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DOCTORAL DISSERTATION

The role of plant-associated bacteria in the remediation of soils and groundwater contaminated with petroleum hydrocarbons

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Preface

The journey started in October 2009, is now nearly over. I had a wonderful time during the last four and a half years. First of all, I would like to thank my main supervisor Prof. Jaco Vangronsveld for giving me the opportunity to conduct research at the Centre for Environmental Sciences at Hasselt University and, moreover, for the help under "the whole way", for discussions, tips and shared knowledge. Secondly, thanks to my co-supervisors Daniel van Der Lelie and Nele Weyens for their support and help in many areas of my PhD education. I would also like to thank prof. dr. Jan Colpaert for his intellectual support of the present study. On top of this, I would like to thank the jury of my PhD thesis for their valuable remarks and comments. In particular, I would like to thank prof. dr Jonathan Van Hamme of Thompson Rivers University (Canada), for his collaboration and support, both theoretically and practically throughout of the current thesis. I am also very grateful to dr. Andrea Franzetti of the University of Milan – Bicocca (Italy) for the elaborate discussion of the data presented in this thesis, as well as the technical support of the current project. Furthermore, I would like to thank dr. Francois Rineau for his input, discussions and technical support during various studies. Finally, I am extremely grateful to the Msc students, Kyriakos Spyriounis, Andrea Pacher and Guy Vossen, who have substantially contributed in this work. During these four and a half years, I met warm, helpful and very bright people who supported me on my way to the Final End. I will not mention anyone by name because I am grateful to all of you for forming extremely nice working conditions. Besides work, I feel the need to thank personally my closest friends from Greece - Niko Balota, Ioanni Dimitriou, Kosma Avgitidi, Thoma Gkoulioura and Petro Bozidi- as well as new friends that I created during these years in Belgium (George, Dino, Ali, Roberto, Raoul, Benedetta, Matteo Daghio, Blanca, Maria Balseiro, Maria Gutierrez, Eleni, Gosia, Anastasia, Thomas, Marie and Makis). I am also grateful to Mr. Guido Sterk CEO of IPM Impact for providing to me his greenhouse and other facilities in order to conduct my greenhouse experiment and to Prof. Hidetoshi Okuyama from Hokkaido University - Japan for providing me *Pseudomonas aureginosa* WatG strain. Last but definitely not least, my final thanks go to my family. Without the love, support and patience of my wife Vivi, this PhD would not have been

finished. Moreover, on 12/2/2102 and 17/10/2013 the birth of our kids Danae and Michail made my life a dream and a source of endless happiness. Thanks to my mother Athina for her love, and emotional and economical support all of these years. Coming to an end, I want to dedicate this thesis to the memory of my father Michail. I owe him everything, and although he is not physically anymore next to me, I feel him closer than ever. His morality, advice and his smile are always in my mind. Although, I know that he cannot read this, however, I am sure that somehow he feels happier…

Summary

Among the wide variety of organic contaminants, in this study diesel has been chosen as the target substrate since its extensive use results in environmental contamination. Research related to microorganisms found at diesel contaminated sites has revealed the susceptibility of diesel constituents to bacterial degradation. Indeed the ability of microbes to utilize hydrocarbons as a source of energy was observed as early as the 1940´s (Zobell 1946). Numerous studies have demonstrated that under controlled and optimal laboratory conditions, it is relatively easy for many species of bacteria to degrade selected fractions of diesel oil, usually the n-alkanes, in a matter of days or weeks so that a specific percentage of oil by weight will disappear in a given period of time. It cannot be said that all diesel oil constituents will disappear at the same rate, given the range of chemical structures that make up this fuel. In nature, conditions are rarely favorable for maximum biodegradation; hence, the rate of diesel oil degradation can be slow and spilled oil may remain in the environment for periods that are longer than desirable. As such, bioremediation technologies can be applied to hasten remediation. Diesel remediation schemes may be improved if the resident microorganisms are able to produce surface-active agents (biosurfactants) and emulsifiers, form bioflims, exhibit chemotactic behavior towards diesel and tolerate heavy metals that are often found as co-contaminants. In field applications, the use of plants to facilitate hydrocarbon removal from soil and groundwater, known as phytoremediation, is an attractive approach. Further, the application of hydrocarbonoclastic bacteria able to enhance the growth of these plants can result in positive synergistic effects.

In the Introduction of this thesis a detailed overview about the role of bacteria, plants and mixed bacterial-plant communities in hydrocarbon biodegradation is given. In Section 2, the overriding objectives of the thesis are summarized. In Section 3, the genotypic and phenotypic characterization of bacterial isolated from poplar trees growing in contact with a diesel-contaminated groundwater plume is presented. Here, 30 bacterial strains belonging to the genera *Arthrobacter*, *Acinetobacter*, *Brevibacterium*, *Micorbacterium*, *Pseudomonas* and *Staphylococcus* were found to show desirable properties both as petroleum hydrocarbon degraders and plant growth promoters. Furthermore, two root endophytes, *Acinetobacter oleivorans* and *Acinetobacter calcoaceticus*, and one

rhizospheric strain, *Staphylococcus aureus,* were selected for a more detailed examination of their biodegradation potential *in vitro* using GC-MC analysis. The selected strains were grown in flasks with liquid minimal medium containing 1000 mg Kg^{-1} filter sterilized diesel oil, and compared to a positive control (*Pseudomonas aerugionosa* WatG). Analyses showed that *Acinetobacter oleivorans* degraded diesel oil up to 40%, *Acinetobacter calcoaceticus* 41%, whilst *Staphylococcus aureus* had the worst degradation potential at 27%. The identification of hydrocarbon degraders exhibiting promising plant growth properties provides evidence that the diesel contaminants at the study side selected for a microbial community rich in plant growth promoters with the ability to degrade aliphatic and aromatic hydrocarbons. This highlights the need for a more inclusive set of criteria when selecting bacteria for designing consortia with a high likelihood of success for use in *in situ* applications. Aside from their degradation potential strains should be evaluated for their ability to produce biosurfactants, their affinity to petroleum hydrocarbons, their biofilm formation potential, their tolerance to toxic solvents and metals, as well as their ability to produce plant growth promoting compounds.

In the 4th Section of this thesis, the whole genome shotgun sequence of the diesel degrading and plant growth promoting root endophyte *Acinetobacter oleivorans* strain PF1 is presented. In the literature there is a scarcity of information relating to the effective turnover of organic contaminants for species of the *Acinetobacter* genus with plant growth-promoting properties. As reflected by the availability of complete genomes available in public databases, most reports on this topic are for species in other genera. For this work, genomic DNA of *A. oleivorans* PF1 was extracted from cells grown at 30^{o} C in LB medium with a Qiagen blood and tissue kit (Qiagen N.V., Hilden, Germany) and a single 316v2-chip was used for sequencing on an IonTorrent PGM (Life Technologies Inc., Carlsbad, CA). In total, 2.8 million reads (mean length 303 bases) generated 853 Mb of data, of which 535,611 reads were assembled using MIRA V3.9.9 into 31 contigs, giving a consensus length of 3,766,014 bp at 43.5× coverage. ORF prediction and gene annotation was carried out using RAST. This strain has a GC content of 38,6% and 3509 genes were arranged into 668 subsystems. In order to find the most closely related genome to use as a reference for gene prediction, the genomes of all fully sequenced and closely

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related bacterial strains on the NCBI database (*Acinetobacter oleivorans* DR1, *Acinetobacter baumanii*: 13 strains, *Acinetobacter pittii* ANC4050, *Acinetobacter calcoaceticus* PHEA-2), along with sequences for *Pseudomonas putida* KT2440 and *Escherichia coli* CFT073, were aligned to the *Acinetobacter oleivorans* PF1 data using progressive MAUVE 2.3.1. Moreover, a new taxonomic metrics tool like average nucleotide identity (ANI), calculated from pair-wise comparisons of all sequences shared between two strains was performed. The gene context of gene clusters and the organization of specific operons was compared using the SeqinR1.0-2 package in R. For each gene and operon of interest the sequence of the genomic DNA fragment (”Query fragment”) was extracted, enlarged by two supplementary genes, one upstream and one downstream, prior to using BLAST to compare sequences to the NCBI bacterial genomes database. Matching sequences and gene coordinates of the best hits were downloaded to a GenBank file, and plotted against the”Query fragments”. Given this, a list of 10 biochemical mechanisms involved in diesel degradation and in plant-growth promotion was built, and validated through the review of 24 published studies, by carrying out keyword searches in various databases, and through identification of 219 "query” genes (37 as single genes and 182 genes that were part of one of 38 operons) that have been shown to code for proteins that are key players in hydrocarbon degradation and plant growth promotion. In conclusion, genome sequence analysis of *A. oleivorans* PF1 has provided us with significant insights into the genomic basis of PF1's: (i) Metabolic capability to degrade aliphatic and aromatic components present in diesel oil, (ii) Affinity and chemotaxis towards hydrocarbon substrates, (iii) responses to hydrocarbons and heavy metal stress and, (iv) key plant growth promotion properties. These findings support the hypothesis that *A. oleiovorans* PF1, based on its hydrocarbon degrading and emulsification abilities, together with its potential to promote host plant growth, is promising candidate for the development of novel strategies for the bioremediation of petroleum-contaminated sites by means of plant growth promoting and hydrocarbon degrading bacteria. Finally, the ANI calculation revealed a 91,83% similarity with *Acinetobacter oleivorans* DR1. The peculiar phenotypic and genotypic nature of strain PF1 together with its ANI estimation prompts for a tentative assumption that it represents a new species with the genus.

Summary

In the 5th Section of this thesis, a greenhouse study is presented in which poplar cuttings [*Populus deltoides x* (*trichocarpa x deltoides*) cv Grimminge] were inoculated with a bacterial consortium consisting of two root endophytes, namely *Acinetobacter oleivorans* PF1 and *A. calcoaceticus* PF2, previously described for their ability to degrade diesel in liquid cultures and exhibit various plant growth-promoting traits *in vitro*. Inoculated and non-inoculated cuttings were exposed to **5000 mg kg^{-1}** and **10000 mg kg^{-1}** diesel oil and the biodegradation rate, plant growth index and microbial activity were monitored for a 10 weeks period. Despite the promising results at the lower concentration, at the higher concentration the inoculum did not exert any positive effect on the plants. Therefore further research is needed to unravel and record all the factors that may affect the application of bacterial co-cultures consisting of sequenced strains. Finally, in the 6th and last Section, the most important conclusions of the current project are summarized and perspectives for future studies are given.

Samenvatting

Aangezien het grootschalig gebruik van diesel ernstige milieuvervuiling tot gevolg heeft, werd diesel in deze studie geselecteerd uit de grote variëteit aan organische contaminanten. Onderzoek omtrent micro-organismen die leven op diesel-gecontamineerde sites heeft aan het licht gebracht dat dieselcomponenten onderhevig kunnen zijn aan bacteriële afbraak. Inderdaad, de capaciteit van micro-organismen om koolwaterstoffen te gebruiken als energiebron werd reeds geobserveerd in 1946 (Zobell 1946). Talrijke studies hebben aangetoond dat, onder gecontroleerde, optimale labocondities, het voor vele bacteriesoorten relatief gemakkelijk is om bepaalde fracties van diesel olie af te breken. De n-alkanen worden meestal afgebroken in enkele dagen of weken, zodat een specifiek gewichtspercentage van de olie zal verdwijnen in een bepaalde tijdsperiode. Door de grote range in chemische structuren waaruit diesel is samengesteld, kan niet gesteld worden dat alle diesel olie componenten aan dezelfde snelheid zullen verdwijnen. Bovendien zijn de van nature *in situ* aanwezige condities zelden optimaal voor een maximale biodegradatie. Vandaar dat de snelheid van diesel olie afbraak traag kan zijn en bijgevolg de contaminaties voor veel langere periodes dan wenselijk in het milieu aanwezig blijven. In dit opzicht kunnen bioremediatie technologiën toegepast worden om de remediatie te versnellen. Diesel remediatie schema's zouden verbeterd kunnen worden als de aanwezige micro-organismen in staat zijn om oppervlakte-actieve agentia (surfactanten) en emulgatoren te produceren, biofilms kunnen vormen, chemotaxis gedrag naar diesel vertonen, en zware metalen, die vaak als co-contaminant aanwezig zijn, kunnen tolereren. In veldtoepassingen is fytoremediatie, meer bepaald het gebruik van planten om de verwijdering van koolwaterstoffen uit bodems en grondwater te versnellen, een interessante techniek. Bovendien kan het gebruik van koolwaterstof afbrekende bacteriën die tegelijkertijd ook de groei en ontwikkeling van hun gastheer kunnen bevorderen, resulteren in een positief synergistisch effect.

In de inleiding van dit doctoraat wordt een gedetailleerd overzicht gegeven van de rol van bacteriën, planten en plant-bacterie gemeenschappen bij de biodegradatie van koolwaterstoffen. Vervolgens worden in het tweede deel de algemene doelstellingen samengevat. De genotypische en fenotypische karakterisatie van de bacteriën, geïsoleerd uit populieren die groeien te midden

van een diesel-gecontamineerde grondwaterpluim, wordt voorgesteld in deel 3. Van de geïsoleerde baceriële stammen, bleken er 30, die behoren tot de genera *Arthrobacter*, *Acinetobacter*, *Brevibacterium*, *Micorbacterium*, *Pseudomonas* en *Staphylococcus*, over interessante eigenschappen te beschikken, zoals petroleum koolwaterstof afbrekende, alsook plantengroei promoverende stammen. Bovendien werden 2 wortel endofyten, *Acinetobacter oleivorans* en *Acinetobacter calcoaceticus*, en 1 rhizosfeer stam, *Staphylococcus aereus*, geselecteerd voor een meer gedetailleerde analyse van hun *in vitro* biodegradatie potentieel, dit gebruik makend van GC-MS. De geselecteerde stammen werden gegroeid in erlemeyers met een vloeibaar minimaal medium dat 1000 mg Kg^{-1} filter-gesteriliseerde diesel bevatte, en werden vergeleken met een positieve controle (*Pseudomonas aerugionosa* WatG). De analyses toonden aan dat *Acinetobacter oleivorans* tot 40% diesel olie kon afbreken, *Acinetobacter calcoaceticus* 41%, en *Staphylococcus aereus* had het slechtste afbraak potentieel, nl. 27%. Het feit dat er stammen geïdentificeerd zijn die enerzijds koolwaterstoffen kunnen afbreken en anderzijds beschikken over veelbelovende plantengroei bevorderende eigenschappen, geeft aan dat de diesel contaminanten op de bestudeerde site een selectie geïnduceerd hebben ten voordele van plantengroei bevorderende stammen met het vermogen om alifatische en aromatische koolwaterstoffen af te breken. Dit benadrukt de noodzaak voor een meer inclusieve set van criteria bij de selectie van bacteriën voor het samenstellen van een consortium dat een hoge kans op succes heeft bij *in situ* toepassingen. Naast hun afbraakpotentieel, zou het interessant zijn de geïsoleerde stammen te testen voor hun capaciteit om biosurfactanten te produceren, hun affiniteit voor petroleum koolwaterstoffen, hun potentieel om biofilms te vormen, hun tolerantie voor toxische solventen en metalen, alsook hun capaciteit om plantengroei bevorderende componenten te produceren.

In het vierde deel van dit werk wordt de 'whole genome shotgun sequence' van de diesel afbrekende en plantengroeibevorderende wortel endofyt *Acinetobacter oleivorans* stam PF1 voorgesteld. In de literatuur is zeer weinig informatie te vinden over de effectieve turnover van organische contaminanten voor species van het *Acinetobacter* genus die ook beschikken over plantengroei bevorderende eigenschappen. Dit blijkt ook wanneer de beschikbaarheid van volledige genomen in publieke databasen wordt aanschouwd: de meeste bevindingen

omtrent deze topic betreffen soorten van andere genera. Voor dit werk werd het genomisch DNA van *A. oleivorans* PF1 cellen (gegroeid bij 30°C in LB medium) geëxtraheerd met een 'Qiagen blood and tissue kit' (Qiagen N.V., Hilden, Duitsland) en een enkel 316v2-chip werd gebruikt voor het sequeneren op een IonTorrent PGM (Life Technologies Inc., Carlsbad, CA). In totaal genereerden 2,8 miljoen reads (gemiddelde lengte 303 basen) 853 Mb aan data, waarvan 535.611 reads geassembleerd werden in 31 contigs (gebruik makend van MIRA V3.9.9). Dit resulteerde in een consensus lengte van 3.766.014 bp met 43,5x coverage. ORF predictie en gen annotatie werden uitgevoerd, gebruik makend van RAST. Deze stam had een GC gehalte van 38,6% en 3509 genen waren geördend in 668 subsystemen. Om het sterkst gerelateerde genoom te vinden, dat kan gebruikt worden als referentie voor gen predictie, werden de genomen van alle volledig gesequeneerde en sterk gerelateerde bacteriële stammen van de NCBI database (*Acinetobacter oleivorans* DR1, *Acinetobacter baumanii*: 13 stammen, *Acinetobacter pittii* ANC4050, *Acinetobacter calcoaceticus* PHEA-2), alsook de sequenties van *Pseudomonas putida* KT2440 en *Escherichia coli* CFT073 gealigneerd met de data van *Acinetobacter oleivorans* PF1, gebruik makend van progressive MAUVE 2.3.1. Bovendien werden via een nieuwe taxonomische tool, zoals 'average nucleotide identity' (ANI), paargewijze vergelijkingen van alle sequenties die in beide stammen voorkomen berekend. De gen context van de gen clusters en de organisatie van specifieke operons werd vergeleken aan de hand van het SeqinR1.0-2 pakket in R. Vooraleer BLAST werd gebruikt om de sequenties te vergelijken met de NCBI bacteriële genoom database, werd voor elk interessant gen of operon de sequentie van het genomisch DNA fragment ("Query fragment") geëxtraheerd, en uigebreid met 2 supplementaire genen: 1 upstream en 1 downstream. De sequenties en gen coördinaten van de beste hits werden gedownload naar de GenBank file en geplot tegen de "Query fragments". Op deze manier werd een lijst opgebouwd van 10 biochemische mechanismen betrokken in diesel afbraak en plantengroeipromotie. Verder werd deze lijst gevalideerd a.h.v. de review van 24 gepubliceerde studies: er werd gebruik gemaakt van keyword searches in verschillende databasen en van identificatie van 219 "query" genen (37 afzonderlijke genen en 182 genen die deel uitmaakten van 1 van de 38 operons)

waarvan werd aangetoond dat ze coderen voor proteïnen die een sleutelrol spelen bij koolwaterstof afbraak en plantengroeipromotie.

Door heel deze genoom sequentie analyze van *Acinetobacter oleivorans* stam PF1 beschikken we over informatie omtrent de genomische basis van zijn (i) metabolische capaciteit om de alifatische en aromatische componenten aanwezig in diesel olie af te breken, (ii) zijn affiniteit en chemotaxis naar koolwaterstofsubstraten, (iii) zijn stressrespons na blootstelling aan koolwaterstoffen en zware metalen, (iv) en zijn belangrijkste plantengroeipromotie eigenschappen. Deze bevindingen ondersteunen de hypothese dat *A. oleivorans* PF1 een veelbelovende kandidaat is voor de ontwikkeling van nieuwe strategieën voor de bioremediatie van petroleum-gecontamineerde sites en dit dankzij zijn capaciteit om koolwaterstoffen af te breken, en zijn emulsificatie en plantengroeipromotie potentieel. Tenslotte bleek uit de ANI berekening dat er slechts 91,83% gelijkenis was met *Acinetobacter oleivorans* DR1. Deze bevinding, tesamen met zijn uitzonderlijke fenotypische en genotypische kenmerken doen ons denken dat het hier mogelijk om een nieuwe species gaat.

In het vijfde deel van dit doctoraat wordt een serre experiment beschreven waarin populier stekken [*Populus deltoides x* (*trichocarpa x deltoides*) cv Grimminge] geïnoculeerd werden met een bacterieel consortium bestaande uit 2 wortelendofyten, namelijk *Acinetobacter oleivorans* PF1 en *A. calcoaceticus* GK1, voorheen reeds beschreven o.w.v. hun diesel afbraak capaciteit in vloeibare cultuur en hun verschillende *in vitro* plantengroei bevorderende eigenschappen. Geïnoculeerde en niet-geïnoculeerde stekken werden blootgesteld aan 5000 mg kg^{-1} en 10.000 mg kg^{-1} diesel olie. De biodegradatiesnelheid, de plantengroei-index en de microbiële activiteit werden gedurende een periode van 10 weken opgevolgd. Ondanks de veelbelovende resultaten bij de lagere concentratie, had het inoculum geen positief effect op de planten bij de hogere concentratie. Daarom is verder onderzoek nodig om alle factoren die invloed kunnen hebben op de toepassing van bacteriële co-culturen bestaande uit gesequeneerde stammen te ontrafelen en weer te geven. Tenslotte worden in deel 6, het laatste deel, de belangrijkste conclusies samengevat en enkele toekomst perspectieven aangehaald.

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ABA	Abscisic acid
ACC	1-aminocyclopropane-1-carboxylic acid
acdS	1-aminocyclopropane-1-carboxylate (ACC) deaminase gene
ACO	1-aminocyclopropane-carboxylate oxidase
ACS	1-aminocyclopropane-carboxylate synthase
ACV	Absorbance of crystal violet
Ade	Adenine
AHLs	Acylhomoserine lactones
alk system	Integral membrane non-heme iron alkane hydroxylases
AlkB	Alkane hydroxylase
AlkM	Alkane monooxygenase
AlkS	Positive regulator for the *alkBFGHJKL* operon and *alk*St genes
AlkT	Rubredoxin reductase
Am	Amicasin
ANI	Average Nucleotide Identity
Ap	Ampicillin
APL	Absorbance of planktonic cells
ARDRA	Amplified rDNA Restriction Analysis
Bc	Bacitracin
BDH	2,3 Butanediol dehydrogenase activity
BF	Biofilm
BH	Bushnell Haas
BP	Boiling point

List of Abbreviations

BPB	Bromophenol blue
BS	Biosurfactants
BTEX	Benzene, toluene, ethylbenzene and xylenes
*bud*A	Acetoin decarboxylase
budB	Acetolactate synthase
budC	Acetoin reductase
CAS	Chrome azurol S
CCA	Canonical Correlation Analysis
Cf	Ciprofloxacin
CFU	Colony Forming Units
CH	Chemotaxis
CHX	Chlorhexidine gluconate
CKs	Cytokinins
CLPs	Cyclic lipopeptides
Cm	Chloroamphenicol
CoA	Acetyl-coenzyme A
CPC	Cetylpyridinium chloride
CVC	Citrus-variegated chlorosis
D_{air}	Diffusivity in air
DAPG	2,4-diacetyl phloroglucinol
DCPIP	2,6-dichlorophenolindophenol
DHZR	Dihydrozeatinriboside
DRO	Diesel range organics
D_w	Diffusivity in water
EA	Emulsification Assay

List of Abbreviations

EB	Endophytic bacteria
EC	Equivalent carbon number
ECM	Ectomycorrhizal
ED	Entner-Doudoroff
EMP	Embden-Meyerhof-Parnas
e-PGPR	Extracellular Plant Growth-Promoting Rhizobacteria
EPS	Extracellular polysaccharides
Er	Erythromycin
FDA	Fluorescein diacetate
FDAH	Fluorescein diacetate hydrolysis
Fur	Ferric uptake regulator
G-6-PDH	Glucose-6-phosphate dehydrogenase
GA_3	Gibberellic acid
GAs	Gibberellins
GDH	Glucose dehydrogenase
GI	Growth index
Gm	Gentamycin
GRO	Gasoline range organics
H	Henry's law constant
HC	Hydrocarbons
HCN	Hydrogen cyanide
HDTMA	Hexadecyltrimetyl ammonium bromide
HEAR	High erucic acid rapeseed
HM	Heavy Metals
HMW	High molecular weight

List of Abbreviations

HR	Hypersensitive
IAA	Indole-3-acetic acid
IaaH	IAM hydrolase
IAAld	Indole-3-acetaldehyde
IaaM	Tryptophan-2-monooxygenase
IAM	Indole-3-acetamide
IAN	Indole-3-acetonitrile
Ipa	Isopentenyladenosine
IPCD	Indole-3-pyruvate decarboxylase
i-PGPR	Intracellular Plant Growth-Promoting Rhizobacteria
IPyA	Indole-3-pyruvate
ISR	Induced systematic resistance
JA/ET	Jasmonate/ethylene
K_d	Partition coefficient
Km	Kanamycin
K_{oc}	Organic-carbon partition coefficient
K_{ow}	Octanol-water partition coefficient
K_s	Soil-water sorption coefficient
LB	Luria Bertani
LEAR	Low erucic acid rapeseed
LMW	Low molecular weight
MAMPs	Microbe-associated molecular patterns
MATH	Microbial adhesion to hydrocarbon
MCP	Methyl-accepting chemotaxis protein
MMO	Methane monooxygenases

List of Abbreviations

MPN	Most Probably Number
MPPS	Multi-process phytoremediation system
MR-VP	Methyl red-Voges Proskauer
MSM	Mineral salts agar medium
MT	Metallothionein
MTA	5´-Methylthioadenosin
MTBE	Methyl tertiary butyl ether
MTR	5´-Methylthioribose
MW	Molecular weight
NA	Natural attenuation
NA	Nicotianamine
Nah	Naphthalene catabolic genes
NAPLs	Non-aqueous phase liquids
NBRI-BPB	NBRIP medium containing bromophenol blue
NCBI	National Center for Biotechnology Information
NCEDs	9-*cis*-epoxycarotenoid dioxygenases
Neo	Neomycin
N-hexanoyl-L-homoserine lactone	AHL molecule C6-HSL
NRPSs	Non-ribosomal peptide synthetases
OD	Optical Density
P	Canonical variate
PAH-RHD	Polycyclic aromatic hydrocarbon oxidation
PAHs	Polycyclic aromatic hydrocarbons
PAL	Phenylalanine ammonia-lyase

List of Abbreviations

PGP	Plant Growth Promoting
PGPA	Plant Growth Promoting Activity
PGPB	Plant Growth Promoting Bacterium
PGPR	Plant Growth Promoting Rhizobacteria
PHCs	Petroleum hydrocarbons
PIPES	Piperazine -1, 4-bis (2-ethanesulfonic acid)
pMMO	Copper-containing methane monooxygenase
*pox*B	Pyruvate dehydrogenase
PPO	Polyphenoloxidase
PQQ	Pyrolloquinoline quinone
PSM	Phytate screening medium
QQ	Quorum Quenching
QS	Quorum sensing
Rf	Rifampicin
RubA	Rubredoxin
RubB	Rubredoxin reductase
S	Aqueous solubility
SAM	S-adenosylmethionine
SAM – synthetase	S-Adenosyl-L-methionine synthetase
SAR	Systemic acquired resistance
Sm	Streptomycin
sMMO	Soluble di-iron methane monooxygenase
ST	Solvent Tolerance/Sucrose Tryptone/Surface Tension
T3SS	Type III secretion systems
TAM	Tryptamine

List of Abbreviations

Tc	Tetracyclin
TCA	Tricarboxylic acid
TCE	Trichloroethylene
TLC	Thin Layer Chromatography
TPH	Total petroleum hydrocarbon
Trp	Tryptophan
TSO	Tryptophan side-chain oxidase
VOCs	Volatile organic compounds
VP	Vapor pressure
X-Gal	Bromo-chloroindolyl beta-D-galactopyranoside
YadA	Autotransporter adhesion
ZR	Zeatin riboside

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SECTION 1

Introduction

List of Abbreviations

Section 1

Microorganisms comprise the biggest part of biomass and biodiversity on our planet. Indeed, according to estimations their number rises up to 10^{30} and this huge number represents a capital for researchers globally that can be further exploited in order to understand the mechanisms and solutions being invented by nature as an auto regulation machinery of its functions. The acquired knowledge holds a catalytic role both socioeconomically and scientifically, seeing that the application and the profits could be expanded in sectors like biofuels production, industrial water treatment, environmental management and protection, agriculture as well as in human health. The industrialization of modern societies and the increasing demand for energy generation to heat our domestic and working areas, to fuel our transportation networks as well as to power fabricating processes has resulted in the extensive exploitation of petroleum hydrocarbons (PHCs), which are the most widespread class of organic contaminants worldwide (Brassington et al. 2007). The negative impacts of pollution caused by increasing use of PHCs on human health and the environment are diverse and are of concern for the scientific community, policy makers and the public. Prolonged exposure to PHCs can initiate detrimental damages to the central nervous system in humans and animals, can result in respiratory system disfunction (asthma and allergic inflammation), disrupt the endocrine system and, as a result, considerably increase the probability of lung, skin, bladder, liver and kidney cancers (Boffetta et al. 1997; Costello 1979; Hutcheson et al. 1996; Locksley 2010; Singh et al. 2004). Often PHC pollution, if it is left untreated, is interwoven with the conversion of agricultural soils to contaminated marginal lands. These lands are increasingly valuable for the production of edible crops, biofuels and fibers. It is critical for both farmers and consumers to retain access to crops able to produce sufficient quantities of consumable high quality food, otherwise a decline in existing yields would directly result in more expensive products. Globally, there is an escalation in the sensitivity of various lands to PHC contamination (Figure 1.1). Over the last two decades, increased ecological awareness about the seriousness of PHC pollution has lead to strengthened legislative measures for environmental protection and reduction in the number of PHC polluted sites. Despite this, a substantial number of ”hot spots” still exist globally that require remediation. Generally, conventional physical and chemical in *situ* and *ex situ* clean–up technologies for

petroleum hydrocarbon remediation involve excavation, air sparging, removal and off-site treatment in biopiles, pump and treat, incineration, slurry- and solid phase reactors, soil washing, soil vapor extraction, asphalt batching, thermal desorption, chemical oxidation, hydrolysis and photolysis (Amatya et al. 2002; Chan 2011; Do et al. 2009; Khan et al. 2004; Zhou et al. 2005); however, experience has demonstrated that these methods are frequently expensive, environmental unfriendly and invasive, laborious, and often only result in an incomplete removal of the pollutants of concern.

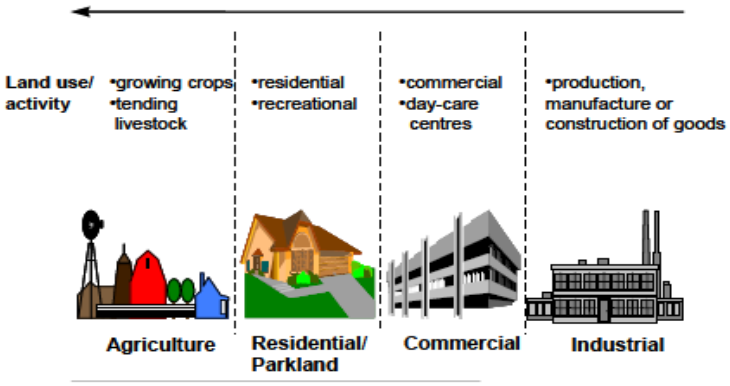


Figure 1.1 Land uses and increasing sensitivity to PHC (source: Canada Wide Standard for PHC, 2008).

Thus, research over the last decade has focused on offering remediation schemes that are moving away from the conventional ones and are mainly based on biological methods, which can be divided into bioremediation s.s., specifically the use of microorganisms (bacteria and fungi), and phytoremediation, which includes methods where vascular plants are used and their associated microorganisms for the cleanup of contaminated sites. Whilst the convergent action of plants and their related microorganisms to remove and degrade petroleum compounds is considered to be advantageous in terms of cost, due to low capital expenditure, and flexibility for *in situ* implementation, there are still numerous aspects about the mechanisms involved that remain the subject of research and debate among members of the scientific community. This Section tries to provide one more piece of information in this complicated puzzle of plant-microbe partnerships with emphasis on the remediation of hydrocarbon contaminated sites mediated by plant-bacteria associations.

Section 1

1.1 Petroleum hydrocarbons

1.1.1 Classification of Petroleum hydrocarbons

Petroleum hydrocarbons are organic compounds comprised of carbon and hydrogen atoms arranged in varying structural configurations with physical and chemical characteristics that vary over orders of magnitude; they are broadly classified in two categories namely, diesel range organics and gasoline range organics, respectively (Figure 1.2). Diesel range organics (DRO) include longer chain alkanes (C_{10}–C_{40}) and hydrophobic chemicals such as polycyclic aromatic hydrocarbons (PAH). Gasoline range organics (GRO) include to mono-aromatic hydrocarbons such as benzene, toluene, ethylbenzene and xylenes (BTEX), and short chain alkanes (C6-C10) with low boiling points (60-170 °C) such as isopentane, 2,3-dimethyl butane, n-butane and pentane (Kamath et al. 2004).

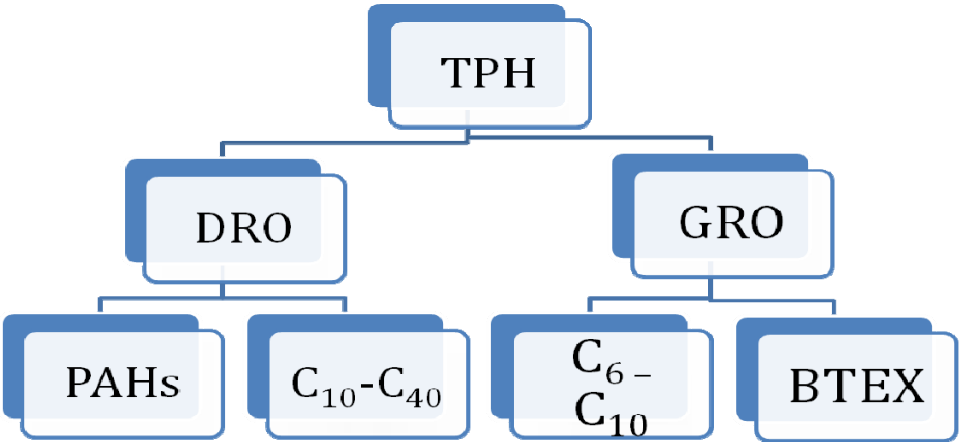


Figure 1.2 Classifications of Petroleum Hydrocarbons.

PHCs can be further subdivided into ***Alkanes***, known as paraffins, with saturated linear or branched-chain structures, ***Alkenes***, known as olefins (unsaturated molecules), ***Alicyclics***, known as naphthenes, which are saturated cyclic structures based on five- and six-membered rings and ***Aromatics,*** which are cyclic structures with conjugated double bonds, mainly based on the six-membered benzene ring. However, it should be underlined that there are alkyl–substituted cyclic, mixed cyclic and mixed polycyclic containing both aromatic and fully saturated rings.

1.1.2 Physico-Chemical characteristics of Petroleum Hydrocarbons

The physicochemical properties of the hydrocarbons, specifically molecular weight, water solubility, specific gravity, vapor pressure, Henry's law constant, diffusivity, organic carbon/water partition coefficient (K_{oc}) and octanol/water partition coefficient (log K_{ow}), are important parameters that govern the behavior and migration of petroleum hydrocarbons in the environment (AHES 1997). Table 1 provides a listing of the chemical properties required to evaluate how a petroleum hydrocarbon partitions in air-water-soil systems.

Table 1.1 Chemical properties of hydrocarbon compounds (source AEHS 1997).

Parameter	**Symbol**	**Units**
Molecular weight	MW	g/mole
Aqueous solubility	S	mg/L
Vapor pressure	VP	atm
Henry's law constant	H	atm-m^3/mole or cm^3/cm^3
Boiling point	BP	^oC
Equivalent carbon number	EC	unitless
Soil-water sorption coefficient	K_s	cm^3/g
Octanol-water partition coefficient	K_{ow}[a]	cm^3/cm^3
Organic-carbon partition coefficient	K_{oc}	cm^3/g
Diffusivity in air	D_{air}	cm^2/s
Diffusivity in water	D_w	cm^2/s

In detail, the **soil-water sorption coefficient (K_s)** shows the tendency of a compound to be adsorbed onto a soil or sediment particle. **K_s** can be measured as the ratio of the amount of the chemical absorbed per unit weight of soil or sediment to the concentration of the chemical in solution. Sorption of non-ionic hydrophobic chemicals is regulated by the **organic carbon partition coefficient (K_{oc})**, and defined by the following equation:

$$K_{oc} = \frac{K_s}{f_{oc}}$$

where:

f_{oc} = fraction of organic carbon [kg organic carbon/kg soil]

The potential for a hydrocarbon component to be absorbed by soil and sediment particles affects migration through soil and aquifer materials as well as migration

from surface water to sediments; this can be described in terms of a **partition coefficient (K_d),** which is defined as the ratio of the concentration of absorbed components to the concentration of aqueous-phase constituents, and it is generally reported in units of milliliters per gram (ml/g). A higher K_d values indicates greater potential for the component to be absorbed by soil, sediment, and aquifer materials. This partition coefficient may be determined empirically or estimated using constituent-specific and sediment- or soil-specific parameters.

The parameters used to calculate K_d for organic constituents are the organic carbon partition coefficient **(K_{oc})**, which measures the selective affinity for soil organic carbon vs. water and the fraction of organic carbon **(f_{oc})** in soil, because K_d is usually expressed as the product of the K_{oc} and f_{oc}.

The Henry's law constant, H, can be defined as an air-water partition coefficient and can be measured as the ratio of a compound's concentration in air to its concentration in water at equilibrium.

$$H = \frac{\text{concentration in air [atm]}}{\text{concentration in water [mole/m}^3\text{]}}$$

It should be underlined that the Henry's law relationship is only valid for dilute solutions where the concentration of a chemical is lower than its water solubility. Thus, when petroleum concentrations in soils and soil-water approach residual saturation this relationship may not be valid.

Solubility is an important property affecting the migration of chemicals in soils, groundwater, and surface water, since the higher the solubility, the greater the tendency of a constituent to dissolve in water. **Volatility** is another important property affecting the mobility and persistence of hydrocarbons. The diffusion coefficient in air, D_{air}, is a measure of the diffusion of a molecule in a gas medium as a result of intermolecular collisions and is defined as:

$$D_{air-B} = \frac{J_B}{\nabla X_B}$$

where:

D_{air-B} = diffusivity of compound B in compound or mixture A (in this case A is the air) [cm^2/s]

J_B = net molar flux of B [$mol/cm^2/s$]

∇X_B = concentration gradient of B [$mol/cm^3/cm$]

The diffusion coefficient in water, $\mathbf{D_{wat}}$, is a function of solute size, temperature, and solution viscosity, while the **Equivalent Carbon Number (EC)** is related to the boiling point of a chemical normalized to the boiling point of the *n*-alkanes. Finally, the **octanol-water partition coefficient**, $\mathbf{K_{ow}}$, is defined as the ratio of the hydrocarbon concentration in the octanol phase to its concentration in the aqueous phase of a two-phase octanol-water system.

$$K_{ow} = \frac{\text{concentration in octanol phase } [\frac{mg}{L}]}{\text{concentration in aqueous phase } [\frac{mg}{L}]}$$

$\mathbf{K_{ow}}$ provides an estimated value of a chemical's hydrophobicity or partitioning tendency from water to organic matter, and thus can be correlated to the compound's water solubility and $\mathbf{K_{oc}}$ (AEHS 1997). A discernible pattern of bonding between adjacent carbon atoms explains the different polarity behavior of aliphatic and aromatic hydrocarbons. Aromatic molecules have ring structures and are basically flat and symmetric with clouds of electrons above and below the plane of the molecule. Aromatic carbon-carbon bonds are termed resonance bonds due to the fact that electrons are shared between multiple carbon atoms. By this logic, the electrons are considered to be participating in several bonds which increase chemical stability. This bonding pattern is responsible for their moderate polarity. On the contrary, aliphatics are non-polar or slightly polar because their bonding pattern does not permit non-uniform distribution of charge to the same degree. Therefore, the polarity of hydrocarbons controls the degree to which these molecules interact both with themselves and with water. Compounds with low boiling points will tend to be more volatile, including many gasoline and diesel constituents (AEHS 1998). Hence, aromatics are more water soluble and less volatile compared to alkanes with a corresponding number of carbons. Moreover, an increase in molecular weight correlates with a decline in volatility, therefore aliphatic compounds are more volatile compared to aromatics (Stroud et al. 2007). Another interesting trait of petroleum hydrocarbons is their large number of *isomers*. Compounds referred to as *isomers* have the same elemental formula but different structural configurations. Generally, an increase in the carbon number increases the number of possible isomers concomitantly. For example, an alkane with six carbon atoms has five possible isomers, while increasing the number of carbons to ten increases the number of possible isomers to twenty-five.

That explains the correlation between the degree of complexity and the high number of isomeric constituents found in petroleum mixtures. Gaining knowledge about these properties of petroleum hydrocarbons is valuable in order to comprehend in depth their attenuation by weathering processes that hydrocarbons incur with time upon their release to the environment.

1.2 The role of Plants and Bacteria in the Degradation of Petroleum Hydrocarbons: an Environmental Perspective

1.2.1 Bioremediation of Petroleum Hydrocarbons

Bioremediation is defined as the use of biodegradative processes (usually by means of bacteria, fungi, earthworms and yeasts) to detoxify, degrade or support the degradation of various pollutants. Bioremediation has proved to be a useful tool for the treatment of hydrocarbon contaminated terrestrial and marine ecosystems (Alarcon et al. 2008; Almeida et al. 2013; Atlas 1995; Atlas and Cerniglia 1995; Ceccanti et al. 2006; Dolores Fernandez et al. 2011; Fan et al. 2014; Gunderson et al. 2008; Hamdi et al. 2007a; Head et al. 2006; Hernandez-Ortega et al. 2012; Margesin et al. 2007b; Menendez-Vega et al. 2007b; Whang et al. 2008; Wolicka et al. 2009). The conceptual parameters that dictate the degree of a hydrocarbons susceptibility to biodegradation can be widely classified into three inter-related categories (Figure 1.3): (a) **microbial properties** (genetic complement, gene regulation and expression, surface hydrophobicity, metabolic diversity and flexibility, substrate uptake or adherence mechanisms, tolerance to metals and other toxic xenobiotics, chemotaxis, biofilm formation); (b) **environmental factors** (presence of oxygen, nutrients, salinity and pressure, temperature, pH, water availability); and (c) **properties of the hydrocarbon substrate** (Banat et al. 2010; Bordoloi and Konwar 2009; Botalova et al. 2009; Bressler and Gray 2003; Calvo et al. 2009; Couling et al. 2010; Hino et al. 1997; Marquez-Rocha et al. 2001; Martinez-Checa et al. 2007; Sikkema et al. 1995).

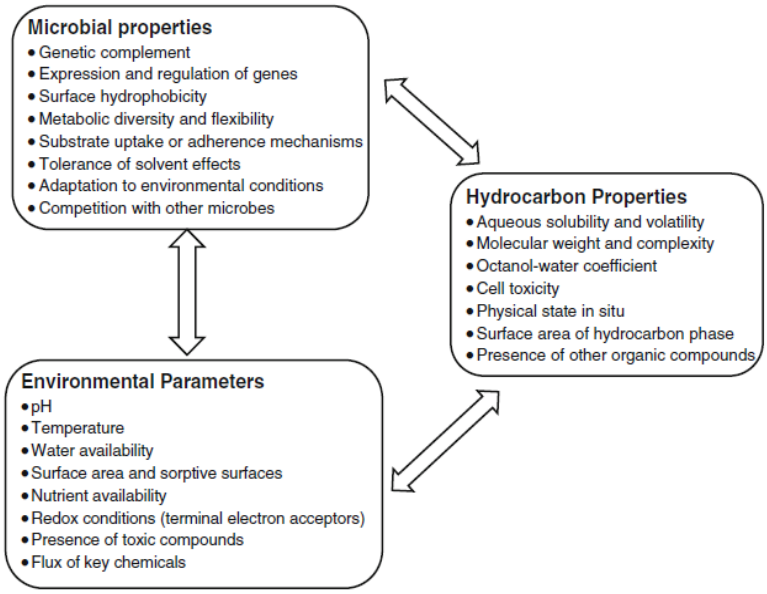


Figure 1.3 Main factors affecting biodegradation of hydrocarbons (adapted from Abbasnezhad et al., 2011).

