plant selects its preferred microbial communities (Siciliano et al., 2001a) but also due to the type of root exudates that select microbial communities and influence their activity (Kowalchuk et al., 2002).

Advantages of using phytoremediation include cost effectiveness, aesthetic advantages, and long-term applicability. Furthermore, the use of phytoremediation as secondary or polishing *in situ*-treatment step minimizes land disturbance and eliminates transportation and liability costs associated with offsite treatment and disposal. Increasing public and regulatory acceptance are likely to extend the use of phytoremediation beyond current application.

1.2.1. Phytoremediation mechanisms

Phytoremediation utilizes physical, chemical, and biological processes to remove, degrade, transform, or stabilize contaminants within soil and groundwater. Hydraulic control, uptake, transformation, volatilization, and rhizodegradation are important processes used during phytoremediation (Fig. 1).

1.2.2. Hydraulic control

Phytoremediation applications can be designed to capture contaminated groundwater plumes to prevent off-site migration and/or decrease downward migration of contaminants. Trees and grasses act as a solar pump removing water from soils and aquifers through transpiration. Downward migration of contaminants due to percolation of rainwater can also be controlled with phytoremediation. Within the upper region of an aquifer, grasses with dense, fibrous root systems are used to transpire water and limit percolation of contaminants through vadose zone.

1.2.3. Phytoextraction

The term refers to the use of pollutant-accumulating plants to remove metals or organics from soil by concentrating them in the harvestable parts of the plant. The process often occurs with

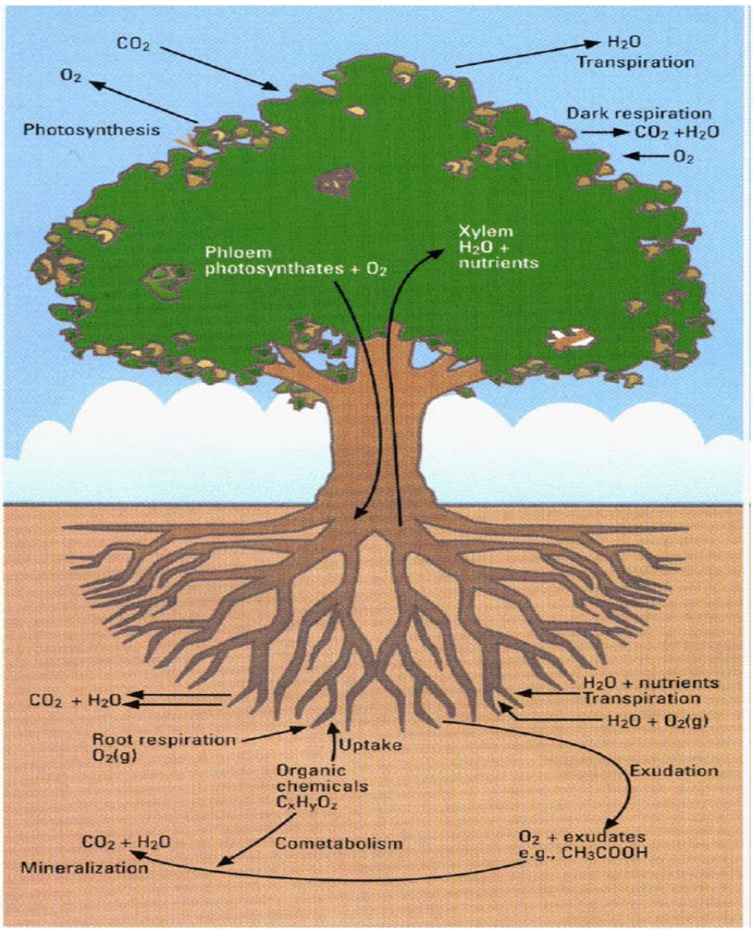


Fig. 1. Scheme of different mechanisms of contaminant removal by plants (Schnoor et al., 1995).

heavy metals, radionuclides and certain organic compounds that are resistant to plant metabolism, by uptake and translocation of such compounds in the soil by plant tissue in a recoverable form. Hyperaccumulation is only possible when plants grow vigorously and produce over 3t dry matter/hectare (Salt et al., 2003; Yateem et al., 1999).

1.2.4. Phytotransformation

Phytotransformation/phytodegradation refers to a process beyond uptake and storage of contaminants. Phytodegradation has been studied extensively to understand the fate of herbicides in crop plants. Several reports on herbicide phytodegradation have been extended to cell cultures of non-crop species including hybrid poplar trees (Bockers et al., 1994; Thompson et al., 1998). In phytodegradation/phytotransformation, contaminants are taken up from soil/water, metabolized in plant tissues and broken up to less toxic or non-toxic compounds within the plant by several metabolic processes via the action of compounds produced by the plant (Burken et al., 2000; Macek et al., 2000; Meagher, 2000).

1.2.5. Phytovolatilization

The natural ability of a plant to volatilize a contaminant that has been taken up through its roots can be exploited as a natural air-stripping pump system. In phytovolatilization volatile chemicals or their metabolic chemical compounds are released into the atmosphere through plant transpiration. Certain organic pollutants that are recalcitrant in subsurface environment react rapidly in the atmosphere with hydroxyl radical, an oxidant formed in the photochemical cycle. Very few contaminants are sufficiently water soluble, non-toxic to plants, and volatile enough to reach atmospheric concentrations that would be of concern in case of evapotranspiration.

1.2.6. Rhizosphere bacteria and phytoremediation (rhizoremediation)

Phytoremediation is not solely a function of plants but must always be considered in combination with the effect of rhizospheric microorganisms (Mackova et al., 2006; Rittmann, 2006). Although they have an inherent ability to detoxify some xenobiotics (i.e. to make them non-

phytotoxic), plants, compared with microorganisms (Eapen et al., 2007), generally lack the mechanisms necessary for the complete degradation/mineralization of toxic compounds. Rhizoremediation is a specific form of phytoremediation involving plants and their associated rhizospheric microorganisms (bacteria and fungi). Rhizoremediation can either occur naturally or can be facilitated by inoculating soil with microorganisms capable of degrading environmental contaminants. Rhizodegradation is responsible for the enhanced removal of total petroleum hydrocarbons from soil (Schwab and Banks, 1993; Carman et al., 1998).

1.2.7. Endophytic bacteria and phytoremediation

Because of the complex plant-rhizobacteria interactions, the use of endophytic bacteria for biodegradation has been extensively explored in the last years (Doty, 2008; Ryan et al., 2008). Endophytic bacteria that colonize the internal tissues of plants without causing a negative effect (Schulz and Boyle, 2006), have less competition for nutrients and are physically protected from adverse changes in the environment (Reinhold-Hurek and Hurek, 1998). However, successful remediation by endophytic bacteria requires the transport of the contaminant to the plant endosphere. Contaminant transport and its distribution in plants have been reported to depend on soil and plant properties and on the physiochemical properties of the contaminant (Sung et al., 2001).

Ryan et al. (2008) listed some of the advantages associated with use of endophytic bacteria in phytoremediation of contaminated soil when compared to the use of plants alone. They include (i) quantitative gene expression of bacterial pollutant catabolic genes can be used to assess the efficiency of remediation process, (ii) genetic engineering of a bacterial catabolic pathway is easier to manipulate than a plant catabolic pathway, and (iii) toxic pollutants taken up

by the plant may be degraded *in planta* by endophytic degraders reducing the toxic effects of contaminants in the environmental soil on flora and fauna. Doty (2008) claimed that a major advantage of using endophytic bacteria over rhizospheric bacteria in phytoremediation is that while a rhizospheric bacterial population is difficult to control, and competition between rhizospheric bacterial strains often reduces the number of the desired cells (unless metabolism of the pollutant is selective), the use of endophytes reduces the problem of competition between bacterial strains.

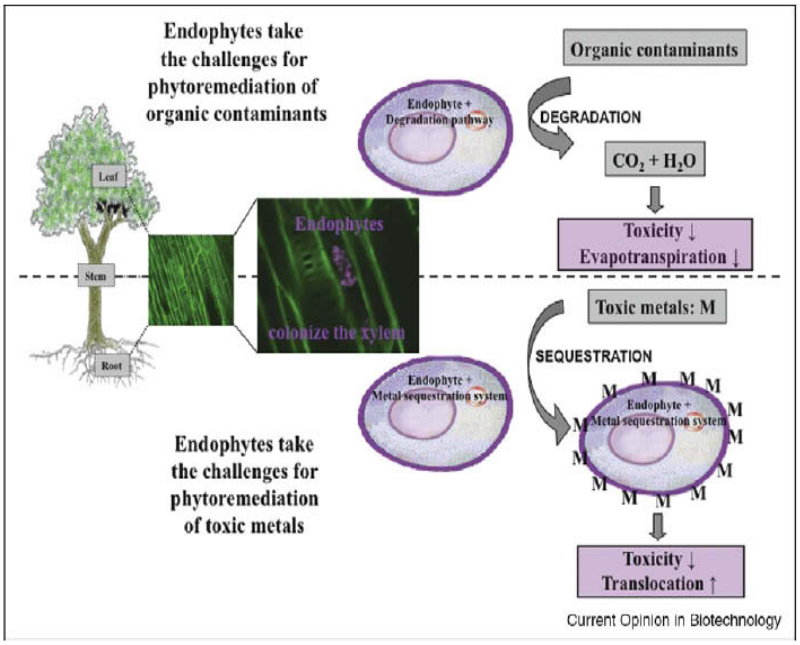


Fig. 2. Role of endophytes in the phytoremediation of soil contaminants (Weyens et al., 2009b)

1.2.8. Transgenic plants and phytoremediation

An exciting alternative to the use of plant-associated bacteria to degrade toxic organic compounds in soil is the use of recombinant DNA technology to generate transgenic plants expressing bacterial enzymes resulting in improved plant tolerance and metabolism of toxic

organic compounds in soil (James and Strand, 2009; van Aken, 2009). A recent study describes the development of transgenic poplars (*Populus*) over expressing a mammalian cytochrome P450, a family of enzymes commonly involved in the metabolism of toxic compounds (Doty, 2008). The engineered plants showed enhanced performance with regards to the metabolism of trichloroethylene and the removal of a range of other toxic volatile organic pollutants, including vinyl chloride, carbon tetrachloride, chloroform and benzene. This work suggests that transgenic plants might be able to contribute to the wider and safer application of phytoremediation. Typically, transgenic plants exhibiting new or improved phenotypes are engineered by the over-expression and/or introduction of genes from other organisms, such as bacteria or mammals. Heterotrophs possess the enzymatic machinery necessary to achieve a complete mineralization of organic molecules; bacterial and mammalian degradative enzymes can therefore be used to complement the metabolic capabilities of plants (Eapen et al., 2007). Transgenic plants for phytoremediation were first developed in an effort to improve heavy metal tolerance; for example, tobacco plants (*Nicotiana tabacum*) expressing a yeast metallothionein gene for higher tolerance to cadmium, or *Arabidopsis thaliana* over-expressing a mercuric ion reductase gene for higher tolerance to mercury (Misra and Gedamu, 1989; Rugh et al., 1996). The first attempts to transform plants for phytoremediation of organic compounds targeted explosives and halogenated organic compounds in tobacco plants (French et al., 1999). A number of studies have shown that transgenic plants are capable of detoxifying herbicides, organic explosives, TCE, and PCBs, using bacterial genes encoding enzymes involved in the detoxification of the target organic contaminant (McGuinness and Dowling, 2009).

1.3. Plant growth-promoting rhizobacteria (PGPR)

Beneficial free-living soil bacteria are generally referred to as plant growth-promoting rhizobacteria and are found in association with the roots of many different plants (Glick et al., 1999). The high concentration of bacteria around the roots, i.e., in the rhizosphere presumably occurs because of the presence of high levels of nutrients (especially small molecules such as amino acids, sugars and organic acids) that are exuded from the roots of most plants, and can then be used to support bacterial growth and metabolism (Bayliss et al., 1997; Penrose and Glick, 2001)

Free living as well as symbiotic PGPR can enhance plant growth directly by providing bio-available phosphorous for plant uptake, fixing nitrogen for plant use, sequestering trace elements like iron for plants by siderophores, producing plant hormones like auxins, cytokines and gibberellins, and lowering of plant ethylene levels (Glick et al., 1999). The use of PGPR in phytoremediation technology is now being considered to play an important role as adding PGPR can aid plant growth on contaminated sites (Burd et al., 2000) and enhance detoxification of soil (Mayak et al., 2004). The properties of plants used for phytoremediation, e.g. biomass production, low level contaminant uptake, plant nutrition and health, are improved by PGPR but it is important to choose PGPR, which can survive and succeed when used in phytoremediation practices. Although the role of PGPR is potentially important in phytoremediation strategies, research in this area, as pointed out by Lucy et al. (2004), is very limited and requires field studies to support greenhouse or growth chamber results.

1.4. Hydrocarbons

The behavior of hydrocarbons in soil depends on physical and chemical properties such as the molecular structure, composition, weight, solubility and affinity for lipids or soils 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.

1.5. Plant species in phytoremediation

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 release root exudates that support the degradation of hydrocarbons (Pilon-Smits, 2005). 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.

Plants should be selected according to the needs of the application, the contaminants of concern and their potential to thrive on contaminated soil. Design requirements should include the use of native plants to avoid introduction of invasive species. Apart from this, vegetation should be fast growing, hardy, easy to plant and maintain.

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

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

[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

The main aim is to ensure that roots expand throughout the entire contaminated zone. Grasses are often planted in tandem with trees at sites with organic contaminants as the primary remediation method. They provide a tremendous amount of fine roots in the surface soil, which is effective at binding and transforming hydrophobic contaminants such as total petroleum hydrocarbons (TPH), benzene, toluene, ethylbenzene, xylenes (BTEX) and polycyclic aromatic hydrocarbons (PAHs). Legumes such as alfalfa (*Medicago sativa*), alisike clover (*Trifolium hybridum*), birdsfoot trefoil (*Lotus corniculatus*), and peas can be used to restore nitrogen to poor soils (Siciliano et al., 2002b).

Plant tolerance to high contaminant concentrations is also a very important factor to keep in mind. Organic compounds can be translocated to other plant tissues (Salt et al., 2003) and subsequently volatilized; they may undergo partial or complete degradation or they may be transformed to less phytotoxic compounds and bound in plant tissues. Collectively, these properties determine whether a contaminant is subjected to phytoextraction, phytodegradation, phytovolatilization or rhizodegradation, although in all cases, the process of phytoremediation begins with the transport of the contaminant to the plant. In general, most organics appear to undergo some degree of transformation in plant cells before being sequestered in vacuoles or bound to insoluble cellular structures such as lignin. In wetland plant species, contaminants can enter through the roots or can partition from the water column directly into plant tissues. The metabolism of certain non-agricultural contaminants such as PAHs, TCE, 2,4,6-trinitrotoluene (TNT), glyceroltrinitrate (GTN) and other chlorinated compounds has been documented (Alkorta and Garbisu, 2001).

The ‘green liver’ model is often used to describe the fate and disposition of organic contaminants (xenobiotics compounds) within plants. Metabolism of foreign compounds in plant

systems is generally considered to be a detoxification process that is similar to the metabolism of xenobiotics compounds in humans (Burken et al., 2000), hence the name 'green liver'. The detoxification of xenobiotics is carried out in three stages: transformation, conjugation and sequestration (Fig. 3). Certain initial reactions such as reduction, oxidation and hydrolysis give rise to compounds that are amenable to subsequent conjugation reactions. Oxidation of lipophilic compounds is very important for increasing the solubility and this step engages specific enzymes such as cytochrome P450 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 live' model is sequestration of conjugated compounds, which is analogous to mammalian excretion.

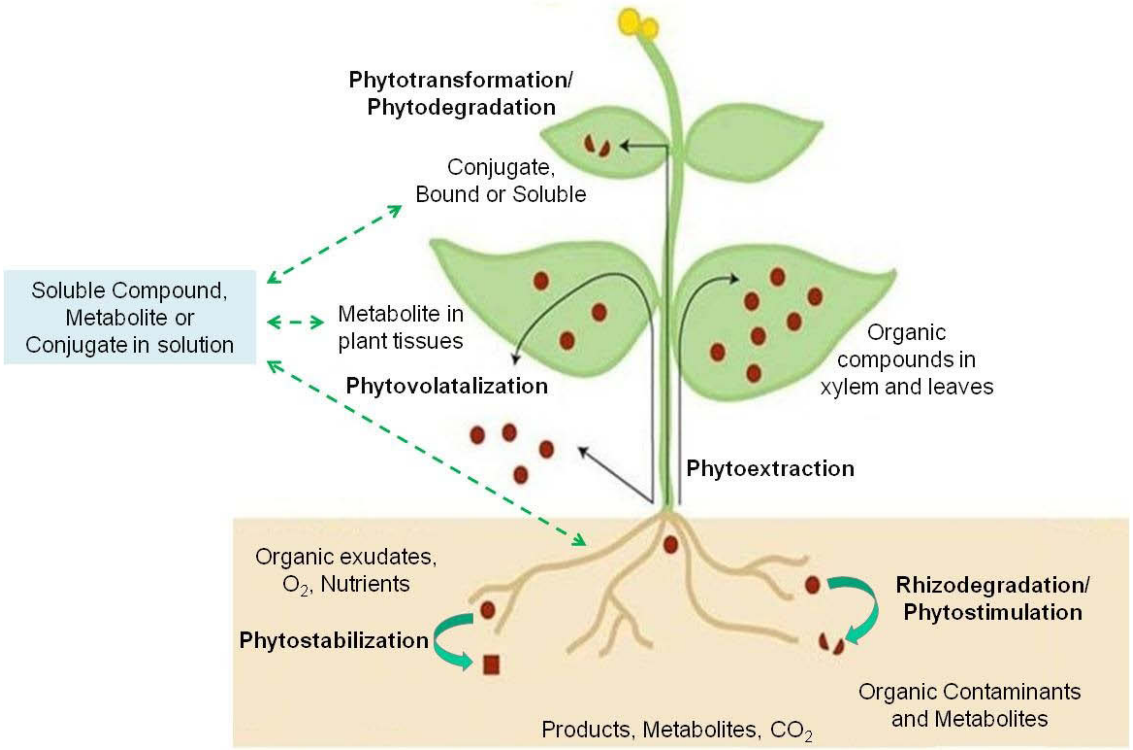


Fig. 3. Schematic representation of mechanism of organic contaminants removal and 'green liver' model for metabolism of xenobiotics in plants. *Dashed lines* Possible transport mechanisms in aquatic plants (adapted from Burken, 2003; Pilon-Smits, 2004).

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 compartmentalization 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 cell wall (Burken, 2003).

1.6. Hydrocarbon degrading bacteria

Bacteria play a major role in phytoremediation process due to their ability to metabolize. Furthermore, various bacteria are able to promote plant growth and to reduce plant stress symptoms. Various bacteria living in the rhizosphere or endosphere of plants have been reported to be involved in the degradation of petroleum hydrocarbons (Table 2).

Bioremediation by bacteria is based on two processes: complete mineralization and cometabloism of hazardous chemicals. 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, ruberdoxin and ruberdoxin reductase, which act as electron carriers between NADH and the hydroxylase for conversion of alkane to alcohol.