Generally, once an active bacterial community begins to remove petroleum hydrocarbons from a contaminated environment, bioavailability (Figure 1.4) (here defined as "quantity of a contaminant which is freely available to cross an organism's (cellular) membrane from the medium the organism inhabits at a given point in time") and bioaccessibility (here defined as the "quantity of the contaminant which is available to cross an organism's (cellular) membrane from the environment it inhabits") determine the degree to which the contaminant can be taken up by the microorganism (Dandie et al. 2010; Semple et al. 2007). According to (Harmsen 2007), bioavailability may be assessed in two complementary ways: (i) by chemical methods (*e.g.*, selective extraction methods), which determine the available fraction of a well defined class of contaminants, and (ii) by biological methods, which expose organisms to contaminated soil. Although a plethora of reports supports the concept that bioremediation efficiency is likely to be more limited by the bioavailability of HCs (Hamdi et al. 2007b; Liste and Alexander 2002; Schwartz and Scow 2001; Shor et al. 2003; Tabak et al. 2003; Wick et al. 2001), the work of Huesemann et al. (2004) raised a strong opposition to that generalization.

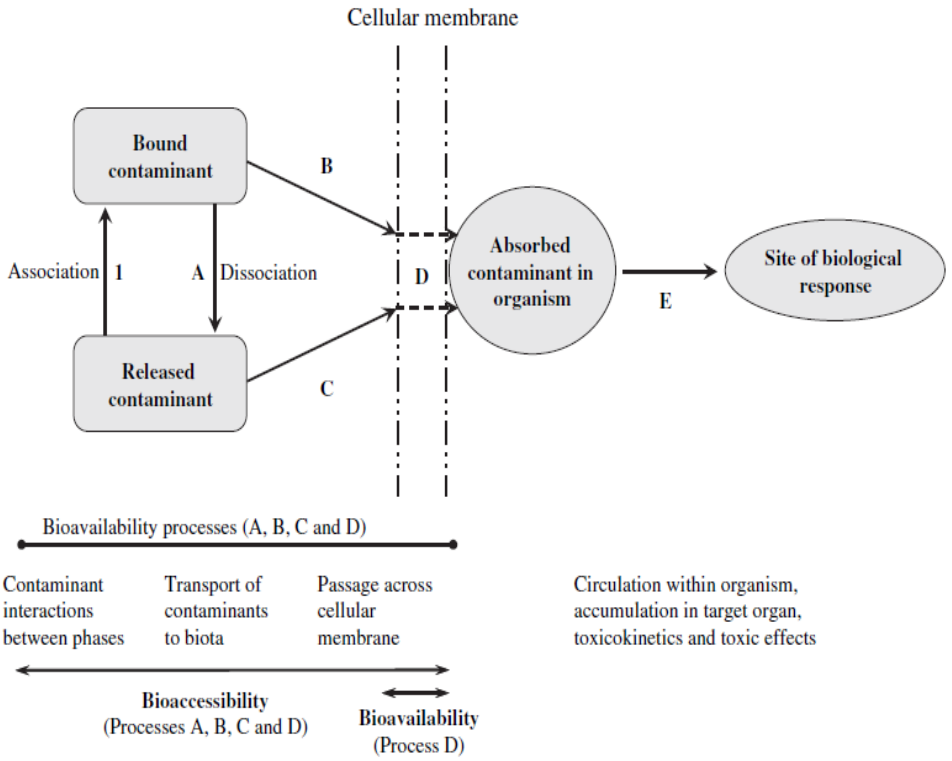


Figure 1.4 Bioavailability and bioaccessibility processes (adapted from Semple et al., 2007).

In a classical experiment conducted 34 years ago, the microbial adhesion to hydrocarbon (MATH) assay was established as a method to determine adhesion of cells to oil droplets and microbial cell surface hydrophobicity (Rosenberg et al. 1980). After that, other quantitative methods with the same aim have also been introduced including the measurement of water contact angles (Reid et al. 1992) and zeta potential (Busscher et al. 1995). Microbial adhesion to hydrophobic surfaces, usually defined as the process of transferring unbound, suspended cells from the aqueous phase to an interface (pure or mixed liquid hydrocarbon or a mixture of hydrocarbons dissolved in a water-immiscible phase), is a prevailing mechanism used by microorganisms to counteract the limited bioavailability of hydrocarbons (Bouchez-Naitali et al. 1999; Hermansson 1999). The role of adhesion in the biodegradation of aliphatic hydrocarbon non-aqueous phase liquids (NAPLs) has been reported by Volkering et al., 1997; however, adherence to hydrocarbons does not necessarily correlate with utilization.

Interestingly, the MATH assay has revealed that the cationic surfactants cetylpyridinium chloride (CPC), poly-l-lysine and chlorhexidine gluconate (CHX), and the long chain alcohols 1-dodecanol and farnesol, promoted the growth of a hydrophilic bacterium *Pseudomonas fluorescens* strain LP6a on an oil–water interface (Abbasnezhad et al. 2008). A recent review concluded that bacterial adhesion can benefit growth on, and biodegradation of, very poorly water-soluble hydrocarbons such as n-alkanes and large PAHs dissolved in a non-aqueous phase (Abbasnezhad et al. 2011).

1.2.2 Biosurfactants, biofilms and chemotaxis: role in improving bioremediation

Bacteria are able to synthesize structurally different organic compounds with surface activity. These compounds are made of amphiphilic molecules in which the hydrophobic moiety is comprised of an acid, peptide cations or anions, mono-, di- or polysaccharides, and a hydrophobic moiety of unsaturated or saturated hydrocarbon chains or fatty acids (Banat et al. 2010). Surface active compounds in biological systems have been classified into two main classes: (a) low-molecular-weight compounds called **biosurfactants**, such as lipopeptides, glycolipids and proteins and (b) high-molecular-weight polymers of polysaccharides, lipopolysaccharides proteins or lipoproteins called **bioemulsifiers** (Neu 1996). Biosurfactants can efficiently reduce surface and interfacial tensions, while bioemulsifiers are amphiphilic and polyphilic polymers that are more effective at stabilising emulsions of oil-in-water, but do not lower the surface tension as much (Smyth et al. 2010a; Smyth et al. 2010b). So far, among the low-molecular-weight biosurfactants, glycolipids such as rhamnolipids, trehalose lipids, sophorolipids, mannosylerythritol lipids, and lipopeptides such as surfactin and fungicin, are the best studied. As previously noted, the drastic reduction of hydrocarbon bioavailability to microorganisms, especially in aged contaminated matrices, is one of the major rate-limiting factors in the bioremediation of hydrocarbon-contaminated ecosystems. It is this barrier that surfactants of bacterial origin can assist in breaching, leading to accelerated biodegradation. Interpreting the interactions between bacteria, contaminants and biosurfactants in a functional perspective leads to a focus on the main natural role of biosurfactants; i.e. their involvement in hydrocarbon

accession and uptake. Microbial surfactants can promote bacterial growth on hydrocarbons by increasing the surface area between oil and water, and also through emulsification and increasing hydrocarbon pseudosolubility through partitioning into micelles (Volkering et al. 1997). In certain cases, this results in a general increase in contaminant bioavailability to degrading microorganisms. Recent reviews provide paradigms of successful biosurfactant applications in bioremediation processes (Lawniczak et al. 2013; Mulligan 2009; Pacwa-Plociniczak et al. 2011). Production of lipopeptides by *Bacillus circulans* (Das et al. 2008), as well as lipopeptides and protein-starch-lipids by two strains of *P. aeruginosa* (Bordoloi and Konwar 2009) has been proven to enhance PAH biodegradadation. Recently, different modes of enhancing petroleum hydrocarbon biodegradation by *Burkholderia multivorans* (NG1) have been reported. In this case, Triton X-100 enhanced bioavailability through emulsification and supported direct interfacial uptake, while the rhamnolipid mixture JBR-515 did not emulsify hydrocarbons, enhancing bioavailability instead through micellar solubilization (Mohanty and Mukherji 2013). It further was demonstrated that in *P. aeruginosa* the uptake of rhamolipid-coated n-hexadecane droplets occurred through a mechanism very similar to pinocytosis (Cameotra and Singh 2009). In contrast, it is well known that the presence of a surfactant may also inhibit biodegradation. Micelle cores can trap organic contaminants, creating a barrier between microorganisms and organic molecules, the result of which is the potential substrate becoming less available (Colores et al. 2000). Biosurfactants are also involved in hydrocarbon uptake by the regulation of cell envelope hydrophobicity and, thus, the attachment and detachment to and from oil droplets. This can be facilitated by exposing the hydrophilic or hydrophobic moieties of cell-bound biosurfactants external to the cell (Rosenberg et al. 1988). Degrading microorganisms may also alter their cell hydrophobicity during growth on hydrocarbons (Franzetti et al. 2008; Tzintzun-Camacho et al. 2012).

Interestingly, it has been concluded that concentration-depending mechanisms are able to regulate both the qualitative and quantitative composition of the bacterial outer envelope in the presence of rhamnolipids (Sotirova et al. 2008; Zhong et al. 2007). In addition, changes in the cell-surface hydrophobicity have been observed to be enhanced by the accumulation of different fatty acids at the

cell surface during *R. erythropolis* NTU-1 growth on hydrocarbons (Chang et al. 2009). Moreover, the addition of chemical surfactants led to the increase of both cell hydrophobicity and the hydrocarbon biodegradation rate in *Burkholderia* spp. (Mohanty and Mukherji 2012). Similar to biosurfactants, the formation of biofilms, which are defined as bacterial communities surrounded by a self-produced polymeric matrix and reversibly attached to an inert or a biotic surface (Costerton et al. 1995), is another successful adaptive mechanism of bacteria to enhance bioremediation processes via improvement of pollutant availability (Johnsen and Karlson 2004; Wick et al. 2002). A study conducted by (Pamp and Tolker-Nielsen 2007) provides evidence for the involvement of biosurfactants in structural biofilm development during cell attachment and microlony emergence. Given this, possible negative effects caused by the application of synthetic surfactants on the structure of biofilms should always take into account (Schreiberova et al. 2012). Generally, biofilm formation is triggered by environmental signals, and growth under nutrient limitation. The presence of flagella is imperative for cells when approaching and moving across surfaces, whereas occasionally outer membrane proteins such as calcium-binding proteins and adhesins mediate the initial steps of attachment. Interestingly, many bacteria produce acylhomoserine lactones (AHLs), molecules which are known to regulate biofilm formation via quorum sensing (QS) behavior (de Kievit 2009; Dickschat 2010; Kjelleberg and Molin 2002; Parsek and Greenberg 1999; Whitehead et al. 2001). Indeed, after microcolony formation, AHL production is required for the formation of a mature biofilm (Rinaudi and Giordano 2010). The biofilm matrix concatenates the cells and confers many key features to the biofilm, including the ability to protect the cells from physical, chemical and biological stresses (Gorbushina and Broughton 2009; Shemesh et al. 2010). The matrix usually consists of extracellular polysaccharides (EPS), but can also include proteins and even DNA (Branda et al. 2005; Rinaudi and Gonzalez 2009; Sutherland 2001). It is pertinent to mention that EPS largely exerts its effect by affecting the porosity, density, water content, charge, hydrophobicity, and mechanical stability of biofilms (Flemming and Wingender 2010). A number of studies raised prominence to the role of bacterial biofilms in bioremediation, with special emphasis on the biodegradation of diesel and naphthalene (Baldi et al. 1999; Chakraborty et al. 2010; Nopcharoenkul et al. 2013; Pepi et al. 2003;

Shimada et al. 2012; Singh et al. 2006; Tribelli et al. 2012b). In addition to the production of biosurfactants and biofilm formation, chemotaxis, defined as the direct movement of microorganisms in favor of, or in opposition to, a gradient of substrate with the aim of finding ideal conditions for growth and survival (Paul et al. 2006), has been reported as one of the most thoroughly characterized signal transduction systems known to mediate peculiar changes in bacterial behavior in response to different environmental stimuli. There are a muster of reviews that assiduously describe the molecular processes and components involved (Baker et al. 2006a; b; Eisenbach and Caplan 1998; Hazelbauer and Lai 2010; Krell et al. 2011; Rao et al. 2008; Wadhams and Armitage 2004). Chemotaxis is a behavioral process that some flagellated bacteria exploit to detect and interact with pollutants present in various environments and can be viewed as a prelude strategy used to facilitate the bioremediation of contaminated soil or groundwater by bringing bacteria in direct contact with the compounds of concern (Ford and Harvey 2007; Marx and Aitken 2000; Pandey and Jain 2002; Parales and Haddock 2004; Strobel et al. 2011). Indeed, the capability of bacteria to sense and swim towards n-hexadecane (Nisenbaum et al. 2013), gas oil (D'Ippolito et al. 2011), as well as various monocyclic and polycyclic aromatic hydrocarbons and their nitro-, amino- or chloro-substitutions has been demonstrated to stimulate degradation of the corresponding hydrocarbons (Bisht et al. 2010; Cunliffe et al. 2006; Fernandez-Luqueno et al. 2011; Gordillo et al. 2007; Grimm and Harwood 1997; Iwaki et al. 2007; Lanfranconi et al. 2003; Law and Aitken 2003; Ortega-Calvo et al. 2003; Pandey et al. 2002; Parales et al. 2000; Peng et al. 2008; Samanta and Jain 2000; Tremaroli et al. 2010; Vardar et al. 2005). Although in these works the chemotactic response function of bacteria has been evaluated qualitatively in semi-solid and liquid media via assays such as the swarm plate assay, and capillary and agarose plug assays, it is worthwhile to mention that bacterial chemotaxis has also been monitored quantitatively (Jeong et al. 2010; Olson et al. 2004). Interestingly, the naphthalene degrading *Pseudomonas putida* PpG7 strain was repelled by vapor-phase naphthalene although the steady state gaseous concentrations were significantly lower than the aqueous concentrations that result in positive chemotaxis (Hanzel et al. 2010). Remarkably, it has been shown that bacteria of the genus *Pseudomonas* host a new form of chemotaxis, whereby they are

attracted directly to particles containing low or high concentrations of toxic aromatic hydrocarbons. Briefly, the chemotactic response is mediated by the McpT chemoreceptor encoded by the pGRT1 megaplasmid. Two alleles of *mcp*T are borne on this plasmid and inactivation of either one resulted to loss of this chemotactic phenotype, while cloning of *mcp*T into a plasmid complemented not only the *mcp*T mutants but also its transfer to other *Pseudomonas* conferred chemotactic response to high concentrations of toluene and other chemicals indicating that chemotaxis towards toxic PAHs at high concentrations is gene-dose dependent (Lacal et al. 2011). Hence, the increase in motility and chemotaxis genes suggest that microbial communities are able to ramp up metabolic pathways that will allow for direct contact with hydrocarbon compounds (Smith et al. 2013).

1.2.3 Remediation Strategies

In case of PHC pollution, *ex situ* and *in situ* bioremediation are the two major methods being applied to attain the restoration of polluted environments (Stroud et al. 2007), however, *in situ* approaches constitute the most prevalently used treatment technologies for ecological restoration of contaminated environments (Jorgensen 2007; Romantschuk et al. 2000). The different approaches used for assessment of the ecological sustainability of *in situ* bioremediation processes have been reviewed by Pandey et al., 2009.

1.2.3.1 Natural Attenuation

Of the *in situ* treatments, natural attenuation (NA) processes have been defined as ''a variety of physical, chemical, or biological processes that, under favorable conditions, act without human intervention to reduce the mass, toxicity, mobility, volume, or concentration of contaminants in soil or groundwater'' (EPA, 1999). A growing body of studies, including modeling (Khan and Husain 2003; Suarez and Rifai 2004; Verginelli and Baciocchi 2013), field experimentation (Bayer et al. 2013; Bockelmann et al. 2001; Chiu et al. 2013; Cho et al. 1997; Cozzarelli et al. 2001; Johnson et al. 2006; Kao and Borden 1997; Lundegard and Johnson 2006; Ouvrard et al. 2013; Sarkar et al. 2005; Tang et al. 2012), and reviews (Scow and Hicks 2005; Smets and Pritchard 2003) provide evidence

that natural attenuation is a promising remediation option for soil, estuarine sediments and groundwater contaminated by PHCs.

In the same context, several other reports (Abreu et al. 2009; Devaull 2007; Lundegard et al. 2008; Pasteris et al. 2002) and reviews (Roling and van Verseveld 2002) have underlined the significant role of subsurface natural attenuation processes in bioremediation. Interestingly, natural attenuation has been shown as an effective bioremediation option for a chronically diesel-oil-polluted site over a long period of time under unfavorably cold conditions (Margesin and Schinner 2001). The recovery of the Gulf of Mexico after the Deepwater Horizon blowout, testifies to the fact that *in situ* bioremediation based on natural attenuation can be successful after large scale spills.

Indeed, quick adaptation of native microflora of the deep sea ecosystem to oil contamination resulted to dominance of bacteria of the order *Oceanospirillales* in the γ-*Proteobacteria*, a group which includes known psychrophilic hydrocarbon degraders and microorganisms from hydrocarbon-dominated environments (Hazen et al. 2010).

1.2.3.2 Biostimulation or Bioaugmentation?

The principle behind biostimulation as a method to increase petroleum hydrocarbon degradation relies on the establishment of a propitious environment for hydrocarbonoclastic bacterial communities through the addition of nutrients (nitrogen & phosphorus) and other supplementary components such as biosurfactants, horse manure, poultry litter, domestic sewage, rice straw biochar and crop residues (Coles et al. 2009; Gallego et al. 2001; Lai et al. 2009; Molina-Barahona et al. 2004; Qin et al. 2013). The adjuvant role of these factors is related either to the metabolic activity of the naturally occurring degrading bacteria or to the bioavailability of petroleum hydrocarbons. Among these biostimulants, addition of nutrients (*e.g* nitrogen, phosphorus) has been shown to improve the degradation potential of native microbial communities (Delille et al. 2004; Garcia-Blanco et al. 2007; Thomassin-Lacroix et al. 2002). Studies at both laboratory and field scales have shown enhanced degradation of petroleum hydrocarbons (diesel oil, pyrene, phenanthrene) based on the addition of biosolids (nutrient –rich organic matter), inorganic fertilizers (rich in N and P) and organic fertilizers (Braddock et al. 1995; Carmichael and Pfaender 1997;

Margesin et al. 2003; Sarkar et al. 2005; Xu and Obbard 2003). More recently, it has been observed that the higher the initial TPH contamination, the more marked was the effect of fertilization on TPH removal (Margesin et al. 2007a). Similar results have been observed in marine environments, however, prudent application of N and P sources is required, given that high nutrient levels can be the causative agent of ecological impairments such as eutrophication (Nikolopoulou and Kalogerakis 2009). Approximately 1-5% N by weight of oil with a ratio of N:P between 5 and 10:1 is applicable for oil spill remediation (Swannell et al. 1996). Moreover, based on a theoretical calculation that conversion of 1g of hydrocarbon to cell materials requires the utilization of 150 mg of nitrogen and 30 mg of phosphorus (Rosenberg and Ron 1996). A number of comparative studies have reported different C:N:P ratios as the most suitable prior to the commencement of *in situ* bioremediation. In this sense, it has been proposed that optimal C:N:P mole-ratios to enhance hydrocarbon removal in soil are at the levels of 100:9:2, 100:10:1, 100:10:5 or 250:10:3 (Zawierucha and Malina 2011). Given that the majority of microbial degraders are aerobic it is assumed that adequate aeration through mechanical tillage, forced aeration and addition of alternative oxygen sources, such as oxygen-releasing compounds ORC®, or agents such as potassium permanganate ($KMnO_4$), hydrogen peroxide (H_2O_2) and ozone (O_3) should stimulate microbial activity and enhance aerobic biodegradation rates (Brown et al. 2003; Goi et al. 2006; Menendez-Vega et al. 2007a; Saito and Magara 2003; Tsai and Kao 2009). Furthermore, the rate of hydrocarbon removal has also been stimulated by generating optimal conditions for other physical factors such as temperature (Horel and Schiewer 2009) and moisture (Zawierucha and Malina 2011).

Recently, the application of non-conventional biostimulation methods has been reported. In one such study, incorporating modified Fenton's reagent as a pre-treatment in combination with inorganic fertilizers used for biostimulation post-treatment, has led to the bioremediation of diesel polluted soil (Andrea Silva-Castro et al. 2013).

Several authors have investigated the impact of *in situ* biostimulation treatments on bacterial diversity aiming to understand the relationship between the dominance, physiology and function of specific genera able to degrade contaminants of concern (Evans et al. 2004; Iwamoto et al. 2000).

These observations suggest that identifying the key players that drive community structure is a prerequisite to comprehend, model, forecast, monitor, and control biostimulation remediation processes (Hazen 2010).

Another variant of bioremediation, defined as bioaugmentation, involves the introduction in adequate numbers of a bacterial population with the necessary catabolic potential to mediate petroleum hydrocarbons degradation (Paliwal et al. 2012; Vogel 1996). Therefore, selection and addition of (a) a pre-adapted bacterial strain, (b) a pre-adapted consortium, (c) genetically engineered bacteria, or (d) catabolic genes packaged in a vector to be transferred by conjugation into indigenous microorganisms, is of paramount importance for any bioaugmentation process (El Fantroussi and Agathos 2005; Singer et al. 2005; Thompson et al. 2005).

Attempts to consider it as an option to treat sites contaminated with petroleum hydrocarbons should always take into consideration several possibilities: a single strain or a known mixed microbial consortium can be introduced; an autochthonous bacterial consortium previously isolated from the polluted soil and cultivated with hydrocarbons as the carbon source can be introduced; or an allochthonous consortium previously drawn from another hydrocarbon polluted site can be introduced (Ueno et al. 2007). In fact, the bioremediation of soils freshly contaminated with petroleum constituents could benefit from the addition of biota primed for hydrocarbon biodegradation (Greenwood et al. 2009). Interestingly, based on the use of selected native strains, bioaugmentation has been shown to successfully speed up the bioremediation soils co-contaminated with diesel oil and various heavy metals (Alisi et al. 2009).

A study conducted to evaluate potential of autochthonous and exogenous microorganisms for bioremediation of a clayey and silty soils polluted with diesel oil revealed that the native consortium was the best option for remediating the silty soil, while a combination of native and exogenous consortia was more effective for remediating the clayey soil (Moliterni et al. 2012). Most recently, the introduction of three exogenous hydrocarbon–degrading consortia (M10, R3, and K52) as a method to increase tolerance of four biomass crop species (Indian mustard, alfalfa, low erucic acid rapeseed [LEAR] and high erucic acid rapeseed [HEAR]) to diesel oil treatments ranging from 6,000 to 24,000 mg/kg dry soil showed that HEAR, after being augmented with M10 consortium consisting of

Rhodoccocus equi, *Betaproteobacterium*, *Enterobacter* sp., *Acinetobacter calcoaceticus*, *Comamonas* sp., and *Pseudomonas alcaligenes*, increased its dry weight compared to controls (Graj et al. 2013).

Despite the satisfactory nature of these results, the major drawback of traditional bioaugmentation remains the successful integration of foreign bacteria into a stable community (Bouchez et al. 2000; El Fantroussi and Agathos 2005; Goldstein et al. 1985; Thompson et al. 2005; vanVeen et al. 1997). Numerous studies have concluded that bioaugmentation, through isolation and reintroduction of hydrocarbon degrading bacteria from a contaminated site, is more effective than *in situ* biostimulation and natural attenuation when applied to sites contaminated with methyl tertiary butyl ether (MTBE), diesel oil and other petroleum hydrocarbons (Bento et al. 2005a; Couto et al. 2010; Liu et al. 2008; Smith et al. 2005).

On the contrary, a 14-week diesel biodegradation field study revealed that biostimulation with a commercial fertilizer was more effective than bioaugmentation (Demque et al. 1997). Further, biostimulation mediated via optimized nitrogen and oxygen supply significantly improved bioremediation of diesel oil-contaminated soil, while microbial inocula (bioaugmentation) did not have an additional effect on the degradation of diesel fuel in boreal soil (Kauppi et al. 2011). The latter observation is in agreement with a previous study (Yergeau et al. 2009), although it does appear that outcomes are site specific. Bioaugmentation with endophytic bacteria with biodegradative capabilties may have benefits compared to conventional bioaugmentation with free-living bacteria, as the endophytes may have more potential to find a suitable niche in an established community due to their association with a plant host.

Further benefits can be achieved if the endophyte transfers metabolic genes for biodegradation to native endophytes (Taghavi et al. 2005). For example, *in situ* bioaugmentation with strain *Pseudomonas putida* W619 reduced trichloroethylene (TCE) evapotranspiration up to 90% under field conditions (Weyens et al., 2009). This result was achieved after the establishment and enrichment of *P. putida* W619-TCE as a poplar root endophyte followed by further horizontal gene transfer of TCE metabolic activity to members of the poplar's endogenous endophytic population (Weyens et al. 2009a).

For more information about the several different techniques developed for bioaugmenting environmental sites (Figure 1.5), with emphasis on oil spills, the reader is referred to the following reviews (Gentry et al. 2004; Hosokawa et al. 2009). Overall, both bioaugmentation and biostimulation have appeared to be effective in enhancing biodegradation of oil hydrocarbons in soil and, in some cases, the simultaneous application of these techniques seems to improve the biodegradation rates more efficiently (Hamdi et al. 2007a; Mrozik and Piotrowska-Seget 2010; Sun et al. 2012a; Taccari et al. 2012; Xu and Lu 2010). In a very recent work, it has been demonstrated that the highest pyrene removal (84.29%) was obtained through a combined bioaugmentation-biostimulation process followed by bioaugmentation (57.86%), biostimulation (50%) and control (37%) processes (Ghaly AE et al. 2013). For a comparison between the merits and demerits the two techniques see Tyagi et al. 2011. Conclusively, the selection of a bioremediation technology in case of petroleum hydrocarbon contamination is not an easy task. The involvement of several factors, such as site conditions, indigenous microbial community composition, and the type, quantity and toxicity of the pollutant present does not allow generalizations and, therefore, a case by case approach seems a rational way to deal with contamination challenges.

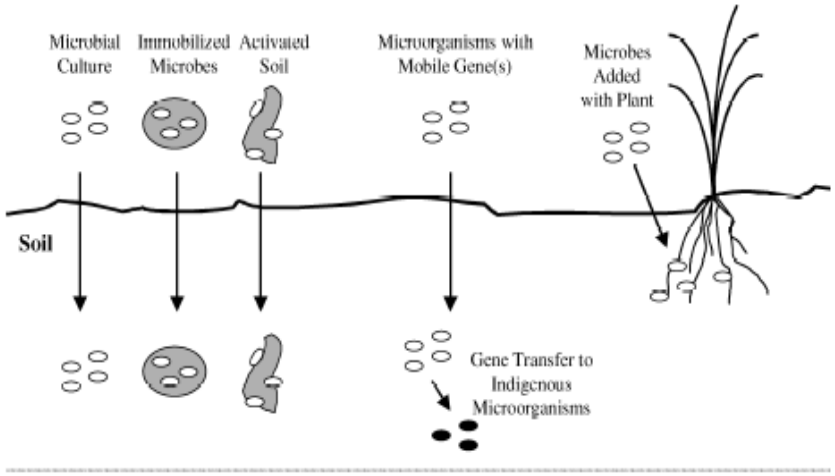


Figure 1.5 Overview of different bioaugmentation technologies to contaminated sites (modified by Gentry et al., 2004).

1.2.4 Genes and enzymes participating in aerobic degradation of hydrocarbons

The success of bioremediation interventions along with promoted bioavailability (*e.g.* production of biosurfactants), and boosted bacterial activity (*e.g.* by biostimulation or bioaugmentation) can be further optimized by involving assiduously characterized bacterial strains carrying the necessary metabolic pathways for the complete removal of various components found in a petroleum mixture. In general, despite the fact that the biodegradation of aliphatic and aromatics can occur also under anaerobic conditions, the most complete degradation of the majority petroleum hydrocarbons is more efficient under aerobic conditions. Figure 1.6 illustrates the basic principle of aerobic degradation of hydrocarbons. Many PHCs are highly biodegradable, while others have intermediate biodegradability or are generally recalcitrant. Biodegradability tends to decrease in the following order: n-alkanes > branched-chain alkanes > branched alkenes > low-molecular-weight n-alkyl aromatics > monoaromatics > cyclic alkanes > polycyclic aromatic hydrocarbons (PAHs) >> asphaltenes (Atlas 1981; Tyagi et al. 2011; Van Hamme et al. 2003).

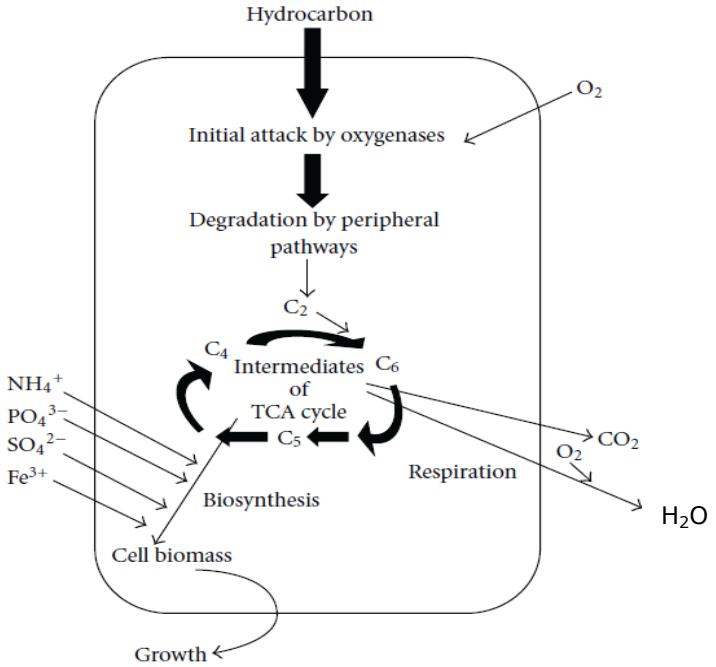


Figure 1.6 Aerobic degradation of hydrocarbons by microorganisms (Das and Chandran 2010).

Section 1

Despite the chemical inertia of alkane molecules, in the presence of O_2 they can be activated by oxygenases and completely oxidized to carbon dioxide and water. Generally, alkane-degradation by bacteria begins with an oxidative attack at the terminal methyl group with the formation of a fatty alcohol, aldehyde and fatty acid. The carboxylic acid can then be combined with CoA and, via ß-oxidation, yields acetyl-CoA that enters the tricarboxylic acid (TCA) cycle. For short-chain length (C_1-C_4) n-alkanes, methane monooxygenases (MMO) are the first enzymes involved in the process. The MMO enzyme family consists of two distinct forms: a soluble di-iron methane monooxygenase (sMMO) and a membrane-bound copper-containing methane monooxygenase (pMMO), enzymes which are encoded by *mmoX* and *pmoA* genes, respectively. Notably, sMMO performs the co-oxidation of saturated, unsaturated, linear, branched and cyclic hydrocarbons, whereas pMMO has a much narrower substrate range, being mostly active against alkanes and alkenes with lengths up to five carbons (Baik et al. 2003; Berthe-Corti and Bruns 2001; Lieberman et al. 2003). Although the heterologous expression of all three components of the sMMO system has been attained (Blazyk and Lippard 2002; Smith et al. 2002), whereas for the highly unstable pMMO complex, attempts to express full-length pMMO subunits in *E. coli* have not been successful, although recently it has been reported the heterologous expression of the pmoB subunit (Smith et al. 2011). Another interesting aspect is that gaseous alkanes are metabolized by strains expressing propane or butane monooxygenases that are related to pMMO or sMMO, respectively. For example, *Gordonia* sp. TY-5 has been reported to be able to use propane as the sole carbon source, but no other gaseous alkane. A complete operon encoding a pMMO—including large and small hydroxylase subunits, an NADH-dependent reductase and a regulatory protein, was cloned and sequenced from this organism. Upon deletion of one of the subunits, the ability of the organism to grow on propane was nullified, corroborating its role in propane oxidation (Kotani et al. 2003). The hydroxylase subunits of propane monooxygenase show relatively high sequence similarity with butane monooxygenase isolated from *Pseudomonas butanovora*, an organism which oxidizes butane to 1-butanol. This butane monooxygenase has been cloned and is similar to sMMO: the hydroxylase subunits α and ß and the regulatory protein B show more than 60%, 50% and 40% amino acid sequence identity,

respectively, to the corresponding subunits of sMMOs (Sluis et al. 2002). Medium-chain length (C_5-C_{16}) alkanes may be oxidized by two main classes of enzymes: integral membrane non-heme iron alkane hydroxylases (*alk* system) and soluble cytochrome P450s. The most thoroughly characterized alkane degradation pathway is encoded by the OCT plasmid carried by *Pseudomonas putida* Gpo1 (van Beilen et al. 2001).

In that system, the *alkBFGHJKL* operon encodes the enzymes necessary for converting alkanes into acetyl-coenzyme A (CoA), while *alkST* encode a rubredoxin reductase (AlkT) and the positive regulator for the *alkBFGHJKL* operon (AlkS). These two operons are located end to end, separated by 9.7 kb of DNA, within which lies *alkN*, a gene coding for a methyl accepting transducer protein that may be involved in alkane chemotaxis. Noteworthy, at this point that of all the genes mentioned, the function of *alkL* remains unknown, although it is suspected to be involved in transport (Figure 1.7). Comparative analysis of insertion sequences in *P. putida* P1 and the previous observation that the G+C content of the *alk* genes is lower than that of both the host strain and the OCT plasmid suggest that the genes are part of an integrated mobile element (Van Hamme et al. 2003). The genetic characterization in *P. putida* GPo1 of the alkane degradation pathway, boosted the research on the field, and since then more than 60 homologues of alkane hydroxylase gene (*alkB*) have been cloned and sequenced in both Gram-positive and Gram-negative bacteria such as *Acinetobacter* sp., *Mycobacterium* sp., *Rhodococcus* sp., *Pseudomonas putida* P1, *P. aureofaciens* and *P. fluorescens*. Furthermore, the *alkB* gene and encoded protein AlkB are diverse in nucleotide and amino acid sequences, respectively (Ratajczak et al. 1998a; van Beilen et al. 2002; Vomberg and Klinner 2000; Wasmund et al. 2009; Whyte et al. 2002). Despite the diversity of organization of the *alkB* genes among alkane-degrading bacteria, the two operons consisting of clusters of *alkB* genes of *P. putida* GPo1 are likely to be transferred via horizontal gene transfer (Rojo 2009). The *alkB* is composed of two soluble electron transfer proteins: rubredoxin (AlkG) and the electron-providing NADH-dependent flavoprotein rubredoxin reductase (AlkT). Using PCR with highly degenerate primers, genes encoding AlkB homologs have been identified in both alkane-degrading α-, ß- and γ-Proteobacteria and high G+C content Gram-positive bacteria (Actinobacteria), such as *Burkholderia cepacia*, *Pseudomonas*

aeruginosa, *Acinetobacter* sp., *Alcanivorax borkumensis*, *M. tuberculosis*, *Prauserella rugosa*, *Dietzia cinnamea*, *Gordonia* sp., and *Rhodococcus erythropolis* (Procopio et al. 2012; Shen et al. 2010; van Beilen and Funhoff 2005). Some bacterial strains like *Acinetobacter* sp. strain M-1 (Tani et al. 2001) and *P. aerugionosa* strains RR1 and PAO1 (Rojo 2009), have been documented to contain more than one alkane hydroxylase gene differentially regulated under specific physiological conditions. In a recent work, shifts in *alkB* abundance linked to the depletion of TPH substrate highlighted the importance of this molecular target as a line of confirmation for hydrocarbon biodegradation (Sutton et al. 2013).

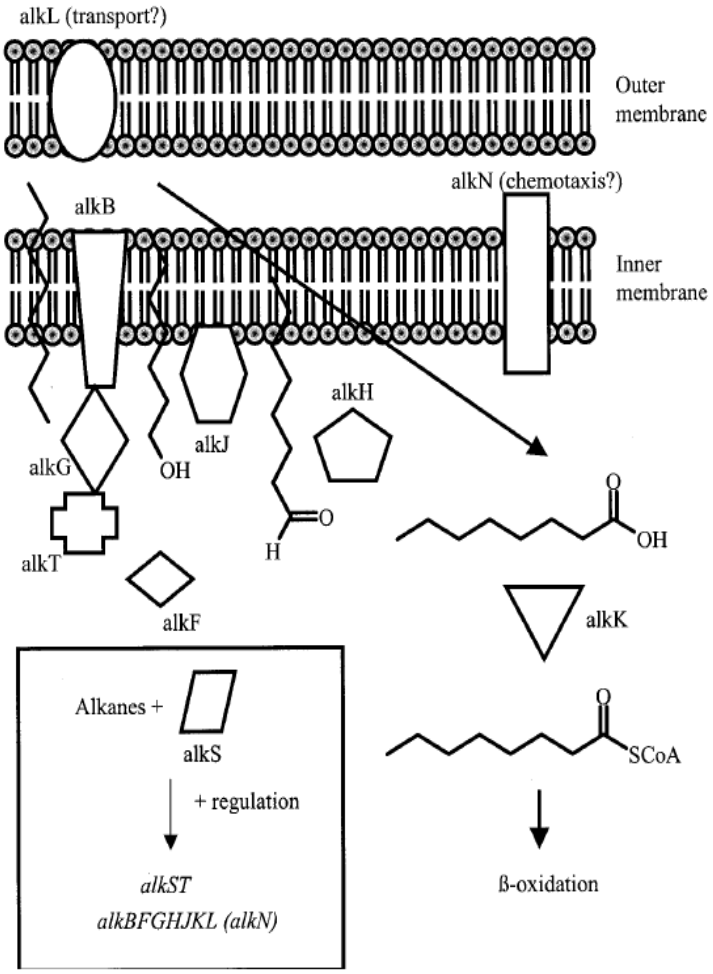


Figure 1.7 Locations and functions of the *alk* gene products in gram negative bacteria (adapted from Van Hamme 2003).

The other class of hydroxylases, facilitating the terminal hydroxylation of medium-chain n-alkanes, encompasses enzymes related to the soluble cytochrome P450 CYP153A1 from *Acinetobacter* sp. EB104 (Maier et al. 2001). Since that enzyme was characterized, various researchers have reported that bacteria belonging to *Mycobacterium*, *Rhodococcus* and *Alkanivorax* genera isolated from various environments such as contaminated soil, groundwater and surface water, use that enzymatic machinery to degrade medium-chain alkanes (Kubota et al. 2005; Liu et al. 2011a; Schneiker et al. 2006; Sekine et al. 2006; Wang et al. 2010). Even though assimilation of alkanes up to C_{20} is reported for bacteria containing AlkB family and cytochrome P450 alkane hydroxylases, there is a scarcity of information on metabolic pathways and enzyme systems that degrade $>C_{20}$ alkanes (Rojo 2009). Usually, the alkane hydroxylases present in bacteria able to degrade alkanes longer than C_{20} are not evolutionary related to known AlkB- and P450-like sequences and include AlmA (a flavin binding monooxygenase able to oxidize C_{20}-C_{32} alkanes) from *Acinetobacter* strain DSM 17874 (Throne-Holst et al. 2007) and LadA from *Geobacillus thermodenitrificans* NG80-2 (Feng et al. 2007), able to generate primary alcohols from C_{15}-C_{36} alkanes. Remarkably, *almA* represents the first cloned gene encoding an enzyme specifically involved in the degradation of n-alkanes with carbon chains longer than C_{40} (Throne-Holst et al. 2007). Genes homologous to *almA* have been identified in and cloned from *Acinetobacter* sp. M-1 (Sakai et al. 1994), and *Acinetobacter baylyi* ADP1 (Vaneechoutte et al. 2006). Similar enzymes, based on sequence homology, have been suggested to be present also in *Marinobacter aquaeolei* VT8, *Oceanobacter* sp. RED65, *Ralstonia* spp., *Mycobacterium* spp., *Photorhabdus* sp., *Psychrobacter* spp., and *Nocardia farcinica* IFM10152 (Wentzel et al. 2007). Lately, a unique functional AlkB-type alkane hydroxylase system has been described that allows growth on long-chain liquid and solid *n*-alkanes in the Gram-positive *Gordonia* strain SoCg (Lo Piccolo et al. 2011). In addition to that, results obtained from the work of Zhang et al., 2011 indicated that *P. aeruginosa* strain DQ8 can metabolize the long-chain alkane n-docosane via the beta-oxidation pathway. Briefly, based on characterization of the metabolites, they proposed that n-docosane could be oxidized at the terminal position to docosanol by a monoxygenase, with further oxidation to docosanoic acid.

Section 1

In contrast to alkanes, the general mode of mono- and polycyclic aromatic hydrocarbon biodegradation requires the presence of bacteria that harbor catabolic genes coding for dioxygenases.

Generally, catabolism of PAHs is triggered by a dioxygenase reaction on one ring that adds hydroxyl groups (OH). Thereafter, the hydroxylated ring is subjected to ring fission, producing a substituted PAH with one ring less than the parental molecule. Subsequent oxygenase reactions are utilized to ultimately mineralize the PAH (Olson et al. 2003). Ring-hydroxylating dioxygenases related to polycyclic aromatic hydrocarbon oxidation (PAH-RHD), such as those encoded by the *nah*, *nod* and *phn* genes in Gram-negative bacteria, and the evolutionarily correlated *nid*, *nir* and *nar* genes in Gram-positive bacteria, catalyze the first step of the PAH degradation pathway (Khan et al. 2001; Larkin et al. 1999; Saito et al. 2000). In this step, dioxygenase catalyzed oxidation of arenes yields vicinal *cis*-dihydrodiols as the early bioproducts by a multicomponent enzyme system (Figure 1.8). Furthermore, these dihydroxylated intermediates may then be cleaved by intradiol or extradiol ring-cleaving dioxygenases through either an ortho-cleavage pathway or a meta-cleavage pathway, leading to central intermediates such as protocatechuates and catechols that are further converted to tricarboxylic acid (TCA) cycle intermediates (Peng et al. 2008). The catalytic component with hydroxylase activity is composed of an alpha subunit of about 50 kDa and a beta subunit of 20 kDa, which assemble in a α3ß3 heteroxamer. Each alpha subunit consists of two domains, the N-terminal Rieske domain, which contains a [2Fe-2S] cluster, and the C-terminal catalytic domain, which contains a mononuclear ferrous ion close to the substrate-binding site. The catalytic component requires electrons in order to activate oxygen at each cycle of hydroxylation of the substrate. Two auxiliary proteins, a ferredoxin and a flavin-containing oxidoreductase, often provide the necessary reductant at the expense of NAD(P)H oxidation (Jouanneau et al. 2011). Genes coding for the catalytic domain of PAH-RHDs (α-subunit) have been broadly used as biomarkers of PAH-degrading potential in various environments, making this subunit a valuable tool for studying RHD biodiversity (Ding et al. 2010; Flocco et al. 2009).

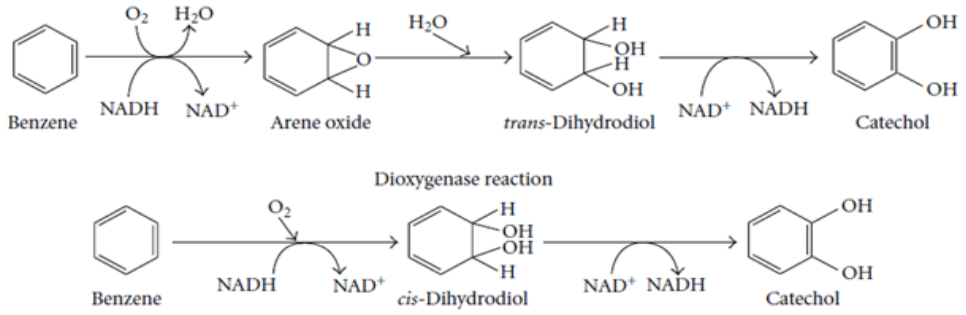


Figure 1.8 Dioxygenase catalyzed oxidation of arenes (modified from Das & Chandran 2010).

Based on amino acid sequence comparisons of the catalytic oxygenase α subunits, four discernible classes have been reported. These are: (a) the naphthalene family which includes Gram-negative bacterial enzymes responsible for the degradation of naphthalene and phenanthrene; (b) the benzoate family encompassing enzymes for the oxidation of aromatic acids; (c) the phthalate class that includes the diverse mono- and dioxygenases (interestingly the majority of the members of this family lack the ß subunits and possess only the reductase component in the electron transport chain); and (d) the toluene/biphenyl class that contains enzymes from both Gram-negative and Gram-positive microbes capable to transform toluene, benzene and chlrobenzenes (Gibson and Parales 2000). Studies on PAH degraders have found RHD-encoding genes in divergent locations in genomes; that is, either on the chromosome or on plasmids (Obayori and Salam, 2010). Historically, the critical point for the analysis of PAH degradation by aerobic bacteria started with the discovery, in *Pseudomonas putida* strain G7, of naphthalene catabolic genes (*nah*) located on the plasmid NAH7 (Simon et al. 1993). After that discovery, several works mainly on *Pseudomonas* species made evident that naphthalene biodegradation occurs via the formation of salicylate as an intermediate. Figure 1.9 illustrates that oxidation of naphthalene follows either the gentisic acid (Grund et al. 1992), or catechol (ortho and/or meta) degradation pathways (Eaton and Chapman 1992) in order to generate compounds for integration in the TCA cycle.

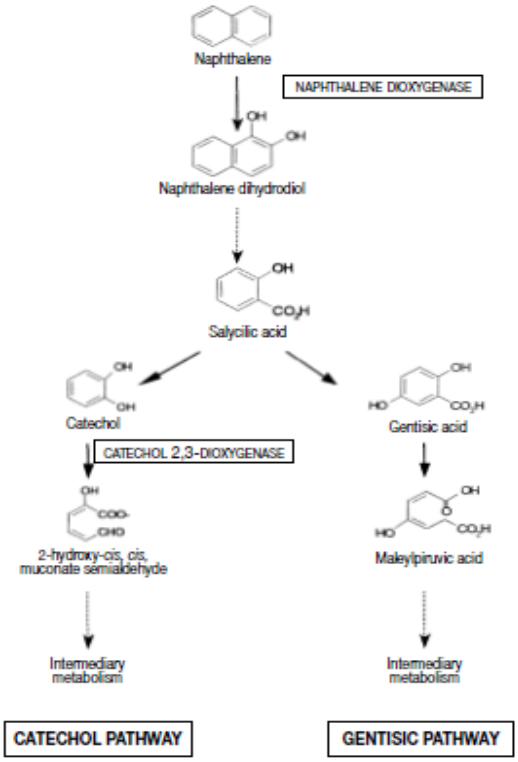


Figure 1.9 Pathways of naphthalene degradation (adapted by Alquati 2005).

In a more general view about the genetics and diversity of dioxygenases involved in the degradation of low molecular weight (LMW) and high molecular weight (HMW) PAH compounds (*e.g.* naphthalene, phenanthrene, anthracene, pyrene, benzo[a] pyrene, benzo[a] anthracene) it is noticeable that both Gram-negative genera like *Pseudomonas*, *Ochrobactrum*, *Polaromonas*, *Sphingomonas*, *Novosphingobium*, *Acidovorax* and *Burkholderia*, and Gram-positive genera like *Mycobacterium*, *Gordonia*, *Bacillus*, *Nocardia*, and *Rhodococcus*, exploiting these enzymes for the degradation of the aforementioned compounds (Alquati et al. 2005; Auffret et al. 2009; Bosch et al. 2000; Brezna et al. 2003; Iwabuchi and Harayama 1997; Jurelevicius et al. 2012; Kulakov et al. 2000; Lee et al. 2011; Ma and Herson 2000; Martin et al. 2013; Mattes et al. 2008; Moscoso et al. 2012; Schuler et al. 2009; Silva et al. 2012; Singh et al. 2013; Singleton et al. 2009; Vazquez et al. 2009; Wongwongsee et al. 2013).

1.2.5 Plants and bacteria for the remediation of petroleum hydrocarbons

Phytoremediation, defined as the use of plants and their associated microorganisms to assimilate, transform, metabolize, detoxify and degrade various toxic inorganic and organic compounds (*e.g.* petroleum hydrocarbons, pesticides, dyes, solvents) found in soil, water, groundwater and air is generally considered as an environmentally friendly, cost effective and socially accepted remediation approach (Alkorta and Garbisu 2001; Kabra et al. 2012; Pilon-Smits 2005; Prasad et al. 2010; Reichenauer and Germida 2008; Salt et al. 1995; Salt et al. 1998; Sandhu et al. 2007; Wenzel 2009). For more information about the advantages and disadvantages of phytoremediation we refer to the following reviews (Arthur et al. 2005; Kuiper et al. 2004; Pandey et al. 2009; Susarla et al. 2002; Vangronsveld et al. 2009). Plants and their associated bacteria are characterized by varied and complex interactions. In these very close plant-bacteria interactions, plants provide nutrients and residency for bacteria, which in exchange can improve applicability and efficiency of phytoremediation in case of sites contaminated by petroleum hydrocarbons. In the following paragraphs, the quotation of selected paradigms will aim to shed light to the field of petroleum hydrocarbons degradation via plants, bacteria and their intimate synergy.

1.2.5.1 Plants and Hydrocarbons remediation

Practically, *in situ* implementation of phytoremediation strategies to restore contaminated sites has several drawbacks. For example, the root zone generally reaches only a few meters down from the soil surface. This, combined with slow growth rates may result in long periods of time passing before the target pollutant is reached, if it is reached at all. The toxicity of the pollutants to the native or introduced vegetation may result in inhibition of seed germination, reduction of photosynthetic pigments, shortening of tissues (root, aerial parts), slackening of nutrient assimilation and disruption of the plant root architecture (Euliss et al. 2008; Meudec et al. 2007; Smith et al. 2006). Hence, selection of plants with increased pollutant tolerance, production of sufficient root and shoot biomass, suitability for various soil types, effective pollutant uptake mechanisms, and appropriate metabolic capabilities to degrade organic pollutants is a requisite for a successful remediation scheme (Wenzel 2009).

Section 1

Initially, the response of plants to HCs present in soil includes uptake, translocation to and accumulation in structures such as roots and shoots. The rates of these processes are typically related to the HC concentration (Lu et al. 2010; Wild et al. 2005). It is known that root uptake of HCs is strongly affected by HCs lipophilicity, a parameter which is expressed as the octanol-water partition coefficient (K_{ow}). HCs with a log K_{ow} < 1 are characterized by high water-solubility, and plant roots do not generally accumulate them at a rate surpassing passive efflux into the transpiration stream with subsequent translocation to the shoot, therefore, that practically means impassability to be taken up by the plant roots (Cunningham and Berti 1993), whereas HCs with a log K_{ow} > 3.5 cannot be taken up and translocated into the plant due to tight sorption onto the soil or root surfaces (Meng et al. 2011). After being transported inside the plant, HCs can be either sequestered in root tissue, or transported into shoots and then to leaves, where they can be stored in the vacuole or volatized into the atmosphere (Reichenauer and Germida 2008).

Over the last years, increasingly compelling evidence has accumulated about the possibility to use plants alone for the remediation of environments polluted by hydrocarbons (HCs) (Euliss et al. 2008; Gerhardt et al. 2009; Liste and Alexander 2000; Newman and Reynolds 2004; Pena-Castro et al. 2006; Peng et al. 2009; van der Lelie et al. 2001; Zhang et al. 2012).

Numerous studies focusing on the discovery of plant species suitable for phytoremediation of petroleum hydrocarbon-contaminated soils have recognized that among others, Italian ryegrass (*Lolium perenne*), sorghum (*Sorghum bicolour*), maize (*Zea mays*), tall fescue (*Festuca arundinacea*), alfalfa (*Medicago sativa* var. Harpe), elephant grass (*Penninsetum purpureum*), bermuda grass (*Cynodon dactylon*), birdsfoot trefoil (*Lotus corniculatus* var. Leo), sunflower (*Helianthus annuus*), southern crabgrass (*Digitaria sanguinalis*), red clover (*Trifolium pretense*), beggar ticks (*Bidens cernua*), sedge species (*Cyperus rotundus*) and leguminous plants may be effective (Ayotamuno et al. 2010; Basumatary et al. 2012; Basumatary et al. 2013; Chaineau et al. 2000; Hall et al. 2011; Huang et al. 2004; Kaimi et al. 2007; Muratova et al. 2008; Parrish et al. 2004; Radwan et al. 1995; Rutherford et al. 2005; Shirdam et al. 2008; Tang et al. 2010a; Wiltse et al. 1998; Yousaf et al. 2010).

In general, the positive influence of leguminous plants is attributed to their ability to increase the nitrogen concentration in soils with high C:N ratio, whereas the positive contributions provided by grasses are correlated with their fibrous root systems, large root surface and deeper penetration into the soil matrix (Gaskin and Bentham 2010; Rezek et al. 2008; Tesar et al. 2002).

Interestingly, it has been reported that the application of an ornamental plant (*Mirabilis jalapa*), characterized by nontrivial tolerance to petroleum contamination, strongly promoted TPH degradation when the concentration of petroleum hydrocarbons in soil was equal to or lower than 10,000 mg/kg (Peng et al. 2009).

Despite the consensus for the beneficial aspects of grasses and leguminous plants, planting trees like willows (*Salix* spp.) and hybrid poplars (*Populus* spp.), is another way to manage remediation of sites with contaminated groundwater (Cook et al. 2010).

This approach can be justified because of: easy propagation, fast and perennial growth, presence of phreatophytic roots that extend to the water table, high water uptake rates, high absorption surface areas, and tolerance to contaminants and flooding (Barac et al. 2009; Euliss et al. 2008; Jordahl et al. 1997; Newman and Reynolds 2004; Widdowson et al. 2005).

A recent review compares the effectiveness of trees and grasses for remediation of PHCs and concludes that only minor differences exist between trees and grasses with respect to average reduction of hydrocarbons (Cook and Hesterberg 2013). Conclusively, phytoremediation is a site-specific remediation method, that's why some contradictory results have been reported regarding the efficiency of this technology in removing contaminants from soil (Joner et al. 2004).

Employing native plant species that are tolerant to high concentrations of TPHs in soil can be a key factor in the success of phytoremediation. Yet, selecting plant species for PHC phytoremediation remains a complex and difficult decision and possibly, both approaches are needed, the one reinforcing the other.

1.2.5.2 Rhizosphere bacteria and HC remediation

The photoautotrophic nature of plants, together with the fact that petroleum mixtures (*e.g.* diesel oil) are poorly soluble in water and not taken up by plants,

means that for efficient PHC degradation, the biocatalytic activities of the rhizospheric microorganisms is essential.

Generally, vegetated soils favour higher microbial numbers and diversity compared to bulk soil (Glick 2010; Haichar et al. 2008; Smalla et al. 2001; Uroz et al. 2010). This effect is thanks to the release of organic compounds by plants in a process commonly referred to as "rhizodeposits"; these compounds can be categorized as exudates, secretions, plant mucilages, mucigel and root lysates (Olson et al., 2003) that are utilized by microorganisms as sources of carbon and energy (Chaudhry et al. 2005).

Further, plants release others organic compounds including terpernes, flavonoids and some lignin-derived components with chemical structures similar to those of PHCs, chemicals which may induce expression of HC-degrading genes in rhizospheric microorganisms (Sun et al. 2010). As such, HC-degrading rhizosphere bacteria may ameliorate plant tolerance to HCs, which is of paramount importance for the recovery of soil health during phytoremediation (Barrutia et al. 2011; Escalante-Espinosa et al. 2005).

Research has also shown that plants, by releasing these organic compounds as well as changing physicochemical and biological properties of the soil, most likely facilitate the attraction of bacteria with desired metabolic activities (Hartmann et al. 2009). As an example, the observed increase of phenolic compounds found in root exudates has been associated with a higher degree of degradation of benzo[a]pyrene in the rhizosphere of *P. australis* (Toyama et al. 2011), while more recently it has been demonstrated that gene abundance and mineralization patterns of HCs by rhizosphere bacteria was substantially affected by the composition of root exudates, since certain compounds (acetate, alanine) were found to be associated with increased mineralization capacity, whilst some others (malonate) decreased mineralization (Phillips et al. 2012).

The beneficial association of bacteria and their host plants in the remediation PHCs at the level of the rhizosphere has been confirmed, and selected paradigms include *Pseudomonas* sp. strain UG14Lr with maize (Chouychai et al. 2012), *Pantoea sp*. strain BTRH79 with *Lolium perenne* (Afzal et al. 2012), *Rhizobium meliloti* strain ACCC 17519 with alflfa (Teng et al. 2011), *Gordonia* sp. strain S2RP-17 with maize (Hong et al. 2011), *Sinorhizobium meliloti* with sorghum (Golubev et al. 2011), *Acinetobacter* sp. strain with ryegrass (Yu et al.

2011), *Pseudomonas* sp. with birdsfoot trefoil (Yousaf et al. 2010), *Azospirillum brasilense* strain SR80 with winter rye and alfalfa (Muratova et al. 2010), *Rhodococcus* sp. strain ITRH43 with Italian ryegrass (Andria et al. 2009), *S. meliloti* strain P221 with sorghum (Muratova et al. 2009), *P. putida* strain MUB1with maize (Chouychai et al. 2009), *P. putida* strain KT2440 and *Mycobacterium* sp. strain KMS with barley (Child et al. 2007a; Child et al. 2007b), *A. lipoferum* sp. and *Pseudomonas* sp. strain GF3 with wheat (Muratova et al. 2005; Sheng and Gong 2006), *R. leguminosarum* with white clover (Johnson et al. 2004), *P. fluorescens* & *P. aureofaciens* with barley (Anokhina et al. 2004) , and *P. putida* strain PCL1444 with barmultra grass (Kuiper et al. 2001).

1.2.5.3 Endophytic bacteria and HC remediation

In a pioneering study, it was shown that the enrichment of bacteria with the appropriate catabolic genes in the endophytic root compartment is correlated with the type and amount of contaminant and also on the genotype of the plant (Siciliano et al. 2001).

Since then, a number of reports have confirmed that endophytic bacteria (EB), rather than rhizosphere or soil bacteria have a better capacity to enhance HC phytoremediation (Barac et al. 2004; Doty 2008; Ryan et al. 2008; Weyens et al. 2010; Yousaf et al. 2011). Bacteria dwelling the internal tissues of plants (roots, stems, leaves) overcome the competition for nutrients and space, and are physically protected from unfavorable environmental conditions (Schulz et al. 2006). Some soil bacteria can penetrate into roots and move into shoots, indicating that these soil bacteria are a source for EB (Germaine et al. 2004), however, it is also observed that some phyllosphere bacteria may be a source of EB (Quadt-Hallmann et al. 1997). In plant–endophyte associations, the plant has to confront the toxic nature of the hydrocarbons; therefore, an endophyte with the ability to mitigate the toxicity of the pollutant seems of utmost importance. Indeed some endophytic bacteria have the potential at first to tolerate and then mineralize hydrocarbons. Hence, this hydrocarbon degrading capacity of endophytic bacteria has being investigated aiming to enhance the remediation potential of trees, herbaceous plants and grasses (Afzal et al. 2011; Barac et al. 2004; Phillips et al. 2008). In addition, other studies have

demonstrated that EB with appropriate degradation pathways are metabolically active in the root and shoot of plants vegetated in diesel contaminated soils (Afzal et al. 2012; Andria et al. 2009).

1.3 Plant Growth Promoting Bacteria and Environmental Development

The contribution of bacteria to plant growth promotion relies on the ability of these microorganisms to alleviate, through various complex mechanisms, unfavorable abiotic and biotic conditions that plants confront during their life cycles.

Although, it has been known for over a century that bacteria can stimulate plant growth and increase productivity, the establishment of the term Plant Growth-Promoting Rhizobacteria (PGPR) did not occur until the mid 1970's when Kloepper and Schroth introduced the term to describe the ability of some bacteria, mainly pseudomonads, to control soil-borne pathogens resulting in the indirect enhancement of plant growth.

Thus, PGPR was originally used to describe only bacteria with biocontrol properties (Bashan and Holguin 1998). Later, (Gray and Smith 2005) introduced more specific terms to reflect the physical sites on a plant where PGPR reside: Intracellular PGPR (i-PGPR – symbiotic bacteria) reside inside plant cells, produce nodules on the surface of a root hair during infection and being localized inside those specialized structures; and extracellular PGPR (e-PGPR – free-living rhizobacteria) live outside plant cells and do not produce nodules, but enhance plant growth through production of signal compounds that directly stimulate plant growth, improve plant disease resistance, or improve mobilization of soil nutrients.

Using as a criterion their propinquity to the plant root, ePGPRs can be further divided into three classes: those living in proximity to, but not in contact with the roots; those dwelling on root surfaces; and those living in the spaces between the cells of the root cortex (Gray and Smith 2005).

Currently, the enhancement of plant growth promotion is attributed to the action of microorganisms that colonize the rhizosphere, whereas beneficial effects are also possible within a framework of endophytic and phyllospheric lifestyles.

Presumably, the different microbial groups use similar mechanisms to benefit their host plants so the collectivized action of rhizospheric, endophytic and

phyllospheric bacteria are thought to all contribute to plant growth promotion. For the rest of this thesis, the term Plant Growth Promoting Bacteria will encompass the aforementioned microbial groups.

There is evidence to suggest that Plant Growth Promoting Bacteria elicit plant growth promotion through indirect and direct action.

Indirect mechanisms include: production of antibacterial or antifungal compounds that prevent infections by pathogens; sequestration of nutrients to prevent pathogen growth (e.g. via siderophore production resulting in a depletion of iron in the rhizosphere); induction of plant systemic resistance mechanisms; and competition for microbial binding sites on roots.

Direct effects include: assisting plants in the acquisition of macronutrients such as nitrogen via fixation (diazotrophy), phosphorus via production of phosphatases and organic acids, iron via siderophores; lowering plant ethylene levels through production of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase; and by producing phytohormones involved in plant growth regulation such as indole-3-acetic acid (IAA), cytokinins, gibberilins, acetoin and 2,3 butanediol (Glick 2010; Long et al. 2008; Taghavi et al. 2009; Weyens et al. 2009b).

Both direct and indirect activities resulting in Plant Growth Promotion have been described for a diverse array of bacteria including, most commonly, *Pseudomonas*, *Bacillus*, *Arthrobacter*, *Azotobacter*, *Azospirillum*, *Burkholderia*, *Enterobacter*, *Gluconacetobacter*, *Herbaspirillum*, *Klebsiella*, *Ochrobactrum*, *Pantoae*, *Rhodococcus*, *Rhizobium*, *Serratia*, *Stenotrophomonas* and *Streptomyces* (Babalola 2010; Barea et al. 2005; Dimkpa et al. 2009).

With rhizobacteria and endophytes, the success of plant growth promotion activities depends on the colonizing ability of bacteria, an ability which is determined by the ability of the bacteria to utilize organic acids rather than sugars for carbon and energy, their chemotaxis behavior, and their production of lipopolysaccharides and proteins (Miransari 2011).

Unraveling the mechanistic details underlying the myriad of plant-bacterial interactions will have a substantial effect on our abilities to contribute to plant welfare and could lead to the development of useful tools for environmentally friendly and sustainable approaches to agriculture and bioremediation.

As such, what is currently understood about direct an indirect plant growth promotion by bacteria is discussed below.

1.3.1 Direct plant growth promoting activity

1.3.1.1. Plant growth-promoting bacteria as biofertilizers

Major deficiencies of mineral nutrients in soils, including nitrogen, phosphorus and iron, can have negative impacts on the growth and yield of terrestrial plants, while the excessive use of conventional chemical fertilizers to alleviate these deficiencies in agricultural systems inevitably leads to serious environmental problems such as leaching, runoff, emission and eutrophication of aquatic bodies (Adesemoye et al. 2009; Flessa et al. 2002; Ma et al. 2007).

As global food demand increases hand in hand with decreases in petroleum reserves, it is critical that sustainable alternatives to chemical fertilizers, for example biofertilizers, are employed by the agricultural sector.

Biofertilizer can be defined as a substance that contains living microorganisms which, when applied to seeds, plant surfaces, or soil, colonize the rhizosphere or the interior of the plant and promotes growth by increasing the supply or availability of primary nutrients to the host plant.

However, it should be underlined that not all PGPR can be considered as biofertilizers, since many PGPR stimulate the growth of their host plants only by helping to control pathogenic organisms (Vessey 2003).

Microorganisms in biofertilizers may promote plant growth through biological nitrogen fixation, phosphorus mineralization/solubilization and iron acquisition, meaning that they could be the core of a strategy for a sustainable crop production by reducing reliance on chemical fertilizers.

Inorganic nitrogen in the form of dinitrogen gas **(N_2)** cannot used by plants for their metabolic needs, despite the fact that N_2 comprises 78% of ambient air and that scarcity is explained by its existence in the form of dinitrogen gas (N_2). Nitrogen is a substantial constituent of the organic molecules in plant cells being found in proteins, nucleic acids, chlorophyll, alkaloids and some hormones.

With the exception of a few microbial genera, all living organisms (including plants) must meet their needs for nitrogen with ammonium (NH^{4+}) or nitrate (NO^{3-}). Biological nitrogen fixation, the reaction that converts atmospheric $N_2(g)$

into bioavailable ammonia, is catalyzed by O_2- sensitive nitrogenases, encoded by *nif* genes, and is carried out exclusively by free-living or symbiotic prokaryotes.

The Mo-nitrogenase, which contains Mo at its active site, is the most commonly found and the most efficient form of the enzyme known. Two alternative nitrogenases, a V-nitrogenase and a Fe-only nitrogenase have also been identified. In these alternative nitrogenases, which are expressed when Mo is limiting, the Mo atom is replaced by V (in the V-nitrogenase) or Fe (in the Fe-only nitrogenase), respectively (Bellenger et al. 2011). Nitrogenase is able to catalyze the following reaction under ambient environmental conditions:

$$\mathbf{N_2 + 8\ H^+ + 8e^- + 16\ MgATP \rightarrow 2\ NH_3 + H_2 + 16\ MgATP + 16\ Pi}.$$

Nitrogen fixing bacteria are called diazotrophs and they can be closely associated with higher plants, often called symbioses, or loosely associated in so called associative interactions.

Symbiotic diazotrophs include: (a) legume symbionts (Gram-negative bacteria); (b) members of the genus *Frankia* that from nodules on roots of woody, dicotyledonous trees and shrubs; and (c) symbiotic diazotrophic cyanobacteria form mutualistic relationships with primitive plants.

The legume symbionts are classified into several genera, with most species belonging to the genera *Rhizobium*, *Sinorhizobium* (Ensifer), *Mesorhizobium*, *Bradyrhizobium*, *Azorhizobium* and *Allorhizobium* (Beattie 2007).

Symbiotic diazotrophic bacteria are characterized by their ability to induce the formation of new organs in their hosts, namely root nodules. The term nodulation, defined as "the formation of nitrogen-fixing root nodules on plant roots" describes the mutualistic relationship between legumes and nitrogen fixing bacteria (Chibucos and Tyler 2009). The outcome of these interactions is the increased transfer of biologically fixed nitrogen to the host plant. The process of nitrogen fixation is energy intensive for the bacterial symbiont, increasing the need for an energy rich and well protected microhabitat.

Within the framework of nodulation, both partners benefit from their association as the plant provides various carbohydrates, amino acids, organic acids, as well as other compounds, to the nitrogen-fixing microorganisms. These microorganisms are chemotactically attracted to root exudates, allowing them to

colonize and multiply both in the rhizosphere and the rhizoplane (Compant et al. 2010).

Remarkably, in legumes, the formation of nitrogen fixing nodules is the aftermath of a root tissue infection; the infection occurs via an infection thread which acts as a carrier that takes the bacteria through the root hair into the root cortex and distributes them to plant cells, with subsequent conversion of the infected cells into the nitrogen-fixing nodule. Within the nodules, rhizobia switch to an endosymbiotic form, bacteroids, with the ability to reduce dinitrogen to ammonia (Broughton et al. 2003; Stougaard 2000). The signaling transaction between legumes and rhizobia includes the secretion of flavonoids from plants that, along with the bacterial activator protein NodD, induce expression of rhizobial nodulation (*nod*) genes. The *nod* genes code for Nod factors that direct nodule formation.

Nod factors are lipochitooligosaccharides consisting of backbones of four or five *b*-1, 4-linked *N*-acetyl-glucosamine residues with an acyl chain at C2 of the non-reducing end that are also decorated with acetyl, sulfonyl, carbamoyl, fucosyl or arabinosyl moieties at defined positions depending on the rhizobial strain (Badri et al. 2009). It is noteworthy to mention that the annual contribution of these actinorhizal plants (legumes) to the total amount of biologically fixed nitrogen is estimated to be 25% in terrestrial ecosystems.

The genus *Frankia* was first isolated in 1886 from non-legume root nodules, the first indication that actinomycetes can consolidate an intimate association with plants, and since then has been found to be widely distributed. The ubiquity of *Frankia* spp. is attributed to their ability to fix nitrogen in the free-living state, a fact which explains their presence in poor soils, even in the absence of suitable hosts (Francis et al. 2010). As in legume symbionts, nodulation is induced by *Frankia* in a host specific manner, however, the nodules that form on over 200 species of actinorhizal plants, such as *Alnus* (alders), *Casuarina*, *Ceanothus* and *Myrica*, as well as on two non-woody species of *Datisca*, are branched. *Frankia* cells do not form bacteroids in the host cell cytoplasm, but rather differentiate the terminal regions of their hyphae into thick-walled, lipid-encapsulated vesicles (Beattie 2007).

Currently, it is believed that the ability of the nitrogen fixing actinomycete *Frankia* to form hopanoid vesicles protects nitrogenase from exposure to oxygen.

The protective mechanism of the vesicles is attributed to the formation of a multilamellar external gas-diffusion barrier around the nitrogen-fixing cells (Pearson et al. 2007; Qin et al. 2011).

Finally, despite their independent lifestyle, many cyanobacteria establish symbiotic relationships with Pteridophytes. These are N-fixing symbioses involving heterocystous cyanobacteria, particularly *Nostoc*, as cyanobionts (Cohen 2011).

After nitrogen, **phosphorus (P)** is the second most important macronutrient required to stimulate early plant growth, strong local root formation and lifelong growth and development (Gaxiola et al. 2011). Globally, most soils are characterized as P-deficient and even in cases of phosphorus abundance it normally exists in an insoluble form resulting in limited uptake by plants. Alterations of phosphorus bioavailability to plant tissues are correlated with depletion and accumulation of inorganic P in the rhizosphere which underlines the significant importance of phosphate mobilization in the rhizosphere (Hinsinger 2001).

The application of chemical fertilizers with soluble inorganic phosphate to soil is a common agricultural practice. Despite this, a large amount of the applied P becomes unavailable as it is sequestered by absorption to soil, or forms precipitates with cations of iron and aluminum in acid soils, and calcium in calcareous soils (Liu et al. 2011b; Park et al. 2011).

Typically, plant cells absorb either the monobasic (H_2PO^{4-}) or dibasic (HPO_4^{2-}) forms of inorganic P via high affinity transporters that are localized in the plant roots, with H_2PO^{4-} being preferred to HPO_4^{2-}. Aside from inorganic P, organic P is a significant P source for plants, comprising an estimated 20% to 80% of total soil P (Tao et al. 2008). The fixation and precipitation of P in soil is dependent on pH and soil type (Rodriguez and Fraga 1999) and the fixed P not taken up by plants can cause environmental problems such as eutrophication and increased soil salinity.

Several reports have shown that soil microorganisms (bacteria, fungi, actinomycetes) are able to solubilize inorganic P, and mineralize organic P,

resulting in greater P availability for plants. Descriptions of these organisms date back to 1903 and it seems that bacteria are more effective than fungi and actinomycetes (Liu et al. 2011b).

The most effective and powerful P solubilizers belong to the genera *Pseudomonas*, *Bacillus*, *Rhizobium* and *Enterobacter* (Hameeda et al. 2008) but there are a substantial number of reports that describe that other genera such as *Achromobacter*, *Agrobacterium*, *Micrococcus*, *Aereobacter*, *Serratia*, *Flavobacterium*, *Citrobacter*, *Klebsiella*, *Erwinia*, *Chryseobacterium*, *Delftia*, *Gordonia* and *Phyllobacterium* also catalyze these reactions (Chen et al. 2006; Liu et al. 2011b).

The mechanism of microbial inorganic phosphorus solubilization includes the production and release of organic acids. It is well documented that gluconic acid and 2-ketogluconic acid seem to play a pivotal role, while other organic acids such as glycolic, oxalic, malonic and succinic acid has have also been implicated in P solubilization (Rodriguez and Fraga 1999).

Other mechanisms of solubilization include release of chelating substances and inorganic acids such as sulphideric, nitric, and carbonic acids (Viruel et al. 2011).

A recent review summarizes the mechanisms of P solubilization as well as the impact of various factors involved (Sharma et al. 2013). As mentioned before the release of organic acids by phosphorus solubilizing bacteria reduces the pH of the culture medium through the direct oxidation of glucose to gluconic acid, and often to 2-ketogluconic acid which occurs on the outer face of the cytoplasmic membrane (Zaidi et al. 2009b).

To illustrate this, a gene from *Erwinia herbicola* was cloned and expressed in *E.coli* HB 101 that was found to code for the enzyme pyrolloquinoline quinone (PQQ) synthase, an enzyme which directs the synthesis of PQQ an essential redox co-factor for the formation of the holoenzyme glucose dehydrogenase. Glucose dehydrogenase (GDH) oxidizes glucose to gluconic acid, which is oxidized by gluconic acid dehydrogenase to 2–ketogluconic acid, and then to 2, 5–diketogluconic acid by 2-ketogluconic acid dehydrogenase (Goldstein and Liu 1987; Kim et al. 2003a).

Moreover, glucose can be taken up by an active mechanism followed by catabolism via an intracellular phosphorylative pathway catalyzed by the

enzymes glucokinase and glucose-6-phosphate dehydrogenase (G-6-PDH). Finally, both pathways turn out to the entry of 6-phosphogluconate into the Entner-Doudoroff (ED) pathway which is major glucose catabolic route in pseudomonades (Buch et al. 2008). As discussed previously, organic P can be a significant source of available P for plants. Phytate (myoinositol hexakisphosphate along with other isomers and lower-order derivatives) is a prevalent form of organic P in soil, representing between 10 and 50% of total organic P (Jorquera et al. 2008; Richardson and Simpson 2011).

Phytate mineralization can be catalyzed by phytases, enzymes which release inorganic orthophosphates from phytic acid and phytates through the hydrolysis of phosphoester and phosphoanhydride bonds (Jorquera et al. 2008; Rodriguez and Fraga 1999). In addition to phytases, there are two other groups of enzymes involved in phosphorus release from organic compounds: non-specific phosphatases, phosphonotases and C-P lyases (Rodriguez et al. 2006).

While phosphatases have nothing to do with the solubilization of inorganic phosphates, it is noteworthy that their synthesis is stimulated when inorganic P is limited (Selvaraj et al. 2008). Studies on the beneficial association of phosphate solubilizing/mineralizing rhizospheric bacteria and their host plants include *Burkholderia gladioli* with *Stevia rebaudiana* (Mamta et al. 2010); *Rhizobium leguminosarum* (Chabot et al. 1998) and *Pseudomonas* sp. with maize (Vyas and Gulati 2009); *Acinetobacter calcoaceticus* with cucumber, Chinese cabbage and crown daisy (Kang et al. 2009); *Rhizobium* sp. and *Bradyrhizobium japonicum* with radish (Antoun et al. 1998); *Enterobacter agglomerans* with tomato (Kim et al. 1998); *Pseudomonas lutea* sp with grasses (Peix et al. 2004); *Acinetobacter rhizosphaerae* with pea, chickpea, maize, and barley (Gulati et al. 2009); *Pantoea* sp. and *Citrobacter* sp with pigeon pea (Patel et al. 2010); and *Serratia nematodiphila* with Black pepper (Dastager et al. 2011).

Besides crop plants, phosphobacteria have been isolated from the rhizosphere of poplar trees (Liu et al. 2011b). Solubilization of immobilized mineral phosphate is not only a trait of some rhizospheric bacteria, there are also reports of endophytic bacteria exhibiting similar activities. Verma *et al.* (2001) showed that endophytic bacteria enhanced phosphate availability to rice plants, during initial colonization, while it has been showed that 49% of the endophytic bacteria

isolated from soybean were able to solubilize mineral phosphate (Kuklinsky-Sobral et al. 2004). Moreover, it was found that the endophyte *Klebsiella oxytoca* had P solubilization traits and promoted growth of the semi-aquatic *Typha australis* (Jha and Kumar 2007). This finding was further supported by Hameeda *et al*. (2008) who showed that bacterial strains in maize rhizospheres could enhance plant growth and grain yield as a result of solubilization of phosphorus.

Like phosphorous, **iron** is abundant in the environment and is essential for redox reactions that take place in all living organisms. As a consequence, iron plays a pivotal role in bacterial cells, where it is required at micromolar concentrations.

Aside from iron metal (Fe^0), iron is generally found in one of two oxidation states. In the presence of high concentrations of oxygen, it exists exclusively in the ferric state (Fe^{3+}), which is not highly soluble and reacts to form highly insoluble hydroxides and oxyhydroxides. Ferrous (Fe^{2+}) iron has a much better solubility, but it only exists under anoxic conditions or at very low pH. In that way, iron is largely unavailable to both plants and microorganisms under normal environmental conditions (Ma et al. 2011).

Microorganisms (bacteria, fungi and yeasts) have developed a mechanism to cope with iron deficiency through the production of organic molecules, called siderophores ("iron carrier" in Greek). Bacterial siderophores are low-molecular-weight compounds (500–1500 daltons) with a high affinity and selectivity for iron (III), and their biosynthesis is typically regulated by the iron levels of the environment.

Expression of siderophore coding genes is down-regulated at the transcriptional level by a dimeric protein, the ferric uptake regulator (Fur). Despite the many different known siderophore structures, they are generally classified based on the type of ferric iron-binding ligand into: **hydroxamates** (e.g., proferrioxamines, ferrichrome, and coprogen), **catecholates** (e.g., enterobactin, vibriobactin, yersiniabactin, and amonabactins), **α-hydroxycarboxylates**, and **mixed ligands** (Zawadzka et al. 2006). A substantial number of siderophores are polypeptides that are synthesized by members of the nonribosomal peptide synthetases multienzyme family.

Nevertheless, many of the hydroxamate- and α-hydroxy acid-containing siderophores are not polypeptides but are assembled instead from alternating dicarboxylic acid and either diamine or amino alcohol building blocks (derived from amino acids) that are linked by amide or ester bonds (Challis 2005). Bacteria excreting siderophores are able to bind Fe^{3+} and deliver it to the cell for conversion to the desirable form, Fe^{2+}. In detail, upon binding the ion, the siderophore-Fe^{3+} complex is subsequently bound by iron-limitation-dependent receptors at the bacterial cell surface and the Fe^{3+} ion is subsequently released and activated in the cytoplasm as Fe^{2+} (Lugtenberg and Kamilova 2009).

The biology, chemistry as well as the enzymatic machinery involved in the biosynthesis of siderophores has been reviewed by (Barry and Challis 2009) and (Hider and Kong 2010). Moreover, bacterial Fe^{3+} siderophore complexation is a trait that, apart from being advantageous for bacterial iron uptake, can improve iron acquisition in several plant species, too. A substantial number of plants, especially in calcareous soils, have evolved strategies to bind bacterial iron-siderophore complexes, transport them into and through the plant prior to reductively releasing the iron for use (Katiyar and Goel 2004; Sharma and Johri 2003).

In addition, some plants generate their own siderophores, phytosiderophores. It is important to discriminate between microbial siderophores and phytosiderophores due to their different metal binding affinities. For example, strategy II plants (*Poaceae*), especially graminaceous species (grasses, cereals and rice), are featured by an iron deficiency-induced enhanced release of non-proteinogenic amino acid phytosiderophores (mugineic acid in barley and avenic acid in oat) (Marchner,1995).

For the biosynthesis of phytosiderophores, L-methionine is a common precursor that is anabolically shuttled through a nicotianamine (NA) intermediate. Specifically, methionine is converted to S-adenosylmethionine (SAM) by SAM synthetase while nicotianamine (NA) is synthesized by nicotianamine synthase. SAM and NA are the direct precursors for phytosiderophores (Lemanceau et al. 2009). In calcareous soils, the ability of graminaceous plants to efficiently chelate ferric iron is attributed to the amine and carboxyl groups of phytosiderophores (Kidd et al. 2009). It has been showed that the efflux of deoxymugineic acid, the primary phytosiderophore from rice and barley,

involves the *TOM1* and *HvTOM1* genes, shedding light to the crucial role of phytosiderophore efflux transporters for iron acquisition in graminaceacous plants (Nozoye et al. 2011) .

In summary, the contribution of bacteria to the more efficient acquisition of essential nutrients like nitrogen, phosphorus and iron is substantial; however, that mechanism of plant growth promotion should not be viewed as one dimensional. In fact the stimulation in plant growth can be correlated also with the production of secondary metabolites like Indole acetic acid (IAA). A recent work, using chromatographic and colourimetric methods, demonstrated that *Rhizobium* species, as well as, symbiotic, and non-symbiotic *Paenibacillus,* yielded high concentrations of IAA in the range of 5.23-0.27 and 4.90-0.19 ppm IAA/mg biomass, respectively (Shokri and Emtiazi 2010). Furthermore, it has been reported that rhizospheric phosphate solubilizing and mineralizing bacteria may stimulate plant growth also through the production of IAA (Shahab et al. 2009).

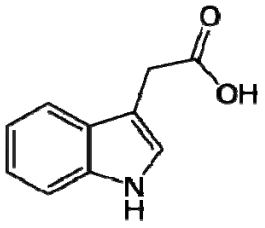
This synergy seems to be widespread among bacteria and its complex nature requires to be addressed in details. Therefore, the following paragraphs aim to elucidate the role and mechanisms of various secondary metabolites produced by bacteria with respect to direct plant growth.

1.3.1.2 Phytohormone modulation

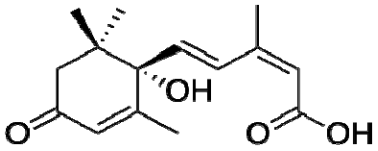
Various elements of plant growth-promotion can be attributed to the capacity of plant-associated bacteria to synthesize phytohormones such as auxins, abscisic acid (ABA), cytokinins, and gibberellins (Figure 1.10), as well as volatile plant growth promoting compounds such as acetoin and 2,3-butanediol.

Examples are production of indole-3-acetic acid (IAA) by *Acetobacter diazotrophicus* and *Herbaspirillum seropedicae* (Bastian et al. 1998); zeatin and ethylene by *Azospirillum* sp. (Strzelczyk et al. 1994); gibberellic acid (GA_3) by *Azospirillum lipoferum* strain op33 (Bottini et al. 1989); and abscisic acid (ABA) by *Azospirillum brasilense* strains Cd and Az39 (Perrig et al. 2005). Indeed, production of phytohormones by plant associated bacteria oftentimes incite plant growth and development in a variety of ways such as cell growth, apical dominance, tropisms, initiation of adventitious and lateral roots, cell and

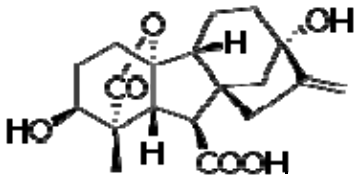
vascular differentiation, stamen development, and both biotic and abiotic stress resistance (Li et al. 2012).



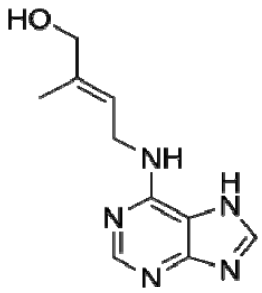
Auxins



Abscisic acid (ABA)



Gibberellins



Cytokinins

Figure 1.10 Structure of most common auxins, abscisic acids, gibberellins and cytokinins.

Although, bacteria also produce other phytohormones and phytohormone-like substances including ethylene, brassinosteroids, oligosaccharines, salicylic acid, and jasmonic acid (Tsavkelova et al. 2006), in this Section emphasis will be given to the significant contribution of auxin, ABA, cytokinins and gibberellins to the aforementioned plant growth alterations.

Plant-associated bacteria – either rhizospheric or endophytic, can interfere with plant development by disturbing the balance of **auxin** (indole-3-acetic acid or IAA). A diverse array of plant growth promoting bacteria are known to produce IAA including species of *Azospirillum*, *Bacillus*, *Bradyrhizobium*, *Enterobacter cloacae*, *Paenibacillus*, *Pseudomonas* and *Rhizobium* (Spaepen and Vanderleyden 2011). The biosynthetic pathways of IAA in bacteria require tryptophan as a precursor and five different mechanisms of synthesis, namely (a) indole-3-pyruvate, (b) indole-3-acetamide, (c) tryptamine, (d) tryptophan side-chain oxidase and (e) indole-3- acetonitrile pathway have been reported and

correlated to the phytopathogenic or phytostimulatory nature of the correspondent bacteria.

Generally, the **indole-3-pyruvate (IPyA)** pathway appears to be more predominant in a broad range of bacteria such as the beneficial bacteria *Bradyrhizobium*, *Azospirillum*, *Rhizobium*, *Pseudomonas putida* and *Enterobacter cloacae*, the pythopathogenic bacterium *Pantoea agglomerans*, and cyanobacteria (Baca et al. 1994; Costacurta and Vanderleyden 1995; Koga et al. 1991; Patten and Glick 2002; Schutz et al. 2003; Sekine et al. 1988; Spaepen et al. 2007b).

This pathway consists of 3 steps that begin with the conversion of tryptophan to **IPyA** by an aminotransferase (transamination). This is followed by the rate-limiting step, decarboxylation of **IPyA** to indole-3-acetaldehyde **(IAAld)** by indole-3-pyruvate decarboxylase **(IPDC),** and finally, **IAAld** is oxidized in **IAA.** The above-mentioned steps can be schematically depicted as follows:

Trp → IPyA → indole-3-acetaldehyde (IAAld) → IAA

The **indole-3-acetamide pathway (IAM)** has been described in all gall forming-bacteria such as *Agrobacterium tumefaciens*, *Pseudomonas syringae*, *Pantoea agglomerans*, but also in symbiotic nitrogen fixing bacteria such as *Bradyrhizobium japonicum* and *Rhizobium fredii* (Sekine et al. 1988; Theunis et al. 2004) and the plant-beneficial *Pseudomonas chlororaphis* O6 (Dimkpa et al. 2012). In this two-step pathway tryptophan **(Trp)** is first converted to **IAM** by tryptophan-2-monooxygenase **(IaaM)**, encoded by the *iaaM* gene, and then **IAM** is converted to **IAA** by an **IAM** hydrolase **(IaaH)**, encoded by *iaaH*. This two-step mechanism is schematically depicted as follows:

(Trp →IAM → IAA)

The **tryptamine (TAM) pathway** in bacteria has been found in some members of the *Azospirillum* genus (Hartmann et al. 1983) and in strains of *Bacillus cereus* (Perley and Stowe 1966).