Table 2. Genera of hydrocarbon degrading bacteria, which grow in the presence of polycyclic aromatic hydrocarbons (PAHs), benzene, toluene, ethylbenzene, xylenes (BTEX) or alkanes

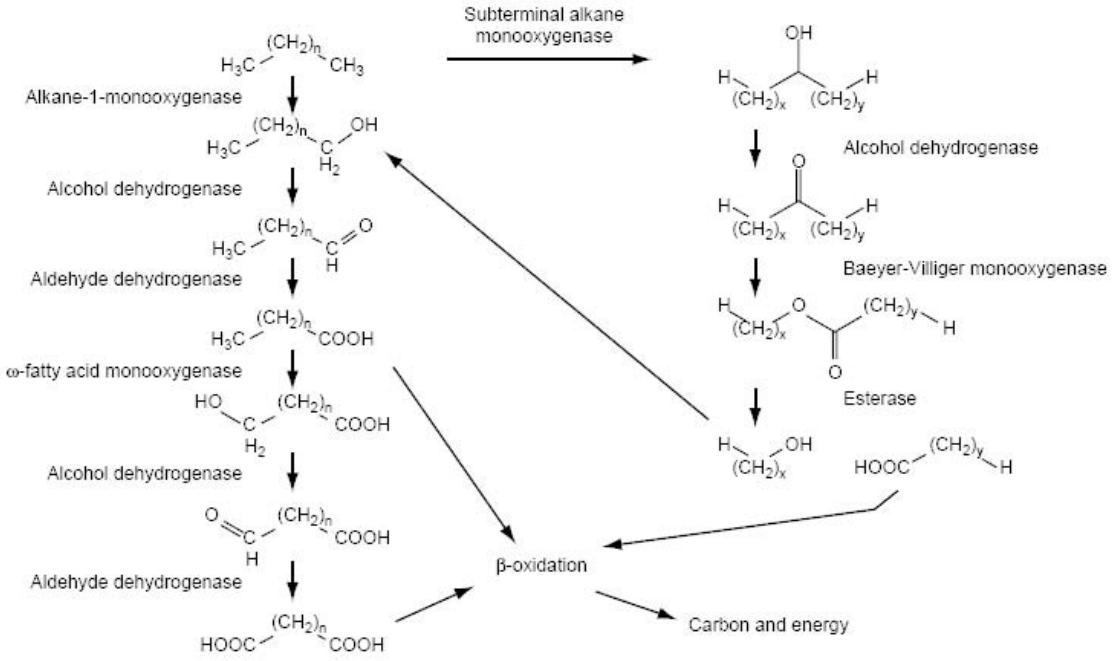
Soil and rhizosphere bacteria[1]		Endophytic bacteria (colonize root, shoot and leaf interior of plant)[2]
Acidovorax	*Methylococcus (only CH_4)*	*Acinetobacter*
Alcaligenes	*Micrococcus*	*Arthrobacter*
Arthrobacter	*Mycobacterium*	*Bacillus*
Achromobacter	*Norcadia*	*Cellulomonas*
Acinetobacter	*Paenibacillus*	*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, 1999; Van Hamme, 2003)

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

In general, the oxidation takes place on one or both terminal methyl group or at a sub-terminal location (**Fig. 4**). 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. 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.



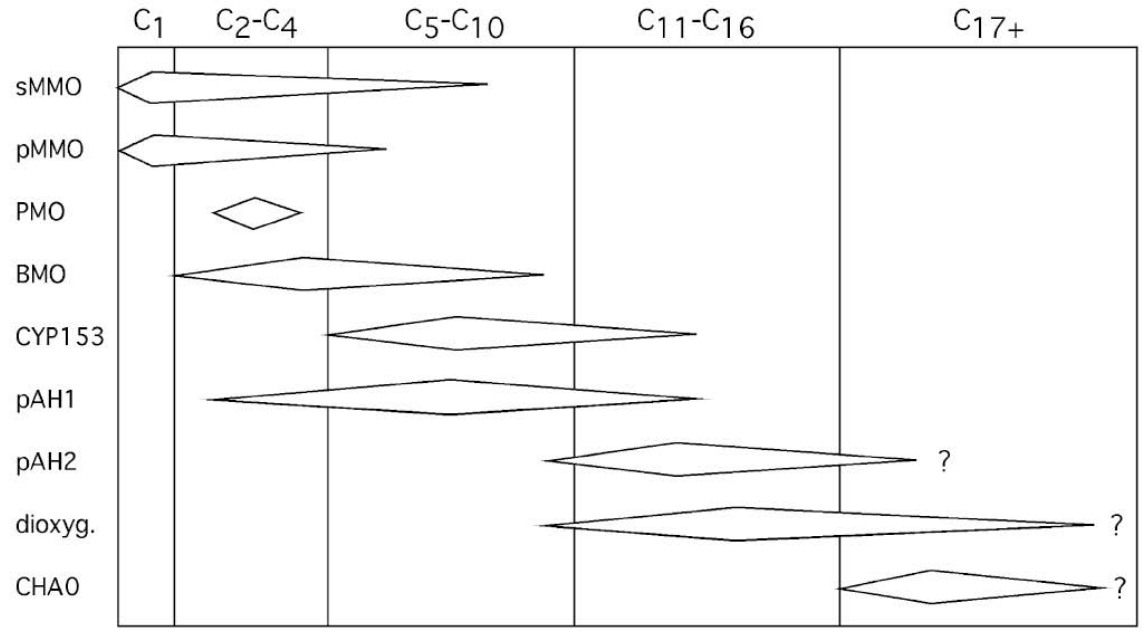
Source: van Beilen *et al.*, 2003

Fig. 4. Metabolic pathway for degradation of alkanes by terminal and sub-terminal oxidation

Anaerobic degradation of hydrocarbons is restricted to anaerobic photo heterotrophic bacteria, Fe (III)-reducing bacteria, Denitrifying bacteria, and sulfate reducing bacteria 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, phenantherene, C_6 n-alkanes, branched alkanes and hydrocarbon mixtures can be also degraded under anaerobic conditions.

1.7. Alkane hydroxylases involved in microbial alkane degradation

Depending on the chain-length of the alkane substrate, different enzyme systems are required to introduce oxygen in the substrate and initiate biodegradation. There are three main categories described by (van Beilen and Funhoff, 2007): C_1-C_4 (methane to butane, oxidized by methane monooxygenase-like enzymes), C_5 -C_{16} (pentane to hexadecane, oxidized by integral membrane non-heme iron or cytochrome P450 enzymes), C_{17}+ (longer alkanes, oxidized by essentially unknown enzymes systems (Fig. 5).



Source : (van Beilen and Funhoff, 2007)

Fig. 5 Overview the substrate range of alkane hydroxylase with respect to alkanes, *sMMO* Soluble methane monooxygenase (Colby et al., 1977); *pMMO* particulate methane monooxygenase (Elliott et al., 1997); *PMO* propane monooxygenase (Kotani et al., 2003); *BMO* butane monooxygenase (Doughty et al., 2006); CYP153 (van Beilen et al., 2006); *pAH1* medium-chain-length integral membrane alkane hydroxylase (van Beilen et al., 2005); (Johnson and Hyman, 2006); *pAH2* long-chain-length integral membrane alkane hydroxylase *dioxy Acinetobacter* sp. M-1 dioxygenase; *CHA0* inferred alkane oxygenase in *P. fluorescens* CHA0 (Smits et al., 2002).

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, while pMMO has a much narrower substrate range, which appears to be restricted to alkanes and alkenes up to C_5 (Beilen and Funhoff, 2005).

The cytochrome P450 alkane hydroxylase enzyme system comprises more than 4000 different enzymes, of which 10-15% is found in prokaryotes. So far only few P450s enzymes have been identified and characterized. 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 C5-C10 alkanes, cyclic compounds and limonene, encoded by CYP153 gene family (Beilen and Funhoff, 2005) and is commonly found in alkane degrading bacteria that lack integral membrane alkane hydroxylase (van Beilen et al., 2006). Class II P450 enzymes are found in the microsome consists of two-component systems comprising a membrane-bound cytochrome P450 and a reductase, and need heme as cofactor. 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 ruberdoxins and electron providing ruberdoxin reductase, and needs iron as cofactor.

1.8. Hydrocarbon analysis

Among many analytical methods that have been used recently for assessment of the *in situ* biodegradation, the most effective ones are based on spectroscopic analyses for example UV-Vis, fluorescence and fourier transform infrared (FTIR) spectroscopy. Spectroscopic methods have also been used for analysis of chemical pollutants that were not identified previously (Weber et al., 2000). The major advantage of spectroscopic methods is their ability for rapid monitoring of the degradation process along with identification of degradation intermediates produced during the metabolic process. UV-Vis spectroscopy has also been integrated successfully with mathematical modeling for determination of the substrate utilization process, microbial activity products and biomass-associated products as indicators of the *in situ* load of different organic chemicals in a test sample (Carvallo et al., 2007). The specific ability of FTIR spectroscopy to distinguish even among very similar chemical structures was used along with the GC-MS studies of biotransformation of 1,1-dichloro-2,2-bis(4-chlorophenyl)ethylene (DDE), a molecule that was earlier considered to be a dead-end product of dichloro-diphenyl trichloroethane (DDT) transformation. In this study, *Pseudomonas acidovorans* strain M3GY was identified for the ability to further transform DDE; however, the transformation products could only be identified using GC-MS-FTIR analysis (Hay and Focht, 1998). Such reports have clearly established the positive impact of different analytical methods on the bioremediation studies including *in situ* bioremediation studies/trials.

1.9. Inoculation and colonization of bacteria

Inoculation of soils or plants with bacteria showing appropriate degradation abilities may be one approach to improve the efficiency of bioremediation applications. Bioaugmentation is the

introduction of microorganisms with specific catabolic abilities into the contaminated environment in order to supplement the indigenous population and to speed up or enable the degradation of pollutants. However, the poor survival of degrading micro-organisms in a usually highly competitive environment has been found to be the most detrimental reason for non optimal performance of bioremediation process (Yousaf et al., 2010). The activity of inoculated microorganisms can be inhibited by a variety of factors including pH and redox, the presence of toxic contaminants, concentration and bioavailability of contaminants, or the absence of key co-substrates. But the key factor to the successful bioremediation is the selection of appropriate bacteria strains that, besides degrading pollutants in pure culture or in a sterile system, are also able to survive in natural soil for long periods of time (Thompson et al., 2005).

Successful establishment of an inoculant strain will depend on many parameters including the type and degree of selective pressure, the complexity of competing microflora, the presence of a compatible plant species and other environmental factors, which might enhance or decrease the survival of a particular strain. Efficient plant colonization by inoculated bacteria is likely to enhance their survival (Shim et al., 2000).

In a series of experiments aiming to develop bacterial inoculants to treat spent metal working fluids in bioreactors three criteria were used to select strains: (i) the relative abundance of the source populations in the target habitat, (ii) tolerance to co-contaminants and (iii) the ability to degrade target contaminants (Gast et al., 2003). The final consortium of four strains was 85% more effective at processing the waste than undefined inocula from sewage.

1.10. Methods to investigate microbial populations in natural environments

Traditional methods to study environmental bacteria are mainly based on cultivation. As only a minor percentage of bacteria are culturable (Amann and Ludwig, 1995), environmental microbiologists have also used novel, molecular, cultivation-independent methods. FISH with targeted oligonucleotide probes has emerged as an invaluable molecular tool for the assessment of the environmental survival of the degrading strain during the bioremediation process (Amann et al., 2001; Aulenta et al., 2004; Caracciolo et al., 2005). From the methodology point of view, **FISH** is based on the sequence-specific *in situ* binding of a fluorescent-labeled probe to the target DNA/RNA, which results in the emission of measurable fluorescence (Thomas et al., 1997). The most important feature of this method is its ability to monitor the target microorganism within an environmental sample without the need for culturability or DNA isolation.

ARDRA is the extension of the technique of RFLP to the gene encoding the small (16s) ribosomal subunit of bacteria. This method is based on PCR amplification of 16S rRNA genes from the total environmental DNA, followed by digestion with a few selected restriction endonucleases that can provide an observable resolution among closely related microbial groups (Ingianni et al., 1997; Jampachaisri et al., 2005). However, the working methodology also requires cloning of 16S rRNA gene amplicon in a suitable vector before restriction digestion of the library clones to prevent the cross-contamination of 16S rRNA gene fragments of different microbial origins. Afterwards, the restricted clones are subjected to electrophoresis and categorized according to the restriction digestion pattern.

T-RFLP analysis is an innovative fingerprinting method (Kitts, 2001; Lukow et al., 2000; Marsh et al., 2000). The method essentially works as ARDRA (described above) but

identifies microorganisms on the basis of terminal restriction fragment (T-RF) (proximal to a fluorescently labeled primer) rather than the total digestion profile (Dunbar et al., 1999; Heuer et al., 1997; Horz et al., 2000). In T-RFLP analysis a target gene is PCR amplified using mixed community DNA (such as soil metagenome) and PCR primers that are usually labeled at the 5 end with some fluorescent dye; this PCR amplification results in the generation of a pool of 5 end labeled amplicons. After amplification, the amplicons are digested and then size separated on an automated gel or capillary sequences.

D/TGGE incorporates the advantageous features of high throughput fingerprinting methods and ability to sequence the selected DNA fragments to determine the taxonomic status of different constituents of the complex bacterial communities (Heuer et al., 1997). Like other fingerprinting methods, D/TGGE also consists of direct extraction of nucleic acids (DNA or RNA), followed by PCR amplifications of the target gene. Later the amplicons are analyzed using electrophoretic separation on gradient gels.

1.11. Abundance and expression of catabolic genes

The efficiency of a phytoremediation process depends mainly on the presence and activity of plant associated microorganisms carrying degradation genes required for enzymatic break-down of contaminants. The list of contaminant degrading bacteria associated with plant rhizosphere is very extensive. For the efficient removal of soil contaminants, not only do microbes with appropriate catabolic genes have to be maintained in the rhizosphere and plant interior but the genes have to be conveniently expressed and be free of the catabolic repression effect, in which microbes use a given carbon or nitrogen source preferentially over others. The relatively recent introduction of molecular techniques for quantifying gene expression from complex

environmental samples has started to create a greater understanding of the roles and diversity of many bacterial populations.

Different methods that have been used to quantify mRNA from environmental samples include in situ hybridization techniques (Beer et al., 2002; Cho and Chae, 2003), RNase protection assays (Taté et al., 1999; Wilderman et al., 2001), Northern blotting (Wouters et al., 2003), and reverse transcription RT-PCR (Alfreider et al., 2003; Pirnay et al., 2002). Real-time PCR assays used for microbial gene expression analysis combine the best attributes of both relative and cRT-PCR, in that they are extremely sensitive, rapid, capable of high throughput, and relatively easy to perform (Ginzinger, 2002; Klein, 2002).

1.12. Objectives of thesis

The main focus of this thesis is on

- the identification of hydrocarbon degrading bacteria with high hydrocarbopn degradation potential
- survival and colonization efficiency of inoculant strains in sterile and non-sterile environments
- to investigate the diversity of indigenous alkane degrading soil bacteria on the basis of cytochrome P450-type and alkane monooxygenase genes in the rhizo- and endopshere of Italian ryegrass and birdsfoot trefoil
- effect of compost amendment and plant species on hydrocarbon degradation and bacterial community structure during alkane degradation
- to assess the role of *Enterobacter ludwigii* strains in hydrocarbon degradation, plant colonization, abundance and expression of CYP153 genes in different plant compartments of three plant species (Italian ryegrass, birdsfoot trefoil and alfalfa).

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Chapter 2

Hydrocarbon degradation and plant colonization by selected bacterial strains isolated from Italian ryegrass and birdsfoot trefoil

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Running Head: Bacterial hydrocarbon degradation and plant colonization

***Published in: Journal of Applied Microbiology (109): 1386-1401 (2010)**

Abstract

Aims: To assess the degradation potential and plant colonization capacity of four alkane degrading strains (ITSI10, ITRI15, ITRH76, and BTRH79) in combination with birdsfoot trefoil and Italian ryegrass and to evaluate the diversity of indigenous alkane-degrading soil bacteria in the rhizo- and endosphere.

Methods and Results: Contaminated soil was prepared by spiking agricultural soil with 10 g diesel fuel kg^{-1} soil. Italian ryegrass (*Lolium multiflorum* var. Taurus) and birdsfoot trefoil (*Lotus corniculatus* var. Leo) were inoculated with four alkane degrading strains. Hydrocarbon degradation (up to 57 %) was observed in all inoculated treatments of vegetated and unvegetated samples. Italian ryegrass in combination with compost and BTRH79 showed highest degradation, while birdsfoot trefoil performed best with compost and strain ITSI10. Cultivation-based as well as cultivation-independent analysis showed that both strains were competitive colonizers.

Conclusions: The combination between vegetation, inoculation with well performing degrading bacteria and compost amendment was an efficient approach to reduce hydrocarbon contamination. Two *Pantoea* sp. strains, ITSI10 and BTRH79, established well in the plant environment despite the presence of a variety of other, indigenous alkane-degrading bacteria**.**

Significance and Impact of Study: The present study suggests that the application of degrading bacterial strains, which are able to compete with the native microflora and to tightly associate with plants, are promising candidates to be used for phytoremediation applications.

Keywords: bioremediation, rhizosphere, degradation

Introduction

Industrial and military activities have led to the widespread contamination of the environment, including air, water and soils. Among hydrocarbon pollutants, diesel fuel is a common contaminant in soil and water, which contains a complex mixture of alkanes and aromatic compounds leaking from storage tanks and pipelines or released from accidental spills (Gallego *et al*. 2001). Remediation techniques are required to clean up soil contaminated with petroleum hydrocarbons. Phytoremediation, i.e. the use of plants and associated microorganisms, is an attractive technology, which involves minimal disturbance of the site to be remediated and is comparably inexpensive (Karthikeyan and Kulakow 2003; Singh and Jain 2003). During phytoremediation, plants enhance microbial activity and contaminant degradation via root exudates providing sources of carbon and energy for rhizosphere microorganisms, which oxidize or degrade organic contaminants (Shim *et al*. 2000). Plant endophytes, bacteria which colonize the endosphere of plants without exhibiting pathogenicity to their hosts, may also be important during phytoremediation. They may either degrade organic contaminants after plant uptake or improve plant stress tolerance (Weyens *et al*. 2009).

Aerobic degradation pathways of alkanes have been described extensively (van Beilen *et al*. 2003; van Beilen and Funhoff 2005). Alkanes usually undergo terminal or sub-terminal oxidation mediated by different alkane hydroxlase systems including methane monooxygenases (*mmo* genes, *pmoA*; Murrell *et al*. 2000), integral membrane non-heme iron alkane hydroxylases (or alkane monooxygenases, *alkB*; van Beilen *et al*. 2003; van Beilen and Funhoff 2005) and cytochrome P450 alkane hydroxylases (CYP153 genes; van Beilen and Funhoff 2005). Oxidation products are further oxidized by alcohol

and aldehyde dehydrogenases and the resulting fatty acids enter the TCA cycle. Anaerobic degradation pathways in terrestrial environments have received less attention. In anaerobic oxidation of alkanes sulfate or nitrate act as electron donors and it has been suggested that alkane activation occurs by the addition of a C group (Widdel and Rabus 2001; Callaghan *et al.* 2009).

Inoculation of soils or plants with bacteria showing appropriate degradation abilities may be one approach to improve the efficiency of bioremediation applications. However, the poor survival of degrading microorganisms in an usually highly competitive environment has been found to be the most detrimental reason for non-optimal performance of bioremediation processes (Ramadan *et al*. 1990; Miethling and Karlson 1996; Rousseaux *et al*. 2003). Inoculant strains can be poor survivors or may loose catabolic activity when inoculated into complex microbial ecosystems (McClure *et al.* 1991; Watanabe *et al.* 1998). Consequently, bioremediation requires appropriate inoculant strains that, besides degrading pollutants in pure culture or in a sterile system, are also able to survive in natural soil for long periods of time (Schwartz *et al.* 2000). Successful establishment of an inoculant strain will depend on many parameters including the type and degree of selective pressure, the complexity of the competing microflora, the presence of a compatible plant species and other environmental factors, which might enhance or decrease the survival of a particular strain. Efficient plant colonization by inoculated bacteria is likely to enhance their survival (Shim *et al*. 2000). There is a need to identify degrading bacteria with high hydrocarbon degradation potential, which also efficiently colonize the environment (e.g. the rhizosphere) of an appropriate plant species to be used for phytoremediation.