The fourth IAA biosynthetic pathway is the **Tryptophan side-chain oxidase pathway (TSO)** pathway, although there are no reports of microorganisms using this pathway being associated with plants. In this pathway tryptophan

bypasses **IPyA** and is directly converted to **IAAld,** which then can be further oxidized to **IAA.**

It should be underlined that this pathway has only been described in *Pseudomonas fluorescens* CHA0 isolated from tobacco plants (Oberhansli et al. 1991). Finally, the last **tryptophan-dependent IAA biosynthetic route** is the **indole-3-acetonitrile (IAN)** pathway. That pathway has been studied in both plants and bacteria, but the vast amount of information comes from plant studies. Despite this, the last step of the IAN pathway, specifically the conversion of **IAN** to **IAA** by a nitrilase, has been observed in bacteria such as *Alcaligenes faecalis*.

The conversion of IAN to IAA via IAM has been attributed to nitrile hydratase and amidase activity in *Agrobacterium tumefaciens* and *Rhizobium* spp., respectively. An overview of the aforementioned mechanisms along with supplemental information describing the tryptophan-independent pathway can be found in (Spaepen et al. 2007a). Isolation of IAA-producing plant beneficial bacteria has been reported from lettuce (Barazani and Friedman 1999), wheat (Kaushik et al. 2000; Sachdev et al. 2009), rice (Araujo et al. 2013; Mehnaz et al. 2001), sugarcane (Mirza et al. 2001), poplar trees (Taghavi et al. 2009), corn and soybean (Cassan et al. 2009), apple trees (Miliūtė & Buzaitė, 2011), eucalyptus (Peralta et al. 2012), cotton (Liu et al. 2013) and sunflowers (Prapagdee et al. 2013).

In studies with poplar, it has been shown that the endophytic bacteria *Enterobacter* sp.638, *Serratia proteamaculans* 568, *Stenotrophomonas maltophilia* R551-3 and *Pseudomonas putida* W619 produce IAA (Taghavi et al. 2009). More recently, it has been shown that IAA producing bacterial endophytes isolated from poplar and willow trees could be used to inoculate crops such as corn, tomato, pepper, squash, sunflower, and grasses to enhance viability, facilitate earlier flowering and fruiting, and increase plant growth (Khan et al. 2012). It is worthwhile to mention that endophytic yeasts isolated from stems of hybrid poplar (*Populus trichocarpa x Populus deltoides*) identified to be species *Rhodotorula mucilaginosa* were observed to be able to produce indole-3-acetic acid (IAA) (Xin et al. 2009a). The activities of IAA producing bacteria is not always positive for the host plant since some of them are plant pathogens and many have no known effect that has been described. The tumor-inducing

phytopathogenic nature of bacteria producing high concentrations of IAA seems to be linked, to a large extent, to the production of IAA via the IAM pathway (Kuklinsky-Sobral et al. 2004; Lee et al. 2004). Albeit, as discussed above, production of IAA can stimulate plant growth, there are indications that the production of high levels of IAA by some bacteria can impair plant development. In studies conducted with bacterial endophytes isolated from field-grown *Solanum nigrum*, it was demonstrated that IAA-producing isolates induce root growth on their natural host, *S. nigrum*, as well as on *Nicotiana attenuata*, another Solanaceous native plant, but only when low quantities of the hormone are released. High levels of bacterial or exogenously applied hormone limited growth (Long et al. 2008). In this context, it would be interesting to explore the counterbalance between microbial and plant IAA production with bacteria able to catabolize IAA. In tomato plants, inoculation with a strain of *Pseudomonas putida* with the ability to degrade IAA *in vitro*, showed that the roots of seedlings grown in the presence of increased concentrations of IAA were significantly longer when seeds were previously treated with *P. putida* (Gravel et al. 2007).

The role of **abscisic acid (ABA)** in higher plants is associated with seed development, root growth and stomatal aperture. Moreover, it is also associated with plant adaptation to various stresses, such as cold, drought and water stress (Guo et al. 2009). Briefly, abscisic acid biosynthesis begins with the oxidative cleavage of the carotenoids 9´-*cis*-violaxanthin or 9´-*cis*-neoxanthin to xanthoxin by plastid 9-*cis*-epoxycarotenoid dioxygenases (NCEDs). Then, xanthoxin is converted to abscisic aldehyde by xanthoxin oxidase, and finally, abscisic aldehyde oxidase catalyses conversion of abscisic aldehyde to ABA (Dodd et al. 2010). Notably, there is a lack of information about the possible pathways for ABA synthesis in bacteria, while, in some fungi it has been suggested that the biosynthesis of ABA takes place in a direct way from farnesyil diphosphate (Siewers et al. 2006). In *Arabidopsis* plants, inoculation with *A. brasilense* Sp 245 had as a result a two-fold enhancement of the plant's ABA content (Cohen et al. 2008). This observation about the ability of bacteria belonging to the genus *Azospirillum* spp. to produce ABA was further supported with experiments in maize (*Zea mays*) plants. In these experiments, inoculation of maize plants with *A. lipoferum* previously treated with fluridone (F), which blocks abscisic acid synthesis of maize seedlings, completely reversed the

detrimental effect of the inhibitor. That is, inoculation with the bacteria increased the ABA content of maize seedlings, accompanied with an enhanced growth compared to those treated with fluridone (Cohen et al. 2009). The potential of soil and plant dwelling bacterial species to synthesize ABA has been documented (Karadeniz et al. 2006; Tuomi and Rosenqvist 1995). Recently, isolation of endophytic bacteria able to produce ABA in chemically-defined culture media has been reported in sunflower (Forchetti et al. 2007) and in the halophyte shrub *Prosopis strombulifera* (Piccoli et al. 2011; Sgroy et al. 2009).

Cytokinins (CKs), are involved in the modulation of several different aspects of plant growth and differentiation, such as cell division, apical dominance, nutrient mobilization, chloroplast development, senescence and flowering (Hare et al. 1997). In a review, (Kamada-Nobusada and Sakakibara 2009) addressed in detail the molecular basis of cytokinin synthesis and underlined the critical role of cytokinins in various developmental processes correlated to plant growth. In addition, the exigent role of cytokinins in the stimulation of plant growth and developmental processes such as root proliferation, apical dominance, nutritional signaling and shoot meristem function seems to be strongly dependent on the presence of active or conjugated forms of cytokinins (Bajguz and Piotrowska 2009).

The discovery in *Agrobacterium tumefaciens* of the *ipt* gene, coding for isopentenyltransferase (Nester et al. 1984), followed by the detection of the same gene in *Arabidopsis* (Kakimoto 2001), concretized the hypothesis that both plants and microorganisms are able to synthesize cytokinins. The prominent role of adenine (ADE) as a precursor of cytokinins production by bacteria was confirmed in experiments with strains of *Azotobacter chroococcum*, *A. beijerinckii*, *A. vinelandii*, *Pseudomonas fluorescens* and *P. putida* (Nieto and Frankenberger 1989).

These studies revealed that *Azotobacter chroococcum* was the most plenteous producer of cytokinins in liquid media, a finding confirmed by (Arshad and Frankenberger 1991). Detection of cytokinins in the culture media of bacteria such as *Halomonas desiderata*, *Proteus mirabilis*, *P. aerugionsa*, *P. vulgaris*, *Klebsiella pneumoniae*, *Bacillus licheniformi*, *Bacillus megaterium*, *B. cereus*, *B. subtilis* and *Escherichia coli* as well as various Cyanobacteria has been reported (Ali et al. 2009; Arkhipova et al. 2005; Hussain and Hasnain 2011; 2012;

Hussain et al. 2010; Karadeniz et al. 2006). While a plethora of articles have referred to the ability of phytopathogenic bacteria to produce cytokinins (Pertry et al. 2009; Stes et al. 2013), the plant growth promotion aspects of cytokinin synthesizing bacteria belonging to the genera *Rhizobium*, *Azotobacter*, *Azospirillum*, *Arthrobacter*, *Bacillus* and *Pseudomonas*, has also been documented (Tsavkelova et al. 2006). Cytokinin-forming PGPR have been isolated from rape and lettuce (Noel et al. 1996), wheat (Timmusk et al. 1999), soybean (de Salamone et al. 2001), and pine (Bent et al. 2001).

Based on experiments in *Arabidopsis*, it has been concluded that cytokinins of bacterial origin have a positive effect in the plant growth (Ryu et al. 2003). In certain cases, enhanced formation of lateral and adventitious roots has been associated with PGPR-synthesized cytokinins (Werner et al. 2003). Application in lettuce and wheat plants of bacteria of the genus *Bacillus* able to produce various cytokinins such as zeatin riboside (ZR), dihydrozeatinriboside (DHZR) and isopentenyladenosine (iPA) resulted in an augmentation of the cytokinin content in both roots and shoots.

This result was interpreted to be an effect of the expanded leaf area of the plants caused by cytokinin producing bacteria. In turn, that finding was associated with the positive effect of cytokinins producing bacteria to the overall plant growth of the corresponding crop plants (Arkhipova and Anokhina 2009; Arkhipova et al. 2007; Arkhipova et al. 2005). Members of the genus *Methylobacterium* are able to produce cytokinins when preserved in liquid cultures (Hellmuth and Kutschera 2008) and, remarkably, cytokinin producing epiphytic pink-pigmented methylotrophic bacteria belonging to the genus *Methylobacterium* isolated from the phyllosphere of crop plants such as sugarcane, pigeonpea, mustard, potato and radish have been shown to enhance seed germination of wheat (Meena et al. 2012).

The capacity of the nodulating rhizobia *Sinorhizobium meliloti* (Gonzalez-Rizzo et al. 2006) and *Mesorhizobium loti* (Okazaki et al. 2007) to produce cytokinins (Sturtevant and Taller 1989) has been linked to the existence of genes sharing homology with the *ipt* gene responsible for the synthesis of isopentenyltransferase in *Agrobacterium tumefaciens* implying that bacterial cytokinin and/or cytokinin-like compounds seem to hold a prominent role for Nod factor-independent nodulation in some legumes (Frugier et al. 2008).

Overproduction of cytokinins in engineered *Shinorhizobium meliloti* strains expressing the *Agrobacterium ipt* gene under the control of different promoters, improved the tolerance of alfalfa to drought stress, while having no effect on nodulation or nitrogen fixation (Xu et al. 2012).

Gibberellins (GAs) comprise another group of phytohormones that includes more than 100 tetracyclic diterpenes with a variety of functions in plant growth and development such as seed germination, seedling emergence, stem and leaf growth, shoot elongation and flowering (Bottini et al. 2004). As with auxins, GAs are also involved in promotion of root growth as well as root hair abundance (Tanimoto 2005).

More recently, a review by (Santner and Estelle 2009) summarized how gibberellin-signaling pathways are key players of an integrated network correlated to hormonal regulation of plant growth. As in higher plants, early steps of the gibberellin biosynthetic pathways in bacteria may be regulated by membrane-related cytochrome P450 monooxygenases, and the late hydroxylative steps catalyzed by soluble 2-oxoglutarate-dependent dioxygenases (Bottini et al. 2004).

However, from reviewing the literature, the indigence of information related to gibberellin metabolism in bacteria in comparison to biosynthetic pathways known for fungi and plants has become obvious. Despite the scarce occurrence of plant-associated bacteria thought to produce gibberellins**,** the presence of the hormone has been reported in chemically defined medium of *Acetobacter diazotrophicus* and *Herbaspirillum seropedicae* (Bastian et al. 1998), *Bacillus pumilus* and *Bacillus licheniformis* spp. (Gutierrez-Manero et al. 2001), *Azospirillum brasilence (Perrig et al. 2007)*, *Burkholderia* sp. (Joo et al. 2009), *Azospirillum* sp., *Azospirillum brasilense* and *Bradyrhizobium japonicum* (Cassan et al. 2009), *Arthrobacter koreensis* (Piccoli et al. 2011) and *Promicromonospora* sp. (Kang et al. 2012b).

The emission of **volatile organic compounds (VOCs)** such as 3-hydroxy-2-butanone (acetoin) and 2,3-butanediol seems to be another important factor within the general framework of plant growth promotion modulated by rhizospheric and endophytic bacteria. The pathway of acetoin biosynthesis and the enzymes involved in the metabolism of 2,3-butanediol have been well studied. In detail, the degradation of glucose or other fermentable carbon

sources via the Embden-Meyerhof-Parnas (EMP) pathway results in the formation of pyruvate. Thereafter, acetoin is produced by first condensing two molecules of pyruvate into α-acetolactate by α-acetolactate synthase, and subsequently α-acetolactate is decarboxylated to acetoin by α-acetolactate decarboxylase. In the last step, acetoin can be converted reversibly to 2,3-butanediol by the action of acetoin reductase (Figure 1.11).

Thereafter, under aerobic conditions, acetolactate is spontaneously converted into diacetyl, which then can be converted into acetoin by the acetoin dehydrogenase protein. Generally, acetoin and its biological analogues 2,3-butanediol and diacetyl are considered as C_4 compounds (Xiao et al. 2010; Xiao and Xu 2007). More recently, the identification of the *budAB* operon, involved in acetoin production in *Serratia plymuthica* strain RVH1, was proven to be positively controlled by external stimuli such *N-(oxohexanoyl)*-L-homoserine lactone (OHHL) quorum sensing signaling molecules (Moons et al. 2011). Production of acetoin and 2,3-butanediol by strains of *Bacillus subtilis* and *Bacillus amyloliquefaciens* has been linked to the growth promotion of *Arabidopsis thaliana* seedlings (Ryu et al. 2003).

The novelty of that work brought to the foreground volatile compound mediated communication between bacteria and plants; moreover, the authors of the study highlighted the involvement of 2,3-butanediol in plant growth and development based on experiments with butanediol-deficient *B. subtilis* mutants. In a review, it was postulated that 2,3-butanediol and acetoin apparently act via cytokinin and ethylene signaling and concluded that a substantial number of root colonizing PGP bacteria are capable of producing these volatile compounds at adequate concentrations to induce changes in plant hosts (Ping and Boland 2004).

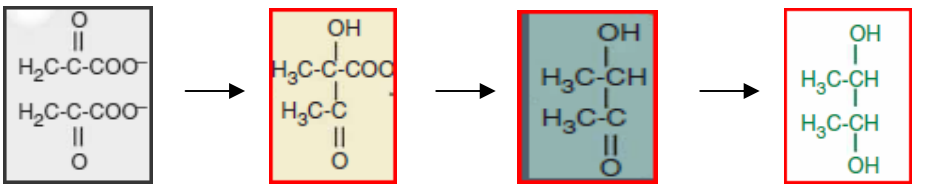


Figure 1.11 Acetoin biosynthesis pathway.

Section 1

The analysis of *Arabidopsis* mutants impaired in hormone signaling has given out possible elicitation of growth promotion by PGPR strains due to involvement in signaling of auxin, salicylic acid, brassinosteroids and gibberellins (Ryu et al. 2005). Genome sequencing has revealed that the sugarcane nitrogen-fixing endophyte *Gluconobacter diazotrophicus* Pal5 harbors acetolactate synthase and acetoin diacetyl reductase genes which are involved in acetoin synthesis pathways (Bertalan et al. 2009). Furthermore, *Enterobacter* sp. 638, an endophytic plant growth promoting gamma-proteobacterium that was isolated from the stem of poplar trees (*Populus trichocarpa x deltoides* cv.H11-11), possesses genes to convert pyruvate to acetolactate.

Remarkably, the strain possesses an additional gene for the conversion of diacetyl into acetoin, a compound which can be finally emitted by the bacterium and converted into 2,3-butanediol by the plant. The existence of the required genes for the conversion of acetoin into 2,3-butanediol in the poplar genome, but not the ones for the biosynthesis of acetoin, seems to indicate that poplar trees rely on endophytic bacteria like *Enterobacter* sp. 638 for the synthesis of the desirable plant growth promoting hormones acetoin and 2,3-butanediol (Taghavi et al. 2010b). More recently, genes potentially responsible for the acetoin synthesis were found in the genome of the plant growth-promoting bacterium (PGPB) *Pseudomonas* sp. UW4, isolated from the rhizosphere of the common reed (Duan et al. 2013). These works point to the capability of bacterial endophytes to mediate plant growth promotion of various plants species via the emission of acetoin and 2,3-butanediol.

On the other hand, members of the genera *Pseudomonas*, *Burkholderia* and *Serratia* assayed for their interaction with *Arabidopsis thaliana* seedlings were found to dictate plant growth via the emission of other bacterial volatiles, with indole, 1-hexanol and pentadecane, apparently being the most prominent (Blom et al. 2011).

1.3.1.3 Stress relief and ACC – deaminase producing bacteria

Ethylene, the simplest olefin, is a phytohormone that regulates various aspects of the plant life cycle, including seed germination, tissue differentiation, formation of root and shoot primordia, root elongation, lateral bud development, initiation of flowering, anthocyanin synthesis, flower opening and senescence,

pollination, fruit ripening, the production of volatile organic compounds responsible for aroma formation in fruits, leaf and fruit abscission, and plant-microbial interactions that are important for plant growth and survival (Sisler et al., 2006). The ethylene biosynthetic pathway in plants involves: **S-Adenosyl-L-methionine synthetase** (SAM – synthetase), 1-aminocyclopropane-carboxylate synthase (ACS), 1-aminocyclopropane-carboxylate oxidase (ACO), 1-aminocyclopropane-carboxylate-*N*-malonyl-transferase (ACC-N-malonyl–transferase), 5´-Methylthioadenosin–nucleosidase (MTA), 5´-Methylthioribose (MTR) kinase and transaminases. Briefly, three key enzymatic reactions are involved in the conversion of methionine to ethylene:

i. Methionine is converted to S-Adenosyl-L-methionine by SAM-synthetase
ii. ACC synthase (ACS) converts S-Adenosyl-L-methionine to ACC
iii. ACC oxidase (ACO) degrades ACC to release ethylene

The conversion of ACC to ethylene catalyzed by ACO is oxygen dependent, and, under anaerobic conditions, ethylene formation is completely suppressed. In this reaction, Fe^{2+} and ascorbate are required as a cofactor and a co-substrate, respectively. Moreover, apart from ACC, ACS also produces 5´-methylthioadenosine (MTA), which is further utilized for the synthesis of new methionine. In this way, high rates of ethylene biosynthesis can be sustained even in conditions of relatively low methionine concentrations.

The review of (Lin et al. 2009) examines information related to the mechanisms of ethylene production, perception, and signal transduction. Among the plethora of effects of ethylene on plant development described earlier, the synthesis of "stress" ethylene by plants as a response to various abiotic (wounding and mechanical stress, water deficiency, salinity, flooding/hypoxia, chilling, ozone) and biotic (pathogenic infection, predation by insects) environmental stresses has been studied.

For example, it has been demonstrated that once tomato seedlings were exposed to salt stress the observed suppression of the growth was linked to increased production of ethylene (Mayak et al. 2004). While bacteria can adjust the biosynthesis of plant ethylene via auxin production (Hardoim et al. 2008; Stearns et al. 2012), it has been suggested that plant growth promoting bacteria containing the enzyme 1-aminocyclopropane-1- carboxylic acid (ACC) deaminase reduce "stress" ethylene levels in plant tissues by two mechanisms:

(i) by cleaving the ethylene precursor ACC to α-ketobutyrate and ammonia or (ii) by inhibiting ACC synthetase and/or ß-cystathionase, both enzymes of the ethylene biosynthesis pathway in plants (Figure 1.12).

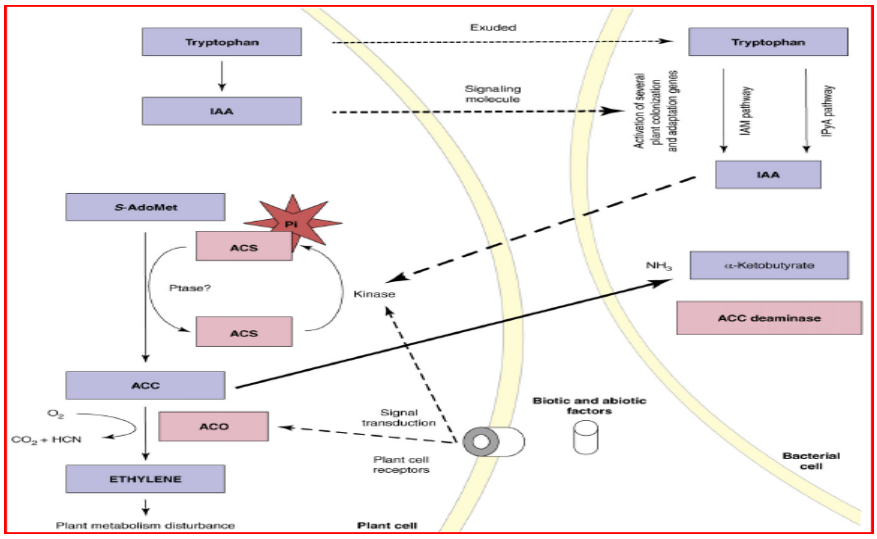


Figure 1.12. Schematic representation of plant ethylene biosynthesis modulation by bacteria (adapted by Hardoim et al., 2008).

In addition, increased ethylene levels are also induced by the presence of environmental contaminants (both trace elements and organic compounds) in the rhizosphere resulting in inhibition of root and shoot growth. Many negative effects on plant growth caused by these environmental suboptimal conditions are thought to be mediated by these increased levels of "stress" ethylene. Therefore, alleviation of stress by means of lowering ethylene production could serve as an alternative and efficient way to improve root elongation and overall plant fitness.

Descriptions about the biochemical reaction mechanisms involved in plant responses to "stress" ethylene, including gene sequences, gene regulation and the structures for key proteins, have been widely reviewed (Glick and Stearns 2011; Glick et al. 2007; Hontzeas et al. 2006). The trait of ACC deaminase producing bacteria to mitigate plant ethylene levels, has been reported in numerous plant growth promoting bacterial strains isolated either from the rhizosphere or the inner tissues of plants that belong to phylogenetically distant genera such as *Alcaligenes* spp., *Bacillus* spp., *Bacillus pumilus*, *Burkholderia*

sp., *Burkholderia phytofirmans*, *Enterobacter* spp., *Enterobacter cancerogenus*, *Enterobacter clocae*, *Erwinia herbicola*, *Klebsiella* sp., *Kibdelosporangium phytohabitans*, *Micrococcus* sp., *Methylobacterium oryzae*, *Pantoea agglomerans*, *Pseudoalteromonas maricaloris*, *Pseudomonas fluorescens*, *Pseudomonas putida*, *Pseudomonas thivervalensi*, *Rhodococcus* sp., *Serratia marcesens* and *Variovorax paradoxus* (Belimov et al. 2005; Belimov et al. 2001; Bharti et al. 2013; Chen et al. 2013; Dastager et al. 2010; El-Tarabily and Youssef 2011; Farajzadeh et al. 2010; Glick et al. 1998; Jasim et al. 2013; Jha et al. 2012; Jorquera et al. 2012; Luo et al. 2012b; Madhaiyan et al. 2007; Onofre-Lemus et al. 2009; Sessitsch et al. 2005; Shahzad et al. 2013; Sun et al. 2009; Vessey 2003; Wang et al. 2012; Xing et al. 2012) as well as, *Azoarcus*, *Azorhizobium caulinodans*, *Azospirillum* spp., *Gluconacetobacter diazotrophicus*, *Herbaspirillum* spp., and others (Dobbelaere et al. 2003).

Since the inhibitory role of ethylene in nodulation is known (Gamalero et al. 2008; Onofre-Lemus et al. 2009), the increased seed nitrogen concentrations of pea (*Pisum sativum*) plants grown in dry soil after inoculation with ACC-deaminase producing *Variovorax paradoxus* 5C-2 was correlated with enhanced nodulation; moreover, N, P, K, Ca, and Mg uptake increased by 16, 81, 50, 46, and 58%, in inoculated plants compared to controls, respectively ((Belimov et al. 2009; Jiang et al. 2012).

These findings regarding the ability of ACC-deaminase producing *Variovorax* species to aid with nutrient availability and uptake are consistent with information found in the complete genome sequence of the metabolically versatile PGP endophyte *Variovorax paradoxus* S110 (Han et al. 2011). In a comparative study, the application of rhizobacterial isolates *P. thivervalensis* (STF3) and *S. marcesens* (STJ5) in combination with NPK chemical fertilizers (CF;250-150-250 kg ha^{-1}) applied as Urea, Diammonium phosphate and Myriate of potash at 50, 75 and 100% recommended levels, increased growth (plant height, root length, fresh biomass, chlorophyll content and root dry weight) and yield of maize plants compared to the non-inoculated fertilized treatments and treatment with rhizobacteria alone.

The improvements in growth and yield following application of rhizobacterial isolates under fertilized conditions could primarily be attributed to their ACC-deaminase activity, an activity which possibly reduced endogenous ethylene

biosynthesis in developing roots of seedlings, resulting in formation of more healthy and longer roots affording higher nutrient uptake efficiency (Shahzad et al. 2013).

In recent years, various reviews demonstrated the conceptual role of ACC-deaminase producing bacteria in the expedition of phytoremediation in soils contaminated with metals, organic compounds, and mixtures of the two. Overall, it is apparent that the use of bacteria having both pollutant degrading and ACC-deaminase abilities is more advantageous than using bacteria equipped with only one of both properties (Arshad et al. 2007; de-Bashan et al. 2012; Gerhardt et al. 2009; Glick 2010; Khan et al. 2013a; Rajkumar et al. 2012; Zhuang et al. 2007).

A number of studies has asseverated that certain rhizosphere and endophytic ACC deaminase producing bacteria have improved the tolerance of various plant species that encountered toxicity due to the increased levels of ethylene under heavy metal stress (Ni, Pb, Zn, Cu, Cd, Co and As) (Belimov et al. 2005; Belimov et al. 2001; Burd et al. 2000; Dell'Amico et al. 2008; Farwell et al. 2006; Nie et al. 2002; Reed et al. 2005; Rodriguez et al. 2008; Zhang et al. 2011a).

The presence of the ACC deaminase producing strains *Enterobacter cloacae* UW4 and *Enterobacter cloacae* CAL2 in a multi-process phytoremediation system (MPPS) was thought to increase biomass production of Tall Fescue (*Festuca arundinacea*) grown on a soil contaminated with oil refinery sludge at a level of 5% (w/w) total petroleum hydrocarbons (TPHs) (Huang et al. 2005).

Similarly, recent research has confirmed that the inoculation of plants with bacteria harboring both ACC deaminase and hydrocarbon-degrading activity improved plant growth and well–being in case of severe contamination and, moreover, appears to be advantageous compared to bacteria that can degrade only the pollutant or promote plant growth (Afzal et al. 2011; Gurska et al. 2009b; Khan et al. 2013b; Yousaf et al. 2011).

It has been found that genetically modified bacteria overexpressing ACC deaminase genes mitigate "stress" ethylene production in a "twisting strategy", whereby they act as both plant growth promoters and biocontrol agents, simultaneously (Wang et al. 2000).

Section 1

The promotion of seed germination and root elongation of the common reed *Phragmites australis* in the presence of copper or polycyclic aromatic hydrocarbons was facilitated in an equal manner by both native and genetically transformed to express a bacterial gene coding for the enzyme 1-aminocycloporpane-carboxylate deaminase *Pseudomonas asplenii* AC strains (Reed et al. 2005).

In recent works (Brigido et al. 2013; Nascimento et al. 2012), it has been reported that inoculation of chickpea (*Cicer arietinum*) plants with *Mesorhizobium ciceri* strains expressing an exogenous 1-aminocyclopropane-1-carboxylate (ACC) deaminase gene (*acdS*), resulted in growth promotion mainly via an increased nodulation activity under saline conditions and reduced chickpea root rot disease susceptibility.

These results accentuate that ACC deaminase can play a pivotal role in facilitating plant-*Rhizobium* interactions under various unfavorable conditions. The consensus about the stimulatory contribution of bacteria containing ACC-deaminase to plant growth has propelled researchers to evolve transgenic plants expressing bacterial ACC deaminase genes.

These plants have been reported result in higher bioremediation efficiency (Nie et al. 2002; Stearns et al. 2005), reduced symptoms during pathogenic infections (Robison et al. 2001), and increased tolerance to flooding and high salt conditions (Grichko et al. 2000; Saravanakumar and Samiyappan 2007; Sergeeva et al. 2006).

Selected paradigms of ACC utilizing rhizosphere and endophytic bacteria interacting with plants under specific stress conditions are summarized in Table 1.2.

In a recent review, it is was reported that regulation of ethylene levels of transgenic plants that express an exogenous ACC deaminase gene in their roots is very similar to that of plants treated with ACC deaminase expressing plant growth-promoting bacteria.

However, the bacterial-treated plants are generally more successful than the transgenic plants, probably due to the fact that, as dicussed earlier, plant growth-promoting bacteria do more for their host plants than partially lower their ethylene levels (Glick and Stearns 2011).

Table 1.2 Stress conditions and ACC utilizing rhizosphere and endophytic bacteria.

PGPB	Test plant & Stress	Experimental site	Mechanisms	References
P. fluorescens YsS6 (E) & *P. migulae* 8R6 (E)	Leaf senescence of *Dianthus caryophyllus* flowers	Glass house	Delay of leaf senescence	(Ali et al. 2012)
A. xylosoxidans Fd2 (R), *S. ureilytica* Bac5 (R), *H. seropedicae* Oci9 (R), & *O. rhizosphaerae* Oci13	Waterlogged *Ocimum sanctum* plants	Glass house	Increased flood tolerance	(Barnawal et al. 2012)
Mesorhizobium ciceri LMS-1	*Fusarium* root rot – Chickpea plants (*Cicer arietinum*)	Glass house	Reduce chickpea root rot disease susceptibility	(Nascimento et al. 2012)
B. epidermidis, *B. iodinum*, *A. nicotianae*, *Zhihengliuella alba*, *M. yunnanensis*, *O. smirnovii*, *B. licheniformis* & *C. Variabile and P. fluorescens* TDK1	Salt stress in canola seedlings and groundnut (*Arachis hypogea*) plants	Laboratory and field conditions	Enhanced root elongation/ dry weight and improved yield parameters	(Saravanakumar and Samiyappan 2007; Siddikee et al. 2010)
Raoultella planticola Rs-2	Salinity stress and cotton plants *(Gossypium hirsutum)*	Glasshouse	Enhanced germination rate of seedlings	(Wu et al. 2012)
B. phytofirmans PsJN	Cold stress and Grapevine plantlets (Vitis vinifera cv)	Laboratory conditions	Enhanced chilling resistance	(Barka et al. 2006; Fernandez et al. 2012; Theocharis et al. 2012)

1.3.2 Indirect plant-growth promoting activity

Interestingly, positive plant growth promotion effects can be indirectly facilitated via the ability of bacteria to suppress the activity of, and control diseases caused by, phytopathogenic microorganisms (Whipps 2001). These indirect effects can be considered as a form of biocontrol, the classical definition of which is, "the use of living organisms to suppress the population density or impact of a specific pest organism, making it less abundant or less damaging than it would otherwise be" (Eilenberg et al. 2001).

As better understanding of plant-microbe interactions as developed, the defintion for biocontrol has been expanded and now includes microbial metabolites and other naturally derived compounds that exhibit antagonistic activity toward a pathogen (Beattie 2007). Mechanistically, rhizosphere bacteria may act as biocontrol agents through competition for nutrients, niche exclusion (Compant et al. 2005; Miethke and Marahiel 2007), antibiosis (Ramos-Gonzalez et al. 2005), biosurfactant production (Banat et al. 2010), manipulation of quorum sensing (QS) molecules (Wu et al. 2009), production of hydrolytic enzymes (Krechel et al. 2002), inhibition of pathogen-produced enzymes or toxins (Bertagnolli et al. 1996), interference with pathogen activity, survival, germination and sporulation (Lugtenberg and Kamilova 2009), bacteriocins (Riley and Wertz 2002), and induction of host defense responses (Bakker et al. 2007; Kloepper et al. 2004).

1.3.2.1 Competition

Impeded growth of pathogenic bacteria due to the simultaneous presence of non-pathogenic plant associated bacteria can be attributed the ability of the latter group to colonize niches more efficiently and sequester nutrients. However, the hypothesis that competition is an important bio-control mechanism still lacks sufficient direct experimental evidence as reviewed by (Lugtenberg and Kamilova 2009). It has recently been demonstrated that treatment of *Arabidopsis thaliana* seedlings with the leaf bacterium *Sphingomonas* sp. against the leaf pathogenic *Pseudomonas syringae* pv. *tomato* prevented the emergence symptoms of infection (Innerebner et al. 2011). Data obtained from the complete genome sequencing of seven strains of the *Pseudomonas fluorescens* group that colonize plant surfaces and function as biological control agents

revealed that the occurrence and expression of type III secretion systems (T3SS) may be used for delivery of effector molecules into eukaryotic host cells. This may explain the prevalence of various strains as plants root colonizers and, therefore, better competitors in natural habitats (Loper et al. 2012).

Together with the enhancement of plant growth in environments with low iron content, it has been also reported that the production, release, and uptake of siderophores by non-pathogenic bacteria is a major repression mechanism of soil pathogens which subsumes in the general framework of competition for limited resources in the same niche. These iron scavenging molecules are metabolites exhibiting high potential for biological disease control, mainly via depriving iron from phytopathogenic bacteria and/or fungi.

It has been hypothesized that this mode of action is effective because biocontrol PGP producing siderophores have a much higher affinity for iron than siderophores produced by fungal pathogens; moreover, as a result of the ability of plants to grow at much lower iron concentrations than the majority of microorganisms, their growth is generally not impaired by the presence of siderophores produced by biocontrol PGP (Schippers et al. 1987).

In addition, plants can bind, take up and then exploit the biocontrol PGPB iron siderophore complexes (Wang et al. 1993). It has been demonstrated that the involvement of bio-control PGPB in the inhibition of both bacterial and fungal pathogens is associated with fluorescent *Pseudomonades* and their competitive advantage is based on pyoverdine-mediated iron uptake (Duan et al. 2013; Loper et al. 2012; Shen et al. 2013). Facilitated antagonism over pathogenic organisms has also been observed for strains of *Serratia fonticola* (Szentes et al. 2013), *Bacillus amyloliquefaciens* (Tan et al. 2013), *Streptomyces* spp. (Xue et al. 2013), *Burkholderia cepacia* (de los Santos-Villalobos et al. 2012), *Bacillus firmus (Chaiharn et al. 2009),* and *Rhizobium meliloti* (Arora et al. 2001). The potential of PGPB producing siderophores to act as biocontrol agents was linked to rhizospheric bacteria and endophytes in a recent work by Wensig et al. (2010). In this work, the production of siderophores by the soybean epiphyte *Pseudomonas syringae* pv. *syringae* was responsible for suppressive activity against *Pseudomonas syringae* pv. *glycinea*, the causal agent of bacterial blight of soybean (Wensing et al. 2010).

1.3.2.2 Antibiosis and Biocontrol

Besides siderophores, the control of plant pathogens relies on production and secretion of microbial secondary metabolites possessing antimicrobial properties. Antibiotics encompass a heterogeneous group of organic, low-molecular-weight compounds that are deleterious to the growth or metabolic activities of other microorganisms (Duffy et al. 2003). Among the antibiotics produced by antagonistic bacteria with the ability to control plant pathogens, strong interest has been ascribed to ammonia, hydrogen cyanide (HCN), 2,4-diacetyl phloroglucinol (DAPG), phenazine-1 carboxylic acid, kanosamine, oligomycin A, oomycin A, pyoluteorin, pyrrolnitrin, viscosinamide, xanthobaccin and zwittermycin.

In some studies the genes encoding for the enzymes responsible for synthesis of antibiotics have been isolated and their regulation, at the transcriptional and post-transcriptional levels, have been studied to facilitate the genetic improvement of antibiotic production in genetically modified strains (Bhattacharyya and Jha 2012; Haas and Keel 2003; Whipps 2001).

Generally, the aforementioned antibiotics inhibit pathogens through disruption of cell wall synthesis, by, impacting membrane structure and function, and through inhibition of the formation of initiation complexes on the small ribosomal subunit (Maksimov et al. 2011). Production of antibiotics has been observed in bacterial strains of *Enterobacter cloacae (Hinton and Bacon 1995)*, *Lysobacter spp. (Islam et al. 2005)*, *Burkholderia spp. (Mao et al. 2006)*, *Pseudomonas fluorescens (Maurhofer et al. 1994)*, *Serratia plymuthica (Grosch et al. 2005)*, *Streptomyces melanosporofaciens (Agbessi et al. 2003)*, *Bacillus spp. (Romero et al. 2004)*, *Paenibacillus polymyxa (Kim et al. 2010)*.

Notably, genome analysis of the leaf-colonizing bacterium *Bacillus sp*. strain 5B6, isolated from a cherry tree, revealed gene clusters possibly involved in biosynthesis of antibiosis-related surfactants, nonribosomal peptides and polyketides that may function as antibiotics similar to bacilysin, difficidin, bacillaene and macrolactin, and for synthesis and transport of bacillibactin, a high-affinity siderophore that may inhibit fungal pathogen growth (Kim et al. 2012). Each antibiotic class has a different biosynthesis mechanism and exhibits another antifungal spectrum. For example, pyrrolnitrin, an antifungal compound produced mainly by *Pseudomonas* and species in other genera, eliminates the

growth of *Botrytis cinerea*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum* (Fernando et al., 2005) (Dwivedi and Johri 2003; van Pee and Ligon 2000). A review by (Chin-A-Woeng et al. 2003) on biocontrol by phenazine-producing *Pseudomonas* strains provides detailed knowledge about the action, biosynthesis, regulation mechanisms and the production of microbial phenazines. Redox-active phenazines are a structurally and functionally diverse group of potent antibiotics that are produced by several PGPR and non-PGPR groups of bacteria, including members of *Streptomyces*, *Pantoea*, *Pectobacterium*, *Burkholderia*, as well as several strains of fluorescent *Pseudomonas* species (Mavrodi et al. 2010).

Suppression of plant pathogens such as *Fusarium oxysporum* and *Gaeumannomyces graminis* has been related to phenazines produced by pseudomonads (Chin-A-Woeng et al. 2003). Even though phenazines failed to antagonize effectively *S. sclerotiorum* (Selin et al. 2010), they were found to be effective against *Sclerotium rolfsii* (Le et al. 2012).

In addition, bioactive phenazines produced by two plant growth promoting rhizobacteria strains, a *Pseudomonas aeruginosa* strain RRLJ 04 and a *Bacillus cereus* strain BS 03, which showed growth promotion and disease control in pigeon pea against *Fusarium udum*, were able to efficiently control fusarial wilt of pigeon pea under gnotobiotic and nursery conditions (Dutta et al. 2014)**.** A recent review by (Mavrodi et al. 2013) presents novel insights about the diversity, frequency and ecological roles of phenazines produced by fluorescent *Pseudomonas* spp.

Another secondary metabolite with profound antifungal activity is 2,4-diacetyl phloroglucinol (DAPG). The production of this polyketide antibiotic has been mainly observed in strains of *Pseudomonas fluorescens* and is cited as one of the most effective suppressive agents against various root and seedling diseases such as black root rot of tobacco, crown and root rot of tomato, soft rot of potato as well as *Pythium* damping-off of cucumber and sugar beet, and take-all of wheat (Landa et al. 2002) and references therein).

In particular, the work of (de Souza et al. 2003) on the effect of DAPG to the oomycete *Pythium* spp. has unveiled that the main target of this antibiotic is the cell membrane and zoospores, whilst phenazines and pyrrolnitrin target the electron transport chain.

Experiments with the DAPG-producing PGPR strain, *Pseudomonas fluorescens* (*phlD*$^+$) have demonstrated that plant species can enrich and support different microbial populations (De La Fuente et al. 2006) and genotypes (Landa et al. 2006) in the rhizosphere.

Recently, genome sequencing of the biocontrol strain *Pseudomonas fluorescens* F113 has revealed the existence of genes responsible for the biosynthesis of DAPG (Redondo-Nieto et al. 2012). Even though the production of antifungal metabolites has been correlated mainly with strains of fluorescent pseudomonades, antifungal secondary metabolites have also been isolated from biocontrol active *Streptomyces* and *Bacillus* (Khamna et al. 2009; Kumar et al. 2009); furthermore, based on a recent review (Palaniyandi et al. 2013), antibiotics produced by actinobacteria have applications as broad spectrum soil fungicides that can be used as alternatives to chemical fungicides such as methyl bromide and metalaxyl.

1.3.2.3 Biosurfactants as antifungal agents

In addition to antibiosis, biosurfactants based on their distinct characteristics are also involved in biological control by acting as antifungal agents or elicitors of induced systemic resistance. In general, biosurfactants are thought to result in the formation of channels in cell walls and cause other disorders like hyphal swellings, increased branching, reduced mycelium growth and sclerotinia formation (Raaijmakers et al. 2006).

Among the many classes of biosurfactants, mainly glycolipids, such as cellobiose lipids, rhamnolipids, and cyclic lipopeptides such as surfactin, iturin and fengycin, provide protection to plants through their antifungal properties against phytopathogenic fungi (Banat et al. 2010).

The potential use of biosurfactants as biological control agents has been mentioned by (Stanghellini and Miller 1997); they described how rhamnolipids can disrupt zoospore membranes and cause lysis of zoospores of many oomycete plant pathogens. Since then, numerous reports have discussed the important role of rhamnolipids against various phytopathogenic fungi.

In particular, (Debode et al. 2007) reported that rhamnolipids produced by *Pseudomonas* spp. facilitate the suppression of *Verticillium* microsclerotia viability.

In their 2008 study, (Perneel et al. 2008) hypothesized that *Pythium hyphae* were broken down after incubation in liquid medium amended with both phenazines and rhamnolipids produced by *Pseudomonas aeruginosa* PNA1; the results indicated that both metabolites acted synergistically in controlling soil-borne diseases caused by *Pythium* spp.

It further has been reported that rhamnolipid biosurfactants apart from their direct antifungal properties by inhibiting spore germination and mycelium growth of *Botrytis cinerea* on grapevines, possibly perceived from the plant as microbe-associated molecular patterns (MAMPs). Notably, the response of the grapevine against this disease was associated with Ca^{2+} influx, mitogen-activated protein kinase activation and reactive oxygen species production as early events.

Induction of plant defenses including expression of a wide range of defense genes, hypersensitive (HR)-like response explained parts of the mechanisms involved in plant resistance. Additionally, rhamnolipids potentiated defense responses induced by chitosan elicitor and by the culture filtrate of *Botrytis cinerea* suggesting that the combination of rhamnolipids with other effectors could participate in grapevine protection against the grey mould disease and in a broader sense (Varnier et al. 2009b).

A review by (Vatsa et al. 2010) highlights the antimicrobial properties of rhamnolipids and the involvement of these molecules in the stimulation of 'immunity' in plants. Recent work by (Sha et al. 2012) demonstrated that cell-free crude rhamnolipids extracts showed activity against colony growth and biomass accumulation of seven fungal plant pathogens including two *Mucor* spp., three *Ascomycota* and two *Oomycetes*.

The antifungal efficiency of cell-free culture medium of rhamnolipid producing bacteria could be attributed to the major component of di-rhamnolipid in this cell-free medium, which is characterized by better lysis traits over mono-rhamnolipid to rupture the spore membranes especially for zoospores producing plant pathogens via the intercalation of RLs within plasma membranes of the zoospore which are not protected by a cell wall (De Jonghe et al. 2005; Varnier et al. 2009a).

Cyclic lipopeptides (CLPs) represent another class of biosurfactants with antifungal activity towards phytopathogenic fungi equally important as

rhamnolipids. In short, biosynthesis of cyclic lipopeptides is governed by non-ribosomal peptide synthetases (NRPSs), encoded by very large gene clusters and composed of modules, one for each amino acid that needs to be incorporated in the oligopeptide. Each module consists of several conserved domains responsible for recognition, activation, transport and binding of the amino acid to the peptide chain. A special thioesterase domain in the last module coordinates cyclization and release of the peptide product.

Due to this unconventional biosynthesis scheme, incorporation of unusual amino acids is possible (D'Aes et al. 2010). Indeed, in some CLPs like iturin A synthesized by *B.subtilis*, it has been demonstrated that the genes responsible for the biosynthesis of the fatty acid side-chains are in the vicinity of the NRPSs (Tsuge et al. 2001).

The results obtained by (Grover et al. 2010) confirm the ability of iturin-producing *Bacillus subtilis* strain RP24 isolated from the rhizoplane of field grown pigeon pea to act as a biocontrol agent against a wide range of phytopathogenic fungi.

Iturin antibiotics increase the membrane permeability of the target microorganism due to the formation of ion channels on the cell membranes thereby increasing the permeability to K^+. In addition to iturin, surfactin is another lipopeptide with well-documented antifungal activity. Secretion of a lipopeptide by *Bacillus licheniformis* strain BC98, identified as surfactin, induced morphological changes in *Magnaporthe gris*ea, inhibiting its further growth (Tendulkar et al. 2007).

Isolation and characterization of Leu7-Surfactin which is a surfactin A from the endophytic bacterium *Bacillus mojavensis* strain RRC 101 indicated that this bacterium can be a useful antagonist to assist in the control of endophytic infections and resulting diseases caused by *Fusarium verticillioides* (Snook et al. 2009). In agreement with the afore mentioned results, (Bacon et al. 2012) recognized also that strains of *Bacillus mojav*ensis produce a higher number of isoforms of surfactin A compared to B and C isoforms.

Genome sequencing has unveiled the genes and regulatory elements putatively involved in the biosynthesis of known cyclic lipopeptides produced by different bacterial genera (de Bruijn et al. 2007; Koumoutsi et al. 2004; Loper and Gross 2007).

For example, the genome of the plant-associated *Bacillus amyloliquefaciens* strain GA1 was found to harbour four gene clusters to direct the synthesis of the cyclic lipopeptide surfactins iturin A and fengycin (Arguelles-Arias et al. 2009). Recent reports also confirmed the existence of biosynthetic gene clusters encoding pathways for secondary metabolite production, such as genes for lipopeptide (surfactin, iturin, and fengycin) biosynthesis. ((Blom et al. 2012; Dunlap et al. 2013).

Pseudomonas spp. comprises another important group of plant-associated bacteria fostering biocontrol activities mediated by the production of cyclic lipopeptides. Although the interaction between glycolipid type biosurfactants and fungi has been the main focus of many studies, the zoosporicidal properties of cyclic lipopeptides have been documented ((Hultberg et al. 2010; Kruijt et al. 2009; Tran et al. 2008). The role of biosurfactants in plant-*Pseudomonas* interactions, emphasizing their potential use as biocontrol agents has been discussed in a review by (D'Aes et al. 2010).

1.3.2.4 Parasitism via the production of hydrolytic enzymes

Since the cell walls of many fungi contain chitin, ß-1,3 glucan and protein, suppression of fungal pathogens can be attained by cell wall lysis via the synthesis of exo-enzymes such as chitinase, cellulase, ß-1,3 glucanase, protease, or lipase, alone or in combination (Maksimov et al. 2011; Neeraja et al. 2010). For example, the potential use of chitinase producing bacteria as biocontrol agents has been demonstrated for diseases such as wilt of cucumber caused by *Fusarium oxysporum f.* sp. *cucumerinum* (Singh et al. 1999), leaf spot of tall fescue caused by *Bipolaris sorkiniana* (Zhang and Yuen 2000), basal drop of lettuce caused by *Sclerotinia minor* (El-Tarabily et al. 2000), sheath blight of rice caused by *Rhizoctonia solani* (Radjacommare et al. 2004), damping off of egg plant caused by *R. solani* (Park et al. 2007), wilt in carnation caused by *F. oxysporum* f. sp. *dianthi* (Ajit et al. 2006), gray mold of chickpea caused by *Botrytis cinera* (Kishore and Pande 2007), and leaf blight of pepper caused by *Phytophthora capsici* (Kim et al. 2008b). Inhibition of pathogenic fungi during developmental stages such as conidial germination and the growth of hyphae has been demonstrated for some *Serratia* and *Bacillus* strains able to produce extracellular chitinases (Popova and Khatskevich, 2004).

A number of reports (Park et al., 2005; Ajit et al., 2006; Kishore and Pande, 2007; Kamil et al., 2007) highlight the inhibition of fungal growth by chitinolytic bacteria and purified chitinase *in vitro* and *in vivo* (Huang et al., 2005; Siwayaprahm et al., 2006; Hariprasad et al., 2011).

Given that fungal cells walls contain ß-1,3-glucan, glucanases represent another significant group of hydrolytic enzymes that may be useful as biocontrol agents. For instance, the production of ß-1,3-glucanases by *Pseudomonas cepacia* (Fridlender et al. 1993), *Streptomyces* sp. (Valois et al. 1996), *Lysobacter enzymogenes* (Palumbo et al. 2005), *Actinoplanes philipinensis* and *Micromonospora chalcea* (El-Tarabily and Sivasithamparam 2006), were found to inhibit the proliferation of phytopathogenic fungi like *Phytophthora fragariae* (raspberry), *Rhizoctonia solani*, *Sclerotium rolfsii* and *Pythium ultimun*, *Pythium* (sugar beet) and *Pythium aphanidermatum* (cucumber), respectively.

Moreover, (Dunne et al. 1998) reported that production of proteases by *Stenotrophomonas maltophilia* resulted in inhibition of *Pythium ultimum* (sugar beet). Some bacterial strains such as *Serratia marcescens*, *Streptomyces viridodiasticus* and *Micromonospora carbonacea* exerting inhibitory action against *Sclerotinia minor* (lettuce), have been found to produce both chitinases and ß-1,3-glucanases (El-Tarabily et al. 2000). Likewise, the study of (Xue et al. 2013), involved the isolation of four *Steptomyces* strains capable of simultaneously producing chitinase, ß-1,3-glucosidase, cellulase and protease when cell wall material of *Verticillium dahliae* was the sole carbon source. It is noteworthy to refer at this point to the observation of (Tanaka and Watanabe 1995), that the synergistic action of chitinases and ß-1,3-glucanases resulted in a more effective inhibition of fungal pathogens compared to the individual enzymes. Regulatory cascades of these lytic enzymes (mainly chitinases and proteases) involve the GacA/GacS system (Corbell and Loper 1995; Gaffney et al. 1994; Sacherer et al. 1994).

1.3.2.5 Quorum sensing and biological control

In many plant-associated bacteria, *N*-acylhomoserine lactone (AHLs) – dependent quorum sensing system (QS) is regulated by protein homologues of LuxI-AHL synthase and LuxR-AHL-response regulator. In this system, when AHLs reach a threshold concentration, the transcriptional complex formed by the

interaction of AHLs with their cognate regulator binds to DNA sequences termed 'lux boxes' upstream of the promoters of genes regulated by QS. This is considered, among other discernible density-dependent behaviors, as the pertinent driver of gene expression for biosynthesis of antibiotics (Bodini et al. 2009).

For example, a quorum sensing system (QS) that utilizes the AHL molecule C6-HSL (N-hexanoyl-L-homoserine lactone) secreted by *Pseudomonas chlororaphis* and *P. aureofaciens* regulates the production of phenazine antibiotics and the biocide hydrogen cyanide (HCN) (Chin-A-Woeng et al. 2001; Wood et al. 1997). In another example, the AHL-regulated synthesis of pyrrolnitrin that was described by (Liu et al. 2007b) was further confirmed by the more recent work of (Muller et al. 2009). Briefly, a comparative analysis under greenhouse conditions between wild type and AHL negative mutants of *Serratia plymuthica*, confirmed that production of pyrrolnitrin in *Serratia plymuthica* HRO-C48 is AHL-dependent. Moreover, it was brought to light that production of extracellular proteolytic and chitinolytic enzymes was positively regulated, as well as a broad spectrum of VOCs produced by HROC48, known to be involved in antifungal activity are, at least in part, regulated by QS mechanisms.

1.3.2.6 Inhibition of pathogen-produced enzymes and/or toxins

To attenuate or prevent the deleterious effects of pathogen-produced enzymes and toxins to plants, some bacteria have been shown to "disarm" key virulence factors through enzymatic degradation. For instance, an extracellular endoproteinase produced by *Bacillus megaterium* B 153-2-2, suppresses the activities of hydrolytic enzymes, such as cellulase, pectin lyase and pectinase, produced by *Rhizoctonia solani* during infection of soybean (Bertagnolli et al. 1996). The presence of the gene responsible for detoxification of albicidin in *Pantoea dispersa* was correlated to reduced pathogenicity of the bacterium *Xanthomonas albilinean* to sugarcane (Zhang and Birch 1997). Remarkably, positive chemotaxis of *Pseudomonas fluorescens* towards fusaric acid (a phytotoxin produced by various *Fusarium* species) along with the ability to hydrolyze it, allowed the colonization of the hyphae together with formation of microcolonies (Bolwerk et al. 2003; de Weert et al. 2004) .

1.3.2.7 Signal interference

Many bacteria orchestrate the expression of multiple pathogenic genes to the concentration of signal molecules via quorum sensing (QS) (Whitehead et al. 2001). The best characterized among these molecules are the N-acyl homoserine lactone signal molecules (AHLs), which are are synthesized by Gram-negative bacteria, whilst, certain oligopeptides and substituted g-butyrolactones are the primary signal molecules found in Gram-positive bacteria. Intriguingly, an AHL system is required for the onset of crown gall disease by *Agrobacterium tumefaciens* in plants, as well for the synthesis of cell-wall degrading enzymes of the pathogen *Erwinia carotovora* (Newton and Fray 2004; Zhang et al. 2002).

The evidence that expression of many virulence factors and establishment of infections between phytopathogenic bacteria and their host plants is regulated by quorum sensing, means that there are opportunites to exploit degradation of AHLs as a biocontrol mechanism. For example, signal interference involving either the inhibition of a QS component or the depletion of the signal itself could result in attenuation of the QS response by Quorum Quenching (QQ) (Dong et al. 2002; Dong et al. 2004; White and Finan 2009).

Two classes of AHL-inactivating enzymes have been identified to date, namely AHL-lactonases which hydrolyse the lactone ring, and AHL-acylases which break the amide linkage (Uroz et al. 2009). AHL inactivation has been reported in α-proteobacteria (e.g., *Agrobacterium*, *Bosea*, and *Ochrobactrum*), ß-proteobacteria (e.g., *Variovorax*, *Ralstonia*, *Comamomonas*, and *Delftia*), and γ-proteobacteria (e.g., *Pseudomonas* and *Acinetobacter*). In the case of Gram-positive bacteria, AHL degradation occurs in both low G + C% strains, i.e., *Firmicutes*, such as *Bacillus*, and in high G + C% strains or actinobacteria, such as *Rhodococcus* and *Arthrobacter* (Cirou et al. 2007; Faure et al. 2009).

Recently, the involvement of volatile organic compounds (VOCs) has been speculated as a novel signal interference mechanism by which some bacteria control diseases (Chernin et al. 2011; Dandurishvili et al. 2011).

1.3.2.8 Bacteriocins

Bacteriocins represent another pillar of antagonistic activity that plant growth promoting bacteria exhibit to minimize phytopathogen proliferation (Riley and

Wertz 2002). These proteinaceous toxins, consisting of ribosomally encoded antimicrobial peptides, are generally antagonistic against similar or closely related strains, though, such ''chemical armory'' can be used to attack sibling cells within a same colony (Be'er et al. 2009; Claverys and Havarstein 2007; Ellermeier et al. 2006; Gonzalez-Pastor et al. 2003).

Representative bacteriocins produced by Gram-negative bacteria include colicin derived from *Escherichia. coli*, pyocins from *Pseudomonas pyogenes* strains, cloacins from *Enterobacter cloacae*, marcescins from *Serratia marcescens* and megacins from *Bacillus megaterium* (Cascales et al. 2007). Interestingly, in addition to related gram positive bacterial species, bacteriocin thuricin 17 isolated from the PGPR *Bacillus thuringenisis* NEB17, was found to inhibit the growth of *Escherichia coli* MM294 (pBS42) (Gray et al. 2006). Production of three bacteriocins by the endophyte *Curtobacterium flaccumfaciens*, isolated from symptomless sweet orange trees, was thought to be a contributing factor in the control of *Xylella fastidiosa*, the causal agent of citrus-variegated chlorosis (CVC) (Lacava et al. 2007).

Genome sequencing of the diazotrophic endophytic bacterium *Gluconacetobacter diazotrophicus* Pal5, revealed the occurrence of bacteriocin anabolic genes in its genome, underpinning that it can promote plant growth both directly and indirectly (Bertalan et al. 2009).

1.3.2.9 Induction of host resistance mechanisms

Another dimension of plant growth promoting bacteria (PGPB), which can provide significant protection against a broad spectrum of phytopathogenic organisms (bacteria, fungi and viruses) is a phenomenon known as induced systematic resistance (ISR). ISR was introduced when it was observed that resistance in carnations can be induced by the rhizobacterium *Pseudomonas* sp. strain WCS417r against *Fusarium* wilt (Vanpeer et al. 1991), and by selected plant growth promoting rhizobacteria against the fungus *Colletotrichum orbiculare* in cucumber (Wei et al. 1996).

Since then, ISR has been established in several plant species against a range of pathogens; however, it should be mentioned that ISR does not presuppose any direct interaction between the resistance-inducing PGPB and the pathogen.

This latter point highlights the main distinction from SAR (systemic acquired resistance), a phenomenon that occurs when plants activate their defense mechanisms in response to a direct contact with a pathogenic agent.
In recent years, it has been found that methyl salicylate, jasmonates, azelaic acid and a specific diterpenoid act as potent mobile signals associated with the activation of systemic acquired resistance (Shah 2009). Moreover, SAR is induced optimally when the plant develops a hypersensitive reaction to repeated assults by the same pathogen, while ISR encompasses a broader range of induced resistance phenomena elicited mainly by non-pathogenic organisms.
A recent work, in grapevine made also evident the involvement of PGPR as elicitors of ISR (Verhagen et al. 2010).
Generally, induced resistance is systemic, in that resistance is induced not only at the primary infection site, but also in distant non-infected tissues (Van Loon and Baker 2006). The ability of endophytic actinobacteria to induce both the SAR and jasmonate/ethylene (JA/ET) pathway has been reported (Conn et al. 2008). Indeed, induction of the JA/ET pathway in *Arabidopsis thaliana* resulted in resistance to the bacterial pathogen *Erwinia carotovora* subsp. *carotovora,* while induction of SAR pathway resulted in resistance to the fungal pathogen *Fusarium oxysporum*.
In addition, the review of (Palaniyandi et al. 2013) provides examples of actinobacteria involed in systemic resistance in various plants. Besides ethylene and jasmonate, other bacterial molecules such as the O-antigenic side chain of the bacterial outer membrane protein lipopolysaccharide, flagellar proteins, pyoverdine, chitin, ß-glucans, cyclic lipopeptide surfactants, and salicylic acid have also been listed to act as elicitors of ISR (Glick B.R., 2012). Notably, the work of (Iavicoli et al. 2003) was the first proof that an antibiotic produced by *Pseudomonas fluorescens* CHA0 strain, can trigger ISR upon root inoculation in *Arabidopsis thaliana* seedlings.
Redundancy of information linked to the ability of *Pseudomonas* and *Bacillus* genera to promote plant growth and to elicit ISR through the aforementioned microbe-associated molecular patterns (MAMPs) can be obtained from the following reviews (Kloepper et al. 2004; van Loon 2007; Van Wees et al. 2008). ISR mediated by plant growth promoting rhizosphere and endophytic bacterial strains can stimulate the production of enzymes related to pathogenesis (PR-

proteins) such as chitinases, lipoxygenases, ß-1,3- glucanases, as well as other defense enzymes including peroxidases, phenylalanine ammonia-lyase (PAL), polyphenoloxidase (PPO) and phytoalexins, strengthening plant responses against various biotic stresses (Bakker et al. 2007; Dutta et al. 2008; Verhagen et al. 2010).

1.4 Conclusions and future perspectives

The use of PHCs has allowed for the development of privileged modern societies, however, the costs of this include severe contamination of seawater, soil and groundwater ecosystems.

Although, a wide range of laboratory and field studies have dealt and indicated that plant-bacteria assisted remediation holds promise as a good treaement strategy of petroleum hydrocarbons polluted environments (Figure 1.13), there are still limiting factors requiring further research in that area.

In particular for bacteria, information gathered over the last decades from various scientific disciplines has been proven extremely helpful and valuable in order to comprehend in depth their function, as well as the interaction between them and their habitats.

Up to 2003, in literature there have been reports favoring culture-dependent approach because because it allows you to investigate degradation and metabolic pathways next to interaction effects with other contaminants and organisms.

For example, Ellis et al. (2003) demonstrated previously that in specific contaminated environments, adapted cultivation-dependent methods even proved to be superior to cultivation-independent methods, because they reflected better the effect of the contaminant on the physiological status of the bacteria (Ellis, Morgan, Weightman, & Fry, 2003).

Indeed, culture based methods have yielded substantial information about interaction between bacteria and petroleum hydrocarbons, however, it should not be underestimated that in that way, it is possible to study less than 1% of microorganisms present in nature, and even if a bacterium is cultured, its role within a community along with the contribution to ecosystem function are not necessarily showed. Moreover, one must keep mind that the possibility to study the genes present in the other 99% might be lost.

Section 1

It seems likely that the uncultured microorganisms encompass diverse microbes that are distantly related to cultured microorganisms. Thus, their investigation requires the use of techniques that sidestep the isolation and cultivation of individual species (Amann et al. 1995).

At present, molecular description based on the so-called "omics" technologies has a pivotal role in all areas of microbial ecology, thus in bacteria mediated biodegradation of hydrocarbons. In this context emerging information from these high-throughput sequencing technologies might explain the large differences in degradation patterns often observed in bioremediation experiments. Yet, care must be taken that, while these tools are able to generate huge amount of data, the genetic composition of a microbial community does not necessarily corresponds to ecosystem function. Conclusively, a sound understanding of the evolved plant-bacteria synergies along with extensive field experiments will fill the gap in knowledge that is still needed in order to design effective remediation schemes based in the ingenuity of bacteria and their host plants.

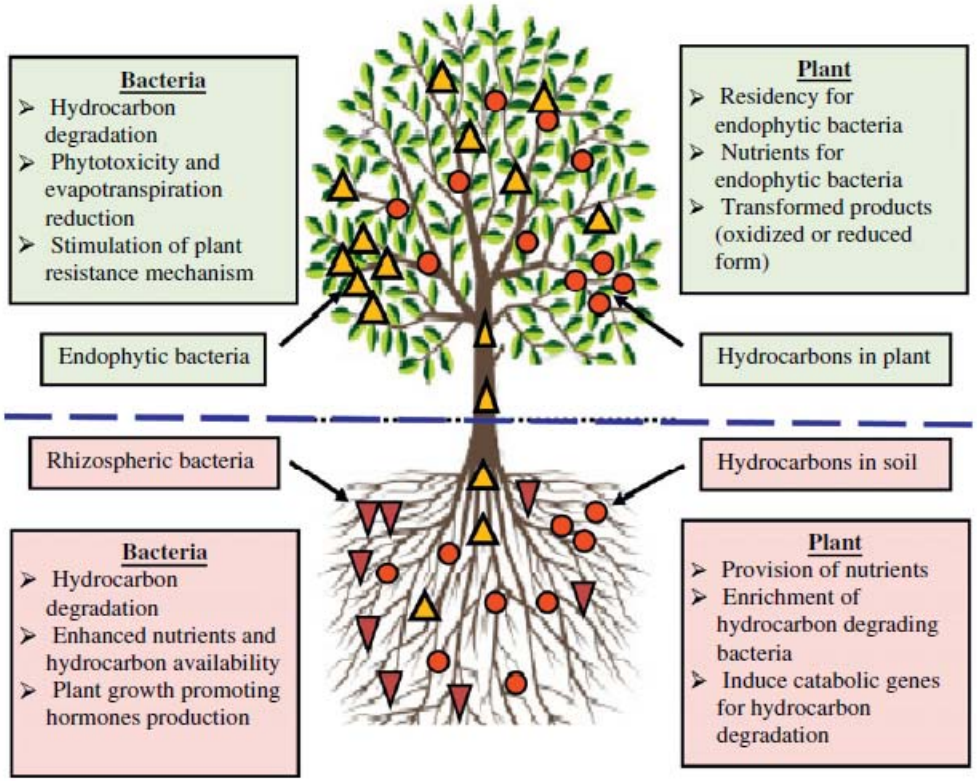


Figure 1.13 Contribution of plants and their associated bacteria to phytoremediation of Petroleum hydrocarbons (adapted from Khan 2013).

SECTION 2

Objectives

Section 2

There is a growing demand for remediation of contaminated soils and groundwater. Indeed, the widespread problems caused to the environment by the intensive use of petroleum hydrocarbons has lead to the need for remediation solutions that are time effective, cheap and environmentally friendly. In view of this, during the last two decades research has concentrated on the utilization of plant-bacteria partnerships as a tool to provide an alternative to or complement for conventional remediation approaches for the restoration of petroleum hydrocarbon contaminated sites. Although, there exists promising results available from laboratory and greenhouse experiments, field applications are not always successful. These failures may arise from a non-integrated approach when implementing remediation strategies are not based on sound science. It is essential to take into account all physical, chemical and biological factors affecting contaminant fate when designing a remediation scheme. For example, when selecting for microorganisms with biodegradative properties compatible with phytoremediation, plant growth potential along with the enzymatic mechanisms to degrade the target contaminants must be considered.

New and emerging -omics technologies such as single cell genomics and metagenomics represent novel tools that generate abundant information that, when analyzed using bioinformatic approaches, can greatly increase scientists' knowledge and ability to design and improve efficient remediation strategies.

Aiming to contribute to the further development of bacteria-enhanced phytoremediation of soils and groundwater contaminated with petroleum hydrocarbons, in this thesis we focused on three main objectives:

i. Isolation and phenotypic and genotypic identification of new plant-bacteria partnerships suitable for phytoremediation of organic contaminants.
ii. Application of –omics technologies (in this case single cell genome sequencing) to further unravel the fundamental mechanisms of diesel degradation and plant-growth promotion in a selected strain
iii. Testing a plant-bacteria system in a model greenhouse trial

In this framework, the topics mentioned in the following Sections were investigated in order to gain a comprehensive understanding of the possible

role(s) of poplar associated bacteria. Hybrid poplar was chosen due to the fact that it possesses phreatophytic roots that extend to the water table, exhibits high water uptake rates and high absorption surface areas, and tolerance to contaminants and flooding. Moreover, it is very suitable for biomass production.

- ✓ Section 3 describes the characterization and identification of the cultivable bacterial populations isolated from the soil, rhizosphere, roots, stems and aerial parts of hybrid poplar trees [*Populus deltoides x* (*trichocarpa x deltoides*) cv Grimminge], growing on a diesel contaminated plume. A wide range of genotypic, phenotypic and biochemical tests were performed to aid with selection of strains harboring both plant-growth promoting and diesel degrading capabilities. Based on these results three strains were selected for a more detailed examination of their biodegradation potential using GC-MS analysis of diesel fuel cultures.

- ✓ Section 4 focuses on the implementation of whole genome shotgun sequencing as a tool to acquire fundamental information about the biodegradation potential and plant-growth promoting properties of a selected strain based on the results obtained in Section 3. IonTorrent PGM (Life Technologies Inc., Carlsbad, CA) and a single 316v2-chip w343 used for sequencing the genome of a root endophyte identified as *Acinetobacter oleivorans* PF1. In Section 3 this strain was shown to possess promising plant growth-promoting traits *in vitro* combined with substantial diesel biodegradation capacity confirmed by GC-MS analysis.

- ✓ Section 5 reports on a greenhouse experiment in which hybrid poplar cuttings [*Populus deltoides x* (*trichocarpa x deltoides*) *cv Grimminge*], inoculated with a bacterial consortium consisting of 2 bacterial strains, namely the root endophyte *A. oleivorans* PF1 and the rhizospheric *Acinetobacter calcoaceticus* GK1, were exposed to two diesel concentrations (5000 mg Kg^{-1} soil - 10000 mg Kg^{-1} soil) spiked in a sandy soil (Figure 2.1). In order to evaluate possible effects of the consortium on the biomass production of poplar, cuttings were also

inoculated in the absence of diesel. As a control, non-inoculated poplar cuttings were used.

- ✓ In Section 6, a general discussion summing up the relevance of our findings together with perspectives for future research is provided.

Figure 2.1 Greenhouse experiment

SECTION 3

Genotypic and phenotypic characterization of cultivable bacterial populations isolated from poplar trees growing on diesel contaminated plume

Section 3

3.1 Introduction

As already mentioned in the introduction, petroleum hydrocarbons are the most widespread class of organic compounds worldwide. Among the various types of petroleum hydrocarbons, diesel oil has been frequently reported as a priority pollutant due to its intrinsic chemical stability, resistance to different types of degradation and toxicity to living biota (Serrano et al. 2008). Furthermore, the presence of high molecular weight compounds in diesel oil are considered a potential health risk due to their possible carcinogenic and mutagenic actions (Bidoia et al. 2010). The main routes of entrance to the environment are via leaking from storage tanks and pipelines, and accidental spills (Bento et al. 2005b). The majority of diesel fuel components consist of saturated aliphatic hydrocarbons (primarily paraffins including n-, iso-, and cyclo-paraffins), and aromatic hydrocarbons (including naphthalenes and alkylbenzenes) obtained from the middle-distillate, gas-oil fraction during petroleum separation (Stroud et al. 2007; Zanaroli et al. 2010) and contains many highly concentrated toxic compounds (Dillard et al. 1997). Diesel oil contains 2000 to 4000 different hydrocarbons (Marchal et al. 2003) with carbon numbers ranging from approximately C_9 to C_{23}. Despite this complexity, diesel oil can be readily degraded by a number of soil microorganisms making it a likely candidate for bioremediation (Adam and Duncan 1999). Diesel fuel oil bioremediation has been demonstrated both in laboratory and field trials (Biggar et al. 1997; Geerdink et al. 1996a; b; Margesin and Schinner 1997; Zytner et al. 2001). Indeed, diesel degradation has been reported for pure bacterial strains like *Pseudomonas stutzeri* (Vazquez et al. 2009), *Pseudomonas aeruginosa* strain IU5 (Hong et al. 2005), *Pseudomonas aeruginosa* DQ8 (Zhang et al. 2011b), *Streptomyces* sp. strain ERI-CPDA-1 (Balachandran et al. 2012), *Rhodococcus erythropolis* (Saadoun 2002), *Rhodococcus erythropolis* strain NTU-1 (Liu and Liu 2011), *Bacillus cereus* (Kebria et al. 2009), *Bacillus subtilis* (Nwaogu et al. 2008), *Exiguobacterium aurantiacum and Burkholderia cepacia* (Mohanty and Mukherji 2008), *Pseudomonas citronellolis* KHA (Sadouk et al. 2009), *Pseudomonas* sp. strain C7 (Luo et al. 2012a), *Brevibacterium* Met-1 (Pavitran et al. 2004), *Gordonia nitida* strain LE31 (Lee et al. 2005), *Rhodococcus baikonurensis* strain EN3 (Lee et al. 2006), *Paenibacillus* sp. (Ganesh and Lin 2009), *Acinetobacter beijerinckii* ZRS (Huang et al. 2013), *Acinetobacter* sp.

strain DR1 (Kang and Park 2010), *Pseudoxanthomonas* sp. strain RN402 (Nopcharoenkul et al. 2013), *Sphingomonas paucimobilis* and *Rhizobium radiobacter (Purwanti et al. 2012)*, and consortia (Moliterni et al. 2012; Owsianiak et al. 2009a; Owsianiak et al. 2009d; Pepi et al. 2003; Rahman et al. 2002; Zhang et al. 2010). Although the plethora of aforementioned studies underlines the ability of different bacterial species to degrade diesel oil, difficulties related to the bioavailability of diesel oil components, the toxicity of the components and the co-presence of inhibitory heavy metals should not be ignored. In this context, in addition to a microorganism being able to metabolise diesel oil components, the production of surface-active agents (biosurfactants) and emulsifiers, the formation of bioflims, chemotactic behavior towards diesel and tolerance to various heavy metals are factors that can give a particular microbial species a competitive advantage.

3.2 Materials and methods

3.2.1 Study area

Samples were obtained from the site of the Ford Motor Company in Genk (Belgium). At this site hybrid poplar [*Populus deltoides* x (*trichocarpa x deltoides*) cv Grimminge] was planted as a means to control a diesel/creosol plume.

3.2.2 Sampling of plant and soil material

Samples of bulk soil, roots and rhizosphere were taken and stored in sterile Falcon tubes (50 mL) filled with sterile 10 mM $MgSO_4$, while stems and leaves were taken and stored in separate plastic bags. Three sets of samples were collected in order to assess the microbial community with culture–dependent methods. For the isolation process, soil samples were diluted up to 10^{-5} in 10 mM $MgSO_4$ solution and plated on 1/10 strength 869 solid medium. Rhizosphere samples were vortexed, roots were removed and serial dilutions up to 10^{-5} were prepared in 10 mM $MgSO_4$ solution and plated on 1/10 strength 869 solid medium (Mergeay et al. 1985). To isolate the endophytic bacteria, the collected plant samples were surface sterilized for 10 (roots) or 5 (stems and leaves) minutes in a 2% (roots) or 1% (stems and leaves) active chloride solution

supplemented with 1 droplet of Tween 80 (Merck) per 100 mL solution, and subsequently rinsed 3 times for 1 min in sterile distilled water. The third rinse solution was streaked on undiluted 869 rich medium to check surface sterility.
If no growth was observed after one week, surface sterilization was considered to be successful. Surface sterile plant samples were macerated for 1 min (roots) or 2 min (stems) in 10 mL 10mM $MgSO_4$ using a Polytron PR1200 mixer (Kinematica A6). Bacterial populations were enumerated as Colony Forming Units (CFU) per gram fresh plant weight following 7-day incubation at 30^o C. After that, the Petri dishes were collected and the different colonies were purified taking into account their colour, size and shape. Based on that work, all bacteria that grew on the Petri dishes were characterized and enumerated. Afterwards, Petri dishes of 1/10 869 diluted rich medium were divided in 4 equal parts and streaked with pure cultures; the selected and characterized bacteria were incubated at 30^o C for one week. Using a sterile toothpick every single colony was transferred into Falcon tubes (15 mL) to which 10 mL of undiluted 869 rich medium was added. Those tubes were incubated at 30^o C under constant agitation at 120 rpm for 5 days. Those samples were used further for DNA extraction and the remnants were stored in glycerol at – 70^o C.

3.3 Characterization of poplar–associated and soil bacteria

3.3.1 Genotypic characterization

After purification, total genomic DNA of all morphologically different bacteria was extracted using the DNeasy® Blood and Tissue kit (Qiagen). A Nanodrop ND-1000 Spectrophotometer (Isogen Life Science) was used for qualitative and quantitative analysis of the extracted DNA. Aliquots (1 µl) of extracted DNA were used for PCR reactions with no further purification in order to amplify a fragment of the 16S ribosomal RNA coding region. Two primers, the universal 1392R (5'-ACGGGCGGTGTGTGTRC-3') and the bacteria-specific 26F (5'-AGAGTTTGATCCTGGCTCAG-3'), were used for the amplification, as described earlier (Barac et al. 2004). PCR products of the 16S rDNA amplification were directly used for Amplified rDNA Restriction Analysis (ARDRA) that was performed as described by Weyens et al. (2009). A PCR purification kit (Qiagen) was used to purify the amplified 16S rDNA of strains with distinct ARDRA

patterns. ARDRA patterns were grouped, and strains with representative patterns were selected for 16S rRNA gene sequencing, as described earlier (Barac et al. 2004; Weyens et al. 2009a). Sequence Match at the Ribosome Database Project was used for nearest neighbor and species identification. Taxa were identified based on the best match in the database (sequence match number > 0.95). In order to verify the identification, a neighbor – joining tree analysis was performed. To assess branch support, bootstrap values were calculated with 1500 pseudo-replicates.

3.3.2 Phenotypic characterization

The isolated strains were screened in triplicate for their ability to utilize diesel fuel as sole carbon and energy source based on established methods such as the 2,6-dichlorophenolindophenol indicator assay and the "Hole plate" method, while their ability to promote plant growth was evaluated directly or indirectly by measuing for a number of compounds and enzyme activities (ACC deaminase, Indole Acetic Acid, inorganic P solubilization, organic P solubilization, acetoin – 2,3 butanediol, organic acids, and siderophores).

3.3.3 Estimation of degradation capacity of diesel by single isolates

Estimation of the single isolates' capabilities to degrade diesel was based on a modified protocol of the 2,6-dichlorophenolindophenol indicator assay (referred as from this point as DCPIP) as described by (Kubota et al. 2008). Briefly, isolated strains were pre-cultured in 5 ml of 869 broth (per liter: 10 g Tryptone, 5 g Yeast extract, 5 g NaCl, 1 g D-glucose, 0,345 g $CaCl_2$; pH 7.0) at 30^o C and 160 rpm orbital shaking until the optical density at 660 nm was > 1.0. After centrifugation at 4,000g for 5 min and washing 3 times with 0.9% saline buffer (9 g of NaCl in one liter of distilled water), the cell density was adjusted to 1.0 at 660 nm. After sterilization, 750 µl of Fe-free W medium (per liter: 2 g $(NH_4)_2SO_4$, 14.3204 g Na_2HPO_4, 5.4436 g KH_2PO_4, 0.5 g NaCl, 0.2465 g $MgSO_4$, 2.78 mg $FeSO_4 \cdot 7H_2O$, 14.7 mg $CaCl_2 \cdot 2H_2O$, 2.01 mg $ZnSO_4 \cdot 7H_2O$, 0.15 mg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.2 mg $CuSO_4 \cdot 5H_2O$, 0.4 mg $CoCl_2 \cdot 6H_2O$ and 1.49 mg $MnSO_4 \cdot 5H_2O$), 50 µl of $FeCl_3 \cdot 6H_2O$ solution (150 µg ml^{-1}) and 50 µl of 2,6-DCPIP solution (100 µg ml^{-1}) were added to 1.5-ml sterile microtubes.

Subsequently, 80 µl of cell suspension and 5 µl diesel sterilized by filtration through 0.45 unit membranes (MILLIPORE Corp. MA, USA) were added to the medium, and the cells were cultivated at 30^{o}C and 120 rpm for 48 h. The assay was performed in triplicate and the colour of the medium was compared visually to four controls; the first control contained no diesel, the second no cells, the third represented the positive control and contained cells of a known diesel degrader (*Pseudomonas aeruginosa* WatG strain), while the fourth one consisted of heat killed cells of WatG (Wongsa et al. 2004). The principle behind this method is that by incorporating an electron acceptor such as DCPIP (Figure 3.1), it is possible to ascertain the ability of a microorganism to utilize a hydrocarbon substrate by simply observing the colour change, in this case, from blue (oxidized) to colourless (reduced) (Bidoia et al. 2010).

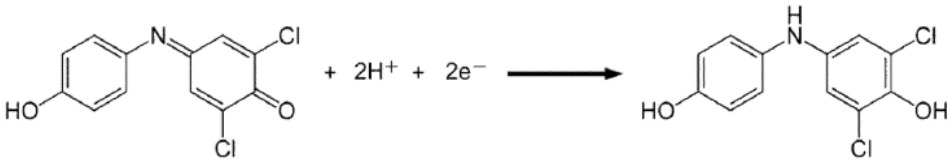


Figure 3.1 DCPIP reduction reaction (adapted from Bidoia 2010).

The reaction was followed for 48 h and examined after 6, 12, 24, 36 and 48 h. Isolates were scored as positive for microbial diesel degrading ability if the DCPIP became colourless and negative if it remained blue.

Although, the 2,6-DCPIP assay can evaluate microbial hydrocarbon-degradation abilities in an accurate, sensitive and simple manner (Hanson et al. 1993; Van Hamme et al. 2000), in order to reduce the likelihood for "false positives", two other protocols namely, the "Hole plate diffusion method" (Saadoun 2002) and a modified Most Probably Number (MPN) technique (Phillips et al. 2006) were also performed.

Briefly, for the "Hole plate" assay, twenty milliliters of mineral salts agar medium (MSM) were poured into Petri agar plates, which then were inoculated with the selected bacteria using a sterile swab. Plugs of 6 mm diameter were removed from the agar and 50µl of filter sterilized diesel were put in the holes. Sterile distilled water was used as the control. Agar plates with the bacterial isolates were incubated at 30^{o}C and the results were recorded after incubation

for 48 h by observing for the appearance of bacterial growth surrounding the holes. For the MPN assay, 20 ml of filter-sterilized diesel was added to wells containing 720 ml Bushnell Haas (BH) mineral salts medium (per litre: 0.2 g $MgSO_4 \cdot 7H_2O$, 0.02 g $CaCl_2 \cdot 2H_2O$, 1.0 g KH_2PO_4, 1.0 g $(NH_4)_2HPO_4$, 1.0 g KNO_3, 0.05 g $FeCl_3 \cdot 6H_2O$; pH 7.0). Each plate was inoculated with 10^{-8} – 10^{-2} serial dilutions (80 ml per well, one dilution per row). A final control row was inoculated with 80 ml BH medium. All plates were incubated in the dark at room temperature. After two weeks, 200 µl of filter-sterilized p-iodonitrotetrazolium violet (3 g l^{-1}) was added to each well of diesel fuel plates, plates were incubated overnight, and positive wells were counted.

3.3.4 Plant growth promotion characteristics

3.3.4.1 Microbial ACC-deaminase activity

For the detection of ACC deaminase production a protocol developed by Belimov et al., (2005) that was slightly modified was used. In detail, 5 µl of glycerol stocked bacterial isolates stored at – 70^o C were inoculated into 96 well plates containing 1 mL of liquid 869 rich medium for 72 h at 30^{o}C with no agitation and harvested by centrifugation at 3000 rpm for 20 minutes at room temperature. The supernatant was discharged and cell pellets were washed twice with 1mL of 10mM $MgSO_4$, re-suspended in 0,5 mL of $MgSO_4$ prior to adding 250 µl of each suspension to 1,2 mL of SMN medium containing 5mM ACC as a sole source of N.

Cultures were incubated for 72 h at 30^o C under a constant agitation of 150 rpm, centrifuged at 3000 rpm for 20 minutes and the supernatants discarded. The pellets were re-suspended in 100 µl of 0.1M Tris–HCl buffer (pH 8.5) and cells were disrupted by the addition of 3 µl of toluene followed by vigorous vortexing for 10 minutes. Then 10 mL of 0.5 M ACC and 100 mL of 0.1 M Tris–HCl buffer (pH 8.5) were added prior to shaking for 10 minutes. Bacterial cell suspensions were incubated for 30 minutes at 30^{o}C under constant agitation at 150 rpm.

Afterwards, 900 µl of 0.56 N HCl and 150 µl of 0.2% 2, 4-dinitrophenylhydrazine reagent were added to the cell suspensions. The mixtures were reacted for 30 minutes at 30^{o}C, supplemented with 700 µl of 2N NaOH. For evaluation purposes the well plates containing no ACC were used as negative controls while

the strains able to convert the colour from yellow to brown were scored as positive.

3.3.4.2 Organic acids production

Bacterial isolates with the ability to produce various organic acids were identified by a colour change of the alizarine red pH indicator from red (pH > 6) to yellow (pH ~ 5 or below) according to the method of (Cunningham and Kuiack 1992). In detail, 5 µl of glycerol stocked bacteria were incubated in 96 well plates containing 1 mL of 869 rich medium at 30^{o} C for 48 h with constant agitation. Then, 20 µl of cell suspension were added to 800 µl of sucrose tryptone medium (ST) which contained the following ingredients (g/L): sucrose, 20g; tryptone, 5g. The medium was supplemented with 10 mL of trace elements solution of the following composition (mg/L): $NaMoO_4$ 20 mg; H_3BO_3 20mg; $CuSO_4$ .5H_2O 20 mg; $FeCl_3$ 100mg; $MnCl_2$.4H_2O 20 mg; $ZnCl_2$ 280 mg. The 96 well plates were kept at room temperature and a change of colour from red to yellow was considered as positive.

3.3.4.3 Microbial Indole Acetic Acid (IAA) Production

A qualitative assay of indole acetic acid (IAA) production by bacterial isolates was performed as described by (Patten and Glick 2002). Briefly, bacteria were inoculated in 96 well plates containing 1/10 diluted 869 rich medium amended with 50 mg mL^{-1} of tryptophan (IAA medium) and incubated at 28^{o}C for 4 days at 150 rpm on a shaker incubator. Then, 1 mL of cell suspension was centrifuged at 3000 rpm for 30 min and 0,5 mL of the supernatant carefully removed, without touching the bacterial pellet, and added into 1 mL of Salkowski reagent (98 mL 35% perchloric acid; 2 mL of 0.5 M $FeCl_3$) and gently vortexed (600 rpm / 5 min). The plates were kept at room temperature for 20 min and bacteria producing IAA were further identified by the development of a pink colour.

3.3.4.4 Siderophore production

The qualitative evaluation for siderophore production used was based on the overlay - CAS assay as described by (Perez-Miranda et al. 2007). Bacteria were incubated in liquid 869 rich medium for 72 h. Then, 10 µl of cell suspensions were introduced into solidified agar containing 284 medium, which stimulates

siderophore production, containing 0 – 0,25 µM and 3 µM of $FeCl_{3,}$, and incubated for 5 days at 30° C. The 284 medium contained (g/L): 6.06 g Tris-HCl, 4.68 g NaCl, 1.49 g KCl, 1.07 g NH_4Cl, 0.43 g Na_2SO_4, 0.2 g $MgCl_2$.$6H_2O$, 0.03 g $CaCl_2.2H_2O$, 0.04 g $Na_2HPO_4.2H_2O$, 10 ml Fe(III) NH_4 citrate solution (containing 48 mg/100 ml) plus trace elements (1.5 mg $FeSO_4.7H_2O$, 0.3 mg H_3BO_4, 0.19 mg $CoCl_2.H_2O$, 0.1 mg $MnCl_2.4H_2O$, 0.08 mg $ZnSO_4.7H_2O$, 0.02 mg $CuSO_4.5H_2O$, 0.036 mg $Na_2MoO_4.2H_2O$, 0.024 mg $NiCl_2.6H_2O$) adjusted to pH 7.
The following carbon sources were added: lactate (0.7 g l^{-1}), glucose (0.5 g l^{-1}), gluconate (0.7 g l^{-1}), fructose (0.5 g l^{-1}) and succinate (0.8 g l^{-1}). The CAS solution was prepared and overlaid over the agar underlay in Petri dishes as described by (Schwyn and Neilands 1987). The overlay medium contained, per liter: chrome azurol S (CAS) 60.5 mg, hexadecyltrimetyl ammonium bromide (HDTMA) 72.9 mg, Piperazine -1,4-bis (2-ethanesulfonic acid) (PIPES) 30.24 g, and 1 mM$FeCl_3 \cdot 6H_2O$ in 10 mM HCl 10 mL. Agarose (0.9%, w/v) was used as a gelling agent and all glassware was cleaned with 30% HNO_3 followed by rinsing with distilled water before use (Cox 1994). After a period of 4 h, the plates were evaluated for a colour change either from blue to purple (siderophores of the catechol type) or from blue to orange/yellow (siderophores of hydroxamate type).