We previously isolated a high number of alkane degrading strains from the rhizo- and endosphere of Italian ryegrass and birdsfoot trefoil (Andria *et al*., unpublished results). Both plant species are able to tolerate high concentrations of the contaminant and are potential candidates to be used for phytoremediation. The objectives of this study were to (i) identify strains with efficient survival, colonization and degrading activity, (ii) to investigate the diversity of indigenous alkane-degrading soil bacteria on the basis of cytochrome P450-type and *alkB* alkane hydroxylase genes in the rhizo- and endosphere, (iii) and to study the effect of compost amendment on hydrocarbon degradation and alkane degrading communities during phytoremediation of diesel fuel using two different plant species.

Materials and Methods

Plant experiment

Four bacterial strains, which were previously isolated from the rhizosphere and endosphere of Italian ryegrass and birdsfoot trefoil (Andria *et al*., unpublished results) were selected based on their high degradation potential of alkanes (tested *in vitro*, data not shown). The strains included two *Pantoea* sp. strains, ITSI10 (an Italian ryegrass endophyte; carrying an unknown gene responsible for alkane degradation) and BTRH79 (a rhizosphere strain from birdsfoot trefoil carrying a cytochrome P450 (CYP 153) gene), and two *Pseudomonas* sp. strains, ITRI15 (an Italian ryegrass endophyte; carrying an unknown gene responsible for alkane degradation) and ITRH76 (an Italian ryegrass rhizosphere strain carrying an alkane monooxygenase (*alkB*) gene). Strains ITRI15 and

ITRH76 were isolated by plating rhizosphere suspensions (ITRH76) or surface-sterilized and macerated root material (ITRI15) on Minimal Salt Basal Medium (Alef, 1994) amended with 1% (v/v) filter-sterilized diesel. Strains BTRH79 and ITSI10 were obtained by plating rhizosphere suspensions (BTRH79) or surface-sterilized and macerated shoots on 10% Tryptic Soy Agar amended with 1% (v/v) filter-sterilized diesel. Strains were evaluated regarding their degradation capacity of diesel as well as of alkanes of different chain lengths.

Three sets of pots were prepared in triplicates: (1) pots planted with Italian ryegrass (IT), (2) pots planted with birdsfoot trefoil (BT) and (3) unvegetated (UV) pots. Both plant species proved in former experiments to tolerate diesel contamination. Diesel fuel contaminated substrate was prepared by spiking agricultural soil (agricultural top soil from Seibersdorf, Lower Austria, Austria; pH 7.4, 27 g sand kg^{-1}, 621 g silt $kg^{-1,}$ 352 g clay kg^{-1} 0.2 mg Corg kg^{-1}) with filter sterilized diesel fuel (10,000 mg kg^{-1} soil). Soils, i.e. sterilized by 30 kGy γ-radiation and non-sterilized, with 10% compost amendment and without compost were used. The sterility of sterilized soil was checked by plating soil suspensions on Tryptic Soy Agar (Merck, Darmstadt, Germany) plates, no growth was observed. Pots with dimensions 13 x 13 x 13 cm were filled with spiked soils and subsequently placed in the greenhouse. Pots were arranged in a completely randomized block design. Seeds of IT and BT were surface sterilized by soaking in 5% sodium hypochlorite solution for 2 min, then in 70% ethanol for 2 min, and were then washed with sterile water for 3 times. Surface-sterilized seeds (100 per pot) were sown. One week after seed germination, plants were thinned to 75 per pot and each pot was inoculated with 25 ml inoculant suspension (app. 10^9 CFU ml^{-1}, cultivated in Luria

Bertani broth at 30°C, centrifuged and resuspended in 0.9% (w/v) NaCl) containing one of the strains described above. For control treatments, spiked soil was treated with 25 ml of 0.9% NaCl instead of inoculum suspension.

Plants were grown at 25°C in the greenhouse (16 h light / 8 h dark) and watered with equal amounts when needed. Plants were harvested at flowering (BT 155 days after germination, IT 90 days after germination). They were cut 2 cm above ground and plant biomass was determined. The isolation of plant-associated bacteria and the isolation of DNA was performed as described below. After the plants were removed from the pots and roots separated from bulk soil, the soil from each pot was thoroughly mixed to obtain homogenized samples for hydrocarbon extraction. These soil samples were then stored at -80°C until further analysis.

Hydrocarbon analysis of soil samples

Total hydrocarbon content (THC) of the soil was measured employing infrared spectroscopy according to ONÖRM S2120 (ÖNORM S 1999; Tesar, 2002). Moist soil material (5 g) was dehumidified with Na_2SO_4 and then extracted with 30 ml 1, 1, 2-trichlorotrifluoroethane ($C_2Cl_3F_3$) by shaking at 180 rpm for 3 h at room temperature. The soil was allowed to settle and an aliquot of supernatant (approximately 3 ml) was purified with aluminium oxide columns. For this, Pasteur pipettes were stuffed with some fibreglass and overlaid by ~8 g aluminium oxide (Al_2O_3, 90 active, neutral (activity I), particle size: 0.063-0.2 mm, Merck). The filtered extract was – after appropriate dilution with $C_2Cl_3F_3$– measured with FT-IR spectroscopy.

Isolation of alkane-degrading bacteria

Alkane-degrading bacteria were isolated from the rhizosphere, shoot interior and root interior of IT, BT and from soil of the UV control. Rhizosphere soil was obtained by agitating roots and sampling the soil still attached to the roots. Subsequently, roots and shoots were carefully washed and surface sterilized with 70% ethanol (IT: 3 min, BT: 5 min), then treated with 1% NaOHCl (IT: 5 min, BT: 6 min), followed by washing 3x with sterile distilled water (1 min each time). Bacteria were isolated on solid Minimal Basal medium (MBM) (Alef 1994) containing 1% (v/v) filter-sterilized diesel as a carbon source. MBM also contained cycloheximide (100 mg L^{-1}) to avoid fungal growth. For the isolation of endophytes, 4 g of surface sterilized roots or shoots were homogenized with a pestle and mortar in 12 ml NaCl solution (0.9%, w/v). The homogenized material was agitated for 1 hour at 30^{o}C. After settling of solid material, serial dilutions up to 10^{-2} were spread on MBM containing 1% diesel. For the isolation of rhizosphere bacteria, the soil slurry was prepared by mixing 3 g soil with 9 ml of 0.9% (w/v) NaCl solution, agitated (180 rpm) for 1 hour at 30^{o}C. After the settlement of soil particles, serial dilutions up to 10^{-3} were spread onto solid MBM containing 1% diesel. Bacterial colonies on each plate were selected randomly and transferred to solid MBM amended with 2% (v/v) filter-sterilized diesel followed by incubation at 30^{o}C for 4 days.

Molecular characterization of isolates

Forty-five colonies from the rhizosphere and 30 colonies from root and shoot interior were randomly selected and their identity with the inoculant strain was confirmed by restriction fragment length polymorphism (RFLP) analysis of the 16S-23S rRNA

intergenic spacer region (IGS) (Rasche *et al.* 2006a). Genomic DNA was extracted from liquid cultures of each selected colony by using the Microbial DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA) following the instructions of the manufacturer. The IGS region was amplified by PCR using the primers IGSfor (5'-TGCGGCTGGATCACCTCCT-3') and IGSrev (5'-GGCTGCTTCTAAGCCAAC-3') (Massol-Deya *et al.* 1995) as described by (Rasche *et al.* 2006a). Digestion of 10 µL of IGS PCR products was performed with 5 U of endonuclease *Alu*I and *Hae*III (Fermentas, Ontario, Canada) at 37^0C for 4 h. The resulting DNA fragments were analyzed by gel electrophoresis in 2% (w/v) agarose gels. Isolates with identical restriction profiles were grouped and compared with the inoculant strain. Representative isolates from each group with IGS profile differing from the inoculant strain were chosen for further analysis. From each IGS type, one representative 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 mentioned above. PCR amplicons were purified with Sephadex™ G-50 (Amersham, Uppsala, Sweden) and used as template for sequence analysis. Partial sequencing of 16S rDNA was performed by AGOWA (http://www.agowa.com/) with primer 518rev (5'-ATTACCGCGGCTGCTGG-3') (Liu *et al.* 1997), resulting in sequences of approximately 500 bp length. Sequences were subjected to BLASTN analysis with the NCBI database.

DNA extraction from shoot, root and soil samples

DNA from rhizosphere soil (0.5 g) was extracted by using FastDNA® Spin Kit for Soil (MP Biomedicals, Solon, OH, USA). DNA was extracted as described by the manufacturer, and was quantified photometrically (Nanodrop ND-1000, Nanodrop Technologies, Wilmington, DE, USA). 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 the FastDNA® Spin Kit for Soil was used.

Terminal Restriction Fragment Length polymorphism (T-RFLP) analysis of alkane degradation and 16S rRNA gene fragments

Cultivation-independent analysis of 16S rRNA genes, alkane monooxygenase (*alkB*) and of cytochrome P450-type alkane hydroxylase genes (CYP153) was performed. For the amplification of 16S rRNA genes primers 8f (5'-AGAGTTTGATCCTGGCTCAG-3') (Weisburg *et al*., 1991) labelled at the 5´ end with 6-carboxyfluorescein (6-fam, MWG) and 1520rev (5'-AAGGAGGTGATCCAGCCGGA-3') (Edwards *et al.* 1989) were used to amplify 16S rRNA genes previously described (Rasche *et al*., 2006b).

For the amplification of the CYP153 genes, primers P450fw1 (5'-GTSGGCGGCAACGACACSAC-3') and fam labeled P450rv3 (5'-GCASCGGTGGATGCCGAAGCCRAA-3') resulting in a 339 bp fragment (van Beilen *et al*. 2006) were used. PCR reactions were performed in 25 µl reactions containing 1x reaction buffer (Gibco BRL, Carlsbad, CA), 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 4% (v/v) DMSO, 1% (w/v) bovine serum albumine (BSA), 1 U of Taq DNA Polymerase (Gibco BRL), 25 ng DNA and 0.2 µM of each primer for the amplification of CYP153 genes.

The following programme was used: 4 min at 95°C; 25 cycles of 45 s at 95°C, 1 min at 58°C, and 1 min at 72°C; 5 min at 72°C; and 4°C until further use.

The *alkB* PCR analysis was performed by using published PCR primers for detection of *alk* genes: primers based on *P. putida alkB* genes: PpalkB-for (5'-TGGCCGGCTACTCCGATGATCGGAATCTGG-3') and fam labeled PpalkB-rev (5'-CGCGTGGTGATCCGAGTGCCGCTGAAGGTG-3') resulting in a 870 bp fragment. PCR reactions were performed in 25 µl reactions containing 1x reaction buffer (Gibco BRL), ingredients as described above, 50 ng DNA and 5 µM of each primer. PCR was carried out as described by (Whyte *et al.* 2002), i.e. 30 cycles of denaturation at 94°C, 1 min of annealing at 60°C, 1 min of extension at 72°C and a final extension of 3 min at 72°C, using a Whatman T1 thermocycler.

Three independent reactions were performed with each sample, which were subsequently pooled to reduce PCR bias. PCR amplified 16S rRNA genes were subjected to T-RFLP analysis using *Alu*I as restriction enzyme, whereas *Taq*I and *Rsa*I were used for restriction of PpalkB-type *alkB* and CYP153 gene fragments, respectively. To obtain the highest number and most even distribution of terminal restriction fragments of *alk* genes, a computer-simulated analysis was utilized in order to select most appropriate restriction enzymes for comparing bacterial communities. Twenty µl restriction mixtures containing 200 ng PCR product, 1x buffer and 1 µl *Alu*I/*Rsa*I/*Taq*I (10 U/µl, Fermentas) were prepared following incubation for 4 h at 37°C. Digestion batches were purified by passage through DNA grade Sephadex G50 (GE Healthcare) columns. Ten µl of purified product were mixed with 10 µl HiDi-Formamide (Applied Biosystems, Carlsbad, CA) and 1 µl 500 ROX^{TM} Size Standard (Applied Biosystems) and denatured at 95°C for 2

min. Detection of FAM-labeled terminal restriction fragments was done by capillary electrophoresis using an ABI 3100 automatic DNA sequencer.

Cloning and sequencing of alkane degradation genes

For a more detailed analysis of the alkane-degrading communities in the rhizosphere of IT and BT soil samples, with and without compost, from uninoculated treatments were further analyzed by a cloning-sequencing approach. Bacterial *alk* genes were PCR amplified with two primer sets as described above: 1) PpalkB-for and PpalkB-rev and 2) P450fw1 and P450rv3. PCR products were purified with a QIAquick gel extraction kit (QIAGEN, Chatsworth, CA, USA) and cloned into pSC-A-amp/kan (Strataclone PCR cloning Kit; Stratagene, Santa Clara, CA) according to the manufacturers' protocol. Twenty-five clones were selected for sequencing from each treatment (i.e. IT with and without compost and BT with and without compost). The primers used for sequencing included the *alk* amplification primers mentioned above. Sequences were compared to Genbank with BLASTN (Altschul *et al.* 1997). Derived sequences were aligned to the existing *alkB* and CYP153 alignment of full length sequences with CLUSTALW by using ARB software package (Altschul *et al*. 1990).

For the calculation of trees, 50% sequence conservation filters for different taxonomic groups were used. These filters exclude highly variable sequence positions that could biase sequence analysis. The neighbor-joining method was performed using the respective of ARB software package (Ludwig *et al.* 1998; Ludwig *et al.* 2004).

Statistical Analysis

T-RFs between 50 and 500 bp and peak heights of ≥ 40 fluorescence units were included in the analysis. T-RFLPs were transformed into numerical data using GenScan 3.7 software. To reduce background noise peaks with intensities higher than three times standard deviation were binned and normalized by dividing all peak areas by the total area of all peaks as described in (Abdo *et al.* 2006) using the statistical program R together with the filtering and binning macro provided at the IBEST homepage (http://www.ibest.uidaho.edu/tools/trflp_stats/instructions.php). Peaks that occurred in at least two replicates and with a percentage higher than 2% were considered as major ones. Non-metric multidimensional scaling using the Primer 5 statistical program (Primer E, Plymouth, UK) was performed for each T-RFLP data matrix.

Data analysis for plant biomass and THC in soil was carried out by using SPSS software package (SPSS Inc., Chicago, IL). The data were first subjected to analysis of variance, and the means [± standard deviation (SD)] were compared using Duncan's multiple range test.

Nucleotide sequence accession numbers

The partial nucleotide sequences of *alk* genes determined in this study were deposited in GenBank data base with accession numbers GU586323 to GU586410 for *alkB* genes and GU586411 to GU586483 for CYP153 genes.

Results

Plant biomass production

Italian ryegrass generally produced more biomass when treated with compost (Table 1). In non-sterilized soil with compost, inoculated treatments showed around 40 % higher biomass production than uninoculated ones, and plant growth was better than in sterilized soil, although differences were not always significant. As we know from previous experiments that birdsfoot trefoil does not grow well in diesel-polluted soil without the addition of compost, only treatments with compost were tested. Also here, the birdsfoot trefoil plants grown in non-sterilized soil performed better than in sterilized soil. Inoculation increased biomass production and the best performing strains were ITSI10, ITRH76 and BTRH79.

Hydrocarbon degradation

Hydrocarbon concentrations remaining in soil showed that inoculation substantially improved degradation in sterilized and non-sterilized soil and degradation rates were generally similar in both soils (Table 2). Generally, pots planted with Italian ryegrass showed better degradation than those planted with birdsfoot trefoil (particularly considering the different times of harvest). Considering degradation efficiencies in only non-sterilized soils, the best performing strains were BTRH79 in combination with Italian ryegrass (and also with birdsfoot trefoil) and ITSI10 in combination with birdsfoot trefoil. Non-vegetated, uninoculated controls showed the lowest hydrocarbon degradation. Inoculation without the addition of compost had a more pronounced effect on the

degradation of diesel than adding compost only. Vegetated treatments with inoculation showed higher degradation than unvegetated treatments with inoculation confirming the positive interaction between plants and the selected bacteria. In uninoculated treatments, higher degradation rates were found in non-sterilized soil than in sterilized soil, indicating that indigenous microorganisms are also capable of degrading diesel but their degradation efficiency was lower than that of inoculated bacteria.

Persistence of inoculant strains in the plant environment

Cultivation-dependent analysis revealed that all inoculant strains competed well in sterilized soil with compost as they showed a high degree of persistence. According to the analysis of isolated strains from Italian ryegrass 75 to 90% and 80 to 100% of the isolates, in rhizosphere and endosphere, respectively, were identical to the inoculant strain. For Birdsfoot trefoil, inoculant strains showed 80 to 100% persistence, both in the rhizosphere and the endosphere.

As strains BTRH79 and ITSI10 showed the highest hydrocarbon degradation activities, we confirmed strain survival in non-sterile soil by cultivation- as well as by cultivation-independent analysis. Analysis of isolates again revealed high persistence even under non-sterile conditions (Fig. 1). In the rhizosphere of Italian ryegrass, 34% and 43% of the isolates, respectively, were identical to the inoculant strains ITSI10 and BTRH79. In the endosphere of Italian ryegrass, colonization of ITSI10 varied from 20% in the root interior to 56% in the shoot interior, whereas strain BTRH79 showed a very high degree of colonization, i.e. 90% in the root interior and 72% in the shoot interior. ITSI10 showed high persistence in the rhizosphere, root and shoot interior of birdsfoot

trefoil i.e. 63%, 89% and 76% respectively. BTRH79 colonization in the rhizosphere of birdsfoot trefoil was 55%, followed by 73% in the root interior and 56% in the shoot interior.

Inoculation also had an effect on the indigenous microbial community (Fig. 1). Furthermore, microbial communities in the rhizosphere and endosphere of plants were different. In the rhizosphere of Italian ryegrass, a *Pseudomonas* sp. (IGS 1, Table 1 Supplementary Information) was dominant, whereas in the endosphere a strain related to *Enterobacter ludwigii* was more abundant (IGS 5, Table 1 Supplementary Information). In the rhizosphere of birdsfoot trefoil again *Pseudomonas* sp. (IGS 1, Table 1 Supplementary Information) was dominant, but in the plant interior more complex communities were encountered.

The persistence of ITSI10 and BTRH79 was also confirmed by T-RFLP analysis. Strain BTRH79 carries a CYP153 alkane hydroxylase gene, and therefore the persistence of this strain was analysed by T-RFLP analysis of that gene. T-RFLP analysis showed that strain BTRH79 efficiently colonized the rhizosphere as well as the endosphere and that the inoculant strain replaced other bacteria containing this alkane hydroxylase gene (Fig. 2a and b). For strain ITSI10, no *alk* gene is known and therefore 16S rRNA-based T-RFLP was used to confirm colonization of this strain. As shown in Fig 2c, d and e, also strain ITSI10 colonized well and represented a dominant member of the rhizosphere and endosphere bacterial community associated with both plant species. Colonization of this strain also induced pronounced community shifts.