3.3.4.5 Acetoin production

The acetoin production of isolated bacteria was qualitatively determined with the Voges–Proskauer test, as described by (Romick and Fleming 1998). According to this test, acetoin end products generated by bacteria are oxidized, in the presence of alpha-naphthol and 40% KOH, to diacetyl, a compound which produces a red colour. Bacteria were grown for 24 h in 96 well plates containing 869 rich medium and then 20 µl of the microbial suspensions were added in wells each containing 1 mL of MR-VP medium. The medium was prepared by dissolving 17 g of methyl red-Voges Proskauer (MR- VP, Sigma) into one liter of distilled water. The solution was mixed well and sterilized by autoclaving at 121° C for 15 minutes. After an incubation period of 24 h at 37°C and 180 rpm agitation, the VP assay was performed by adding, in order, 10 µl L-arginine solution (Sigma, 10 mg ml^{-1} distilled water), 10 µl Barritt's A reagent (Fluka, alpha - naphthol 50 mg ml^{-1} in pure ethanol), and 25 µl Barritt's B reagent

(Fluka, KOH 40% w/v in distilled water) to each well. The samples were exposed to oxygen and allowed to stand for 20 minutes and the development of a pink to red colour was evaluated as positive, while no colour change, or development of a slight yellow colour, was interpreted as negative.

3.3.4.6 Butanediol dehydrogenase activity

Screening for 2,3 butanediol dehydrogenase activity (BDH) was based on a qualitative filter assay (Nicholson 2008). Briefly, cells streaked onto gridded sterile membrane filters (47–mm diameter, 0, 45 μm pore size, Whatman) were placed on Lysogeny Broth (LB) plates and incubated overnight at 30^{o}C. The filters were carefully floated, colony side up, on 1 ml of a solution of the redox dye 2,6-dichlorophenolindophenol (DCPIP; 4 mg/ml [w/v] in 0.1 M potassium phosphate buffer, pH 7.0) and incubated at room temperature until colonies were stained blue (10 to 15 min). Excess DCPIP was blotted from the filter and the filter floated on 1 ml of 2,3-butanediol (0.1 M in 0.1 M potassium phosphate buffer, pH 7.0). BDH-positive colonies turned white within 2 min, whereas BDH colonies that remained blue were marked as negative.

3.3.4.7 Phytase activity

Phytase–producing bacteria were assayed for phytase activity as previously described (Jorquera et al. 2008). Overnight cultures were streaked onto phytate screening medium (PSM; 10 g l^{-1} D-glucose, 4 g l^{-1} Na-phytate, 2 g l^{-1} $CaCl_2$, 5 g l^{-1} NH_4NO_3, 0,5 g l^{-1} KCl, 0,5 g l^{-1} $MgSO_4 \cdot H_2O$, 0,01 g l^{-1} $FeSO_4 \cdot 7H_2O$, 0,01 g l^{-1} $MnSO_4 \cdot H_2O$, 15 g l_{-1} agar, pH=7). After incubation at 28^{o}C for 4 days the appearance of clear zones around the colonies was noted as an indication of phytate mineralization.

3.3.4.8 Inorganic P solubilization

The phosphate-solubilizing abilities of the isolated strains were assayed on plates following a slightly modified protocol described by Schmid et al. (2009). Overnight cultures of the strains were washed twice with PBS buffer and then 10 μl were plated onto National Botanical Research Institute's phosphate growth medium (NBRIP) contained (per liter): glucose, 10 g; $Ca_3(PO_4)_2$, 5 g; $MgCl_2$ $6H_2O$, 5 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; KCl, 0.2 g, and $(NH_4)_2SO_4$, 0.1 g (Nautiyal,

1999). Development of a zone of clearance around colonies incubated at 28° C for up to 7 days was recorded as an indication of phosphate solubilisation. Additionally, NBRIP medium containing bromophenol blue (designated NBRI-BPB) contained (per liter): glucose, 10 g; $Ca_3(PO_4)_2$, 5 g; $MgCl_2 \cdot 6H_2O$, 5 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; KCl, 0.2 g, $(NH_4)_2SO_4$, 0.1 g, and bromophenol blue (BPB), 0.025 g was inoculated and incubated for 48 h at 28°C. Autoclaved, non inoculated medium served as control. Colour change from purple to yellow indicated phosphate solubilisation. In both experiments the pH of the media was adjusted to 7.0 before autoclaving.

3.3.5 Other traits

Selected strains found positive both as diesel degraders and potential plant growth promoters were further screened for a number for other desirable traits such as: antibiotic sensitivity, production of pectinases, solvent and heavy metal tolerance, motility (swimming, swarming, twitching), biofilm formation in presence of diesel fuel, chemotaxis towards diesel and hexadecane, quorum sensing signalling, and production of high and low molecular weight biosurfactants when diesel fuel and hexadecane were used as growth substrates.

3.3.5.1 Antibiotic sensitivity.

The antibiotic sensitivity of selected strains against antibiotics was assayed with the Kirby–Bauer method using Mueller-Hinton agar plates (Beef Extract 2 g L^{-1}, Casein 17,5 g L^{-1}, Starch 1,5 g L^{-1}, Agar 17 g L^{-1}; final pH 7.3 ± 0.1, incubation at overnight at 37°C) as described by Sriram et al. (2011). The following antibiotics were tested: Ampicillin (Ap-10 µg/disk), Amicasin (Am-30 µg/disk), Bacitracin (Bc-10 µg/disk), Ciprofloxacin (Cf-5 µg/disk), Chloroamphenicol (Cm-30 µg/disk), Erythromycin (Er-15 µg/disk), Gentamycin (Gm-10 µg/disk), Tetracyclin (Tc-30 µg/disk), Kanamycin (Km-25 µg/disk), Neomycin (Neo-30 µg/disk), Rifampicin (Rf-5 µg/disk) and Streptomycin (Sm-10 µg/disk). Amikacin, Bacitracin, Gentamycin and Neomycin were used as disk formula, while the other ones were prepared in the laboratory. Table 3.5 illustrates antibiotic sensitivity as shown by zone of inhibition around the disc impregnated with the corresponding antibiotic. Strains were categorized into three groups

(resistant-R, intermediate resistant-I, susceptible-S) based to the diameter of the inhibition zone taking into account CLSI standards (CLSI, 2007).

3.3.5.2 Heavy Metal and Solvent tolerance

Tolerance of strains to various heavy metals [$CdCl_2 \cdot H_2O$, $Pb(NO_3)_2$, $CuSO_4 \cdot 5H_2O$, $ZnSO_4 \cdot 7H_2O$, $NiSO_4 \cdot 6H_2O$] was tested on casamino-acid agar plates (Mathe et al. 2012). Briefly, casamino-acid agar plates (casamino-acid 5 g L^{-1}; agar 17 g L^{-1}) containing different concentrations of heavy metal salts were spot inoculated with bacterial cell suspensions (5µl, OD_{600} = 1) and incubated at 30°C for 2 days. In addition, the behaviour and bacterial tolerance to organic solvents including hydrocarbons like n-hexane, octane, dodecane and a mixture of n-hexane / cyclohexane, was evaluated according to the method described by (Oh et al. 2012).

3.3.5.3 Motility Test

Swimming and swarming were evaluated on 0.2 % and 0.5 % semi-solid agar plates (Kang Y.S. et al. 2011). Cells were grown overnight in Nutrient Broth (NB) medium, and subsequently 2µl of each suspension (OD_{660} =0.2), was spotted on the NB agar medium (approx. 20ml medium). The diameter (mm) of the bacterial zone was measured after 24 and 72 h of inoculation, respectively. Twitching motility was assessed on LB plates containing 1% agar.
Bacterial cultures were stabbed to the bottom of the plates, and after 72 h twitch zones were measured (Poritsanos et al. 2006). For the 3 motility assays, 5 replicates were analyzed.

3.3.5.4 Pectinolytic and cellulolytic activity

Screening of pectinase activity was performed as described earlier (Jha and Kumar 2007), while determination of the cellulolytic activity of bacterial isolates was performed as per the method of (Soares et al. 2012).

3.3.5.5 Catalase and Oxidase Activity

Both oxidase and catalase activity of the strains were evaluated based on the protocol described by (Kang et al. 2011a). For oxidase, 50 µL of hydrogen peroxide 3% (v/v) solution was applied to a fresh culture smeared on a glass

slide. Production of bubbles in the presence of hydrogen peroxide is an indication of catalase activity, while oxidase activity was investigated by adding 10 µL of 1% (w/v) N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride onto a fresh culture on a glass slide The reaction was considered positive if the colour changed to violet or purple within 20 sec.

3.3.5.6 Bioassays for detection of quorum sensing (QS) molecules

Selected bacterial strains were grown under constant agitation (100 rpm) in Lysogeny Broth (LB) (tryptone 10 g L^{-1}, yeast extract 5 g L^{-1}, NaCl 10 g L^{-1}, pH= 7) for 48 h at 30°C until stationary phase. Culture broths were centrifuged in 50 mL tubes at 4000 rpm for 55 min, and supernatants were extracted twice with dichloromethane 1:2 (v/v). Subsequently, dichloromethane extracts were dried under vacuum at T < 40°C to avoid the alteration of metabolites. After that the following bioassays were performed:

I. **Lux screen assay:** The presence of putative AHL signal molecules was initially detected using the *Escherichia coli* lux-based bioreporter pSB536, pSB401 and pSB1075, thus covering a wide range of AHL signal molecules (Swift et al. 1997; Winson et al. 1998). *E. coli* pSB536, grown in LB 0,5% NaCl supplemented with ampicillin 50 µg ml^{-1}, was used to detect short chain AHLs (C4-HSLs); *E. coli* pSB401, grown in LB 0,5% NaCl supplemented with tetracycline 10 µg ml^{-1}, was used to detect mid chain AHLs (C6-C8); *E. coli* pSB1075, grown in LB 0,5% NaCl supplemented with tetracyclin 10 µg ml^{-1}, was used to detect long chain AHLs (C10-C14). 200 mL of all culture broths were extracted as previously described. Extracts were solubilised in 350 µL acetonitrile, and aliquots of 50 µL were then applied to white 96-well microtitre plates. Plates were left to dry in a fume hood for 1 hour, then 200 µL of overnight cultures of bioreporters diluted 1/10 were added to each well. Specific homoserine lactones (sol. 20 µg ml^{-1}) were added to selected wells as control: 2 µL C4-HSL for pSB536, 4 µL C6-HSL for pSB401 and 2 µL OC12-HSL for pSB1075. The microplates were incubated at 37°C and luminescence was measured using an image viewer after 3 h incubation for pSB536, and 6 h for pSB401 and pSB1075.

II. **Thin Layer Chromatography (TLC) overlay:** Dichloromethane extracts were tested for the detection of QS molecules by using a TLC-overlay test

with the *Agrobacterium tumefaciens* NTL4 bioreporter, a bioreporter which detects a broad range of AHLs with high sensitivity (Steindler and Venturi 2007). Aliquots of supernatant extracts were also applied to C-18 RP-TLC plates (20 cm x 20 cm; MERCK) and a mobile phase of 60% (v/v) aqueous methanol was used to separate the extracts. The TLC plates were overlaid with 100 mL of ATGN (minimal salts supplemented with glucose) (Tempe et al. 1977), soft agar (0.6% w/v) supplemented with 0.5% glucose, 40 µg ml^{-1} X-Gal (Bromo-chloroindolyl beta-D-galactopyranoside), antibiotics (streptomycin 50 µg ml^{-1}; tetracycline 5 µg ml^{-1}) and the biosensor *Agrobacterium tumefaciens* NTL4 (pCF218; pCF372) (Fuqua and Winans 1996). The NTL4 AHL bioreporter expresses a *lacZ* fusion in response to medium-chain length AHLs. The TLC plates were kept in a sterile container and incubated at 30° C for 24-48 h. The appearance of light blue spots indicated activation of quorum-sensing mechanisms.

III. Plate T-streak: All strains were tested using the T-streak assay with CV026 as biosensor (Steindler and Venturi 2007).

3.3.5.7 Biofilm formation

Selected strains positive for diesel degradation based on the 2,6 DCPIP assay were analyzed for their ability to form biofilms in polystyrene and polypropylene plates according to the protocol described by (Tribelli et al. 2012a), slightly modified by using diesel and hexadecane as carbon sources. In detail, overnight cultures grown in LB medium (Lysogeny Broth) were used to inoculate (3 µl - OD_{600}= 0.3) 282 µl of W minimal medium supplemented with filter sterilized diesel and hexadecane at 5%. Then, 300 µl of each culture were added to 96 microplate wells and developed for 7 days at 30^{o}C without agitation in a wet chamber to prevent evaporation. After one week of incubation, planktonic cells were removed gently from the wells and transferred into an Eppendorf tube, and the OD_{595nm} was measured (absorbance of planktonic cells: APL). The remaining planktonic cells into the well were solubilised by adding 100 µl of $MgSO_4$. After 20 minutes, the solubilised cells were discharged and attached cells were dyed with 25µl of 1% crystal violet aqueous solution; plates were incubated for 20 minutes to stain the biofilm-forming cells, after which the unbound crystal violet solution was removed and plates were gently rinsed 3 times with tap water.

Subsequently, the dye was extracted with 200 µl 96% ethanol for 20 min and transferred to flat bottom microtiter plates in order to measure the absorbance at 550 nm (absorbance of crystal violet: ACV). All measurements were been performed in triplicate in a FLUOstar Omega microplate reader. The adherence index was defined as follows:

$$\text{Adherence Index} = \frac{\text{ACV (Absorbance of crystal violet at 550nm)}}{\text{APL (absorbance of planktonic cell at 595nm)}}$$

3.3.5.8 Chemotaxis

The chemotaxis response of strains towards diesel oil and hexadecane was evaluated with a swarm plate assay as previously described (Lanfranconi et al. 2003). Two microliters of exponentially grown cultures were spot inoculated onto the centre of soft agar swarm plates containing H1 medium (composition, in g l^{-1}: K_2HPO_4, 11.2, KH_2PO_4, 4.8, $(NH_4)_2SO_4$, 2, $MgSO_4.7H_2O$, 0.246, $FeSO_4.7H_2O$, 5.10–4, pH=7) plus 0,01% Triton X-100, 0,2% of the corresponding hydrocarbons and 0,3% agar. After incubation at 30° C for 48 h the plates were inspected for the development of swarm rings.

3.3.5.9 Production of Biosurfactants

A preliminary screen was done in order to determine whether the isolated strains produced biosurfactants in the presence of diesel oil. The evaulation was based on a series of well established methods; namely a Surface Tension measurement (ST), Emulsification Assay (EA), drop collapsing test, oil displacement test, and lipase production assay. ST and EA measurements were carried out as previously described (Franzetti et al. 2008). Briefly, samples of whole culture broths (with cells) or culture filtrates (without cells) were used; ST analyses were performed with the Du Nouy ring method using a K-8 tensiometer (Kruss, Hamburg, Germany). For EA determination a 3 mL sample was vortexed while the same amount of kerosene was added. After this, the tube was vortexed for an additional 2 min and the mixture was allowed to settle for 24 h. The emulsification activity (E_{24}%), given as the percentage of middle emulsion phase normalized to the total volume, was calcuated. All determinations were performed in triplicate. The other methods were carried out as previously described (Sriram et al., 2011). A biosurfactant production medium of 100 ml in a 500-ml Erlenmeyer flask was inoculated with 3% freshly prepared culture and

incubated at 30° C for 5 days under constant agitation at 160rpm. The production media contained (g L^{-1}): $(NH_4)_2SO_4$, 10; NaCl, 1.1; KCl, 1.1; $FeSO_4.7H_2O$, 2.8 x 10-4; $K_2HPO_4.3H_2O$, 4.4; KH_2PO_4, 3.4; $MgSO_4$, 0.5; Yeast Extract, 0.5; trace elements solution, 0.5 ml L^{-1} and diesel oil, 2% (v/v) as the sole carbon source. The trace elements solution composed (g/L): $CaCl_2$, 0.24; $ZnSO_4$, 0.29; $MnSO_4$, 0.17 and $CuSO_4$, 0.25. After the production media were autoclaved, a trace elements solution and diesel oil were added by filtering through a 0.22 μm pore membrane (Millipore, USA) and a 25mm syringe filter with a 0.45μm membrane PTFE filter (VWR),respectively. For the drop collapse test, 2μl of mineral oil was added to each well of a 96-well microtitre plate and allowed to equilibrate for 1 h at 37° C. Then 5μl of culture supernatant was placed at the centre of the wells over the oil layer and the shape of the oil drop was examined after 1 min. The formation of flattened drops was considered as positive for biosurfactant production, while drops that remained intact in round form were considered to be negative for biosurfactants production. For the oil displacement test, 15μl of weathered crude oil was added to Petri plates filled with 40ml of distilled water. Afterwards, 10μl of cell free culture supernatant was carefully placed on the centre of the oil film. After 30 s of incubation at room temperature the diameter of a clear halo zone was measured. Finally, for the lipase assay, agar plates containing 2% Tween 80, 2.5% agar and 0.5% methyl red were prepared and wells were cut using a sterile puncher. Then, 20μl of cell free culture supernatant was added to each well and incubated overnight at 28° C. Appearance of a clearance zone around the well was scored as a positive.

3.4 Biodegradation kinetic experiments

In order to investigate the physiology of diesel utilization and the degradation rate, 3 strains based were selected based on the previous assays and grown in LB (Lysogeny Broth) (Sambrook and Russel 2001) for 24 h at 30°C under constant agitation at 150rpm. The cells were collected, washed twice with 0.9% saline buffer and re suspended in BH2 mineral medium (K_2HPO_4 1.32 g L^{-1}, KH_2PO_4 1 g L^{-1}, NH_4Cl 0.81 g L^{-1}, $NaNO_3$ 0.84 g L^{-1}, $FeSO_4{\cdot}7H_2O$ 0.01 g L-1, $MgSO_4{\cdot}7H_2O$ 0.42 g L^{-1}). For each culture, 20 mL (OD_{600nm}=1) were inoculated into 100-mL flasks and then filtered sterilized diesel was supplied as carbon and energy source at an initial concentration of 1.0 g L^{-1} (w/v) and incubated at

30°C under shaking at 180 rpm. At each fixed time, two flasks were sacrificed and used for OD600nm measurements to monitor biomass production, and residual hydrocarbon determinations were made. Abiotic controls and controls with heat killed cells controls were included. Cells were heat killed by autoclaving at 121°C for 30 minutes and 30 ppm of $HgCl_2$ was added. Hydrocarbons extractions were carried out with dichloromethane (1:1 v/v) using o-tertphenyl (40 mg/L) as an internal standard. Analyses were carried out by GC/MS (US EPA 2007 <http://www.epa.gov/sw-846/pdfs/ 8270d.pdf>).

3.5 Statistical analysis

Our data consist of eight groups, each of them consisting of a number of variables, varying from one to ten. Our main question of interest is whether these groups are correlated with each other. The method that is widely known for cases where two sets of variables are examined for possible correlation is the Canonical Correlation Analysis (CCA). CCA is a method used for making inference in cases with cross-covariance matrices. Suppose that we have two vectors X = (X_1, ..., X_n) and Y = (Y_1, ..., Y_m) of random variables, and there are correlations among the variables. Canonical-correlation analysis can be used to find linear combinations of the Xi and Yj which have maximum correlation with each other. The way this method examines for possible correlation between the sets of variables is by creating linear composites of the respective variable sets (X, Y), i.e. creating some single variable that represents the Xs and another single variable that represents the Ys. More specifically: Given a linear combination of X variables: $F=f_1X_1+f_2X_2+ \ldots +f_nX_n$, and a linear combination of Y variables: $G=g_1Y_1+g_2Y_2+ \ldots +g_mY_m$. The correlation we are interested in here is between the linear combinations (variates F and G) created for both sets of variables and is called canonical correlation. The number of canonical correlations between two variates is equal to the number of variables in the smaller set. Usually not all of the canonical correlations will be statistically significant.

The set of the null (H_0) and the alternative (H_1) hypothesis are framed in the following way:

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H_0: There is no (canonical) correlation between the two sets of variables
H_1: There is (canonical) correlation between the two sets of variables

The conclusion to this hypothesis set is drawn by the yielded p-value. If this is less than 5%, then we conclude that the null hypothesis must be rejected. On the other hand, if this value is greater than that, then we do not have enough information to reject the null hypothesis.

3.6 Results

In total 344 bacterial strains were isolated and genotypic analysis showed the occurrence of 22 different genera. Among these, based on the 2,6 DCPIP assay (3.2.3), 30 strains belonging to the following genera: *Arthrobacter*, *Acinetobacter*, *Pseudomonas*, *Brevibacterium*, *Staphylococcus* and *Microbacterium*, were positive diesel oil degradation *in vitro*. These strains were also found to be phenotypically positive for a wide range of plant growth-promoting tests (Table 3.1).

In Table 3.2 the upper part of the second column shows the raw canonical coefficients for the variables in the Plant Growth Promoting Activity (PGPA) group, while the lower part of shows the respective canonical coefficients for variables in the Solvent Tolerance (ST) group. The last line of the table indicates the canonical correlation between the two groups of variables along with its p-value. The p-value is equal to 0.017 (<0.05), thus the canonical correlation is significant and the null hypothesis (H_0) is rejected. In order to have a graphical representation of the results of the CCA, we may look at Figure 3.2. At the left hand side of the graph the variables consisting of the PGPA group are presented, while the variables in the ST group are shown on the right side. The linear combination of the variables in each group (noted with P and V, respectively, in the figure) is structured in such a way that the correlation coefficient between them is maximized. The coefficient of the first canonical correlation coefficient is 0.81, while the correlation coefficients between the original variables and their canonical pairs are also indicated. With respect to the variability in the PGPA group explained by their canonical pairs, *i.e.* the canonical variate (P), this is

12,0%. The variables in the ST group are concerned, and 27.4% of their total variability is explained by their canonical pairs.

Table 3.2 Plant growth promoting activity of 2,6 DCPIP positive strains.

	Plant Growth Promoting Activity							
	ACC Deaminase	IAA	Acetoin	Org. Acids	Inorganic P	Phytase	SID	Pectinase
Arthrobacter sp.	+	+	-	-	-	+	+	+
Acinetobacter calcoaceticus	+	-	-	-	+	+	+	+
Acinetobacter calcoaceticus	+	-	-	-	+	+	+	+
Acinetobacter calcoaceticus	+	-	-	-	+	+	+	+
Staphylococcus aureus	+	+	+	-	+	+	+	+
Acinetobacter oleivorans	+	+	-	+	+	+	+	+
Acinetobacter venetianus	+	-	-	-	+	+	+	+
Acinetobacter rhizosphaerae	+	-	-	-	+	+	+	+
Acinetobacter calcoaceticus	+	-	-	-	+	+	+	+
Pseudomonas sp.	+	-	-	+	+	+	+	+
Pseudomonas putida	+	-	-	-	-	+	+	+
Pseudomonas putida	+	-	-	-	+	+	+	+
Pseudomonas reinekei	+	-	-	-	-	+	+	+
Pseudomonas fluorescens	+	-	-	-	-	+	+	+
Staphylococcus epidermis	+	+	-	+	+	-	-	-

Table 3.1 Plant growth promoting activity of 2,6 DCPIP positive strains (continued).

	Plant Growth Promoting Activity							
	ACC Deaminase	IAA	Acetoin	Org. Acids	Inorganic P	Phytase	SID	Pectinase
Pseudomonas sp.	+	+	-	-	-	+	+	+
Pseudomonas koreensis	+	+	-	-	+	+	+	+
Staphylococcus aureus	+	+	-	-	+	+	+	+
Staphylococcus aureus	-	+	-	-	-	+	+	+
Staphylococcus aureus	+	-	-	-	+	+	+	+
Acinetobacter calcoaceticus	+	-	-	-	+	-	+	-
Pseudomonas brassicacearum	+	-	+	-	-	+	+	+
Staphylococcus aureus	+	-	-	+	-	+	+	+
Pseudomonas sp.	+	-	-	-	-	+	+	+
Acinetoabacter calcoaceticus	+	-	-	-	+	+	+	-
Acinetobacter sp.	+	-	-	-	+	+	+	-
Acinetobacter sp.	+	-	-	-	-	+	+	-
Brevibacterium sp.	+	-	-	-	-	-	+	-
Microbacterium hydrocarbonoxydans	+	+	-	+	+	-	-	-
Microbacterium hydrocarbonoxydans	+	+	-	-	+	-	-	-

In Table 3.3 the upper part of the second column shows the raw canonical coefficients for the variables in the Plant Growth Promoting Activity (PGPA) group, while the lower part of shows the respective canonical coefficients for variables in the Biofilm (BF) group. The last line indicates the canonical correlation between the two groups of variables along with its p-value. The p-value is equal to 0.05, thus the canonical correlation is significant and the null hypothesis (H_0) is rejected.

In order to have a graphical representation of the results of the CCA, we may look at Figure 3.3. At the left hand side of the graph the variables consisting of the PGPA group are presented, while the variables in the BF group are shown on the right hand side. The coefficient of the first canonical correlation coefficient is 0.84, while the correlation coefficients between the original variables and their canonical pairs are also indicated.

With respect to the variables in the PGPA group, the variability explained by their canonical pairs, *i.e.* the canonical variate (P), is 5,23%. As far as the variables in the BF group are concerned, 33,6% of their total variability is explained by their canonical pairs.

In Table 3.4 the upper part of the second column shows the raw canonical coefficients for the variables in the Biosurfactants (BS) group, while the lower part of shows the respective canonical coefficients for variables in the Quorum Sensing (QS) group. The last line of the table indicates the canonical correlation between the two groups of variables along with its p-value. The p-value is equal to 0.04 (<0.05), thus the canonical correlation is significant and the null hypothesis (H_0) is rejected. In order to have a graphical representation of the results of the CCA, we may look at Figure 3.4.

At the left hand side of the graph the variables consisting of the BS group are presented, while the variables in the QS group are shown on the right. The coefficient of the first canonical correlation coefficient is 0.78, while the correlation coefficients between the original variables and their canonical pairs are also indicated. With respect to the variables in the BS group, the variability explained by their canonical pairs, *i.e.* the canonical variate (P), is 9,53%. As far as the variables in the QS group are concerned, 100% of their total variability is explained by their canonical pairs.

In Table 3.5 the upper part of the second column shows the raw canonical coefficients for the variables in the Biosurfactant (BS) group, while the lower part of shows the respective canonical coefficients for variables in the Solvent Tolerance (ST) group. The last line of the table indicates the canonical correlation between the two groups of variables along with its p-value. The p-value is equal to 0.03 (<0.05), thus the canonical correlation is significant and the null hypothesis (H_0) is rejected. In order to have a graphical representation of the results of the CCA, we may look at Figure 3.5. At the left hand side of the graph the variables consisting of the BS group at presented, while the variables in the ST group are shown at the right hand side of it. The coefficient of the first canonical correlation coefficient is 0.86, while the correlation coefficients between the original variables and their canonical pairs are also indicated. With respect to the variability of the variables in the BS group explained by their canonical pairs, *i.e.* the canonical variate (P), is 8,5 %. As far as the variables in the ST group are concerned, 34.6% of their total variability is explained by their canonical pairs.

In Table 3.6 the upper part of the second column shows the raw canonical coefficients for the variables in the Solvent Tolerance (St) group, while the lower part of shows the respective canonical coefficients for variables in the Biofilm (BF) group. The last line of the table indicates the canonical correlation between the two groups of variables along with its p-value. The p-value is equal to 0.03 (<0.05), thus the canonical correlation is significant and the null hypothesis (H_0) is rejected. In order to have a graphical representation of the results of the CCA, we may look at Figure 3.6. At the left hand side of the graph the variables consisting of the ST group at presented, while the variables in the BF group are shown at the right hand side of it. The coefficient of the first canonical correlation coefficient is 0.86, while the correlation coefficients between the original variables and their canonical pairs are also indicated. With respect to the variability of the variables in the ST group explained by their canonical pairs, *i.e.* the canonical variate (P), is 38,1 %. As far as the variables in the BF group are concerned, 66,5% of their total variability is explained by their canonical pairs.

In Table 3.7 the upper part of the second column shows the raw canonical coefficients for the variables in the Solvent Tolerance (St) group, while the lower part of shows the respective canonical coefficients for variables in the Heavy

Metals (HM) group. The last line of the table indicates the canonical correlation between the two groups of variables along with its p-value. The p-value is equal to 0.01 (<0.05), thus the canonical correlation is significant and the null hypothesis (H_0) is rejected. In order to have a graphical representation of the results of the CCA, we may look at Figure 3.7. At the left hand side of the graph the variables consisting of the ST group at presented, while the variables in the HM group are shown at the right hand side of it. The coefficient of the first canonical correlation coefficient is 0.62, while the correlation coefficients between the original variables and their canonical pairs are also indicated. With respect to the variability of the variables in the ST group explained by their canonical pairs, *i.e.* the canonical variate (P), is 19,8 %. As far as the variables in the HM group are concerned, 9,8% of their total variability is explained by their canonical pairs.

In Table 3.8 the upper part of the second column shows the raw canonical coefficients for the variables in the Biosurfactants (BS) group, while the lower part of shows the respective canonical coefficients for variables in the Biofilms (BF) group. The last line of the table indicates the canonical correlation between the two groups of variables along with its p-value. The p-value is <0.01, thus the canonical correlation is significant and the null hypothesis (H_0) is rejected.

In order to have a graphical representation of the results of the CCA, we may look at Figure 3.8. At the left hand side of the graph the variables consisting of the BS group at presented, while the variables in the BF group are shown at the right hand side of it. The coefficient of the first canonical correlation coefficient is 0.88, while the correlation coefficients between the original variables and their canonical pairs are also indicated. With respect to the variability of the variables in the BS group explained by their canonical pairs, i.e. the canonical variate (P), is 8,2 %. As far as the variables in the BF group are concerned, 20,5% of their total variability is explained by their canonical pairs.

In Table 3.9 the upper part of the second column shows the raw canonical coefficients for the variables in the Chemotaxis (CH) group, while the lower part of shows the respective canonical coefficients for variables in the Heavy metals (HM) group. The last line of the table indicates the canonical correlation between the two groups of variables along with its p-value. The p-value is 0,04 (<0.05), thus the canonical correlation is significant and the null hypothesis (H_0) is

rejected. In order to have a graphical representation of the results of the CCA, we may look at Figure 3.9. At the left hand side of the graph the variables consisting of the CH group at presented, while the variables in the HM group are shown at the right hand side of it. The coefficient of the first canonical correlation coefficient is 0.59, while the correlation coefficients between the original variables and their canonical pairs are also indicated. With respect to the variability of the variables in the CH group explained by their canonical pairs, i.e. the canonical variate (P), is 51,9%. As far as the variables in the HM group are concerned, 19,7% of their total variability is explained by their canonical pairs. It is worthwhile to mention that the more the variables in a set, the less the variability then is explained by the canonical pairs. For example, in the pair of Chemotaxis (CT) and Heavy Metals (HM) we see that CT has two variables and almost 52% of their variability is explained, whereas HM has four variables and 18% of the respective variability can be addressed to their canonical pairs. This is because the more the variables in a set, the more information – and thus the variability - that exists and hence the more difficult it is to capture this variability. Conversely, when we have only a few variables, it is easier to explain most of the variability.

Table 3.2 CCA between variables of groups Plant growht promoting activity (PGPA) & Solvent Tolerance (ST).

Group	Variables	Raw canonical coefficients
Plant Growth Promoting Activity	ACC Deaminase	0.013
	IAA	-0.66
	Acetoin	-0.24
	Inorganic Acids	1.02
	Organic P	1.17
	Phytase	-0.04
	SID	3.14
	Pectinase	-1.15
Solvent Tolerance	Sol. Hexane	-1.11
	Sol. Octane	0.49
	Sol. Dodecane	-0.27
	Sol. nHx	0.87
	Canonical correlation (p-value)	*0.81 (0.017)*

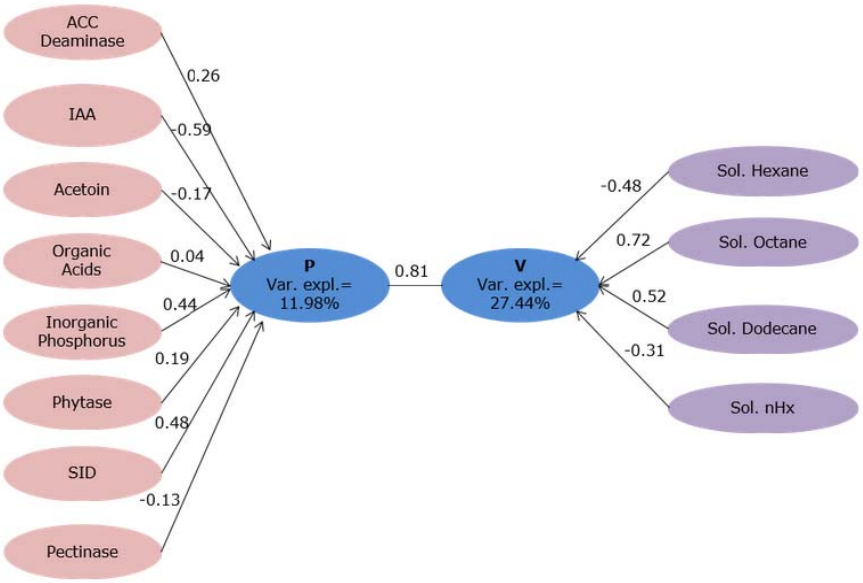

Figure 3.2 Schematic represantation of CCA between variables of groups of Plant growth promoting activity (PGPA) and Solvent Tolerance (ST).

Table 3.3 CCA between variables of groups PGPA & BF.

Group	Variables	Raw canonical coefficients
Plant Growth Promoting Activity	ACC Deaminase	-0.14
	IAA	-0.04
	Acetoin	0.05
	Inorganic Acids	0.60
	Organic P	0.63
	Phytase	-1.85
	SID	4.79
	Pectinase	-1.13
Biofilm Tests	Adherence Index polypropelene Hexane	-0.02
	Adherence Index polystyrene Hexane	0.03
	Adherence Index polypropelene Diesel	-0.23
	Adherence Index polystyrene Diesel	0.04
	Canonical correlation (p-value)	*0.84 (0.05)*

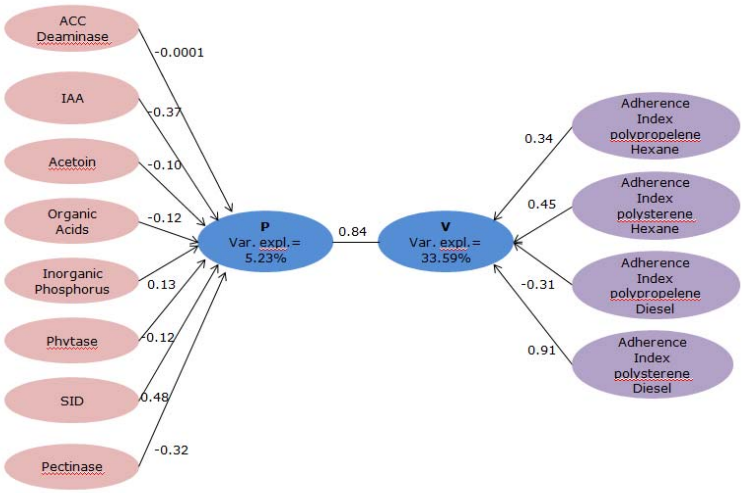

Figure 3.3 Schematic representation of CCA between variables of groups of PGPA and BF.

Table 3.4 CCA between variables of groups BS & Quorum sensing (TLC overlay).

Group	Variable	Raw canonical coefficients
Biosurfactants	Drop collapse SN	0.83
	Drop collapse Pellet	0.26
	Oil displacement SN	-0.19
	Oil displacement Pellet	-0.05
	Emulsification Hexane SN	-1.46
	Emulsification Diesel SN	4.16
	Emulsification Hexadane SN	.
	Emulsification Diesel Pellet	-0.03
	Lipase SN	1.48
	Lipase Pellet	.
Quorum Sensing	TLC Overlay	-2.08
	Canonical correlation (p-value)	*0.78 (0.04)*

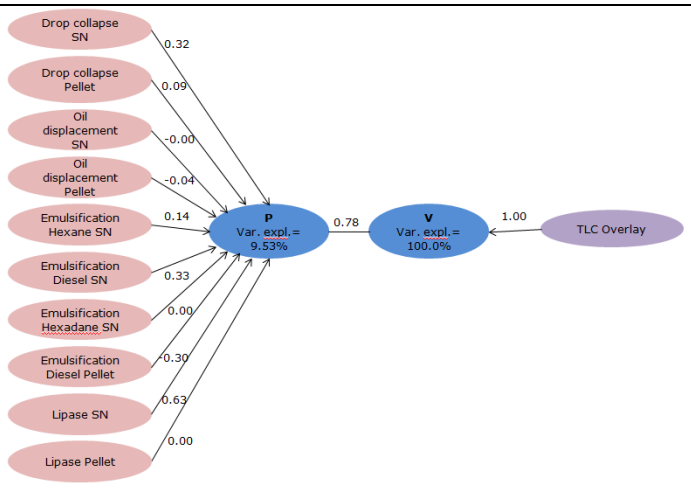

Figure 3.4 Schematic representation of CCA between variables of groups of BS and QS.

Table 3.5 CCA between variables of groups Biosurfactants (BS) & Solvent Tolerance (ST).

Group	Variable	Raw canonical coefficients
Biosurfactants	Drop collapse SN	0.51
	Drop collapse Pellet	0.12
	Oil displacement SN	-0.63
	Oil displacement Pellet	-0.40
	Emulsification Hexane SN	-2.27
	Emulsification Diesel SN	2.89
	Emulsification Hexadane SN	.
	Emulsification Diesel Pellet	2.12
	Lipase SN	1.41
	Lipase Pellet	.
Solvent Tolerance	Sol. Hexane	-0.06
	Sol. Octane	0.04
	Sol. Dodecane	0.57
	Sol. nHx	-0.13
	Canonical correlation (p-value)	*0.86 (0.03)*

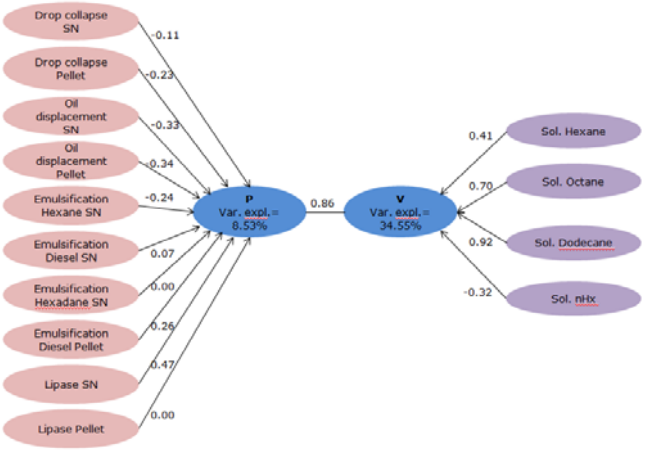

Figure 3.5 Schematic representation of CCA between variables of groups of BS and ST.

Table 3.6 CCA between variables of groups Solvent Tolerance & Biofilm Tests.

Group	Variable	Raw canonical coefficients
Solvent Tolerance	Sol. Hexane	-0.35
	Sol. Octane	0.46
	Sol. Dodecane	-0.07
	Sol. nHx	0.09
Biofilm Tests	Adherence Index polypropelene Hexane	0.02
	Adherence Index polystyrene Hexane	-0.01
	Adherence Index polypropelene Diesel	-0.11
	Adherence Index polystyrene Diesel	0.02
	Canonical correlation (p-value)	*0.86 (0.03)*

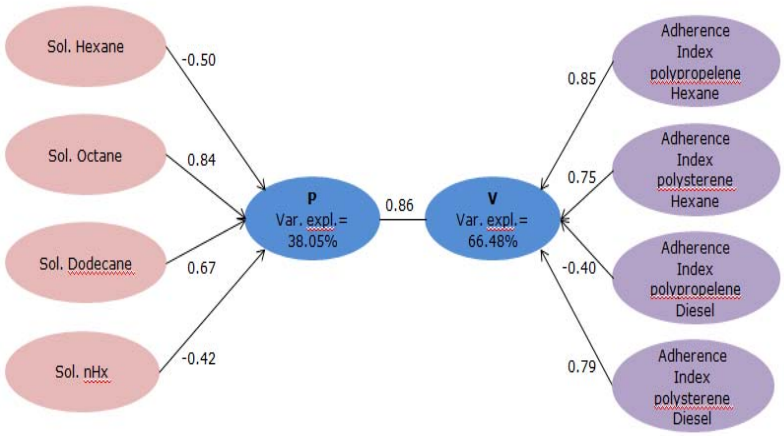

Figure 3.6 Schematic representation of CCA between variables of groups of ST and BF.

Table 3.7 CCA between variables of groups Solvent Tolerance (ST) & Heavy Metals (HM).

Group	Variable	Raw canonical coefficients
Solvent Tolerance	Sol. Hexane	1.47
	Sol. Octane	0.01
	Sol. Dodecane	0.59
	Sol. nHx	-1.33
Heavy Metals	Pb	-5.73
	Cu	2.72
	Zn	0.84
	Ni	-0.78
	Canonical correlation (p-value)	*0.62 (0.016)*

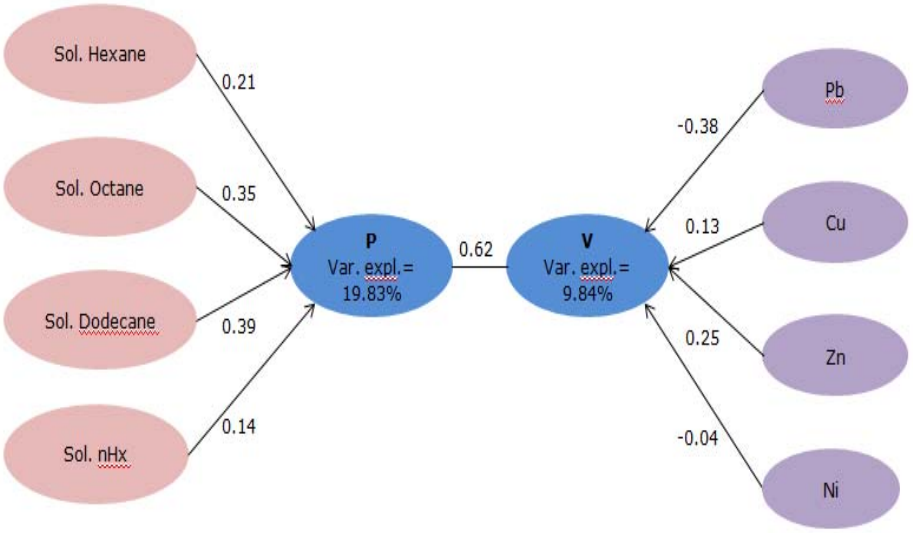

Figure 3.7 Schematic representation of CCA between variables of groups of Solvent Tolerance (ST) and Heavy Metals (HM).

Table 3.8 CCA between variables of groups Biosurfactants (BS) & Biofilm Tests (BF).

Group	Variable	Raw canonical coefficients
Biosurfactants	Drop collapse SN	-0.08
	Drop collapse Pellet	-0.72
	Oil displacement SN	0.03
	Oil displacement Pellet	0.12
	Emulsification Hexane SN	1.19
	Emulsification Diesel SN	-1.01
	Emulsification Hexadane SN	.
	Emulsification Diesel Pellet	2.07
	Lipase SN	-0.48
	Lipase Pellet	.
Biofilm Tests	Adherence Index polypropelene Hexane	0.02
	Adherence Index polystyrene Hexane	-0.07
	Adherence Index polypropelene Diesel	0.14
	Adherence Index polystyrene Diesel	0.04
	Canonical correlation (p-value)	*0.88 (<0.01)*

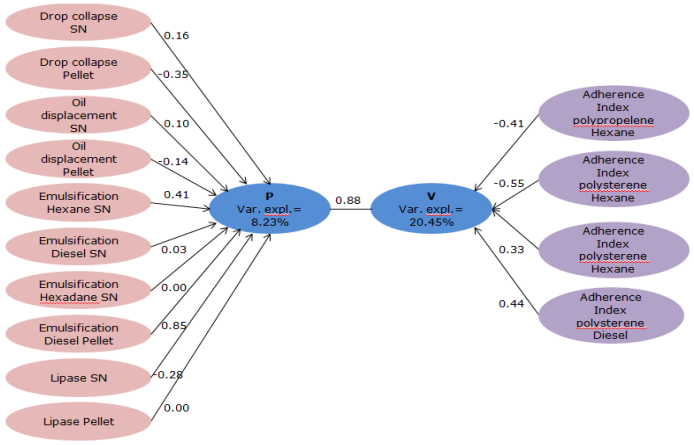

Figure 3.8 Schematic representation of CCA between variables of groups of BS and BF Tests.

Table 3.9 CCA between variables of groups Chemotaxis (CH) and Heavy metals (HM).

Group	Variable	Raw canonical coefficients
Chemotaxis	Diesel	1.85
	Hexadane	-1.79
Heavy Metals	Pb	-3.08
	Cu	-1.71
	Zn	1.11
	Ni	1.52
	Canonical correlation (p-value)	*0.59 (0.042)*

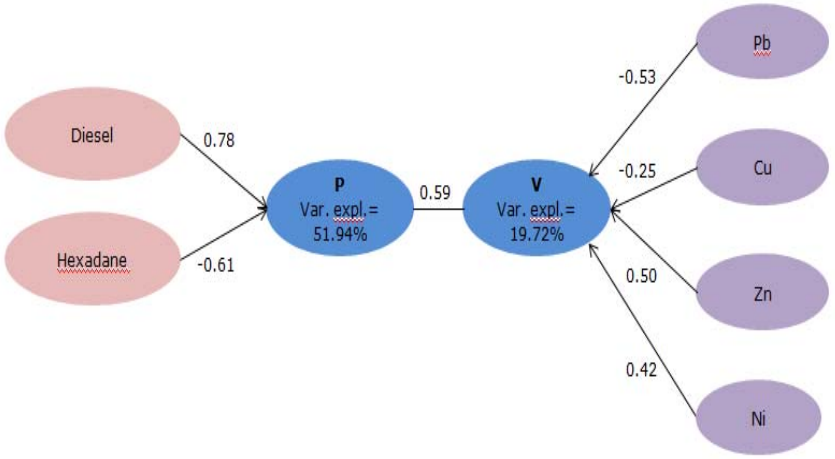

Figure 3.9 Schematic representation of CCA between variables of groups of Chemotaxis (CH) and Heavy metals (HM) Tests.

Section 3

Using the results of the 2,6 DCPIP assay (Figure 3.10) as a criterion, two root endophytes namely, *Acinetobacter oleivorans* and *Acinetobacter calcoaceticus* along with a rhizosphere bacterium identified as *Staphylococcus aureus*, were further selected for a more in depth examination of their diesel degradation abilities.

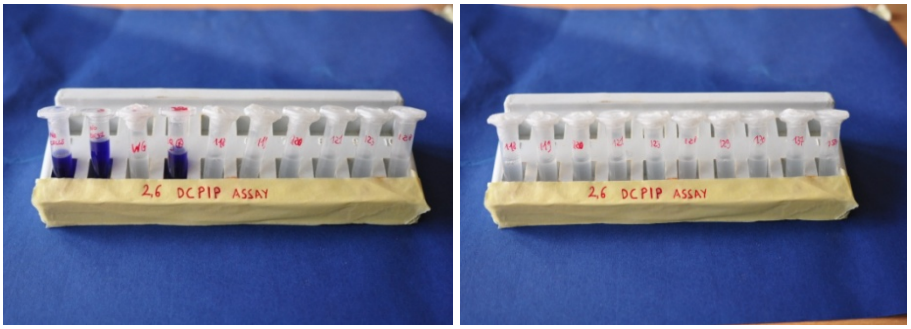

Figure 3.10 2,6 DCPIP decolourization process from blue (oxidized) to colourless (reduced), was evaluated as positive for microbial diesel-degrading ability.

During the biodegradation experiments, hydrocarbon concentration and OD_{600nm} were measured over time. Figure 3.11 illustrates that, among the selected strains, *Acinetobacter calcoaceticus* showed the best degradation properties and degraded about 41% of the initial hydrocarbon (1 g L^{-1}) after 10 days of incubation and the OD_{600nm} increased to 0.44 (Figure 3.10). *Acinetobacter oleivorans* (Figure 3.1) degraded 40% of the initial diesel over 10 days of the experiment and the OD_{600nm} increased to 0.42, while *Staphylococcus aureus* exhibited the weakest degradative properties degrading only 27% of the supplied hydrocarbons, even though the OD_{600nm} reached 0.81. Throughout this experiment, *Pseudomonas aeruginosa* WatG was used as positive control due to its ability to degrade diesel hydrocarbons. This strain showed best degradation abilities and degraded 48% of the total hydrocarbons reaching an OD_{600} of 0.55. The abiotic control and the heat killed cells controls did not show significant hydrocarbon loss during the experiment. Table 3.10 shows that nC17/Pristane and nC18/Phytane ratios decreased during the experiment for all the tested strains.

Table 3.10 nC17/Pristane and nC18/Phytane ratios during 10 days of incubation.

	nC17/Pr 0 days		nC17/Pr 10 days		nC18/Ph 0 days		nC18/Ph 10 days	
	Ave	Err	Ave	Err	Ave	Err	Ave	Err
Acinetobacter oleovorans	1.55	0.08	0.65	0.09	2.38	0.16	0.90	0.08
Acinetobacter calcoaceticus	1.55	0.50	0.65	0.76	2.38	0.34	0.90	0.53
Staphylococcus aureus	1.48	0.46	0.35	0.75	2.33	0.21	0.69	0.52
Pseudomonas aeruginosa WatG	1.51	NA	0.61	NA	2.28	1.01	0.66	1.10

NA, not available data.

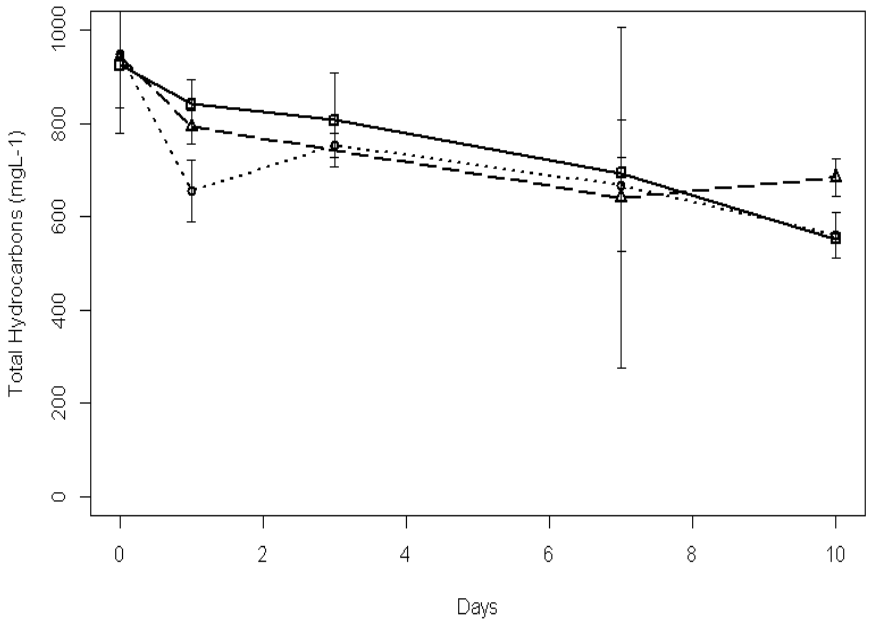

Figure 3.11 – Hydrocarbons concentration during the biodegradation test. (□) *Acinetobacter oleovorans*; (○) *Acinetobacter calcoaceticus*; (▵) *Staphylococcus aureus.*

Section 3

The three selected strains also produced of biosurfactants (Table 3.11), exhibited quorum sensing and motility, biofilm formation and chemotaxis in the presence of diesel and hexadecane (Table 3.12), tolerance to various metals and solvents (Table 3.13), and antibiotic sensitivity (Table 3.14). Furthermore, *Acinetobacter oleivorans*, *Acinetobacter calcoaceticus* and *Staphylococcus aureus* showed promising plant growth promoting traits (Table 3.15). These findings provide evidence that diesel contamination selects for a community rich to plant growth promoters as well as various desirable traits like production of biosurfactants, biofilm, chemotaxis, together with tolerance to solvent and metals at escalated concentrations.

Table 3.11 Production of biosurfactants (R: Root endophyte, RH: Rhizospheric strain, SN: supernatant, Pel: pellet, Hex_: hexadecane, D_: diesel).

Code	Species	Drop collapse_SN	Drop collapse_Ped	Oil displacement_SN	Oil displacement_Pel	Emulsification Hex_SN	Emulsification D_SN	Emulsification Hex_PB	Emulsification D_PB
R	*Acinetobacter oleivorans*	+	-	1,7 cm	-	77,78%	20%	20%	20%
R	*Acinetobacter calcoaceticus*	-	-	0,6 cm	-	55,56%	20%	20%	20%
RH	*Staphylococcus aureus*	+	-	1,3 cm	-	66,67%	20%	20%	90%

Table 3.12 Quorum Sensing and Motility assays

Code	Species	Biofilm	Chemotaxis Diesel	Chemotaxis hexadecen	pSB401	pSB536	pSB1075	T-Streak	TLC	Swimming	Swarming	Twitching
R	*Acinetobacter oleivorans*	+	+	-	-	+	-	+	+	26,6mm	7,8mm	11,6mm
R	*Acinetobacter calcoaceticus*	+	+	-	+	+	-	+	+	24,2mm	9,6mm	9,6mm
RH	*Staphylococcus aureus*	-	+	-	+	+	-	-	+	8,3mm	0mm	0mm

Table 3.13 Test of tolerance to metals and solvents (NG: non growth at the corresponding solvent, -2/-3/-4/-6/: growth observed at the solvents at serial dilution 10^{-2},10^{-3}, 10^{-4} and 10^{-6}, respectively).

Code	Species	n-Hexane	Octane	Dodecane	n-Hexane:cyclohexane	Heavy metal Pb	Heavy metal Cu	Heavy metal Zn	Heavy metal Ni	Heavy metal Cd
R	*Acinetobacter oleivorans*	-2	-6	-6	-3	1mM	1mM	1,5mM	1mM	0mM
R	*Acinetobacter calcoaceticus*	-2	-6	-6	-3	1mM	1,5mM	1mM	1mM	0mM
RH	*Staphylococcus aureus*	NG	-3	-4	NG	2mM	2mM	2mM	1,5mM	0mM

Table 3.14 Antibiotic sensitivity (R: resistant, I: intermediate resistant and S: susceptible).

		Antibiotics sensitivity											
Code	Species	Ap	Am	Bc	Cf	Cm	Er	Gm	Tc	Km	Neo	Rf	Sm
R	*Acinetobacter oleivorans*	-(R)	17(S)	-(R)	16(I)	16(I)	14(I)	17(S)	14(R)	14(I)	14(I)	12(R)	12(I)
R	*Acinetobacter calcoaceticus*	-(R)	17(S)	-(R)	18(I)	-(R)	12(I)	17(S)	10(R)	14(I)	13(R)	14(R)	18(S)
RH	*Staphylococcus aureus*	-(R)	23(S)	-(R)	20(I)	-(R)	14(I)	17(S)	18(I)	20(s)	15(I)	16(R)	20(S)

Table 3.15 Plant growth promoting characteristics

Code	Species	ACC deaminase	IAA	Acetoin	Organic acids	Inorganic P	Phytase	Siderophores	Phytase
R	*Acinetobacter oleovorans*	+	+	-	+	+	+	+	+
R	*Acinetobacter calcoaceticus*	+	-	-	-	+	+	+	-
RH	*Staphylococcus aureus*	+	-	-	+	-	+	+	-

3.7 Discussion

Recently, among the various approaches to treat soils contaminated with hydrocarbons, the application of bacteria with the ability to degrade these contaminants while promoting growth of their host plants has been gaining acceptance (Teng et al. 2011; Weyens et al. 2009b).

It has been reported that application of hydrocarbonoclastic rhizospheric bacteria exhibiting ACC deaminase activity resulted in greater plant biomass production and diesel degradation than bacteria exhibiting only diesel degradation traits (Afzal et al. 2011).

Similarly, inoculation of the rhizobacterium *Gordonia* sp. S2RP-17 in diesel-contaminated soil populated with *Zea mays* resulted in enhanced diesel degradation, ACC deaminase activity and siderophore production (Hong et al. 2011). These reports underline the crucial role of ACC deaminase for reducing plant stress caused by the presence of diesel oil.

Overall, the existence of healthy plants living in symbiosis with bacteria directly or indirectly promoting plant growth is considered an asset for plant-bacteria enhanced remediation of diesel contaminated sites. Of course, other factors such as the availability of diesel, the co- presence of toxic metals, the presence of toxic solvents and soil nutrient deficiencies can result in failure to achieve maximal remediation.

The results of the work presented here revealed a community of soil, rhizospheric and endophytic bacteria belonging to genera such as *Pseudomonas*, *Acinetobacter*, *Staphylococcus*, *Arthrobacter*, *Brevibacterium* and *Microbacterium* able to utilize diesel oil. Although strains of these genera have been routinely associated with hydrocarbon-contaminated environments (Hennessee and Li 2010; Moliterni et al. 2012; Moore et al. 2006; Pavitran et al. 2004; Ryan et al. 2008; Schippers et al. 2005; Shukor et al. 2009; Van Hamme et al. 2003), there is scarcity of information about their ability to produce ACC deaminase, IAA, organic acids and siderophores, and to solubilise inorganic phosphorus and mineralize organic phosphorus.

This work fills this gap and demonstrates that alongside with the potential to degrade complex mixtures of hydrocarbons such as diesel, bacteria isolated from a diesel contaminated plume exhibit a number of desirable plant growth promoting features. Hence, these results provide evidence that diesel

contamination may select for a community rich in plant growth promoters. In addition, the majority of the strains were positive for the production of biosurfactants, the formation of biofilms, the existence of quorum sensing signalling, positive chemotaxis towards diesel, and tolerance to various solvents and metals.

In order to understand in more detail the behaviour of the community, all of the isolates were screened for their ability to degrade hexadecane as well as diesel fuel in order to unravel discernible patterns between aliphatics and aromatics hydrocarbon use. A more in depth analysis of the biodegradation potential of 3 selected strains, namely *Acinetobacter oleivorans*, *Acinetobacter calcoaceticus* and *Staphylococcus aureus*, confirmed their ability to degrade diesel oil in liquid cultures.

The novelty of this work is based on the proposal of an integrated screening protocol which gives emphasis at one hand on the metabolic routes, bioavailability limitations, improved resistance to a variety of stresses such as solvents and heavy metals, biofilm formation, motility, chemotaxis response associated with quorum sensing signalling, and on the other hand takes into account the plant growth promoting features of the isolated bacteria.

Therefore, in that way it is envisaged that the combination of resistances to environmental stresses and catabolic phenotypes in appropriate bacterial strains, together with improved survival characteristics in host plants, can be expected to yield plant-bacteria partnerships with significantly enhanced performance to remediate petroleum hydrocarbon polluted ecosystems. In conclusion, the targeted selection of plant-associated endophytic and rhizospheric bacteria as a mean to foster the degradation of toxic petroleum compounds of concern like diesel requires a holistic approach.

SECTION 4

Whole genome shotgun sequencing of the diesel degrading and plant growth promoting root endophyte *Acinetobacter oleivorans* strain PF1

Section 4

4.1 Introduction

The *Acinetobacter* genus, which belongs to the class of Gammaproteobacteria has been in the epicenter of bioremediation studies due to the significant capacity of its members to effectively adapt to and degrade various recalcitrant organic compounds, which quite often are the causal agents of severe contamination of terrestrial and aqueous environments (Ahmad et al. 2012; DiCello et al. 1997; Ishige et al. 2000; Liu et al. 2009; Luckarift et al. 2011; Mara et al. 2012; Rocha et al. 2013; Sun et al. 2012b; Throne-Holst et al. 2007; Zhan et al. 2012). In addition, strains belonging to *Acinetobacter* genus are capable of utilizing diesel fuel as carbon and energy source and have been found to be useful for bioremediation (Huang et al. 2013; Kang et al. 2011b; Lee et al. 2012; Mara et al. 2012; Marin et al. 1995; Phrommanich et al. 2009; Singh and Lin 2010; Su et al. 2008; Vaneechoutte et al. 2009). Despite this, the use of a single bacterial strain to clean up diesel-contaminated soil is often not successful. Low bioremediation efficiencies may result from limited survivability of the inoculated strain caused by factors such as the presence of toxic heavy metals (Sandrin and Maier 2003), limited substrate bioavailability (Mrozik and Piotrowska-Seget 2010), as well as incorrect pH, temperature and/or competition with other strains. Given this, when selecting bacterial strains for bioremediation it is important to understand not only its biodegradative capacity, but to also understand its ability to form biofilms, engage in quorum sensing signalling (Kang et al. 2011b; Tribelli et al. 2012a), produce biosurfactants (Kaczorek et al. 2012), and respond chemotactically to pollutants (Lacal et al. 2011). These additional traits, if present, could substantially increase the probability of a bacterial inoculant being useful for *in situ* bioremediation. In the case of phytoremediation, selecting bacterial strains that interact well with the planted/seeded plants is essential. It has been reported that *Acinetobacter* spp. are able to directly and indirectly promote plant growth. Two rhizospheric bacteria, *A. calcoaceticus* genomovar *rhizosphaerae* strains IH9 and OCI1, isolated from the rhizosphere of grasses, have been shown to solubilise phosphorus *in vitro* (Peix et al. 2009), and *Acinetobacter rhizosphaerae* strain BIHB 723 expresses an array of plant-growth promoting properties (Gulati et al. 2009; Huddedar et al. 2002). Work by Indiragandhi et. al (2008) described, for the first time, the existence of diazotrophic

Acinetobacter species. This was followed up with work by Sachdev et al. (2010) who proved, using an acetylene reduction assay, that *A. baumannii* strain LRFN53, is able to fix nitrogen. Similarly, *in vitro* characterization of *Acinetobacter* isolates from the rhizhosphere of wheat plants revealed that the majority of these bacteria were able to fix nitrogen, solubilise P and Zn, and produce siderophores. These works, it is evident that bacteria belonging to the *Acinetobacter* genus are involved in direct plant growth promotion. Other *Acinetobacter* strains promote plant growth in an indirect manner by suppressing the growth of phytopathogenic fungi such as *Phytophthora capsici*, *Fusarium graminearum* and *Rhizoctonia solani* (Liu et al. 2007a), and phytopathogenic bacteria such *Ralstonia solanacearum*, a bacterial strain which causes tomato wilt (Xue et al. 2008). A screen of 31 *Acinetobacter* isolates obtained from the rhizosphere of Pearl millet (*Penisetum glaucum*) showed the plant growth-promoting potential of these isolates, via both direct and indirect mechanisms. For example, *Acinetobacter* sp. PUCM1007 inhibited the growth of the fungal phytopathogen *Fusarium oxysporum in vitro* under iron limited conditions (Rokhbakhsh-Zamin et al. 2011). Generally, the application of plant growth-promoting bacteria during phytoremediation of hydrocarbon contaminated sites is not a common practice (Gerhardt et al. 2009), however, it has been recently suggested that the addition of plant growth-promoting bacteria is a simple and effective strategy for enhancing plant growth and phytoremediation activity in presence of petroleum (Glick and Stearns 2011; Guo et al. 2012a; Gurska et al. 2009b; Hong et al. 2011; Tang et al. 2010b; Tara et al. 2014). Endophytic bacteria can support their host plants by reducing pollutant-induced stress (Weyens et al. 2010); hence, inoculation of plants with endophytic bacteria rather than free living bacteria holds promise as an efficient diesel remediation technology (Afzal et al. 2013). For more information about the use of plants in conjunction with hydrocarbon degrading and plant growth-promoting bacteria (PGPB) the reader is referred to the following reviews (Fester et al. 2014; Khan et al. 2013a). Although it has been demonstrated that *Acinetobacter calcoaceticus* strain P23 is able to degrade hydrocarbons, including aromatic phenols and aliphatic alkanes, and promote the growth of *Lemna aoukikusa* (Yamaga et al. 2010), there is scarcity of reports about effective turnover of organic contaminants along with enhanced plant growth-

promotion among species of *Acinetobacter* genus. It appears as reflected by the availability of complete genomes available in public databases, that most reports are for species in other genera. In this context, light has been shed to the hydrocarbon degradation capabilities of various strains like *Pusillimonas* sp. T7-7 (Cao et al. 2011), *Dietzia cinnamea* P4 (Procopio et al. 2012), *Acinetobacter venetianus* RAG-1 (Fondi et al. 2012), *Acinetobacter* sp. DR1 (Jung et al. 2010), *Pseudomonas aeruginosa* SJTD-1 (Liu et al. 2012), *P. aeruginosa* DQ8 (Gai et al. 2012), *Alcanivorox borkumensis* (Schneiker et al. 2006), as well as to plant growth-promoters like *Pseudomonas* sp. UW4 (Duan et al. 2013), *Agrobacterium tumefaciens* CCNWGS0286 (Hao et al. 2012), *Enterobacter* sp. SP1 (Zhu et al. 2012), Rahnella aquatilis HX2 (Guo et al. 2012c), *Bacillus* sp. JS (Song et al. 2012), *Pseudomonas putida* S11 (Ponraj et al. 2012).

4.2 Materials and Methods

4.2.1 Whole genome shotgun sequencing of *A. oleivorans* PF1

Genomic DNA of *A. oleivorans* PF1 was extracted from cells grown at 30^{o} C in LB medium with a Qiagen blood and tissue kit (Qiagen N.V., Hilden, Germany) and a single 316v2-chip was used for sequencing on an IonTorrent PGM (Life Technologies Inc., Carlsbad, CA). Prior to sequencing DNA was enzymatically digested and sequencing adaptors ligated using an Ion Xpress Plus Fragment Library Kit (Life Technologies Inc., Burlington, ON) according to the manufacturer's instructions. Adaptor-ligated DNA was size selected to a target length of 480 bp on a 2% E-Gel Size Select agarose gel, and Agencourt MAPure XP beads (Beckman Coulter, Mississauga, ON) were used for purification steps. An Ion Library Quantitation Kit was used to calculate the library dilution factor prior to amplification and enrichment with an Ion PGM Template OT2 400 kit on an Ion OneTouch 2 system. An Ion Sphere Quality Control Kit was used to quantify the percent enriched Ion Sphere Particles prior to sequencing with an Ion PGM 400 Sequencing Kit. In total, 2.8 million reads (mean length 303 bases) generated 853 Mb of data, of which 535,611 reads were assembled using MIRA V3.9.9 (Chevreux et al. 2004) into 31 contigs, giving a consensus length of 3,766,014 bp at 43.5× coverage. ORF prediction and gene annotation was

carried out using RAST (Aziz et al. 2008). This strain has a GC content of 38,6% and 3509 genes were arranged into 668 subsystems using RASTA.

4.2.2 Phylogenetic tree construction

In order to find the most closely related genome to use as a reference for gene prediction, the following procedure was followed. The genomes of all fully sequenced and closely related bacterial strains on the NCBI database (*Acinetobacter oleivorans* DR1, *Acinetobacter baumanii*: 13 strains, *Acinetobacter pittii* ANC4050, *Acinetobacter calcoaceticus* PHEA-2,), along with sequences for *Pseudomonas putida* KT2440 and *Escherichia coli* CFT073, were downloaded and aligned to the *Acinetobacter oleivorans* PF1 data using progressive MAUVE 2.3.1 (Darling et al. 2010). A Newick tree was generated using MEGA6 (Tamura et al. 2013). Moreover, a new taxonomic metrics tool like average nucleotide identity (ANI), calculated from pair-wise comparisons of all sequences shared between two strains was performed (Goris *et al.* 2007). Briefly, the ANI calculator estimates the average nucleotide identity using both best hits (one-way ANI) and reciprocal best hits (two-way ANI) between two genomic datasets and typically, the ANI values between genomes of the same species are above 95%, while values below 75% are not to be trusted.

4.2.3 Gene Cluster comparisons

The gene context of gene clusters and the organization of specific operons was compared using the SeqinR1.0-2 package in R (Charif and Lobry 2007). For each gene and operon of interest the sequence of the genomic DNA fragment (”Query fragment”) was extracted, enlarged by two supplementary genes, one upstream and one downstream, prior to using BLAST to compare sequences to the NCBI bacterial genomes database. Matching sequences and gene coordinates of the best hits were downloaded to a genebank file, and plotted against the ”Query fragments”.

4.2.4 Identification and annotation of genes involved in hydrocarbon degradation, plant growth promotion, and heavy metal tolerance.

A list of 10 biochemical mechanisms involved in diesel degradation and in plant-growth promotion was built by reviewing 24 published studies, carrying out

keyword searches in the MetaCyc and NCBI databases (when no article was available), and through identification of 219 "query" genes (37 as single genes and 182 genes that were part of one of 38 operons) that have been shown to code for proteins that are key players for each of the mechanisms of interest (see Appendix B). Protein sequence for each "query" gene was obtained from the most phylogenetically related genome on the NCBI database and BLAST (tBlastn, cutoff 10, no filter) was used to compare to the genome of *Acinetobacter oleivorans* PF1. Hits were translated into protein sequences, and checked for two parameters: the presence of the same PFAMs as the query sequence, and a similar name as the query sequence in the top 10 hits in a Psi-Blast (NCBI). This generated a list of 132 "subject" genes in the genome of *Acinetobacter oleivorans* PF1. In the case of metallothioneins, whose sequences are extremely divergent, a Perl script was written to check all of the sequenced strain's protein sequences for the presence of a bacterial MT signature sequence (KCACTTCLC).

4.3. Results and Discussion

4.3.1 Genetic basis of alkane degradation in *Acinetobacter oleivorans* PF1

Conceptual models of *n*-alkane degradation pathways and the genes involved have been described in detail in a variety of organisms and have been found to be diverse in both the regulation and clustering of alkane degradation genes. Interestingly, a single bacterial strain may carry multiple genes that code for different enzymes for similar degradative functions. It is generally accepted that the most detailed examinations of alkane degradation pathways were performed for the OCT plasmid carried by *Pseudomonas putida* Gpo1 (van Beilen et al. 2001), where the first step of *n*-alkane oxidation is catalysed by a three-component alkane hydroxylase complex, consisting of a non-hem integral-membrane alkane monooxygenase and two soluble proteins, a rubredoxin and a rubredoxin reductase. A similar three-component alkane hydroxylase complex, consisting of an alkane mono-oxygenase (AlkM), a rubredoxin (RubA), and a rubredoxin reductase (RubB), has been characterised in *Acinetobacter* sp. strain ADP1 (Geissdorfer et al. 1999; Ratajczak et al. 1998a; b).

In strain PF1 the *alk* operon is partially present. In fact, among the 11 genes found in the genome of *Pseudomonas putida* Gpo1, 9 are also present in the genome of *Acinetobacter oleivorans* PF1. However, they are not organized in an operon, but are instead spread throughout the genome.

The most potent gene of the operon, *alk*B (alkane monooxygenase) is present in PF1 and Figure 4.1 shows that, according to its position, it is under the control of the AraC transcription regulator, a regulator which has been described for members of the *Acinetobacter* genus (Van Hamme et al. 2003).

Besides *alk*B, analysis revealed the presence of the aldehyde dehydrogenase *ald1* gene, a gene which has been shown to be involved in long-chain alkane degradation (Ishige et al. 2000) and which has an exact match (e-value=0.0) to the *ald*1 protein sequence of *Acinetobacter calcoaceticus* (Van Hamme et al. 2003).

The gene context for *ald1* in PF1 is quite similar to *Acinetobacter calcoaceticus* DSM 30006 and *Acinetobacter oleivorans* DR1 (Jung et al. 2010). Unfortunately, *Acinetobacter* sp. strain M-1, where it is known that *ald*1 codes for a protein with alkane degradation activity, has not yet been sequenced so it was not possible to compare the gene context in this "reference" organism.

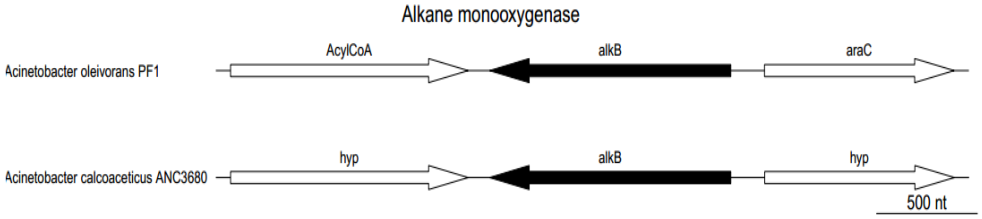

Figure 4.1 Schematic representations of the *alkB* regions in *A. oleivorans* PF1 and *A. calcoaceticus* ANC3680.

The gene is under the control of the ArsR transcriptional factor, which is an inhibitor of the transcription of the arsenic (and antimony) resistance operon. Another interesting feature of the *Acinetobacter oleivorans* PF1 genome is the presence of the flavin-binding monooxygenase gene, closely related to the *alm*A gene in *Acinetobacter calcoaceticus strain* NIPH13. Relatively recently, a gene designated *alm*A, which encodes a putative monooxygenase of the flavin-binding family, was identified from *Acinetobacter* sp. DSM 17874 (Throne-Holst et al., 2007), and *alm*A homologues have also been found in other isolates of the

genus *Acinetobacter* (Wentzel et al. 2007), including *Acinetobacter* sp. M-1 (Sakai et al. 1994), and *Acinetobacter baylyi* ADP1 (Vaneechoutte et al. 2006). This gene encodes the first experimentally confirmed monooxygenase to be involved in the metabolism of long chain *n*-alkanes. Notwithstanding, a flavin-binding dioxygenase has also been reported (Van Hamme et al. 2003).

4.3.2 Genetic basis of PAH degradation in *Acinetobacter oleivorans* PF1

A number of bacterial species are known to degrade PAHs and most of them are isolated from contaminated soil or sediments. *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Sphingomonas* spp., *Mycobacterium* spp., *Haemophilus* spp., *Rhodococcus* spp., *Paenibacillus* spp., *Paracoccus* spp, *Terrabacter* spp., *Marinobacter* spp., *Alcaligenes* spp., *Microbacterium* spp., *Gordonia* spp., *Stenotrophomonas* spp., *Ochrobactrum* spp., and *Acinetobacter* spp., are some of the commonly studied PAH-degrading bacteria (Haritash and Kaushik 2009; Wongwongsee et al. 2013).

Aerobic PAH metabolic pathways resulting from bacterial degradation include the ortho- and meta-pathways, gentisate, o-phthalate, the upper and lower pathways, the Evans and Kiyohara pathways, and the beta-ketoadipate pathway (Harwood and Parales 1996; Juhasz and Naidu 2000; Kanaly and Harayama 2000; Khomenkov et al. 2005; Khomenkov et al. 2008; Kim et al. 2008a; van Herwijnen et al. 2003). Since, the bacterial degradation of naphthalene (Annweiler et al. 2000), phenanthrene (Pinyakong et al. 2003a; Pinyakong et al. 2003b) has been well investigated, the main routes for degradation of aromatic compounds like naphthalene and phenanthrene were chosen for further investigation in *Acinetobacter oleivorans* PF1.

The lower naphthalene degradation pathway is present. In more details, although the meta-cleavage pathway is incomplete, the analysis has given out the presence of all genes related to the ortho-cleavage pathway. There are several genes duplicated for most of the degradation steps, with best hits in *Acinetobacter* sp. NIPH817, *A. calcoaceticus*, *A. baumanii*, *A. pittii*, *A. oleivorans* DR1, *Acinetobacter* sp. WC743 and *Acinetobacter* sp. GG.

The gentisate pathway is also present, assuming that the first step (B1) can be performed by the proteins coded for by the *nahAaAbAcAd* genes found in the PF1 genome.

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The upper naphthalene degradation pathway is incomplete in PF1 since it lacks the *nah*C and *nah*D genes, however, it is possible that other genes are present that fulfill the same function because the remainder of the pathway is present down to and including the TCA cycle. The first steps of the phenanthrene degradation pathway share steps with the upper naphthalene catabolic pathway, while the lower catabolic pathway is based on 1-hydroxy napthoic acid degradation. Sequencing did not reveal the presence of genes involved in the lower pathway.

Bacterial laccases have been implicated in the degradation of PAHs (Ausec et al. 2011), so it is possible that *Acinetobacter oleivorans* PF1 empolyes laccase for PAH degradation given that it harbours a bacterial laccase gene closely matching to the one in *A. baumanii*. Laccase production was not monitored for *Acinetobacter oleivorans* PF1, but this would be an interesting study to carry out.

4.3.3 Emulsification of hydrocarbons by *Acinetobacter oleivorans* PF1

Acinetobacter species are known to produce high molecular weight biosurfactants, for example emulsan and alasan. Emulsan, produced by the *Acinetobacter venetianus* strain RAG-1 (Dams-Kozlowska et al. 2008), is a non-covalently linked complex of a lipoheteropolysaccharide and a protein that interfaces between cell membranes and oil (Fig.4.2). A 27 kb gene cluster, *wee*, encodes the genes (*wza*, *wzb*, *wzc*, *wzx*, wzy) for emulsan biosynthesis (Nakar and Gutnick 2001).

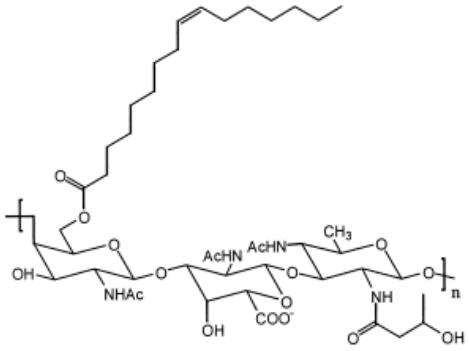

Figure 4.2 Structure of the emulsan-like polymer

The polysaccharide moiety, called apoemulsan, consists of various sugar components such as D-galactosamine, D-galactosaminuronic acid and diamino-dideoxy glucosamine (Dams-Kozlowska et al. 2008; Nakar and Gutnick 2001). Fatty acids make up 12% of this biopolymer and resulting its amphipathic nature. *Acinetobacter radioresistens* strain KA53 produces alasan (Navonvenezia et al. 1995), an anionic polysaccharide containing covalently bound alanine and three proteins (15 kDa, 31 kDa and 45 kDa) present in similar molar amounts (Toren et al. 2001), and releases the biosurfactant into extracellular fluid during the stationary phase. Genome annotation revealed that that *wee* operon is partially present in *A. oleivorans* PF1 (Fig. 4.3). In Fig. 4.3, genes coloured "black" are involved in the "classical" apoemulsan biosynthesis pathway and are located at the start and the end of the *wee* operon. These genes do not allow for complete emulsan synthesis, but theoretically are sufficient for the synthesis of a capsular polysaccharide described by Dams-Kozowska et al. (2008). (Dams-Kozowska et al. 2008). Genes coloured "grey" in Fig. 4.3 are not emulsan-related; but their position indicates that they belong, for *A. oleivorans* PF1, to the same operon.

Moreover, one gene (*hyp*) coding for a hypothetical protein is present and has similarity to the *A. baumanii wzy* gene, which is responsible for the polymerisation of membrane bound apoemulsan. Interestingly, there appears to be a gene cluster insertion, from *weeK* to *wzx*, which is involved in sialic acid generation, a sugar responsible of bacterial pathogenicity and is not apparently linked to biosurfactant activity. Another interesting feature of the *Acinetobacter oleivorans* PF1 gene cluster illustrated in Fig. 4.3 is that there is no transcription factor upstream of the operon.

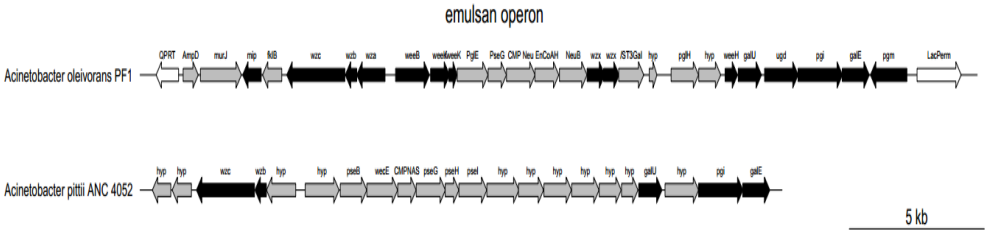

Figure 4.3 Organization of the emulsan operon in *Acinetobacter oleivorans* PF1 and *A. pittii* ANC 4052 ("black" genes are present in the classical *wee* operon, the "grey" ones are not present in the operon, while the "white" ones are located outside the operon).

4.3.4 Chemotaxis and biofilm formation in *Acinetobacter oleivorans* PF1

The *Acinetobacter oleivorans* PF1 genome harbors 3 gene clusters involved in chemotaxis behavior. The *che*W/*pil*J/*pil*H/*pil*G cluster (on contig 8) shows 94% similarity with the same operon in *A. oleivorans* DR1. pil*G*/*H*/*J* codes for a complex that is necessary *Pil*A pilin protein export required for motility (Wu et al. 1998). The association of *che*W with pil*G*/*H*/*J* suggests that motility is linked to detection of a ligand (Sourjik and Berg 2004). A query using a methyl-accepting chemotaxis protein (MCP) matches with a gene, annotated as a type IV *pil*J, which is neighboring a *che*A gene. In *A. oleivorans* DR1, there is also one MCP that is also annotated as type IV *pil*J (Fig. 4.4).

These findings suggest that PF1 may be chemotactic towards alkanes, since the MCP is encoded by a *tlp*S gene, reported for possible role in alkane chemotaxis (Smits et al. 2003; van Beilen et al. 2001). The second cluster is found on contig 4, where 2 copies of the archaic type IV sigma fimbrial gene cluster (protein precursor/papD/usher/adhesin precursor/export) are found.

This operon is also duplicated in *Acinetobacter baumanii*, and has been shown to be involved in biofilm formation and fimbria formation. Finally the third cluster, *pca*F/*pca*K, is also present on contig 4, and it shows similarity to the *pca*RFK gene cluster from *Pseudomonas putida* (Harwood et al. 1994), a cluster which is involved in chemotaxis (*pca*K) and degradation (*pca*F) of 4-hydroxybenzoate. One major difference is that *Acinetobacter oleivorans* PF1 lacks the transcriptional regulator *pca*R. It is worthwhile to note that the latter cluster is immediately upstream of the *nah* operon, so it is possible that PF1 is chemotactic to PAHs, although this has not been tested. With respect to acyl-homoserine lactone synthesis, molecules that are important for quorum sensing and biofilm formation (Huang et al. 2009), PF1 has two genes on contig 4 coding for autoinducer synthesis proteins, as well as a 2-homoserine/homoserine lactone efflux protein, with the same gene context as many of the *Acinetobacter calcoaceticus/baumanii*

complexes.

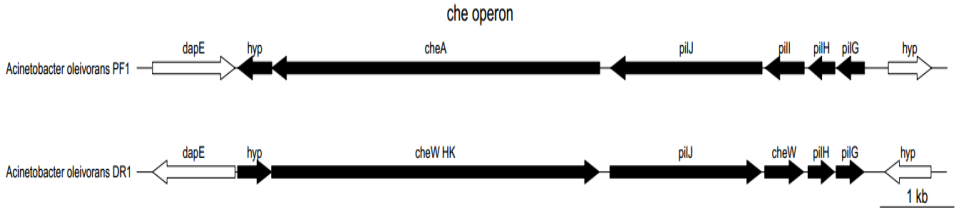

Figure 4.4 Chemosensory signalling system of *Acinetobacter oleivorans* PF1 ("black" genes are present in the operon of a characterized chemotactic organism, while the "white" genes are outside of the operon)

4.3.5 Plant growth-promoting traits

4.3.5.1 Acetoin and 2,3-butanediol biosynthesis and catabolism in *Acinetobacter oleivorans* PF1

Biosynthesis of acetoin and 2,3-butanediol requires the presence of four genes namely, *pox*B (pyruvate dehydrogenase), *bud*A (acetoin decarboxylase), *bud*B (acetolactate synthase) and *bud*C (acetoin reductase) (Taghavi et al. 2010a). The genome of *Acinetobacter oleivorans* PF1 lacks the *pox*B gene, but there are 3 other genes bearing the same PFAM domains, meaning that it may be able to perform this function among others. It also lacks the *bud*A gene, but has *bud*B, and *bud*C. The latter, codes for a protein that can both degrade or synthesize butanediol, called acetoin reductase or 2,3-butanediol dehydrogenase (EC 1.1.304). With respect to acetoin and 2,3-butanediol catobolism, the operon in *Acinetobacter oleivorans* PF1 is slightly different compared to the one described in *Pseudomonas putida* (Huang et al. 1994), where the 2,3-butanediol dehydrogenase and the acetoin-cleaving system are simultaneously induced during growth on butanediol or acetoin. All coding genes are found in one operon on contig 4, and this operon is homologous with the one from *A. calcoaceticus*; furthermore it is under the control of *Aco*R, the transcriptional activator of the acetoin dehydrogenase operon. Overall, the differences between *A. oleiovorans* PF1 and *P. putida* operons are the following: different transcriptional regulatory elements are used, specifically *aco*R in *A. oleivorans* and *aco*X in *P. putida*; the presence of a probable membrane protein, STY1534, in *Acinetobacter oleivorans* PF1; the presence of a second (S)-acetoin specific

2,3-butanediol dehydrogenase; and finally the presence of a dihydrolipoamide dehydrogenase component of the acetoin dehydrogenase system (EC 1.8.1.4).

4.3.5.2 Phosphorous uptake in *Acinetobacter oleivorans* PF1

Organic P may constitute 4-90% of the total soil P (Zaidi et al. 2009a), and such P can be released from organic compounds in soil by various enzymes. Among these enzymes, phytases play a dominant role. Hence, the presence of phytate esterase in the PF1 genome implies that the strain might have phytase activity and cause the release of phosphorus via phytate degradation, which is the major component of organic P in soil. In addition to organic phosphatases, the strain was screened for the presence of genes related to enzymes responsible for inorganic P solubilization. An inorganic pyrophosphatase that may be involved in soil phosphorus uptake from inorganic sources was identified. This enzyme can also be involved in the early steps of lipid biosynthesis, DNA synthesis, or redistribution of internal P sources, so its exact role in *Acinetobacter oleivorans* PF1 is not known.

4.3.5.3 Indole acetic acid (IAA) production in *Acinetobacter oleivorans* PF1

The genome of *A. oleiovorans* PF1 contains genes homologous to genes of the indole-3-pyruvic acid pathway in *A. calcoaceticus*. However, the PF1 pathway appears to be incomplete since it lacks the last two genes that appear to be present in other *A. baumanii* and *A. calcoaceticus* species (Patten and Glick 1996).

4.3.5.4 ACC- deaminase production in *Acinetobacter oleivorans* PF1

The genome of *Acinetobacter oleivorans* PF1 possesses a gene responsible for the production of ACC-deaminase, and analysis has revealed that the closest hit is to *A. calcoaceticus* RUH2202 (Fig. 4.5). The gene is under the control of a TetR transcriptional regulator which is associated with a MFS transporter and a membrane fusion component of a tripartite multidrug resistance protein complex possibly involved in the secretion of ACC.