Cultivation-independent analysis of alkane degrading microbial communities

Cultivation-independent analysis of alkane degraders showed that both uninoculated plants hosted different communities carrying the *alkB* gene (Fig. 3). The *alkB* genes were detected in the rhizosphere of both plants, in the root interior of Italian ryegrass but not in the endosphere of birdsfoot trefoil. The number of T-RFs in the rhizosphere of Italian ryegrass ranged from 17 (with compost) to 18 (without compost) and in the root interior from 17 (with compost) to 20 without compost. The number of T-RFs in the birdsfoot trefoil rhizosphere ranged from 17 (with compost) to 23 (without compost). Without compost amendment different *alkB* communities were found in the rhizosphere of both plants as well as in the root interior of Italian ryegrass, however, after compost addition the rhizosphere communities grouped together (Fig. 3). This indicates that some compost bacteria may have established in the rhizosphere. Endosphere *alkB* communities of Italian ryegrass were not affected by compost amendment.

PCR amplicons of the CYP153 gene were obtained from all rhizosphere and root interior samples. The total number of T-RFs found in the rhizosphere of Italian ryegrass (with and without compost) was 8, whereas the numbers in the root interior ranged from 7 (with compost) to 10 (without compost). The number of T-RFs in the rhizosphere of birdsfoot trefoil ranged from 6 (without compost) to 10 (with compost) and in the root interior from 5 (without compost) to 9 (with compost). The plant compartments of both plants hosted different CYP153 communities (Fig. 4). Compost amendment did not affect CYP153-containing populations associated with Italian ryegrass, but did influence those associated with birdsfoot trefoil (Fig. 4).

Phylogenetic analysis of diversity of alkane degrading genes

Detailed analysis of *alkB* and CYP153 sequences was performed to obtain information on the diversity of alkane degrading bacteria in uninoculated, contaminated soils planted with Italian ryegrass and birdsfoot trefoil. Phylogenetic relationships of alkane degradation genes are shown in Figs. 1 and 2 (Supplementary Information). However, as degradation genes are frequently prone to horizontal gene transfer, our analysis does not show taxonomic relationships of the bacteria hosting these genes. A total of 88 *alkB* and 73 CYP153 sequences were obtained from clone libraries prepared from the rhizosphere soil DNA (from both IT and BT, with and without compost). Overall *alkB* sequence homologies ranged from 71% to 99% (identity of nucleotides) to published sequences, only four had 100% identity to published *alkB* sequences derived from *Arthrobacter* sp. (FJ014910, FJ014913, FJ014915). The *alkB* sequences grouped in two major groups (Fig. 1 Supplementary Information). The majority of group 1 sequences were most similar to those from *Pseudomonas aeruginosa* (AJ344079), *Pseudomonas putida* (AJ245436) and *Alcanivorax* sp. (EU853354), whereas group 2 sequences clustered with sequences from *Acidisphaera* sp. (AY817739) and *Kordiimonas gwangygensis* (EU853377, EU853378). Group 1 contained the majority of sequences and most of them were from the birdsfoot trefoil rhizosphere with and without compost amendment, and some sequences derived from the Italian ryegrass rhizosphere with compost. Sequences in group 2 were mostly from the Italian ryegrass rhizosphere with compost and few sequences were from Italian ryegrass without compost.

CYP153 sequence homologies ranged from 72% to 99% (identity of nucleotides) to published sequences. The CYP153 sequences grouped in four major groups (Fig. 2

Supplementary Information). The majority of group 1 sequences showed high homologies to those from *Rhodococcus* sp. (DQ847173), whereas group 2 sequences showed highest homology to *Sphingopyxis* sp. sequences (FJ014891). Group 3 sequences were more similar to those from *Rhodococcus erythropolis* (AJ833997, AJ833998) and group 4 sequences were related to *Dietzia* sp. (FJ435362). Groups 1 and 2 contained the majority of sequences, most of them derived from the birdsfoot trefoil rhizosphere with and without compost and some sequences from the Italian ryegrass rhizosphere. Sequences in groups 3 and 4 were from the Italian ryegrass rhizosphere with and without compost amendment.

Discussion

Contaminating substances such as hydrocarbons generally inhibit plant growth. The primary inhibiting factors are considered to be toxicity of low molecular weight compounds and hydrophobic properties that limit the ability of plants to absorb water and nutrients (Kirk *et al.* 2005). In our study both plants, Italian ryegrass and birdsfoot trefoil, produced higher biomass when treated with compost and alkane degrading strains as compared to control treatments. The application of compost is known to confer beneficial effects (Tesar *et al.* 2002; Antizar-Ladislao *et al.* 2006; Williamson *et al.* 2009), which are probably due to the input of nutrients and microorganisms that participate in the breakdown of pollutants (Tesar *et al.*, 2002). It is well known that plant-associated microorganisms can promote plant growth by many different mechanisms – by providing nutrients, plant growth hormones or by antagonizing pathogens (Kiely *et al.* 2006; Shaw *et al.* 2006; Danhorn and Fuqua 2007). In the case of promoting the performance of

plants growing in contaminated environments, plant-associated strains have to be able to survive in this toxic environment. The ability of a bacterial strain to degrade the contaminant is required for persistence but is also important for reducing the concentration of the contaminant. Consequently, in phytoremediation processes plants may benefit from the associated microflora by direct plant beneficial effects but also by a less toxic soil environment. In this study compost amendment along with inoculation increased biomass production (up to 40%) of both plants. This suggests that the presence of hydrocarbon-degrading microorganisms reduced the toxicity due to degradation of the pollutant as also previously reported (Hutchinson *et al*. 2001; Escalante-Espinosa *et al*. 2005).

Vegetation, compost amendment and inoculation led to decreased total hydrocarbon levels, however, inoculation with degrading strains had the greatest effect on contaminant degradation. Maximum hydrocarbon removal (57%) was observed with both *Pantoea* strains. Strain BTRH79 performed best in combination with Italian ryegrass, whereas strain ITSI10 showed highest degradation in combination with birdsfoot trefoil (47%). The higher degradation in vegetated than in non-vegetated soil may be explained by either better colonization of the inoculant strains due to the presence of the plant and/or due to the stimulation of the degradation activity by root exudates or a better aerated environment (Juhanson *et al.* 2007; Truu *et al.* 2007). Our results are in agreement with previous studies (Gunther *et al.* 1996; Kaimi *et al.* 2006; Kaimi *et al.* 2007) reporting a better performance of inoculant strains in planted than in unplanted soils.

Inoculation of contaminated sites with degrading microbes is a promising strategy to improve phytoremediation processes. However, inoculant strains are frequently rapidly

out-competed by the natural microflora, which is generally better adapted to its environment (Cunningham *et al.* 1996). Consequently, the competitive ability of inoculants strains for colonization is besides their degradation capacity an important criterion. The ability of an inoculant strain to degrade the contaminant as well as to tightly associate with the plant might positively influence its competition against the resident microflora. In our study, we were interested in identifying strains with both, high degradation and colonization capacity. Cultivation-dependent and -independent analysis confirmed that the best degrading strains, i.e. ITSI10 and BTRH79, competed well in non-sterilized soils. In general, both strains colonized well the rhizosphere as well as the plant interior of both plants. However, strain ITSI10, which showed higher hydrocarbon degradation in combination with birdsfoot trefoil, also colonized this plant better than Italian ryegrass. In addition, the strain was more successful in colonizing the root and shoot interior of birdsfoot trefoil than colonizing Italian ryegrass endophytically. Similarly, strain BTRH79 performing better in combination with Italian ryegrass colonized well the rhizosphere and in particular the endosphere of that plant. We assume that the ability of a strain to colonize a plant endophytically correlates with its ability to associate with that plant as efficient rhizosphere colonization is a requirement for endophytic colonization (Compant *et al.* 2010). Our data further indicate that the colonization of inoculated strains is influenced by the plant, probably because plants favour the establishment of specific bacteria (Kowalchuk *et al.* 2002). Generally, the establishment of inoculated strains correlated with degradation rates. Although the resident microflora was able to reduce hydrocarbon levels to some extent, the inoculant strains had the ability to establish well in the plant environment and to degrade the

contaminant more efficiently. Our results indicate that both, ITSI10 and BTRH79 are good competitors, however, additional data are required to predict their performance with other plant species or soil environments.

It is known that different plants select distinct microbial communities (Kowalchuk *et al.* 2002; Kuske *et al.* 2002), but little is known about the functional diversity of plant-associated microbial communities and plant-specific effects on alkane degraders. Most studies on alkane degrading communities have relied on cultivation (Prince 1993; Lloyd-Jones *et al.* 1999) and cultivation-independent approaches have been rarely applied. In this study we considered only better known alkane hydroxylases involved in aerobic oxidation processes, and did not take potential alternative alkane degradation pathways including anaerobic processes into account. We found various alkane hydroxylase genes despite the fact that we used an agricultural soil, which was spiked with diesel, and not a soil, which has been contaminated for a long time. This is in agreement with Kloos *et al.* (2006), who reported that alkane-degrading bacteria are wide-spread in many, also uncontaminated environments. Cultivation-independent analysis revealed that Italian ryegrass and birdsfoot trefoil, both sampled at flowering, hosted distinct alkane-degrading communities. In association with both plants, *alkB*- as well as CYP153-containing microorganisms were detected, but different subtypes of alkane degradation genes were encountered. Similarly, distinct alkane degrading bacteria colonized the rhizosphere and root interior. Endophytes usually derive from the rhizosphere environment, however, due to distinct growth conditions different populations establish (Compant *et al.* 2010). Interestingly, a higher diversity (richness) of endophytic alkane degraders was encountered in the root and shoot interior than in the rhizosphere. The

expression of *alkB* genes of bacteria colonizing the root and shoot interior has been previously shown and was also increased when plants were grown in a diesel-containing soil (Andria *et al.* 2009). Although this indicates an active role of alkane degrading endophytes in degradation, it is not clear whether they contribute substantially to the degradation process or whether they are important in reducing the toxicity of hydrocarbons taken up by the plant.

Rhizosphere soils of Italian ryegrass and birdsfoot trefoil grown without compost amendment hosted distinct *alkB* gene-containing communities, however, they were similar in treatments receiving compost. It is therefore likely that the applied compost contained *alkB*-containing bacteria, which established well in the rhizosphere of both plants. In compost alkane-degrading bacteria are likely to be present and to be involved in degradation processes of plant litter. However, our results indicated that these strains were presumably not able to enter plants or to establish within plants as endophyte populations of plants grown in compost-treated soil were distinct. In agreement with this, sequence analysis of *alkB* genes showed that sequences clustered in two groups. Group 2 contained only sequences derived from the Italian ryegrass rhizosphere, whereas group 1 comprised sequences derived from the birdsfoot trefoil rhizosphere and sequences derived from Italian ryegrass grown in soil treated with compost possibly including strains derived from compost (grouping in group 1). Compost amendment did not affect bacterial communities containing the CYP153 gene associated with Italian ryegrass but did influence those associated with birdsfoot trefoil. This indicates that strains present in compost may have established in the rhizosphere of birdsfoot trefoil but not in that of Italian ryegrass.

In conclusion, alkane-degrading strains were selected, which were able to degrade hydrocarbon concentrations in a contaminated soil. In particular, the combination between vegetation, inoculation with well performing strains and compost amendment was the most efficient approach to reduce pollutant levels. Two strains, *Pantoea* sp. ITSI10 and BTRH79, established well in the plant environment despite the presence of a various other indigenous alkane-degrading bacteria. We suggest that the application of degrading bacterial strains, which are able to compete with the native microflora and to tightly associate with plants, is a promising strategy for the phytoremediation of polluted soils.

Acknowledgements

The authors would gratefully acknowledge the Higher Education Commission of Pakistan for financial support. We also thank Anton Grahsl for help with the greenhouse experiment and Levente Bodrossy, Yao Pan and Stefan Pfeiffer for discussions about phylogenetic analysis.

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Table 1. Dry shoot weight of Italian ryegrass (IT) and birdsfoot trefoil (BT)

		Dry shoot weight (g)			
Plant	**Inoculant strain**	**With compost**		**Without compost**	
		Sterile soil	**Non-sterile soil**	**Sterile soil**	**Non-sterile soil**
IT	Uninoculated	8.60±1.54^{cde}	9.10±1.50^{cde}	7.80±1.37^{e}	8.50±2.18^{de}
	Pantoea sp. ITSI10	12.00±2.24^{a-d}	13.20±2.37^{a}	9.40±1.76^{b-e}	10.70±2.08^{a-e}
	Pseudomonas sp. ITRI15	11.10±2.12^{a-e}	13.00±1.78^{ab}	10.20± 1.85^{a-e}	11.00±0.50^{a-e}
	Pseudomonas sp. ITRH76	11.50±2.59^{a-e}	13.00±1.97^{ab}	9.90±1.60^{a-e}	11.10±0.75^{a-e}
	Pantoea sp. BTRH79	12.30±1.94^{abc}	13.20±2.01^{a}	10.20±2.16^{a-e}	11.40±2.18^{a-e}
BT	Uninoculated	15.90±2.45^{c}	16.70±1.43^{bc}	ND	ND
	Pantoea sp. ITSI10	20.70±2.90^{ab}	21.70±2.34^{a}	ND	ND
	Pseudomonas sp. ITRI15	16.30±2.40^{bc}	17.70±2.99^{abc}	ND	ND
	Pseudomonas sp. ITRH76	18.70±2.96^{abc}	21.90±2.07^{a}	ND	ND
	Pantoea sp. BTRH79	18.80±2.10^{abc}	21.30±1.81^{a}	ND	ND

Data are means (n=3) ± SD.
'ND' not determined.
Means with different letters are significantly different at a 5 % level of significance.

Table 2. Hydrocarbon concentrations in soil vegetated with Italian ryegrass (IT), birdsfoot trefoil (BT) and unvegetated (UV) soils

			Hydrocarbon concentration (g kg^{-1} soil)			
Plant	**Inoculant strain**	**Initial value**	**With compost**		**Without compost**	
			Sterile soil	**Non-sterile soil**	**Sterile soil**	**Non-sterile soil**
IT	Uninoculated	10	9.21 ± 0.92^{p}	8.17 ± 0.31^{no}	9.36 ± 0.14^{p}	8.82 ± 0.64^{op}
	Pantoea sp. ITSI10	10	$5.86 \pm 0.34^{b-e}$	$5.85 \pm 0.79^{b-e}$	$7.1 \pm 0.19^{h-m}$	$6.85 \pm 0.44^{e-m}$
	Pseudomonas sp. ITRI15	10	5.37 ± 0.29^{bc}	$6.14 \pm 0.51^{b-h}$	$6.92 \pm 0.14^{f-m}$	$7.09 \pm 0.21^{h-m}$
	Pseudomonas sp. ITRH76	10	$6.09 \pm 0.04^{b-h}$	5.47 ± 0.28^{bc}	$6.51 \pm 0.36^{d-k}$	$7.40 \pm 0.10^{j-n}$
	Pantoea sp. BTRH79	10	$5.93 \pm 0.39^{b-f}$	4.35 ± 0.21^{a}	$6.32 \pm 0.10^{c-i}$	5.67 ± 0.35^{bcd}
BT	Uninoculated	10	$9.07 \pm 0.30^{o-p}$	$7.45 \pm 0.05^{k-n}$	$8.78 \pm 0.62^{o-p}$	$7.54 \pm 0.23^{k-n}$
	Pantoea sp. ITSI10	10	5.52 ± 0.29^{bcd}	5.15 ± 0.51^{ab}	ND	ND
	Pseudomonas sp. ITRI15	10	$6.28 \pm 0.10^{c-i}$	$6.16 \pm 0.81^{b-h}$	ND	ND
	Pseudomonas sp. ITRH76	10	$6.14 \pm 0.33^{b-h}$	$6.04 \pm 0.22^{b-g}$	ND	ND
	Pantoea sp. BTRH79	10	$6.12 \pm 0.79^{b-h}$	5.50 ± 0.28^{bcd}	ND	ND
UV	Uninoculated	10	9.37 ± 0.16^{p}	$9.09 \pm 0.12^{o-p}$	9.29 ± 0.12^{p}	9.19 ± 0.44^{p}
	Pantoea sp. ITSI10	10	$6.86 \pm 0.53^{e-m}$	$6.71 \pm 0.55^{e-l}$	7.56 ± 0.35^{lmn}	$7.37 \pm 0.03^{j-n}$
	Pseudomonas sp. ITRI15	10	$7.23 \pm 0.13^{i-n}$	$6.98 \pm 0.26^{g-m}$	7.67 ± 0.59^{lmn}	7.57 ± 0.64^{lmn}
	Pseudomonas sp. ITRH76	10	$6.39 \pm 0.25^{c-j}$	$6.79 \pm 0.40^{e-l}$	$7.04 \pm 0.67^{g-m}$	$7.45 \pm 0.14^{k-n}$
	Pantoea sp. BTRH79	10	$7.36 \pm 1.85^{j-n}$	$6.71 \pm 0.34^{e-l}$	7.86 ± 0.48^{mn}	$6.73 \pm 1.32^{e-l}$

IT*= harvested 90 days after germination
BT*= harvested 155 days after germination
Data are means (n=3) ± SD
'ND' indicates not determined
Means with different letters are significantly different at a 5 % level of significance.

Supplementary Information Table 1. IGS types of bacterial strains isolated from rhizosphere, root and shoot interior of non-sterilized soil with compost, planted with IT and BT, uninoculated and inoculated with ITSI10 and BTRH79.

IGS Type	16S rRNA gene similarity (NCBI accession number / %)	Phylogenetic group	*alk* gene type
IGS-1	*Pseudomonas sp.* hyss58 (FJ613311 / 99)	*Gammaproteobacteria*	PpalkB
IGS-2	*Stenotrophomonas* sp. (AY259519 / 99)	*Gammaproteobacteria*	PpalkB
IGS-3	Uncultured *Rhizobium* sp. (EU375051 / 99)	*Alphaproteobacteria*	PpalkB
IGS-4	*Pantoea agglomerans* (DQ122346 / 99)	*Gammaproteobacteria*	PpalkB
IGS-5	*Enterobacter ludwigii* (AJ853891/ 97)	*Gammaproteobacteria*	Cyp450
IGS-6	Uncultured *Sphingobium* sp. (FJ193550 / 99)	*Alphaproteobacteria*	-
IGS-7	*Pseudomonas* sp. A_wp02262 (AY214348 / 93)	*Gammaproteobacteria*	PpalkB
IGS-8	*Novosphingobium* sp. Rr 2-17 (EU984513 / 98)	*Alphaproteobacteria*	Cyp450
IGS-9	Uncultured *Pseudomonas* sp. (AY834319 / 99)	*Gammaproteobacteria*	-
IGS-10	*Pantoea agglomerans* strain (DQ122346 / 99)	*Gammaproteobacteria*	Cyp450
IGS-11	*Pseudomonas* sp. DVS6dlb (AY864639 / 99)	*Gammaproteobacteria*	Cyp450
IGS-12	*Pantoea* sp. (DQ122350 / 97)	*Gammaproteobacteria*	-
IGS-13	*Arthrobacter* sp. 16.22 (DQ157987 / 99)	*Actinobacteria*	Cyp450
IGS-14	*Pseudomonas* sp. TUT1394 (AB308445 / 97)	*Gammaproteobacteria*	PpalkB
IGS-15	*Pseudomonas anguilliseptica* (AM263520 / 99)	*Gammaproteobacteria*	Cyp450
IGS-16	*Rhizobium* sp. 28/2 (DQ310471 / 100)	*Alphaproteobacteria*	-
IGS-17	*Rhodococcus koreensis* strain (EF010629 / 99)	*Actinobacteria*	Cyp450
IGS-18	Uncultured *Sphingobium* sp. (FJ193550 / 99)	*Alphaproteobacteria*	-
IGS-19	*Bacillaceae bacterium* C19 (AY504445 / 99)	*Firmicutes*	Cyp450
IGS-20	*Alcaligenes xylosoxidans* (AJ491846 / 99)	*Betaproteobacteria*	-
IGS-21	*Pseudomonas sp.* (FJ015027 / 96)	*Gammaproteobacteria*	-
IGS-22	*Pseudomonas fluorescens* strain (GU367870 / 98)	*Gammaproteobacteria*	-
IGS-23	*Uncultured bacterium* (EU515727 / 93)	-	PpalkB
IGS-24	*Uncultured Sphingobium sp.* (FJ193550 / 99)	*Alphaproteobacteria*	Cyp450
IGS-25	*Rhizobium sp.* HGR6 (GQ483458 / 99)	*Alphaproteobacteria*	-
IGS-26	*Novosphingobium* sp. NIY3 (AB360760 / 99)	*Alphaproteobacteria*	Cyp450
IGS-27	*Microbacterium hydrocarbonoxydans* (AJ698726 / 100)	*Actinobacteria*	-
IGS-28	*Gordonia amicalis* strain LH3 (EF424581 / 99)	*Actinobacteria*	PpalkB/Cyp450
IGS-29	*Microbacterium* sp. Atl-19 (EF028128 / 98)	*Actinobacteria*	-
IGS-30	*Cellulomonas* sp. (AM403591 / 99)	*Actinobacteria*	-

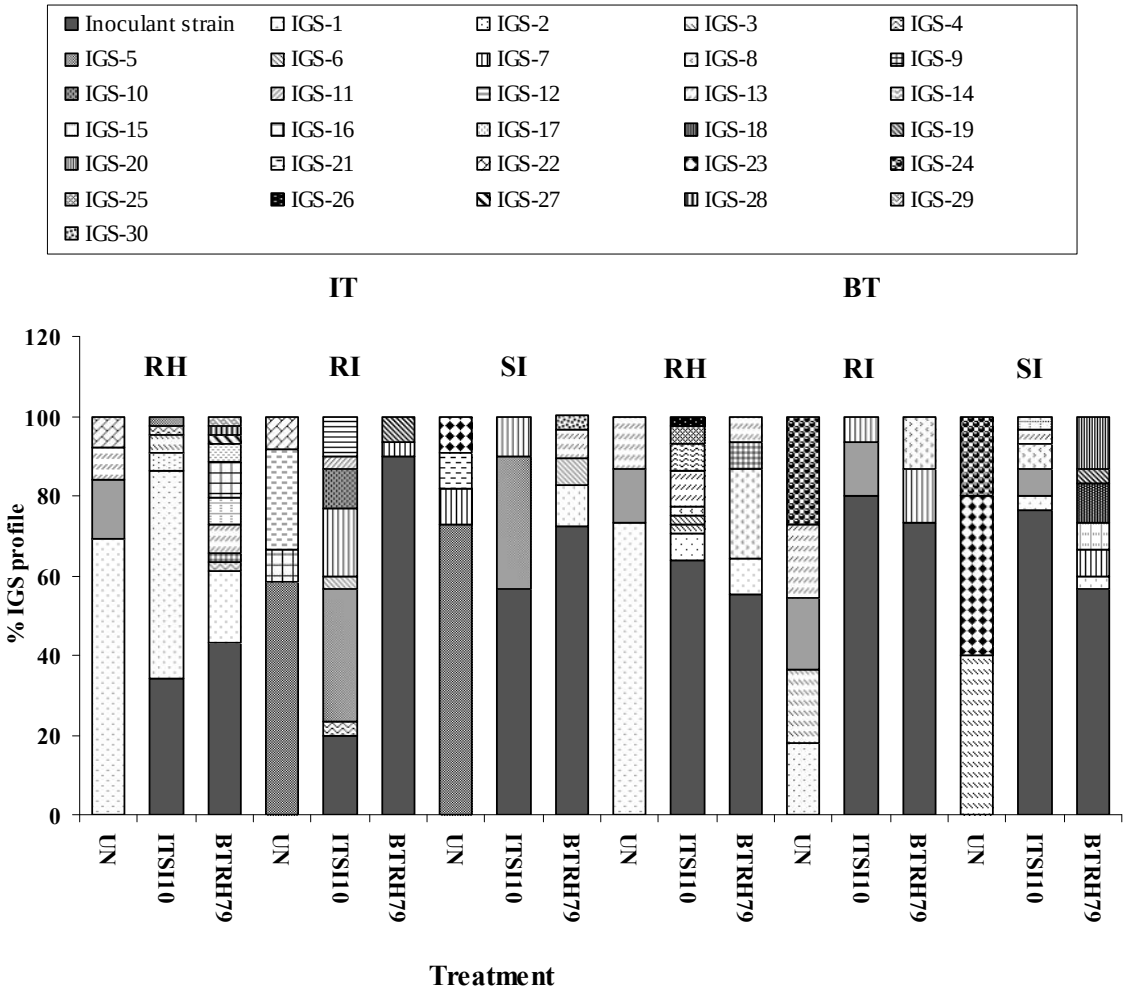

Fig 1. Persistence of inoculant strains (ITSI10 and BTRH79) applied onto Italian ryegrass (IT) and birdsfoot trefoil (BT), grown in non-sterilized soil, analysed by cultivation-dependent analysis. Rhizosphere (RH), root interior (RI), shoot interior (SI).

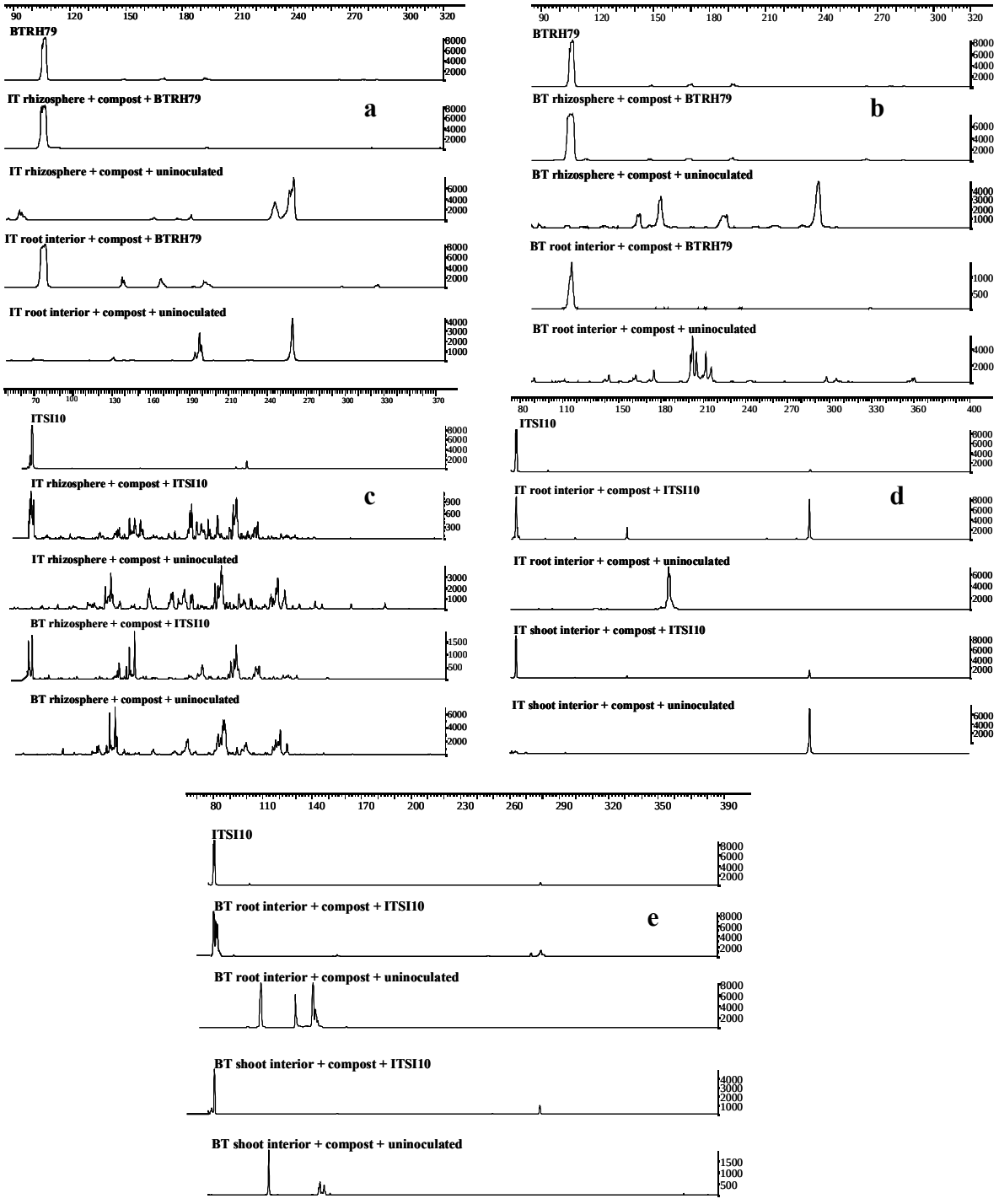

Fig. 2 (a) Electropherograms of CYP153-based T-RFLP fingerprints of microbial communities found in the rhizosphere and root interior of Italian ryegrass inoculated with BTRH79; **(b)** Electropherograms of CYP153-based T-RFLP fingerprints of microbial

communities found in the rhizosphere and root interior of birdsfoot trefoil inoculated with BTRH79; **(c)** Electropherograms of 16S rRNA gene-based T-RFLP fingerprints of microbial communities found in the rhizosphere of Italian ryegrass and birdsfoot trefoil inoculated with ITSI10; **(d)** Electropherograms of 16S rRNA gene-based T-RFLP fingerprints of microbial communities found in the root interior of Italian ryegrass inoculated with ITSI10; **(e)** Electropherograms of 16S rRNA gene-based T-RFLP fingerprints of microbial communities found in the root interior of birdsfoot trefoil inoculated with ITSI10**.**

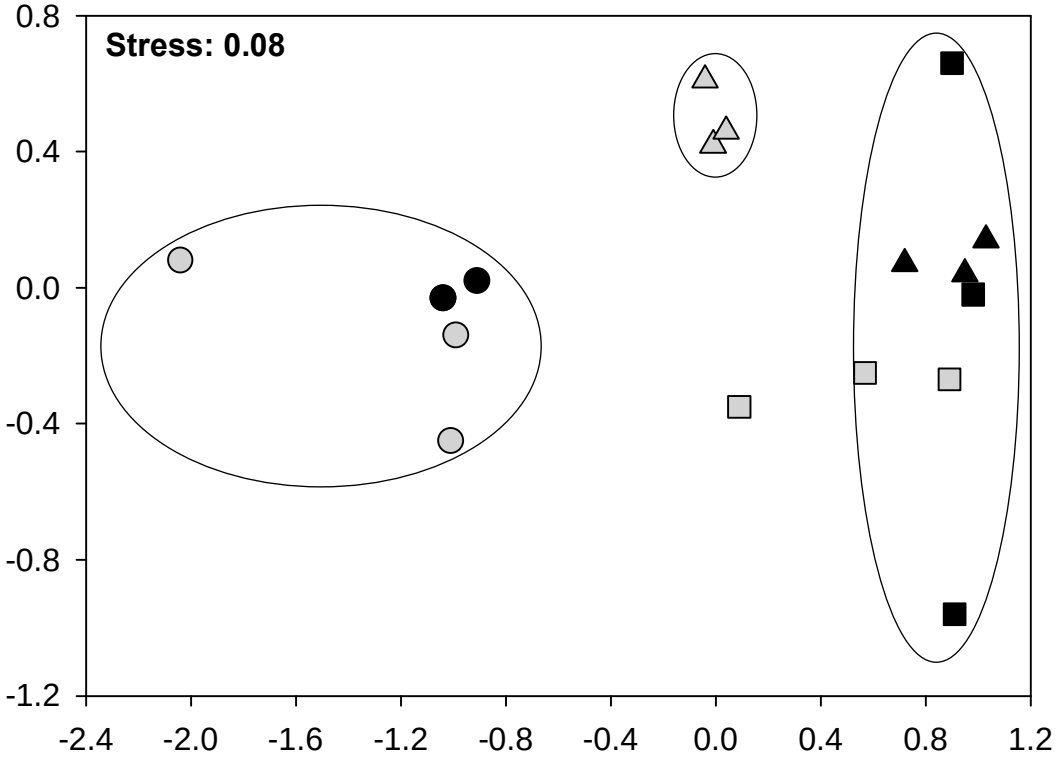

Fig 3. MDS ordering of data generated from *alkB* T-RFLP profiles obtained from soil and root samples collected from Italian ryegrass and birdsfoot trefoil grown in non-sterilized and uninoculated soil, ▲ IT rhizosphere without compost, ▲ IT rhizosphere with compost, ■ BT rhizosphere without compost, ■ BT rhizosphere with compost, ● IT root interior without compost, ● IT root interior with compost

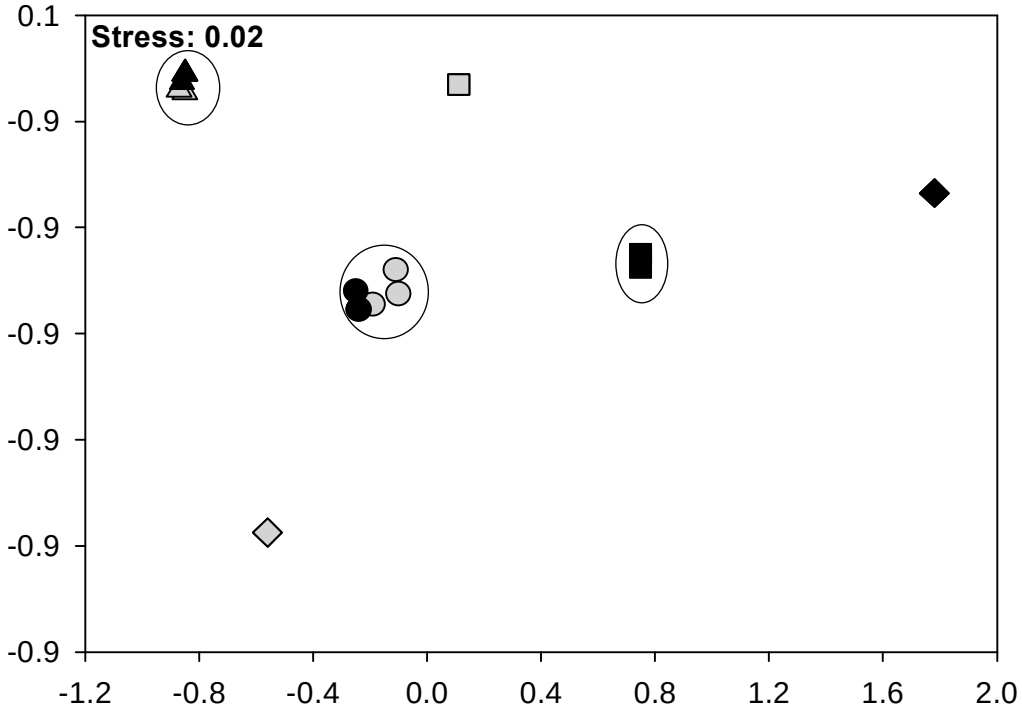

Fig. 4. Multidimensional scaling of data generated from CYP153 *alk* gene T-RFLP profiles obtained from soil and root samples, collected from of Italian ryegrass and birdsfoot trefoil, grown in non-sterilized, uninoculated soil. ▲ IT rhizosphere without compost, ▲ IT rhizosphere with compost, ■ BT rhizosphere without compost, ■ BT rhizosphere with compost, ● IT root interior without compost, ● IT root interior with compost, ♦ BT root interior without compost, ♦ BT root interior with compost

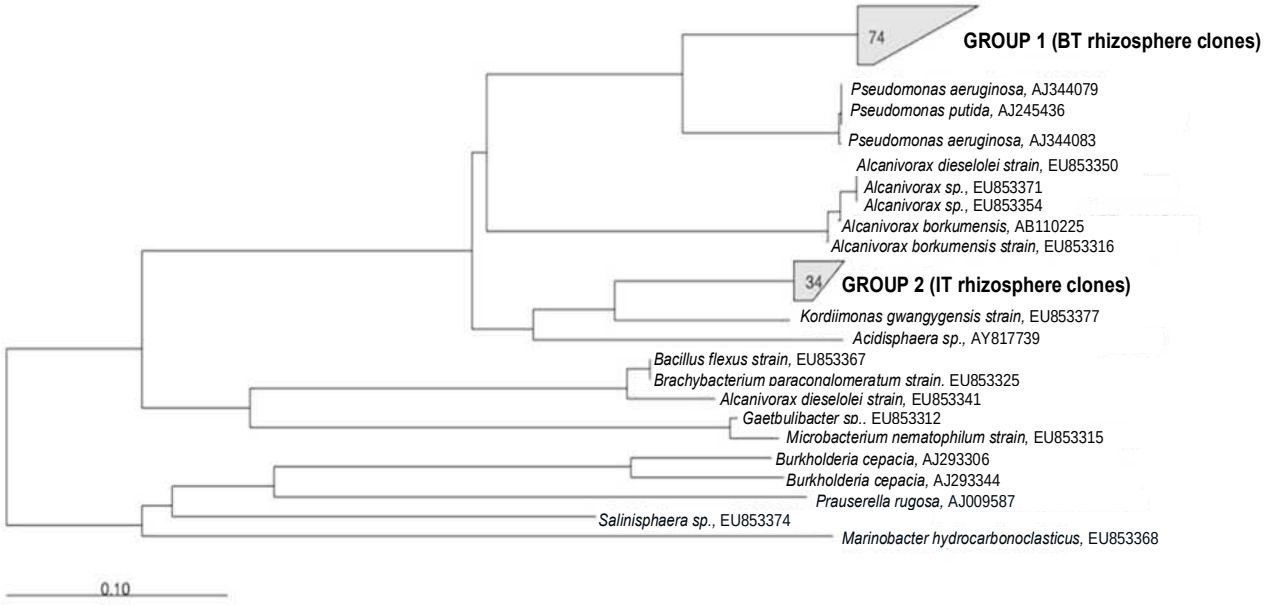

Fig. 1 Supplementary Information. Neighbour-joining phylogenetic tree showing diversity of *alkB* genes in the rhizosphere of Italian ryegrass and birdsfoot trefoil grown in non-sterilized, uninoculated soil. Nucleotide sequences derived from the cloned PCR products obtained by cultivation-independent analysis, from rhizosphere isolates as well as from published sequences. Sequences from the database are labelled with name of the strain and GenBank accession number.

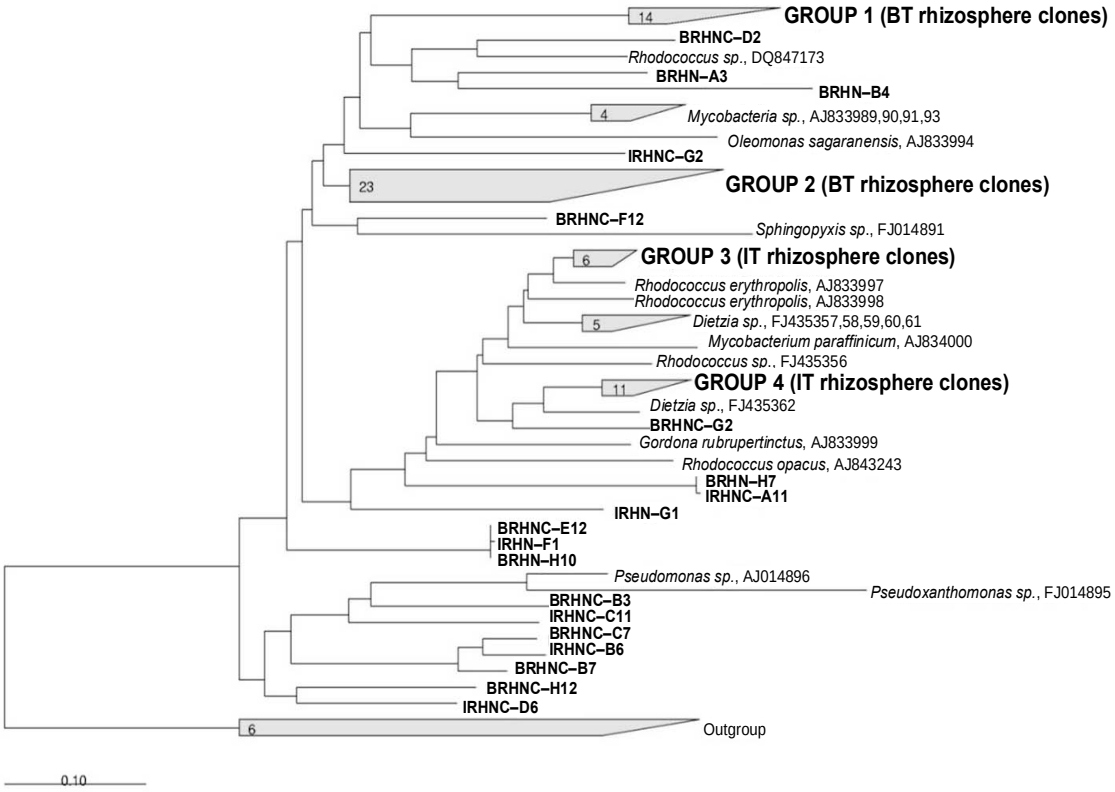

Fig. 2 Supplementary Information. Neighbour-joining phylogenetic tree of CYP153 genes in rhizosphere of Italian ryegrass and birdsfoot trefoil grown in non-sterilized, uninoculated soil. Nucleotide sequences derived from the cloned PCR products obtained by cultivation-independent analysis, from rhizosphere isolates as well as from published sequences. Sequences from the database are labelled with name of the strain and GenBank accession number. The clone sequences from this study are shown bold and abbreviated as IRHN (IT rhizosphere without compost), IRHNC (IT rhizosphere with compost), BRHN (BT rhizosphere without compost) and BRHNC (BT rhizosphere with compost).

Chapter 3

Hydrocarbon degradation, plant colonization and gene expression of alkane degradation genes by endophytic *Enterobacter ludwigii* strains

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Journal: Environmental Pollution

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Running Head: Hydrocarbon degradation by *Enterobacter ludwigii* strains

***Submitted to:** Environmental Pollution

Abstract

The genus *Enterobacter* comprises a range of beneficial plant-associated bacteria showing plant growth promotion and/or biocontrol activity. *Enterobacter ludwigii* belongs to the *Enterobacter cloacae* complex and has been reported to include plant-associated strains with plant growth-promoting and biocontrol capacities. To assess the role of *Enterobacte*r endophytes in hydrocarbon degradation, plant colonization, abundance and expression of CYP153 genes in different plant compartments, three plant species (Italian ryegrass, birdsfoot trefoil and alfalfa) were grown in sterile soil spiked with 1% diesel and inoculated with three endophytic *Enterobacter ludwigii* strains. Results showed that all strains were capable of hydrocarbon degradation and efficiently colonized the rhizosphere and plant interior. Two strains, ISI10-3 and BRI10-9, showed highest degradation rates of diesel fuel up to 68% and performed best in combination with Italian ryegrass and alfalfa. All strains principally expressed the CYP153 gene in all plant compartments, indicating an active role in degradation of diesel in various plant compartments.

Keywords: *Enterobacter ludwigii*, endophytes, hydrocarbon degradation, gene abundance, gene expression

Introduction

Plants interact with a great diversity of microorganisms, including enteric bacteria. These interactions, which are lined by the characteristics of both, host plant and bacteria, result in associative, commensal, symbiotic, or parasitic relationships between both partners. Members of the *Enterobacteriaceae* are distributed in many environments, with some being saprophytes and others being parasites of plants and animals. Several studies have shown that *Enterobacteriaceae* may have beneficial effects on plant development when they are associated with plants (Lodewyckx et al., 2002; Taghavi et al., 2009). They may improve plant growth via nitrogen fixation, suppression of plant pathogens and production of phytohormones and enzymes involved in the metabolism of growth regulators such as ethylene, 1-aminocyclopropane 1-carboxylic acid (ACC), auxins and indole-3-acetic acid (IAA) (Gyaneshwar et al., 2001; Kämpfer et al., 2005; Taghavi et al., 2009). Organisms such as *Enterobacter radicincitans*, *E. arachidis*, *E. oryzae*, and *Enterobacter* sp. CBMB30, which were isolated from the wheat phyllosphere, groundnut rhizosphere, poplar and rice endosphere, respectively, are known as plant growth-promoting bacteria (Lee et al., 2006; Peng et al., 2009; Taghavi et al., 2009; Madhaiyan et al., 2010).

In previous experiments we repeatedly isolated *Enterobacter*-related strains from the rhizosphere and endosphere of plants (Italian ryegrass and birdsfoot trefoil) grown in diesel-contaminated soils (Yousaf et al., 2010a). Further characterization revealed that several strains belong to *Enterobacter ludwigii*. This species is known for its clinical relevance as most isolates have been isolated from clinical specimens (Hoffmann et al., 2005). *E. ludwigii* belongs to the *E. cloacae* complex, which has been frequently isolated

from nosocomial infections; however, it is not clear whether *E. ludwigii* is a true pathogen or has a rather commensal character (Paauw et al., 2008). Generally, few studies on *E. ludwigii* are available, but it has been reported as a plant-associated bacterium with plant growth-promoting and biocontrol capacities (Shoebitz et al., 2009).

Global industrialization over the past years has resulted in numerous sites with strong contamination of the soil with persistent organic and inorganic contaminants. Aliphatic hydrocarbons (e.g. diesel fuel and engine oils) make up a substantial proportion of substances found at contaminated sites (Stroud et al., 2007). The use of plants and their associated microorganisms for the treatment of hydrocarbon-contaminated soils has attained increasing acceptance as a viable clean-up technology (Lelie et al., 2001). The efficiency of a phytoremediation process depends mainly on the presence and activity of plant-associated microorganisms carrying degradation genes required for the enzymatic break-down of contaminants. The rhizosphere and plant endosphere have been reported to host pollutant-degrading bacteria (Siciliano et al., 2001; Andria et al., 2009;) and highly diverse alkane degrading bacteria containing alkane degrading genes have been isolated from the plant environment (Kaimi et al., 2007). Expression analysis of alkane monooxygenase (*alkB*) and a cytochrome P450 hydroxylase (CYP153 gene) indicated degradation in the rhizosphere as well as in the plant interior (Powell et al., 2006; Andria et al., 2009; Afzal et al., 2011).

In this study we characterized in detail selected alkane degrading *Enterobacter* strains, which were previously isolated from Italian ryegrass and birdsfoot trefoil (Yousaf et al., 2010a) and identified as *E. ludwigii*. In plant experiments, we studied in detail the hydrocarbon degradation and plant colonization capacities of these strains.

Materials and Methods

Characterization of bacterial strains

Three strains, IRI10-4, BRI10-9 (root endophytes) and ISI10-3 (shoot endophyte), which were previously isolated from Italian ryegrass (IRI10-4, ISI10-3) and birdsfoot trefoil (BRI10-9) (Yousaf et al., 2010a) were selected. These strains have the capacity to degrade alkanes and contain a cytochrome P450 type alkane hydroxylase (CYP153) gene. Almost complete 16S rRNA sequences were determined for selected strains using the primers and conditions described by Coenye et al. (1999). Based on 16S rRNA gene phylogenetic analysis these strains were considered to belong to the *Pantoea* — *Enterobacter* clade. In order to provide stronger support for the description of these strains, *rpoB* gene sequence analysis was performed using the primers and conditions as described by Brady et al. (2008).

Phylogenetic analysis was done as described by Brady et al. (2008). Briefly, the sequences were aligned using CLUSTAL_X (Thompson et al., 1997) and overhangs were trimmed. The program MODELTEST 3.7 (Posada and Crandall, 1998) was then applied to the datasets to determine the best-fit evolutionary model. Maximum-likelihood and neighbour-joining analyses were performed using Phyml (Guindon and Gascuel, 2003) and PAUP 4.0b10 (Swofford, 2000), respectively, by applying the models and parameters determined by MODELTEST.

ACC deaminase activity

ACC deaminase activity of the bacterial strains was tested on minimal medium containing 0.7g ACC L^{-1} as sole nitrogen source, as described by (Kuffner et al., 2008).

Plant experiment

For the plant experiment three sets of pots were prepared in triplicate: (1) pots planted with Italian ryegrass (IT) (*Lolium multiflorum* var. Taurus), (2) pots planted with birdsfoot trefoil (BT) (*Lotus corniculatus* var. Leo) and (3) pots planted with alfalfa (AL) (*Medicago sativa* var. Harpe). Agricultural soil (agricultural top soil from Seibersdorf, Lower Austria, Austria; pH 7.4, 27 g sand kg^{-1}, 621 g silt $kg^{-1,}$ 352 g clay kg^{-1}, 2.4 g C_{org} kg^{-1}) was sterilized by 30 kGy γ-radiation and amended with 10% compost. The sterility of sterilized soil was checked by plating soil suspensions on Tryptic Soy Agar (Merck) plates, no growth was observed. Before sowing, soil was amended with filter-sterilized diesel fuel (10,000 mg kg^{-1} soil). Pots with dimensions 13 x 13 x 13 cm were filled with spiked soils and subsequently placed in the greenhouse. Pots were arranged in a completely randomized block design. Seeds of IT, BT and AL were surface sterilized by soaking in 5% sodium hypochlorite solution for 2 min, then in 70% ethanol for 2 min, and were then washed with sterile water for 3 times. Surface-sterilized seeds (200 per pot) were sown. One week after seed germination, plants were thinned to 170 per pot and each pot was inoculated with 100 ml inoculant suspension (app. 10^9 CFU ml^{-1}, cultivated in Luria Bertani broth at 30°C, centrifuged and resuspended in 0.9% (w/v) NaCl) containing one of the strains described above. For control treatments, spiked soil was treated with 100 ml of 0.9% NaCl instead of inoculum suspension. Plants were grown at 25°C in the greenhouse (16 h light / 8 h dark) and watered with equal amounts when needed.

Plants were harvested at two growth stages. First harvest was done after 42 days of seed germination and second harvest at flowering (IT 102 days after germination, BT and

AL 150 days after germination). Plants were cut 2 cm above ground and remaining plants were harvested to obtain root and rhizosphere samples. Plant biomass was determined. After the plants were removed from the pots and roots separated from bulk soil, the soil from each pot was thoroughly mixed to obtain homogenized samples for hydrocarbon extraction. These soil samples were then stored at -80°C until further analysis.

Hydrocarbon analysis of soil samples

Total hydrocarbon content (THC) of the soil was measured employing infrared spectroscopy as described previously (Yousaf et al., 2010a).

Detection and enumeration of inoculant strains

The rhizosphere soil was collected by gently sampling the soil closely attached to root surface. Subsequently, roots and shoots were carefully washed and surface sterilized as described by Yousaf et al. (2010a), replacing distilled sterile water by DEPC-treated water. The efficacy of surface sterilization was checked by plating shoots and roots, and aliquots of a final rinse on LB plates, no colonies were observed after 3 days of incubation, ensuring the surface sterilization efficiency.

For the isolation of alkane degrading rhizosphere bacteria, the soil slurry was prepared by mixing 5 g soil with 15 ml of 0.9% (w/v) NaCl solution, agitated (180 rpm) for 1 hour at 30^{o}C. After the settlement of soil particles, serial dilutions up to 10^{-4} were spread onto solid Minimal Basal medium (MBM) (Alef, 1994) containing 1% (v/v) filter-sterilized diesel. For the isolation of endophytes, 3 g of surface sterilized roots or shoots were homogenized with a pestle and mortar in 12 ml NaCl solution (0.9%, w/v). The

homogenized material was agitated for 1 hour at 30°C. After settling of solid material, serial dilutions up to 10^{-3} were spread on MBM containing 1% (v/v) filter-sterilized diesel. Bacterial colonies on each plate were selected randomly and transferred to solid MBM amended with 2% (v/v) filter-sterilized diesel followed by incubation at 30°C for 4 days. Thirty colonies of each treatment were randomly selected and their identity with the inoculant strain was confirmed by restriction fragment length polymorphism (RFLP) analysis of the 16S-23S rRNA intergenic spacer region (IGS) (Rasche et al., 2006a). Isolates and inoculant strains had identical restriction patterns.

Extraction of DNA and RNA

DNA from rhizosphere soil (0.5 g) was extracted by using FastDNA® Spin Kit for Soil (MP Biomedicals, Solon, Ohio, USA), whereas RNA was isolated with RNA Power Soil Total RNA isolation Kit (MO Bio Laboratories) as described by the manufacturer, and was quantified photometrically (Nanodrop ND-1000, Nanodrop Technologies, Wilmington, DE, USA). 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 the FastDNA® Spin Kit for Soil (MP Biomedicals, Solon, Ohio, USA) was used. RNA was isolated by using RNEASY Plant Mini Kit (Qiagen). 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 16S rDNA (Rasche et al., 2006b).

Quantitative analysis of the abundance and expression of CYP153 genes

Reverse transcription was performed with 150-200 ng RNA, the specific primer P450rv3 (van Beilen et al., 2006) and Omniscript Reverse Transcriptase (Qiagen) according to the manufacturer's instructions. Abundance and expression of CYP153 genes were quantified by real-time PCR using an iCycler IQ (Biorad). Standards for qRT-PCR were generated by serial dilution of stocks containing purified CYP153 plasmid from a clone. The number of copies of the target gene in a ng plasmid DNA was determined, and then a serial dilution was prepared from 10^8 to 10^1 copies to use as an external standard curve (r^2 > 0.95), allowing determination of the number of copies of the gene in each sample of DNA and cDNA. Analyses were performed in triplicate and gene copy numbers were calculated as described by Powell et al. (2006). Reaction mixtures (25 µl) contained 5 µl of Q Mix (Evergreen), 2.5 µl 10mg/ml BSA, 1 µl DMSO, 2.6 µl 5 µM of each primer, 50-100 ng of DNA/cDNA template and RNase free water. Thermal cycling conditions were: 3 min 95°C followed by 40 cycles of 95°C for 25 s, 58°C for 25 s, 72°C for 45 s followed by a melting curve from 50 to 100°C. Besides melting curve analysis, PCR products were examined on 2% agarose gels. No primer dimers were detected.

To test possible inhibitory effects on quantitative PCR amplification caused by co-extracted humic substances, the optimal dilution for each DNA/cDNA extract was determined by pre-experiments (data not shown). CYP153 gene copy numbers were quantified relative to a standard curve of a positive control and were normalized to the copy number of control plants. Statistical analysis was based on Duncan's multiple range test using SPSS software package (SPSS Inc., Chicago, IL).

Results

Characterization of hydrocarbon degrading strains

Fig. 1 shows the results from the phylogenetic analysis of the strains based on *rpoB* gene nucleotide sequence. The strains analyzed in this study were assigned to *E. ludwigii*. We used *rpoB* based sequences in order to provide stronger support for the description of taxonomic position of these strains, because on the basis of 16S rDNA phylogenetic tree, the taxonomic position of these strains was not clear (data not shown).

Hydrocarbon degradation

The effect of plants and inoculation on diesel fuel degradation was determined 6 weeks (first harvest of IT, BT and AL), 14 weeks for IT and 21 weeks for AL and BT (second harvest) after germination (Table 1). The degradation of hydrocarbons in soil with inoculation was significantly higher ($p<0.05$) than in uninoculated controls at both harvest times. At the first harvest the maximum decrease in hydrocarbon content was observed with strain ISI10-3 in combination with IT (48%) and with AL (40%), followed by BRI10-9 in combination with AL (38%). At the second harvest strain BRI10-9 showed maximum hydrocarbon degradation in combination with IT (68%). Strain ISI10-3 showed 65% hydrocarbon decrease in association with IT and 60% with AL. Generally, strains ISI10-3 and BRI10-9 showed higher hydrocarbon removal at both harvest times and IT performed better than AL and BT.

Plant biomass production

Results for shoot and root biomass of IT, AL and BT grown in contaminated and non-contaminated soil are shown in Table 2. Diesel contamination in soil had an inhibitive effect on plant growth. All three plant species produced less shoot and root biomass in soil when grown in the presence of diesel. Plant biomass was generally lower at the first harvest compared to the second harvest. Biomass production was significantly higher in inoculated treatments than in uninoculated contaminated treatments. More shoot biomass was produced in the inoculated treatments as compared to the control at the first harvest (56% compared to 34%) and second harvest (76% compared to 53%). Inoculation also led to significantly higher root biomass. Strains ISI10-3 and BRI10-9 led to significantly higher root and partly also shoot dry weight than strain IRI10-4, which correlates with the ACC deaminase activity found in the strains ISI10-3 and BRI10-9.

Cultivation-dependent analysis of colonization

Results from microbial plate counts are given in Table 3. The microbial numbers in rhizosphere soil were higher at the first harvest than at the second harvest for all strains and plant combinations with exception of IRI10-4 and BRI10-9 in association with birdsfoot trefoil, where microbial numbers were lower at the first harvest than at the second harvest. At the first harvest, strain ISI10-3 colonized best and showed highest colonization (2.3×10^8 cells g^{-1} dry soil) in the rhizosphere of IT followed by AL. At the second harvest, the highest microbial numbers (4.5×10^7 cells g^{-1} dry soil) were observed for BRI10-9 in combination with IT followed by ISI10-3. These results clearly showed that strain ISI10-3, originally isolated from the shoot interior of Italian ryegrass, better

colonized the rhizosphere of IT, BT and AL at both harvest times. The second best rhizosphere colonizer was BRI10-9, originally isolated from the root interior of birdsfoot trefoil. The population size of inoculant strains in the rhizosphere ranged from 10^4 to 10^8 cells g^{-1} dry soil (first harvest) and from 10^5 to 10^7 cells g^{-1} dry soil at second harvest.

In the root interior, highest colonization was observed in the endorhiza of IT and BT. Strain IRI10-4, originally isolated from the root interior of Italian ryegrass, better colonized IT roots at the first harvest , whereas at the second harvest BT roots were better colonized. Microbial numbers ranged from 10^3 to 10^7 cells g^{-1} dry root at the first harvest and 10^4 to 10^7 cells g^{-1} dry root at the second harvest. All strains were capable of colonizing the shoot interior. Strain ISI10-3 (a shoot endophyte) showed significantly higher shoot colonization than other strains. Highest colonization was observed in the shoot interior of IT. Microbial numbers gradually increased from the first harvest to the second harvest time.

Quantification and expression of CYP153 genes

Real-time PCR of the CYP153 gene was used to quantify the population size of alkane degrading bacteria by a cultivation-independent analysis (Tables 4 and 5). Generally and in agreement with cultivation-based results, bacterial CYP153 gene abundance in the rhizosphere was highest at the first harvest (up to 1.1 x 10^9 copies g^{-1} dry soil) and decreased in all treatments towards the flowering stage. CYP153 gene abundance was lower in the endosphere and increased towards the second harvest (Table 4). Among different treatments, IT hosted the highest abundance of alkane degrading bacteria. Overall, the highest gene abundance at both harvest points, in the rhizosphere and shoot

interior, was observed with strain ISI10-3 and IT. However, in the root interior IRI10-4 showed significantly higher gene abundance with IT and BT at the first and at the second harvest time, respectively.

All strains principally expressed CYP153 genes in the rhizosphere and endosphere of all three plant species, indicating an active role in hydrocarbon degradation (Table 5). The differences between strains and plant species in regard to CYP153 gene expression followed essentially the same pattern as CYP153 gene abundance. The comparison between samples taken at different harvest times showed that the total number of bacteria, measured via CFU count and real-time PCR, decreased with time especially in the rhizosphere and root interior. Even though the gene expression also decreased with time, higher CYP153 expression was still observed in all plant compartments. The results showed that bacterial abundance and gene expression was affected by strain, plant type and plant environment. In BT and AL average activities were higher in endosphere than in the rhizosphere. Highest activities (transcripts / abundance) were calculated for ISI10-3 in combination with IT and BT as compared to other strains (Fig. 2). However, activity was generally depended on the strain and was affected by the plant and the sampling time.

Discussion

Recently, several studies have reported that human pathogens belonging to the *Enterobacteriaceae* such as *Salmonella enterica* and *Escherichia coli* may colonize plants (reviewed by Holden et al., 2009). Furthermore, plants frequently serve as hosts for many other enteric bacteria including *Erwinia, Pectobacterium, Pantoea* and

Enterobacter, which may colonize as epiphytes, endophytes and/or pathogens. The genus *Enterobacter* comprises a range of beneficial plant-associated bacteria showing plant growth promotion and/or biocontrol activity (Taghavi et al., 2009; Madhaiyan et al., 2010). However, various *Enterobacter* members, in particular bacteria belonging to the *E. cloacae* complex including *E. ludwigii*, are known for their potential pathogenicity to humans, although a commensal character for bacteria belonging to this complex except for *E. cloacae* has been suggested (Paauw et al., 2008). This is supported by the fact that *E. ludwigii* has not been isolated only from clinical samples but also from plants, where these strains showed plant growth promotion (Shoebitz et al., 2009). In this study we taxonomically characterized selected *Enterobacteriaceae* strains, which were isolated previously from Italian ryegrass and birdsfoot trefoil grown in a diesel-contaminated soil. Three strains (IRI10-4, ISI10-3 and BRI10-9) were characterized in detail and showed to belong to *E. ludwigii*.

As our strains showed hydrocarbon degradation activities in preliminary plate assays, we tested in this study, whether these *E. ludwigii* strains are able to degrade hydrocarbons in a soil environment or to colonize plants efficiently. To the best of our knowledge this is the first report of hydrocarbon degradation by *E. ludwigii*. We were particularly interested in strains, which were isolated from the plant interior, as they have several advantages for phytoremediation applications. Facultative endophytes generally can colonize the rhizosphere soil as well as the plant endosphere (Weyens et al., 2009). Furthermore, endophytes may protect plants against the inhibitory effects of high concentrations of hydrocarbon and may promote plant growth by e.g. reducing ethylene levels with ACC deaminase activity (Glick, 2003; Sheng et al., 2008). All strains we

tested showed substantial hydrocarbon degradation, however, strains showed different degradation capacities, although they all contained the same type of alkane hydroxylase gene. Generally, strains ISI10-3 and BRI10-9, showed higher degradation capacity than IRI10-4. The lower degradation activity correlated with plant colonization and the comparably low degradation of strain IRI10-4 can be explained by the low abundance, particularly in the rhizosphere and the shoot interior. These results are in agreement with our previous findings (Yousaf et al., 2010a), where we observed that those strains, which showed high hydrocarbon degradation rates, were also efficient colonizers.

Highest degradation was found with Italian ryegrass, although this plant was (due to its rapid growth) harvested seven weeks earlier than birdsfoot trefoil and alfalfa. This indicates that different plants stimulate degrading strains and degradation activity differently. The higher degradation with Italian ryegrass may be explained by enhanced stimulation of degradation activity by root exudates or a better aerated environment (Juhanson et al., 2007; Truu et al., 2007). Grasses have a fibrous root system, which can penetrate soils providing a large surface area for bacteria to colonize. Consequently, generally more bacterial cells were found to be associated with Italian ryegrass than with other plants. A higher degradation rate was found until the first harvest time, which then decreased until the second harvest time. This may be due to the degradation of easily degradable components of hydrocarbons, but might be also related to the fact that the number of degrading bacteria decreased with time, at least in some plant compartments.

Contaminating substances such as hydrocarbons generally inhibit plant growth (Yousaf et al., 2010b). The primary inhibiting factors are considered to be toxicity of low molecular weight compounds and hydrophobic properties that decrease the ability of

plants to absorb water and nutrients (Kirk et al., 2005; Kechavarzi et al., 2007). Diesel is one of the most phytotoxic and persistent fuel types that contaminate soils and its negative influence on shoot and root biomass has been documented in several studies (Hou et al., 2001; Palmroth et al., 2002). In our study contamination led to a strong reduction in shoot and root biomass, however, inoculation significantly reversed this effect. Up to more than 76% shoot and up to 93% more root biomass was produced in inoculation treatments as compared to the uninoculated controls. More biomass increase occurred between the first and second harvest than between inoculation and the first harvest. As the abundance of alkane degrading bacteria decreased with time, the most likely reason for the higher biomass production in the second stage in comparison to the control treatments is the lower hydrocarbon concentration leading to reduced toxicity for the plants. Inoculated bacteria might have promoted plant growth directly or indirectly by reducing hydrocarbon levels. Both strains (ISI10-3 and BRI10-9) showing ACC deaminase activity were more efficient in plant growth promotion as well as in hydrocarbon degradation. The bacterial enzyme ACC-deaminase can reduce ethylene levels produced by plants under stress and therefore may alleviate stress symptoms leading to better plant growth (Glick, 2003). Our results are in agreement with previous studies (Gurska et al., 2009; Afzal et al., 2011) reporting enhanced root growth and hydrocarbon degradation with strains having ACC-deaminase activity. Plant growth, especially root growth is important in the context of phytoremediation, as the rhizosphere plays an important role in catabolic activity and survival of associated microorganisms (Juhanson et al., 2009).

In phytoremediation, hydrocarbons are degraded mainly by soil and plant-associated microbial communities and it has been suggested that the phytoremediation potential correlates with the number of pollutant-degrading bacteria in the plant environment (Glick, 2003; Liste and Prutz, 2006; Muratova et al., 2008). Successful application of plant-microbe systems for rhizoremediation relies on in-situ establishment of a high level of degrading bacteria (Liu et al., 2007). The results from our study showed that *E. ludwigii* strains were able to efficiently colonize the rhizo- and endosphere of Italian ryegrass, birdsfoot trefoil and alfalfa over a period of 150 days. The best hydrocarbon degrading strains, i.e. ISI10-3 and BRI10-9, colonized all plants well, however, microbial numbers decreased with time. Strain IRI10-4 (a root endophyte) showed higher colonization in the root interior than other strains, whereas strain ISI10-3 (a shoot endophyte) showed higher colonization in the shoot interior. Similar observations were also previously observed (Rosenblueth and Martinez-Romero, 2006; Andria et al., 2009), who postulated that endophytes are generally better able to colonize plant interior.

Our results revealed that the abundance and expression of CYP153 genes of all *E. ludwigii* strains involved in hydrocarbon degradation varied distinctly between different strains, plants species, plant developmental stages and plant compartments (Tables 4 and 5). Bacterial CYP153 gene abundance and expression was highest in the rhizosphere at the first harvest in all treatments. This can be related to enhanced root exudation and high amounts of nutrients in the rhizosphere for bacterial growth and co-metabolism of alkane degradation (Olson et al., 2003; Bürgmann et al., 2005; Hai et al., 2009). The gene abundance and expression was lower in the endosphere at initial stages but increased with

time. This indicates that inoculated bacteria first establish in the rhizosphere and then reach the plant interior at a later stage. Strain ISI10-3 showed highest abundance and expression in rhizosphere and shoot interior, however, in the root interior IRI10-4 showed significantly higher gene abundance and expression than other strains. This might be because IRI1-4 was originally isolated from the root interior and ISI10-3 from the shoot interior. All strains principally expressed alkane degrading genes in all plant compartments, indicating an active role in degradation of diesel in various plant compartments. The average activities (transcripts / abundance) were variable and depended on the inoculant strain, plant species and time of analysis. Some strains generally showed high activity in the shoot interior, which was also previously reported by Andria et al. (2009).

In conclusion this study revealed, that *E. ludwigii* strains efficiently interact with various plant species, efficiently colonize the rhizosphere as well as the plant interior, at least under the conditions tested, and are able to promote plant growth. Furthermore, all strains efficiently degraded hydrocarbons, especially strains ISI10-3 and BRI10-9 performed best, both in terms of plant growth promotion and hydrocarbon degradation. The close interaction with plants and hydrocarbon degradation activities suggest that these strains do not have pathogenic characteristics, although this assumption requires further testing.

Acknowledgements

The authors would greatly acknowledge the Higher Education Commission of Pakistan for financial support. We also thank Anton Grahsl for the help with the greenhouse experiment and Levente Bodrossy for discussions about phylogenetic analysis.

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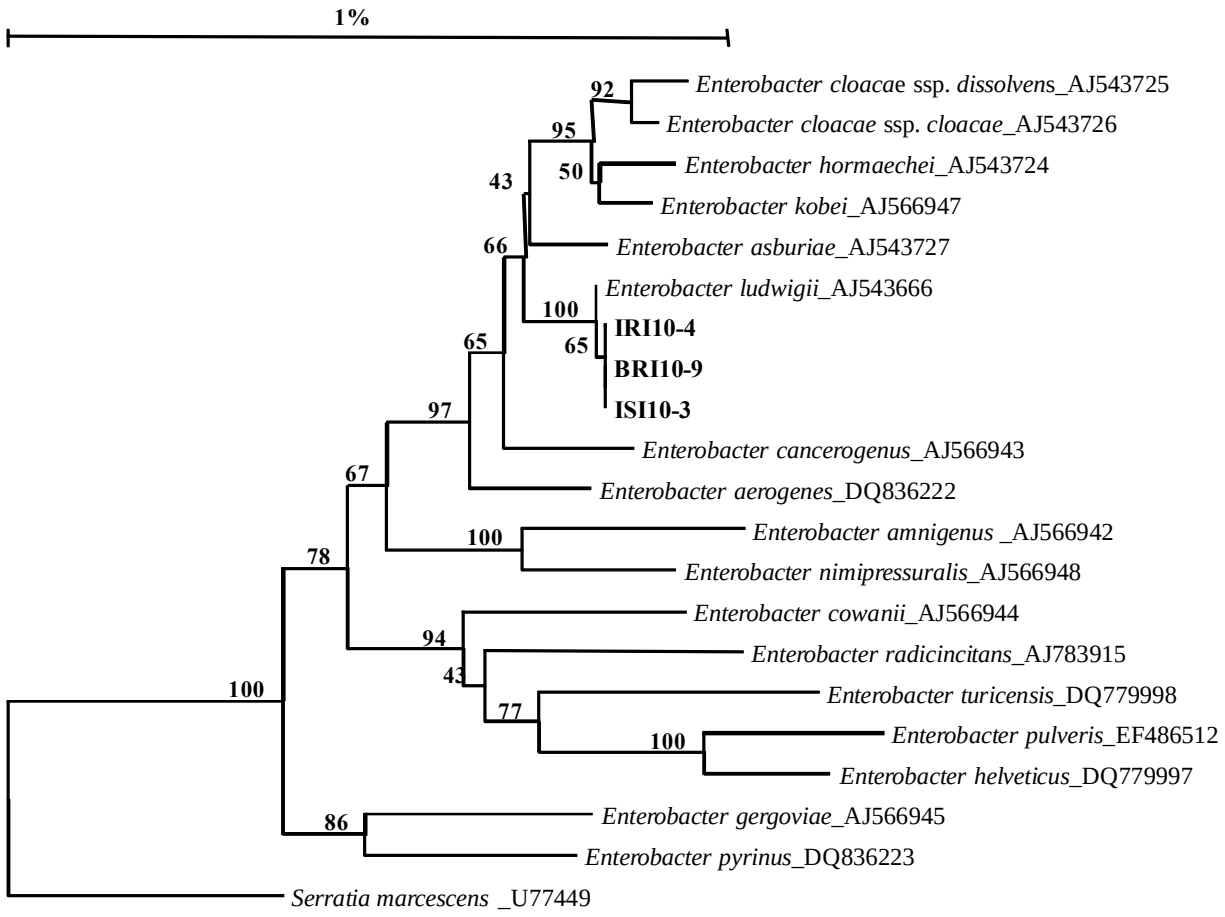

Fig. 1. Neighbor joining tree of *Enterobacter* species based on *rpoB* sequences showing the phylogenetic position of strains IRI10-4, BRI10-9 and ISI10-3.

Table 1. Hydrocarbon concentrations in soils vegetated with Italian ryegrass (IT), birdsfoot trefoil (BT) and alfalfa (AL). Data are means (n=3), standard deviations are presented in parentheses. Means with different letters are significantly different at a 5 % level of significance in each column.

Plant	Treatment	Hydrocarbon concentration (g kg^{-1} soil)				
		Initial value	1st harvest[a]	% decrease	2nd harvest[b]	% decrease
IT						
	Control (+D)	10	9.48^{fg} (0.33)	5	8.04^{h} (0.26)	20
	ISI10-3	10	5.23^{a} (0.31)	48	3.51^{ab} (0.33)	65
	IRI10-4	10	6.98^{d} (0.40)	30	5.07^{ef} (0.44)	49
	BRI10-9	10	6.56^{bcd} (0.34)	34	3.24^{a} (0.30)	68
BT						
	Control (+D)	10	8.90^{f} (0.44)	11	8.05^{h} (0.28)	20
	ISI10-3	10	6.81^{cd} (0.46)	32	4.29^{cd} (0.41)	57
	IRI10-4	10	8.13^{e} (0.28)	19	7.09^{g} (0.58)	29
	BRI10-9	10	6.87^{cd} (0.40)	31	4.36^{cd} (0.56)	56
AL						
	Control (+D)	10	9.76^{g} (0.32)	2	8.08^{h} (0.34)	19
	ISI10-3	10	5.96^{b} (0.34)	40	4.03^{bc} (0.33)	60
	IRI10-4	10	6.78^{cd} (0.50)	32	5.69^{f} (0.43)	43
	BRI10-9	10	6.16^{bc} (0.44)	38	4.78^{de} (0.29)	52

[a] 6 weeks after germination
[b] at flowering stage; IT 14: weeks after germination; BT and AL 21: weeks after germination

Table 2. Shoot and root dry weight of Italian ryegrass (IT), birdsfoot trefoil (BT) and alfalfa (AL). Data are means (n=3), standard deviations are presented in parentheses. Means with different letters are significantly different at a 5 % level of significance in each column.

Treatment	IT		BT		AL	
	1st harvest [a]	2nd harvest [b]	1st harvest	2nd harvest	1st harvest	2nd harvest
shoot biomass (g dry weight)						
Control (-D)	11.6^{a} (1.3)	21.0^{a} (0.6)	2.0^{a} (0.3)	14.2^{a} (0.5)	4.0^{a} (0.3)	15.4^{a} (1.1)
Control (+D)	3.4^{c} (1.1)	6.1^{d} (0.5)	0.9^{b} (0.4)	2.4^{c} (0.7)	0.7^{c} (0.2)	2.0^{c} (1.0)
ISI10-3	7.1^{b} (1.0)	13.1^{b} (0.6)	1.3^{b} (0.2)	6.0^{b} (0.6)	1.6^{b} (0.3)	8.1^{b} (1.1)
IRI10-4	6.4^{b} (1.2)	10. 9^{c} (0.7)	1.1^{b} (0.2)	5.5^{b} (0. 7)	1.5^{b} (0.3)	3.0^{c} (1.2)
BRI10-9	6.9^{b} (1.2)	13.0^{b} (0.6)	1.1^{b} (0.4)	6.6^{b} (0.7)	1.6^{b} (0.3)	7.9^{b} (1.3)
root biomass (g dry weight)						
Control (-D)	6.3^{a} (0.6)	16.4^{a} (0.9)	0.7^{a} (0.1)	5.0^{a} (0.4)	0.3^{a} (0.1)	7.0^{a} (0.2)
Control (+D)	2.9^{c} (0.6)	4.7^{d} (0.9)	0.2^{b} (0.2)	0.5^{c} (0.2)	0.1^{b} (0.1)	0.2^{d} (0.1)
ISI10-3	6.1^{a} (0.8)	12.4^{b} (0.8)	0.6^{a} (0.2)	1.9^{b} (0.3)	0.3^{a} (0.1)	2.8^{b} (0.2)
IRI10-4	4.6^{b} (0.7)	5.7^{cd} (0.7)	0.3^{b} (0.1)	1.6^{b} (0.2)	0.1^{b} (0.1)	0.4^{d} (0.2)
BRI10-9	5.3^{ab} (0.6)	6.5^{c} (0.9)	0.6^{a} (0.1)	1.8^{b} (0.3)	0.2^{ab} (0.1)	1.2^{c} (0.2)

[a] 6 weeks after germination
[b] at flowering stage; IT 14: weeks after germination ; BT and AL 21: weeks after germination

Table 3. Colony forming units (CFU) in the rhizosphere (RH), root interior (RI), shoot interior (SI) of Italian ryegrass (IT), birdsfoot trefoil (BT) and alfalfa (AL). Data are means (n=3), standard deviations are presented in parentheses. Means with different letters are significantly different at a 5 % level of significance in each column.

Treatment	CFU/g dry weight RH			CFU/g dry weight RI			CFU/g dry weight SI		
	IT	BT	AL	IT	BT	AL	IT	BT	AL
					1st harvest [a]				
ISI10-3	2.27E+08 [a] (3.08E+06)	1.09E+07 [g] (2.67E+05)	1.02E+08[b] (2.19E+06)	2.89E+07[b] (1.05E+06)	1.08E+03[d] (1.33E+02)	1.33E+05[d] (7.36E+03)	1.90E+05[a] (2.72E+04)	8.61E+04[b] (3.36E+04)	5.42E+04[c] (7.09E+03)
IRI10-4	4.14E+07 [f] (1.72E+06)	5.95E+04 [h] (7.60E+04)	5.52E+07[d] (2.72E+06)	4.96E+07[a] (5.99E+06)	7.68E+04[d] (2.62E+03)	1.46E+07[c] (7.36E+05)	1.61E+03[e] (3.87E+02)	3.85E+04[cd] (4.70E+03)	1.75E+04[de] (4.79E+02)
BRI10-9	4.76E+07 [e] (2.51E+06)	4.70E+04 [h] (6.09E+04)	6.68E+07[c] (2.50E+06)	1.90E+04[d] (8.89E+02)	8.10E+04[d] (1.74E+03)	2.58E+06[d] (1.29E+05)	9.72E+03[de] (3.06E+03)	ND	2.86E+04[cde] (6.16E+02)
					2nd harvest [b]				
ISI10-3	4.18E+07[ab] (6.94E+06)	2.36E+07[c] (2.04E+06)	3.96E+07[b] (1.49E+06)	2.10E+06[e] (1.53E+05)	9.21E+06[c] (1.14E+06)	8.89E+04[f] (1.54E+04)	1.07E+06[a] (1.23E+05)	2.46E+05[c] (3.45E+04)	9.98E+05[b] (3.83E+04)
IRI10-4	2.76E+06[d] (2.02E+05)	1.82E+06[d] (5.22E+05)	3.80E+06[d] (2.65E+05)	1.11E+05[f] (1.14E+04)	6.57E+07[a] (2.56E+06)	2.09E+07[b] (1.39E+06)	6.79E+04[de] (1.03E+03)	4.67E+04[de] (5.16E+03)	1.16E+05[d] (8.17E+03)
BRI10-9	4.46E+07[a] (2.90E+06)	3.02E+06[d] (3.16E+05)	3.02E+06[d] (2.46E+05)	7.33E+05[ef] (1.15E+05)	5.22E+06[d] (3.09E+05)	9.10E+05[ef] (1.54E+04)	3.19E+04[e] (5.12E+03)	1.38E+04[e] (1.46E+03)	2.52E+04[e] (7.36E+02)

[a] 6 weeks after germination

[b] at flowering stage; IT 14: weeks after germination ; BT and AL 21: weeks after germination

Table 4. CYP153 gene abundance in the rhizosphere (RH), root interior (RI), shoot interior (SI) of Italian ryegrass (IT), birdsfoot trefoil (BT) and alfalfa (AL). Data are means (n=3), standard deviations are presented in parentheses. Means with different letters are significantly different at a 5 % level of significance in each column.

Treatment	CYP genes abundance (copies/g dry weight) RH			CYP genes abundance (copies/g dry weight) RI			CYP genes abundance (copies/g dry weight) SI		
	IT	**BT**	**AL**	**IT**	**BT**	**AL**	**IT**	**BT**	**AL**
				1st harvest [a]					
ISI10-3	1.12E+09[a] (1.06E+08)	4.34E+08[c] (3.22E+07)	1.04E+09[a] (3.93E+07)	2.59E+07[c] (2.29E+06)	1.13E+04[d] (1.04E+02)	5.16E+05[d] (1.94E+05)	2.93E+06[a] (3.16E+05)	3.25E+05[b] (7.63E+04)	2.00E+05[bc] (8.40E+04)
IRI10-4	4.38E+08[c] (5.75E+07)	3.22E+06[d] (5.82E+05)	3.84E+08[c] (7.15E+07)	9.68E+07[a] (4.52E+06)	7.97E+04[d] (8.15E+04)	4.38E+07[b] (1.32E+07)	5.68E+03[c] (1.01E+03)	7.75E+04$_c$ (4.70E+03)	5.52E+04[c] (5.20E+03)
BRI10-9	6.76E+08[b] (4.72E+07)	3.57E+06[d] (5.34E+05)	1.05E+09[a] (8.13E+07)	4.01E+04[d] (1.27E+04)	3.64E+04[d] (2.47E+03)	2.62E+06[d] (5.78E+05)	1.54E+04[c] (3.20E+03)	ND	6.62E+04[c] (4.13E+03)
				2nd harvest [b]					
ISI10-3	9.68E+08[a] (4.26E+07)	1.92E+08[c] (4.18E+07)	9.04E+08[b] (3.66E+07)	2.66E+06[d] (1.29E+06)	9.52E+06[c] (3.29E+05)	1.39E+05[d] (3.15E+04)	2.96E+07[a] (5.56E+06)	3.49E+06[b] (3.47E+05)	3.20E+06[b] (1.28E+06)
IRI10-4	8.68E+07[d] (2.63E+06)	4.64E+06[e] (4.65E+05)	8.31E+06[e] (4.69E+05)	2.22E+05[d] (1.37E+05)	5.07E+07[a] (5.44E+06)	3.87E+07[b] (4.54E+06)	8.65E+04[b] (1.33E+04)	7.47E+04[b] (1.39E+04)	4.43E+05[b] (2.14E+04)
BRI10-9	9.36E+08[ab] (1.83E+07)	8.24E+06[e] (7.62E+05)	7.79E+06[e] (8.14E+05)	5.59E+05[d] (2.21E+04)	3.36E+06[d] (2.46E+05)	9.30E+05[d] (4.62E+04)	6.02E+04[b] (1.42E+04)	4.18E+04[b] (1.09E+04)	5.35E+04[b] (1.04E+04)

[a] 6 weeks after germination

[b] at flowering stage; IT 14: weeks after germination ; BT and AL 21: weeks after germination

Table 5. CYP153 gene expression in the rhizosphere (RH), root interior (RI), shoot interior (SI) of Italian ryegrass (IT), birdsfoot trefoil (BT) and alfalfa (AL). Data are means (n=3), standard deviations are presented in parentheses. Means with different letters are significantly different at a 5 % level of significance in each column.

Treatment	CYP genes expression (copies/g dry weight) RH			CYP genes expression (copies/g dry weight) RI			CYP genes expression (copies/g dry weight) SI		
	IT	BT	AL	IT	BT	AL	IT	BT	AL
				1st harvest [a]					
ISI10-3	6.66E+08[a] (3.07E+07)	8.89E+07[c] (6.19E+06)	1.14E+08[b] (8.42E+06)	5.47E+06[c] (5.04E+05)	6.38E+03[e] (3.56E+02)	2.07E+05[e] (4.16E+04)	1.17E+06[a] (6.71E+04)	1.07E+05[b] (4.24E+03)	1.13E+05[b] (4.98E+03)
IRI10-4	4.81E+07[c] (4.64E+06)	5.92E+05[d] (2.10E+04)	3.76E+07[c] (2.49E+06)	9.27E+06[a] (4.91E+05)	1.50E+04[e] (1.41E+04)	7.28E+06[b] (4.35E+05)	1.27E+03[c] (4.52E+01)	2.07E+04[c] (5.98E+02)	2.57E+04[c] (2.12E+04)
BRI10-9	9.51E+07[bc] (3.78E+06)	8.60E+05[d] (6.22E+04)	1.01E+08[bc] (6.58E+06)	1.48E+04[e] (3.06E+03)	5.20E+03[e] (3.99E+03)	7.73E+05[d] (4.33E+04)	9.58E+03[c] (2.95E+02)	ND	5.07E+04[c] (5.75E+02)
				2nd harvest [b]					
ISI10-3	4.17E+08[a] (5.93E+07)	4.98E+07[d] (1.56E+06)	1.30E+08[c] (5.86E+06)	5.72E+05[d] (5.72E+04)	1.19E+06[c] (4.42E+04)	4.78E+04[e] (4.00E+03)	1.04E+07[a] (4.78E+05)	1.05E+06[b] (8.67E+04)	1.13E+06[b] (4.62E+04)
IRI10-4	1.09E+07[d] (1.33E+05)	6.85E+05[e] (2.80E+05)	1.01E+06[e] (3.12E+05)	5.87E+04[e] (4.95E+03)	8.14E+06[a] (3.36E+05)	5.28E+06[b] (3.34E+05)	1.23E+04[c] (4.20E+02)	1.05E+04[c] (5.64E+02)	5.04E+04[c] (3.95E+03)
BRI10-9	1.66E+08[b] (4.21E+06)	2.85E+06[e] (2.80E+05)	2.37E+06[e] (1.35E+05)	8.94E+04[e] (7.23E+03)	6.87E+05[d] (4.47E+04)	4.49E+05[d] (4.75E+04)	1.32E+04[c] (5.40E+02)	7.55E+03[c] (2.95E+02)	1.17E+04[c] (3.93E+02)

[a] 6 weeks after germination

[b] at flowering stage; IT 14: weeks after germination ; BT and AL 21: weeks after germination

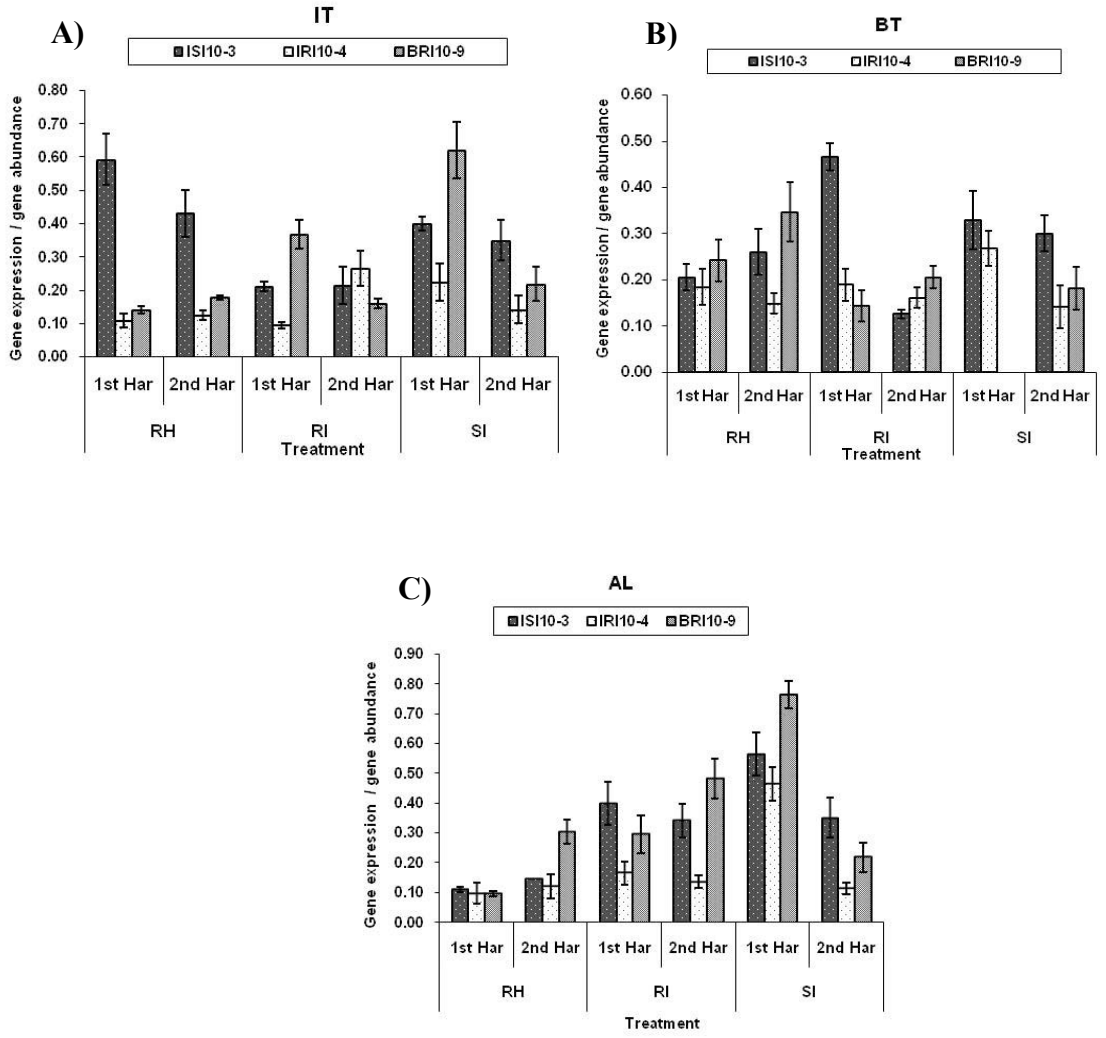

Fig. 2. Mean values of ratio of CYP153 gene expression / abundance in the rhizosphere (RH), root interior (RI), shoot interior (SI) of A) Italian ryegrass (IT), B) birdsfoot trefoil (BT) and C) alfalfa (AL). 1st harvest: 6 weeks after germination, 2nd harvest at flowering stage, IT: 14 weeks after germination; BT and AL: 21 weeks after germination. Data are means (n=3), error bars indicate standard deviation.

Chapter 4

General Conclusions

Phytoremediation is a broad term that is used to describe the use of plants and their associated microbes to reduce the volume, mobility and toxicity of contaminants in soil, groundwater or other contaminated media. Using microorganisms and plants in remediation is generally considered as a safe and inexpensive method for the removal of hazardous contaminants. Plants have an inherent ability to detoxify some xenobiotics in soil by direct uptake of the contaminants, followed by subsequent transformation using enzymes. The microorganisms have the primary catalytic role in degrading or mineralizing various contaminants and converting them into non-toxic by-products during phytoremediation. Plant endophytes, bacteria which colonize the endosphere of plants without exhibiting pathogenicity to their hosts, are also important during phytoremediation. They may either degrade organic contaminants after plant uptake or improve plant stress tolerance.

Indigenous microorganisms present at the contaminated site are often inhibited by the toxic level of these contaminants, or often do not possess the required catabolic capabilities to completely degrade the contaminants. In such cases inoculation of soils or plants with bacteria showing appropriate degradation abilities increases the efficiency and rate of degradation. The first work phase in this PhD study was aimed on selecting efficient hydrocarbon degrading strains and plants. A particular objective was to study the colonization of inoculant strains in the rhizosphere and endosphere of plants using cultivation-dependent and -independent approaches as well as the diversity of alkane-

degrading bacteria. The results showed that the inoculant strains were able to degrade hydrocarbon concentrations in a contaminated soil. Vegetation, compost amendment and inoculation led to decrease total hydrocarbon levels. Maximum hydrocarbon removal (57%) was observed with two *Pantoea* strains. Strain BTRH79 performed best in combination with Italian ryegrass, whereas strain ITSI10 showed highest degradation in combination with birdsfoot trefoil. Cultivation-dependent and cultivation-independent analysis confirmed that the best degrading strains colonized well the rhizosphere as well as the plant interior of both plants. We assume that the ability of a strain to colonize a plant correlates with degradation rates. Our findings further indicated that the colonization of inoculant strains is influenced by the plant, probably because plants favor the establishment of specific bacteria. We found that Italian ryegrass and birdsfoot trefoil hosted distinct alkane-degrading communities. In association with both plants, *alkB* as well as CYP153 containing microorganisms were detected, but different subtypes of alkane-degrading genes were encountered. These findings suggest that the application of degrading bacterial strains, which are able to compete with the native microflora and to tightly associate with plants, is a promising strategy for the phytoremediation of hydrocarbon-polluted soils.

The second work phase was intended to assess hydrocarbon degradation, colonization, CYP153 gene abundance and expression of selected *Enterobacter ludwigii* inoculant strains in different compartments of Italian ryegrass, birdsfoot trefoil, and alfalfa in sterilized, diesel spiked soil. *E. ludwigii* belongs to the *Enterobacter cloacae* complex, which is known for its potential pathogenicity for humans, but has been also reported to include plant-associated strains with plant growth-promoting and biocontrol

capacities. Until recently, the potential of *E. ludwigii* in remediation of hydrocarbon contaminated soils has not been explored. Although various chemical and microbiological aspects of petroleum oil and alkane biodegradation have been relatively well studied, there is a general lack of knowledge concerning the abundance and expression of functional genes involved.

Our results showed that all tested *E. ludwigii* strains, which were originally isolated from a contaminated environment, were capable of hydrocarbon degradation. Strains ISI10-3 and BRI10-9 showed highest hydrocarbon degradation (up to 68%) in combination with Italian ryegrass and alfalfa. The higher degradation with these plants (as compared to birdsfoot trefoil) may be due to better colonization of inoculant strains or due to stimulation of degradation activity by root exudates or a better aerated environment. In our study contamination had a clear effect on shoot and root biomass. For all three plant species significantly higher shoot and root biomass were produced, when inoculated with alkane degrading strains than in uninoculated contaminated controls. The endophyte strains were capable of colonizing efficiently the rhizosphere and plant interior, which lead to efficient hydrocarbon degradation. Our results revealed that the bacterial CYP153 genes can be expressed not only in rhizosphere but also in the plant interior. The abundance and expression of CYP153 genes of all *E. ludwigii* strains involved in hydrocarbon degradation varied distinctly between different strains, plants species, plant developmental stages, and plant compartments. The abundance and expression of CYP153 genes can be correlated with hydrocarbon degradation, as maximum hydrocarbon degradation was observed with those treatments where maximum gene expression was found. The inoculation of endophyte strains resulted in better

survival of plants which might be due to plant growth-promoting or alkane-degrading activities.

Future prospects and recommendations

The increase in the environmental contamination through infiltration of petroleum products, both in water and on land, has led to a progressive deterioration of environmental quality. Phytoremediation of crude oil is cost effective and environmentally sound, and is therefore often the best means of removing contaminants from the polluted environment. Phytoremediation integrates the tools of many disciplines. As each of the disciplines advances and as new cleanup needs arise, opportunities for new remediation techniques will emerge. Recent advances in science and engineering show promise for developing new techniques, as illustrated by the following examples:

- Understanding microbial processes. As novel biotransformations become better understood at ecological, biochemical, and genetic levels, new strategies will become available for phytoremediation. Advances in understanding microorganisms may also improve the effectiveness of phytoremediation approaches in meeting clean-up standards. The uptake and metabolism of organic compounds sometimes stop at concentrations above clean-up standards. Research on various genetic capabilities and their regulation is very active today and may lead to overcome such microbiological limitations.
- Increasing the population of soil microbial microorganisms containing pollutant-degrading and plant growth-promoting (particularly producing ACC deaminase) genes in polluted soil environments by inoculating seeds or roots. In contaminated

soils, some indigenous bacteria are able to degrade the contaminant, but the rates of natural attenuation are slow and therefore unacceptable to devise remediation strategies. Thus there is a need to improve *in situ* biodegradation of hydrocarbons. The remediation efficiency can be enhanced by introducing selected strains with intrinsic properties, such as high pollutant resistance, stimulating the activity of indigenous microbes. In both cases, the strong limitations are that the contaminated sites are usually lacking in nutrients and do not permit rapid growth of introduced degrading bacteria therefore; their potential bioremediation activities are not fully expressed. Introduced bacteria are also easily out-competed by indigenous communities. Thus, further research in rhizosphere biology is needed for the development of molecular and biotechnological approaches to achieve an integrated management of soil microbial populations. The ability of phytoremediation technologies ability to make further strides will depend on how quickly the regulators become convinced of the efficacy of the technology. Secondary plant metabolites can play an important role in phytoremediation. With regard to the metabolism of organic xenobiotics compounds by plants, the whole process is based on a mechanism originally developed to cope with different allelopathic compounds of natural origin. Through exudates and root turnover, many plant products enter the root zone. Some of these compounds supply soil microorganisms with energy, some act as carbon source and some can even serve as inducers of the degradation pathway.

- Genetic manipulation of organisms might be a new breakthrough in terms of accelerated removal of contaminants from soil environments. 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 xenobiotics degradation from microbes or other eukaryotes to plants will further enhance their potential for remediation of these dangerous groups of compounds.

- Innovating engineering techniques for supplying materials that stimulate microorganisms are pushing the boundaries of phytoremediation. For instance, the innovation of gas sparging has substantially expanded capabilities of aerobically degrading petroleum hydrocarbons. Research is ongoing into optimizing ways to supply materials other than oxygen. Such research will pave the way for emerging bioremediation applications.
- Promoting contact between contaminants and microbes. New techniques for promoting contaminant transport to the organisms include high-pressure fracturing of the subsurface matrix, solubilization of the contaminants by injecting heat (via steam, hot water or hot air), and, perhaps addition of surfactants. Discovery of improved methods for dispersing the microorganisms may also enhance microbial contact with the contaminants and lead to more effective phytoremediation.

- Further studies on improving the expression of catabolic genes in the rhizosphere and in the selection of the best plant-microbe combinations will have to be translated into field strategies that can demonstrate usefulness of this approach. The developments in functional genomics will be useful to identify the genes expressed in rhizosphere and plant interior, while the use of promoters to drive gene expression specifically at the root-soil interfaces will allow the engineering of microorganisms for beneficial purposes.
- The utilization of endophytes in the biodegradation of pollutants is an emerging field that has not been widely explored. Advances in this field will have to be followed by better knowledge about the absorption and transport of the toxic chemical by plants.
- Exploring the molecular communication between plants and microbes, and exploiting this communication to achieve better results in the elimination of contaminants, is fascinating area of research. These studies may reveal the mechanisms underlying microbe-plant interactions.

To meet all these criteria and successfully implementation of phytoremediation technology, a multidisciplinary approach and basic knowledge in microbiology, biochemistry, physiology, ecology and genetics are required. Moreover, much information about the biotic and abiotic factors controlling the growth and metabolic activities of plant and microorganisms in polluted environments is necessary.

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