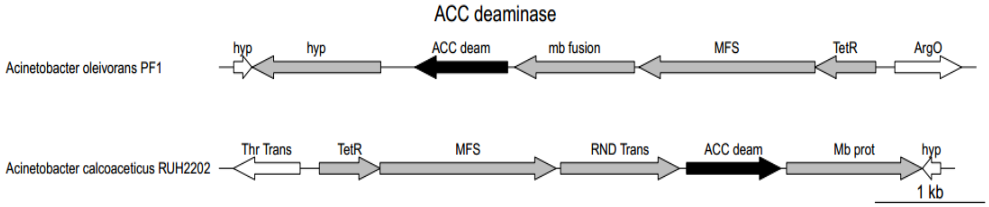

Figure 4.5 Gene context of *A. oleivorans* PF1. Black is the ACC deaminase gene, grey other genes present in the operon, while the white ones are outside the operon

4.3.5.5 Siderophore biosynthesis in *Acinetobacter oleivorans* PF1

The *A. oleivorans* PF1 genome carries 2 operons putatively involved in the biosynthesis of 2 different siderophores. The secretion of acinetobactin, a catechol type siderophore which has been described for *Acinetobacter baumannii* strain ATCC 19606 (Dorsey et al. 2004), seems possible as PF1 harbours 4 *bau* genes. However, PF1 lacks *bas*C, *bas*D, and has only one copy of *bau*D. Some supplementary genes are present in the operon (piuB: iron-uptake factor, and 2 hypothetical proteins). It is interesting to note that this operon, which occurs as a whole in the sequenced strain, has highest homology with a strain of *A. calcoaceticus*. In the latter organism, the operon is split at two separate locations (with 4000 bp in between; *hyp*/*bauB*/*bauD*/*bauE* on one side, *piuB*/*bauA*/*hyp*/*tetR* on the other). The fact that in *A. oleivorans* strain PF1 those two separate locations are closer to each other makes it more likely that they are transcribed together, possibly generating a siderophore with different properties. The *bas*, *bau* and *bar* genes needed in the production, transport and secretion of acinetobactin, respectively, are located in a 26.5-kb chromosomal region harboring seven operons has been reported (Mihara et al. 2004), however, this locus does not include an entA ortholog coding for a 2,3-dihydro-2,3-dihydroxy-benzoate dehydrogenase, an enzyme is involved in the last step of the conversion of chorismate into 2,3-dihydroxybenzoic acid (DHBA), which is essential for the biosynthesis of the catechol moiety of siderophores such as enterobactin in *Escherichia coli* (Gehring et al. 1998; Walsh et al. 1990). Notably, the enterobactin biosynthesis operon is present in PF1 and matches exactly (100% coverage, >93% identity at the nucleotide level) the operon in *A. calcoaceticus* NIPH13 (Penwell et al. 2012).

Overall, these findings suggest that *Acinetobacter oleivorans* PF1 is similar to *A. calcoaceticus* with respect to enterobactin production, but that the acinetobactin synthesis genes are arranged differently.

4.3.5.6 Heavy metal tolerance in *Acinetobacter oleivorans* PF1

In order to exclude toxic metals, bacteria have evolved various defensive mechanisms (Gadd 1992). To investigate the presence of heavy metal tolerance mechanisms in *Acinetobacter calcoaceticus*, Canovas et al. (2003) used genes involved in heavy metal tolerance in *Pseudomonas putida* as a template and, interestingly, all of the genes examined were found to be very similar (Canovas et al. 2003). One important group of molecules for heavy metal resistance are cysteine rich, heavy metal-binding proteins, the metallothioneins MTs. MTs are gene encoded polypeptides involved in heavy metal stress amelioration and have been found in a wide variety of plants, animals and lower eukaryotes (Cobbett and Goldsbrough 2002), as well as bacteria (Turner and Robinson 1995).

There is one bacterial metallothionein (MT) present in the *P. putida* genome. Since, MT sequences are extremely divergent, it is not possible to identify them using BLAST. Instead, coding genes must be identified by searching for specific amino acid "signature" sequences (*e.g*: "CXXCRTTXXC" where X is any amino acid). In this study, a signature search showed that no MTs were present in the sequenced genome of *Acinetobacter oleivorans* PF1.

Another mechanism exploited by bacteria to avoid the toxic effects of heavy metals is metal extrusion. For chromium (Cr) efflux, two operons containing Cr efflux genes (*chrA*) were found in PF1, and one of them contains 2 *chrA* repeats. This appears to be the same in *A. baumanii*. With regards to Arsenic (As) efflux, one operon was found to contain the As efflux genes (*arsRBCH*) downstream from one of the Cr efflux operons.

Again, it seems to be the same in *A. baumanii*. Next to these, the *czc* operon (*czcABCD*), which is responsible for Co-Zn-Cd efflux, is found. Although the *czrCBA* operon, which is responsible for Cd-Zn efflux is absent, there is a Me^{2+} P-type ATPase that can code for a Pb-Cd-Zn-Hg efflux protein. Finally, no genes were found for Co-Ni efflux (*cnrCBA*) or Ag-Cu efflux (*cusCBA*).

4.4 Conclusion

Genome sequence analysis of *A. oleivorans* PF1 has provided us with significant insights into the genomic basis of PF1's:

i. Metabolic capability to degrade aliphatic and aromatic components present in diesel oil.
ii. Affinity and chemotaxis towards hydrocarbon substrates
iii. Responses to hydrocarbons and heavy metal stress
iv. Key plant growth promotion properties

These findings support the hypothesis that *A. oleiovorans* PF1, based on its hydrocarbon degrading and emulsification abilities, together with its potential to promote host plant growth, holds promise as a very promising candidate for the development of novel strategies for the bioremediation of petroleum-contaminated sites by means of PGP and hydrocarbon degrading bacteria.

SECTION 5

Greenhouse experiment

Section 5

5.1 Introduction

Poplar trees (genus *Populus*; family *Salicaeae*) are woody C3 (uses the Calvin cycle for synthesis of cellular components) dicotyledons (have net-veined leaves) that are deciduous (loses leaves yearly) and dioecious (having both male and female individual trees) (Tuskan et al. 2004). Poplars are often grown on artificial tree plantations, especially in countries with insufficient natural resources and where exploitation of forests is expensive (Confalonieri et al. 2003). Poplars possess a number of positive features making them attractive for agroforestry. Specifically: (a) they grow in plots unsuitable for traditional agricultural crops, (b) they require several years of growth in platation prior biomass harvest, mitigating the effects unfavorable growth conditions during single years, (c) they allow for growth of a larger number of wild species compared to field crops when grown in plantations; and (d) the *Populus* genus includes a large number of species and hybrids that are able to grow under range of environmental conditions (Kutsokon 2011). In addition to their use in managed forestry applications poplars are also useful for remediation of ecosystems contaminanated with hazardous pollutants (Doty et al. 2007).

Numerous studies refer to the suitability of poplar trees for phytoremediation of both persistent organic pollutants such as solvents, herbicides, polycyclic aromatic hydrocarbons (PAHs), explosives (Aitchison et al. 2000; Burken and Schnoor 1997; Gordon et al. 1998; Kacalkova and Tlustos 2011; Liu and Schnoor 2008; Mueller and Shann 2006; Thompson et al. 1998a; Thompson et al. 1998b; 1999; Van Aken et al. 2004a; Wang et al. 2004), and metals (Di Baccio et al. 2003; Dos Santos Utmazian and Wenzel 2007; Komarek et al. 2008; Laureysens et al. 2004; Laureysens et al. 2005; Robinson et al. 2000; Sebastiani et al. 2004; Wang and Jia 2010), or environments contaminated with both organic pollutants and metals. For example, Doni et al. (2012) used horse manure fertilizer and *Populus nigra* (var. *italica*) to bioremediate about 0.5 ha of soil co-contaminated with metals (Pb, Cr, Cd, Zn, Cu and Ni) and organic contaminants, such as polychlorobiphenyls and petroleum hydrocarbon. After one year,reductions of about 80% total petroleum hydrocarbon (TPH), and 60% polychlorobiphenyls (PCBs) and total metals were observed (Doni et al. 2012).

Poplar trees are also suitable candidates for diesel fuel phytoremediation in subarctic conditions (Palmroth et al. 2002).

Section 5

Improved reductions in petroleum toxicity have been achieved with the addition of oxygen and soil amendments to the rhizosphere when using poplar trees for phytoremediation (Rentz et al. 2003). In addition, specific hybrid poplar genotypes have been found to be effective for *in situ* remediation of soils heavily contaminated with petroleum hydrocarbons (Zalesny et al. 2005). Poplar roots are able to propagate at the water table, a property that was found to be beneficial for remediation of creosote-contaminated water over a four year growing season (Widdowson et al. 2005). Overall, when taking a phytoremediation strategy from the lab scale to practical field applications requires careful selection of appropriate plant species possessing suitable attributes. Poplar trees, due to their phreatophytic nature, grow quickly and produce relatively high biomass levels, a benefit for phytoremediation. Of course, it is critical that either the plant roots grow to the depth of the target contaminants, or the contaminated media move within range of the plants. Figure 5.1, illustrates the root depths of a few plants employed in phytoremediation and it is evident that poplars occupy a larger and deeper root zone compared to other species. It has been reported that poplar trees rooting depth potential can be in the range between 0,8-2,43 m (Dobson and Moffat 1995) in comparison to other plant species such Alfalfa, grasses and Indian mustard. Other advantages for using poplar trees in phytoremediation are ease of planting, their ability to transfer oxygen to the root zone for potential aerobic mineralization of organics, and their contribution to the accumulation of organic carbon from root debris in the rhizosphere, debris which subsequently hinders the movement of hydrophobic organics and provide nutrients to microorganisms. Enriched rhizospheric and endophytic bacterial communities of toluene-degrading bacteria have been reported in a BTEX – contaminated groundwater plume at a site planted with hybrid poplars (Barac et al. 2009). In this particular case, the numbers of toluene-degrading rhizospheric and endophytic bacteria were below the detection limit once the BTEX contaminated plume was remediated, illustrating the selective pressures exerted by exogenous nutrient sources. Table 5.1 is a brief summary of successful bioremediation projects for various organics such as toluene, MTBE, BTEX, explosives (TNT, RDX, HMX) using endophytic bacteria and poplar trees.

Table 5.3 Paradigms of poplar-associated endophytes with potential for bioremediation application.

Plants used	Compound	Bacteria used	Reference
Poplar (*Populus*)	Toluene	*Burkholderia cepacia* Bu61 (pTOM-Bu61)	(Taghavi et al. 2005)
Poplar (*Populus* cv. Hazendans)	MTBE, BTEX	*Pseudomonas* sp.	(Germaine et al. 2004; Moore et al. 2006)
Poplar (*Populus deltoides* x *nigra* DN34)	Explosives (TNT, HMX)	*Methylobacterium populi* BJ001	(Van Aken et al. 2004b)
Poplar (*Populus*)	TCE	*Enterobacter* sp. strain PDN3	(Kang et al. 2012a)
Poplar *(Populus deltoides* cv. Grimminge*)*	TCE	*Pseudomonas putida* W619	(Weyens et al. 2009a)
Hybrid poplar (*Populus deltoides* × *Populus nigra*)	PAH	*Burkholderia fungorum* DBT1	(Andreolli et al. 2013)
Poplar *(Populus nigra* cv. Brandaris)	Diesel fuel	*Achromobacter xylosoxidans*	(Tesar et al. 2002)

Although Jordahl et al. (1997) demonstrated that BTX (benzene, toluene, and xylene)–degrading microorganisms were five times more common in the rhizosphere of poplar trees than in the bulk soil when grown in uncontaminated soil, they did not quantify the direct effect of petroleum hydrocarbon contamination on fine root dynamics or the relative effects on soil properties. More recently the effects of varying concentrations of total petroleum hydrocarbons (TPH) and nutrients, on the spatial and temporal patterns of fine root production of hybrid poplar (*P. deltoides* × *P. petrowskyana* C. V. Griffin) have been investigated (Gunderson et al. 2008).

Interestingly, it was observed that fine root production increased linearly up to approximately 5000 mg kg^{-1} TPH, and then remained constant despite the potential for toxicity at higher concentrations.

A field trial, aiming to evaluate the response of different poplar clones to heavy metals and how metal uptake and its accumulation by plants can be affected by soil microorganisms, investigated soil microbial communities that were collected in proximity to the roots of large and small poplar plants (Gamalero et al. 2012); some taxa were always present, two species (*Chryseobacterium soldanellicola*

and *Variovorax paradoxus*) were only found in the soil where poplars (large or small) were growing, independently from the plant size, while bacterial strains of the genus *Flavobacterium* were prevalent in the soil with large poplar plants. Similarly, (Ulrich et al. 2008) found a high phylogenetic diversity of bacterial endophytes colonizing the aerial parts of poplar trees and showed a significant influence of the plant genotype on the endophytic bacterial communities.

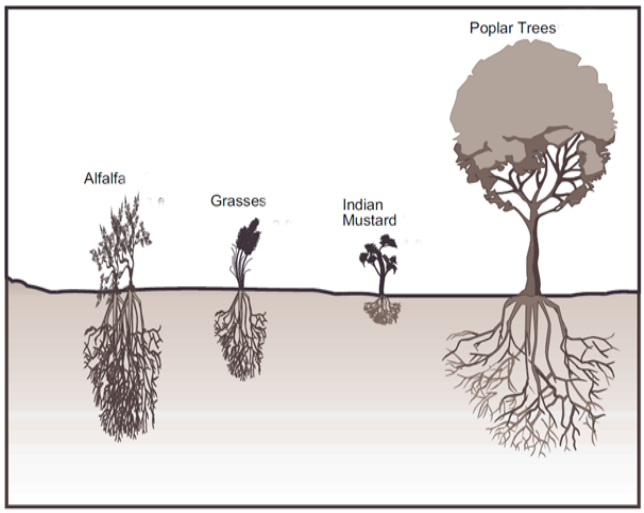

Figure 5.1 Root depth of various plant species involved in phytoremediation (Adams et al. 2000)

Endophytic root colonization of poplar trees follows a general model where initially bacteria move towards the plant roots either passively via soil water fluxes, or actively via specific induction of flagellar activity by plant-released compounds. Then, non-specific adsorption of bacteria to roots occurs, followed by anchoring that result in firm attachment of bacteria to root surfaces. Specific or complex interactions between bacteria and the host plant, such as the secretion of root exudates, may arise resulting in the induction of bacterial gene expression. Microscopic studies have been used to observe a similar phenomenon in other plant species (Germaine et al. 2004; Taghavi et al. 2009). Therefore, the acquired knowledge on the abundance and composition of bacterial endophyte communities is an indispensable for maximizing phytoremediation potential and plant health. A number of studies have

addressed the growth promoting potential of endophytes found in poplar trees by assaying for the production of ACC – deaminase, indole acetic acid (IAA) and the provision of fixed-nitrogen. Genera such as *Burkholderia*, *Sphingomonas*, *Pseudomonas*, *Serratia*, *Stenotrophomonas*, *Rahnella*, *Acinetobacter*, and *Rhizobium* (Doty et al. 2005; Doty et al. 2009; Taghavi et al. 2009; Xin et al. 2009b) have been found to be important in this respect.

Genome sequencing of *Enterobacter* sp. 638, which was isolated from the stem of a 10-year-old hybrid poplar (*Populus trichocarpa* × *P. deltoides* cv. H11-11) and known to improve poplar growth on marginal soils by as much as 40%, has revealed a remarkable interaction between this endophyte and its poplar host. Specifically, strain 638 has been found to produce plant growth promoting compounds only in the presence of plant synthesized compounds, such as sucrose, in the growth medium (Taghavi et al. 2010b).

Genome analysis revealed the presence of genes encoding putative proteins involved in root adhesion such as hemagglutinins, curli fibres, autotransporter adhesins (YadA), type I and IV pili, cellulose biosynthesis and capsular polysaccharide biosynthesis. Diesel fuel, on entering the terrestrial environment, will spread and seep into the soil. The downward migration of diesel fuel through the soil profile is generally limited due to the physical properties of the fuel (Adam and Duncan, 2002).

Specifically, diesel fuel will be adsorbed in the organic rich surface soil, impeding downward migration. This renders diesel fuel contaminated soil a likely candidate for phytoremediation as the contaminant plume is normally is held in the surface soil and within the rooting zone of most plant species.

As such, this type of contaminant is a good candidate for endophyte-assisted phytoremediation. The goal of this study was to determine if the inoculation of poplar cuttings with two previously characterized root endophytes (Section 3 & 4) would improve diesel oil biodegradation and promote plant growth.

5.2 Material and Methods

5.2.1 Bacterial strains and plant material

Poplar cuttings [*Populus deltoides x* (*trichocarpa x deltoides*) *cv Grimminge*] 30 cm long and about 1.0 cm in diameter were placed in aerated tap water for 3 weeks in order to allow for root formation and the development of fully

expanded leaves. The cuttings were inoculated with a bacterial consortium consisting of two root endophytes, namely *A. oleivorans* PF1 and *A. calcoaceticus* GK1, previously described for their ability to degrade diesel in liquid cultures and exhibit potential plant growth-promoting traits. Fresh cultures were grown in 869 rich medium (Mergeay et al. 1985) at 30^{o} C in a rotary shaker (200 rpm), until an approximate absorbance (A_{660}) value of 1 was reached. Afterwards, cells were collected by centrifugation (6000 rpm for 20 min at 4^{o} C), washed twice in physiological saline solution (NaCl 0.9% w/v), and re-suspended in the original volume of saline buffer. Cuttings were inoculated and kept under greenhouse conditions for 4 days in 1800 mL of half-strength sterile nutrient Hoagland's solution (Hoagland and Arnon 1950) to which 200 mL of the consortium was added (1/10 dilution – approximately 10^8 cells mL^{-1}). Uninoculated control plants were placed in the same solution without bacteria for the same time period. Plastic pots filled with 4 kg of sterile sandy soil were spiked with diesel oil from a petrol station (density = 0.833 g ml^{-1}) at two concentrations, namely 5000 mg kg^{-1} soil and 10000 mg kg^{-1} soil, respectively.
The amounts of added diesel were based on preliminary data from a range finding test performed for 3 weeks. To achieve a better distribution of diesel the soil was homogenized for 10 min using a concrete mixer. After a stabilization period of one week, poplar cuttings were planted on top of a drainage system (Figure 5.2), and the pots were placed in a greenhouse under the following conditions: temperature 25/18° C day/night, relative humidity 60/80 % day/night and a photosynthetic photon flux density of 400 µmol photon m^{-2} s^{-1} provided by natural illumination with cold white lamps (Philips SON-T AGRO 400, Belgium). At planting, 400 ml of half strength Hoagland's solutions was added and during the experimental period both planted and unplanted pots were bottom watered as required to maintain a 1-cm depth in the pot plates. In addition, once every two weeks 100 ml of half strength Hoagland's solution was added to the planted pots.

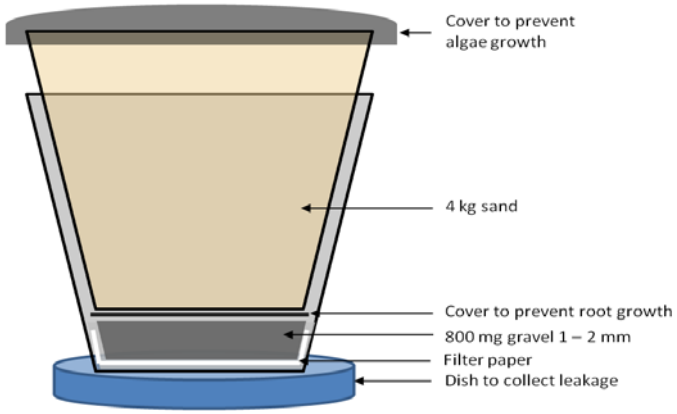

Figure 5.2 Drainage growth system was composed of filter paper and 800 mg gravel (1-2 mm). Cuttings were in 4 kg of sand and a dish was placed at the bottom to collect percolating water.

5.2.2 Experimental design and conditions

In order to investigate whether the endophytic consortium improved diesel degradation and promoted the production of plant biomass in the absence of diesel, 10 different treatments were conducted as shown in Table 5.2.

Table 5.4 Description of the treatments

Condition 1	Condition 2	Condition 3	Condition 4
Soil spiked with 5000 mg/Kg diesel	Soil inoculated with bacteria and 5000 mg kg^{-1} diesel	Soil planted with poplar cuttings and 5000 mg kg^{-1} diesel	Soil planted with poplar cuttings, inoculated with bacteria and 5000 mg kg^{-1} diesel
Condition 5	Condition 6	Condition 7	Condition 8
Soil spiked with 10000 mg kg^{-1} diesel	Soil inoculated with bacteria and 10000 mg kg^{-1} diesel	Soil planted with poplar cuttings and 10000 mg kg^{-1} diesel	Soil planted with poplar cuttings, inoculated with bacteria and 10000 mg kg^{-1} diesel
Condition 9		Condition 10	
Soil planted with poplar cuttings without bacteria and diesel		Soil planted with poplar cuttings inoculated with bacteria and without diesel	

For every condition 40 pots were employed, 10 for every sampling time (Day 0, 2 weeks, 6 weeks, 10 weeks) as shown in Figure 5.3.

Figure 5.3 Management of experimental conditions

5.2.3 Analytical Methods

Samples of sand–diesel mixture to be analyzed were prepared as follows: 4 kg of mixture from each pot were divided into quarters and each quarter was mixed individually. Two quarters were then mixed to form halves. Finally, the two halves were mixed to form a homogenous matrix. This procedure was repeated twice until the samples were adequately mixed. From each homogenized sample, 20g of sand were sampled from the selected contaminated pots and transferred in 25-ml glass tubes. These samples were stored at -20^{o} C before analysis. Diesel oil extraction performed as described by (Gandolfi et al. 2010). In detail, 2 g of sampled soil were added to 30 mL of n-hexane, sonicated for 30 min and filtered with anhydrous sodium sulphate. The extract was dried and resuspended in 10 mL n-hexane. The extracts were analyzed by GC/FID, using an Agilent Technologies 6890 N gas chromatograph equipped with a 30 m HP-5 column (0.32 mm I.D., 0.25 lm film thicknesses, Agilent Technologies, Santa Clara, CA, USA). GC analyses were performed according to two different oven temperature programmes, as follows: 40° C for 2 min, a 12 C/min ramp from 40° C up to 300° C and 300^{o} C for 5 min. For diesel oil quantification, the total area of the chromatogram was integrated, instead of evaluating single peaks. All experiments were carried out in three-fold replicates for every experimental condition

5.2.4 Microbial Activity

Microbial activity was measured through hydrolysis of fluorescein diacetate (FDA), as previously described by (Adam and Duncan 2001). Briefly, 1 g of soil

was transferred in a 125-ml Erlemeyer flask with 50ml of 60mM Na-Phosphate buffer, prepared by dissolving 0.477 moles of monosodium phosphate and 0.523 moles of disodium phosphate in 1L of distilled water (pH= 6.5-7.5). After that 500μl of freshly prepared FDA solution (stored at -30°C), was added. The FDA solution was prepared by mixing 20mg of FDA Powder in 10ml acetone, and flasks were swirled and incubated for 3 hours at 37^{o} C. In order to stop the reaction, 2ml of acetone was added to the flasks and a small amount was extracted, poured into a cuvette and the absorbance was measured at 490nm. All experiments were carried out in five-fold replicates for every experimental condition.

5.2.5 Analysis of plant growth

Before addition of diesel oil to the sandy soil, total plant biomass, M_0 (fresh weight), was measured. Plants were harvested and identical quantifications were done at the 2nd, 6th and 10th wk treatments for final plant biomass (M_1). The growth index was calculated as the (M_1 - M_0)/M_0, ratio. All experiments were carried out in ten-fold replicates for every experimental condition.

5.3 Statistical analysis

Statistical analysis of the data was through the Statistical package R version 3.1.0. Based on the fact that a Shapiro test showed that values were not normally distributed, a Wilcoxon Rank test was used to compare the means between the various treatments at 2, 6 and 10 weeks.

5.4 Results

5.4.1 Diesel degradation in pots containing sand spiked with 5000 mg kg^{-1} of diesel

Diesel biodegradation in pots containing 5000 mg kg^{-1} of diesel, at 2, 6 and 10 weeks in under 4 different experimental conditions (unplanted and uninoculated soil (control); inoculated and non-planted soil; planted soil without bacteria; and planted inoculated soil) was quantified as shown Figure 5.4. At 2 weeks, a higher percentage of degradation was measured for the non inoculated poplar

cuttings (35,72%) compared to the other conditions , followed by the inoculated poplar (33,30%), the inoculated non planted soil (19,33%), and controls (17,17%) (significance level p = 0.2). At 6 weeks, a higher percentage of degradation was measured for the inoculated poplar cuttings 43,46%, followed by 37,87% for the poplar cuttings alone, 35,31% for the inoculated soil, and 24,96% in controls (significance level:p=0.1). After 10 weeks, 54,57% degradation was measured in the poplar cuttings, 50,31% in the inoculated poplars, 40,31% in the inoculated soil, and 45,73% in the control (significance level p=0.1).

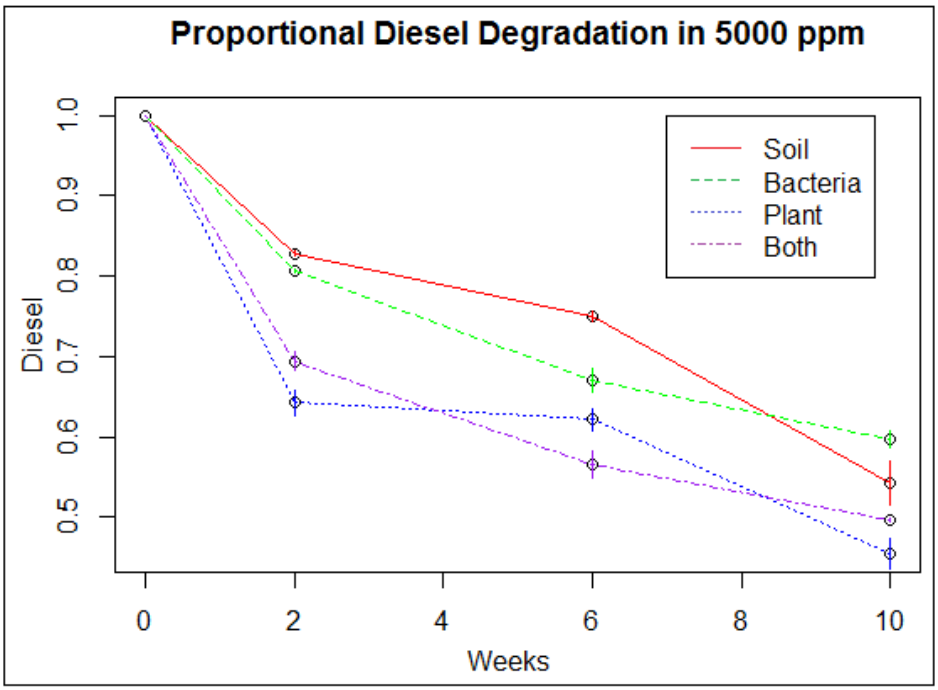

Figure 5.4 Diesel degradation at **5000 mg kg-1** measured by GC–FID in unplanted soil (control), inoculated unplanted soil (bacteria), planted non inoculated (Plant), and *Acinetobacter*-inoculated cuttings (Both) sandy pots at 2, 6 and 10 weeks (Error bars represent the standard deviation of the mean).

5.4.2 Diesel degradation in pots containing sand spiked with 10000 mg kg^{-1} of diesel

Similar to the previous condition the proportional biodegradation pattern of diesel in soil spiked with 10000 mg kg^{-1} diesel, at 2, 6 and 10 weeks in the 4

different conditions, namely unplanted and uninoculated soil (control), inoculated with bacteria and non-planted soil, planted soil without bacteria, and planted inoculated soil is reported in Figure 5.5. Interestingly, the biodegradation was constantly higher in the non-inoculated poplar cuttings compared to the inoculated ones. At 2 weeks degradation was measured in the non inoculated poplar cuttings at 36,50%, followed by the inoculated poplar with 25,19%, while in the inoculated non-planted soil it was 16,40%, and in the control 12,38%. At 6 weeks, the highest percentage of degradation was measured for the non-inoculated poplar cuttings 41,37%, followed by 30,11% for the inoculated poplar cuttings, 25,58% in the inoculated soil, and 15,96% for the controls. Finally at 10 weeks, 59,72% degradation was measured for the poplar cuttings, 57,75% for the inoculated poplars, 40% for the inoculated soil, and 49,14% in the controls. For diesel degradation in sand spiked at 5000 and 10000 mg kg^{-1} the difference of the mean between Poplar cuttings and Poplar-inoculated cuttings, seems to be non-significant (significance level p=0.1 for both tests).

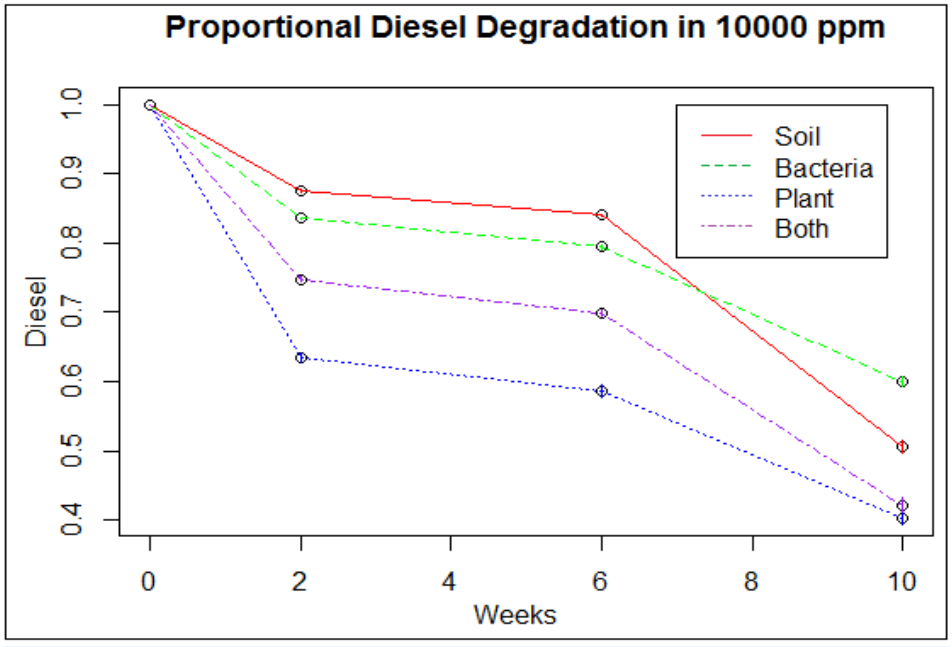

Figure 5.5 Diesel degradation at **10000 mg kg-1** measured by GC–FID in unplanted soil (control), inoculated (bacteria), planted non inoculated (Plant), and *Acinetobacter*-inoculated cuttings (Both) at 2, 6 and 10 weeks (Error bars represent the standard deviation of the mean).

5.4.3 Plant growth analysis

In order to quantify the growth index (GI) of poplar cuttings after 2, 6 and 10 weeks of growth without (control) or with diesel added at 5000 mg kg^{-1} and 10000 mg kg^{-1} , as well as in the absence and in the presence of the bacterial inoculum was measured. In more detail, Figure 5.6 shows that at 2 weeks the GI in the control increased as much as 14,95%, while for inoculated cuttings in the absence of diesel the GI was 29,54% (significance level:p=0.1). Diesel amendement exerted negative effects for both inoculated and non-inoculated cuttings.

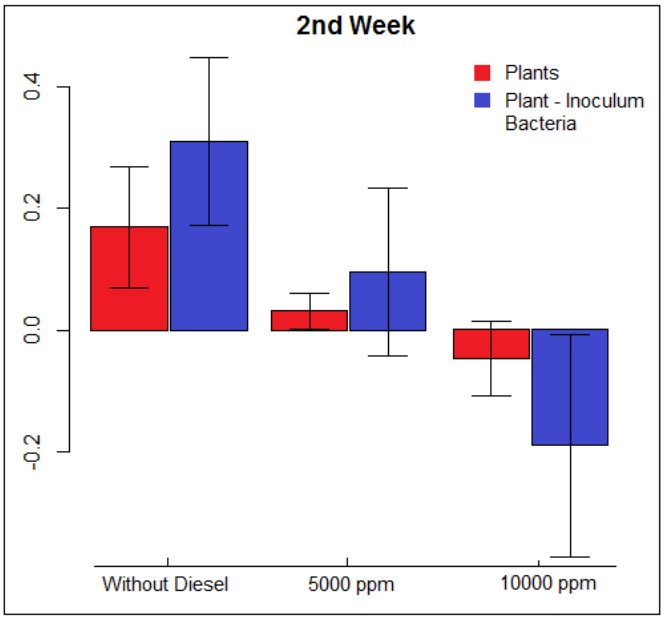

Figure 5.6 Total biomass growth index at 2 weeks (Error bars represent the standard deviation of the mean).

At 5000 mg kg^{-1} the inoculated cuttings increased their GI by 8,49%, while the non-inoculated by 2,66, however that is not statistically significant (significance level: p0.1). On the contrary, at 10000 mg kg^{-1} the GI of the non-inoculated cuttings was reduced 5,82%, whereas for the inoculated was 18,33% (p-value=0.06). At 6 weeks, Figure 5.7 shows that the GI for the control increased by 49,84% and for the inoculated 85,3% (significance level $p<0.01$). Remarkably, at 5000 mg kg^{-1} the GI for inoculated cuttings is increased by 59,95%, while in the non-inoculated cuttings diesel exerted a negative effect

and the reduction of GI is measured to be 15,33% (significance level: p=0.05). At the higher concentration, diesel was detrimental for both non-inoculated and inoculated cuttings. In fact for the former is measured equal to 18,86% and for the latter 30,4%.

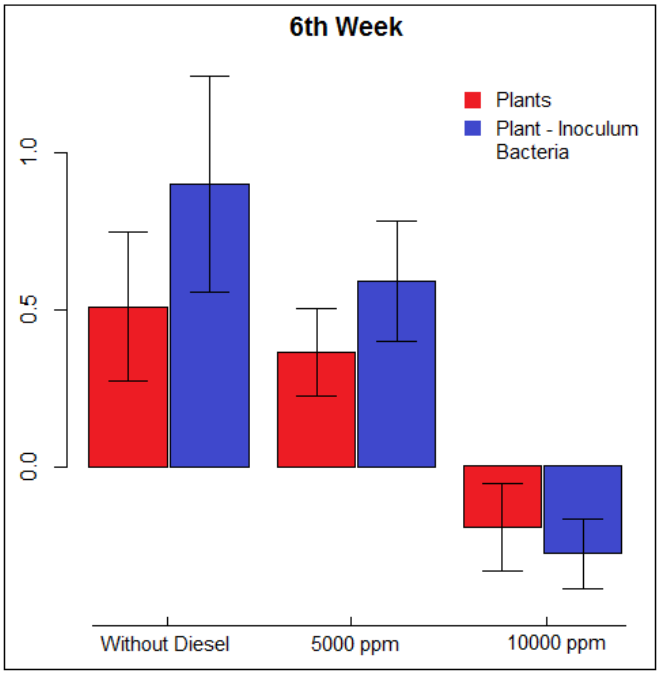

Figure 5.7 Total biomass growth index at 6 weeks (Error bars represent the standard deviation of the mean).

Interesting results emerged after 10 weeks both in the absence and presence of diesel (Figure 5.8). Specifically, in the absence of diesel at 2 and 6 weeks the inoculated cuttings had higher growth yields compared to the control, while at 10 weeks the control had significantly higher GI (112%) compared to the inoculated sample at 50,75% (significance level:p <0,01). Similarly, after 6 weeks of exposure to 5000 mg kg^{-1} diesel, the GI of inoculated cuttings was higher than the non-inoculated ones. Briefly, the GI of inoculated cuttings increased by 24,19% and the GI of the non-inoculated increased by 3,25%, a statistically significant difference (significance level:p=0,02). At 10000 mg kg^{-1} exposure, both non-inoculated and inoculated cuttings exhibited a decrease in total biomass at 6 weeks. The negative effect in the non-inoculated cuttings was

33,86% and for the inoculated was 50,33%. This interesting pattern is also statistically significant (significance level:p=0,03).

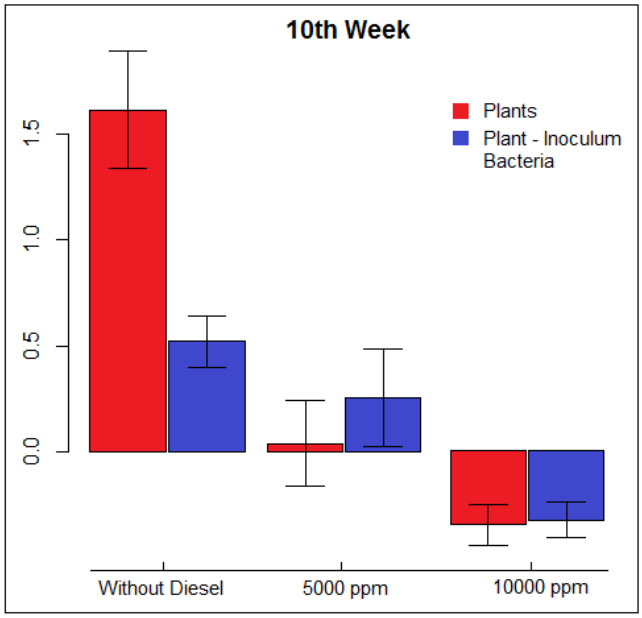

Figure 5.8 Total biomass growth index at 10 weeks (Error bars represent the standard deviation of the mean).

5.4.4 Microbial activity

In order to comprehend better the interaction between bacteria and plants at every experimental time point, microbial activity was monitored using fluorescein diacetate (FDA). At 2, 6 and 10 weeks, enzymatic activity in soil samples was assayed and measured spectrophotometrically as mg of reaction end-product h^{-1} g^{-1} dry soil. Fluorescein diacetate hydrolysis (FDAH) of colourless FDA to coloured fluorescein is catalyzed by both free and membrane-bound bacterial enzymes such as proteases, lipases and esterases (Dick 1997). In order to decipher whether the absence or presence of diesel oil at the two benchmark concentrations had an effect on the microbial activity with subsequent impact on the biodegradation rate and the growth index (GI), FDA was measured at 0, 2, 6 and 10 weeks. At 5000 mg kg-1 (Figure 5.9) the difference of the mean between poplar cuttings (Plant) and poplar-inoculated cuttings (Both) at 6 and 10 weeks were non-significant and significant,

respectively (p-value=0.05933, p-value=0.03175). At 10000 mg kg-1 (Figure 5.10) the differences of the means between poplar cuttings (Plant) and poplar-inoculated cuttings (Both), at 6 and 10 weeks were non-significant (p-value=0.402, p-value=0.675).

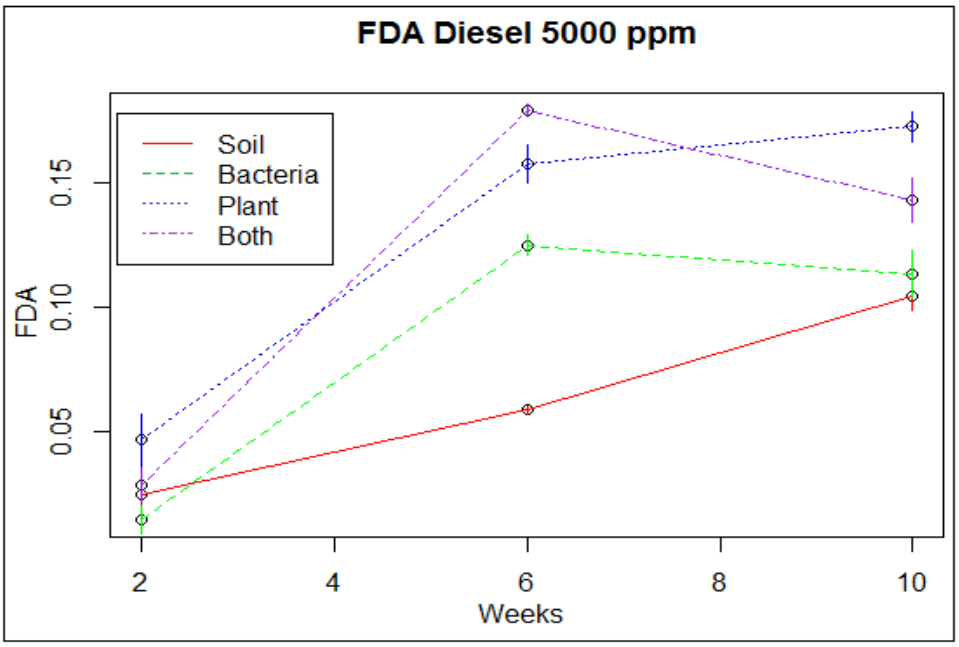

Figure 5.9 FDA at 5000 mg kg^{-1} diesel (Error bars represent the standard deviation of the mean).

The FDA in the absence of diesel oil was also measured to provide more information about the correlation between microbial activity and the amendment of diesel on the biodegradation rate and plant growth (Figure 5.11). The differences of the means between poplar cuttings (plants) and inoculated poplar cuttings (both) at 6 and 10 weeks were non significant (p-value=0.6752, p-value=0.5476).

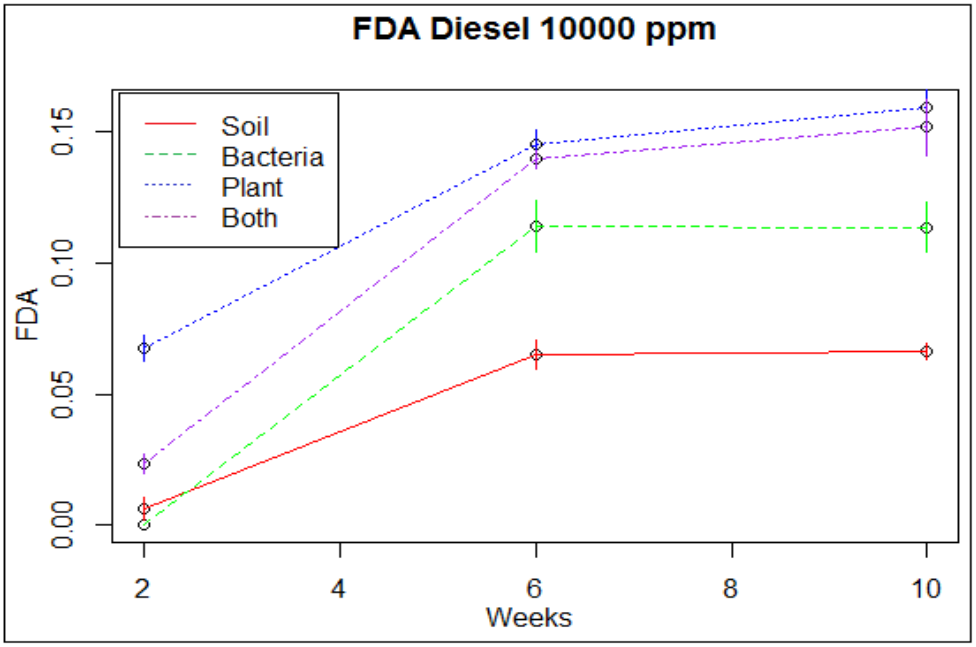

Figure 5.10 FDA at 10000 mg kg^{-1} diesel (Error bars represent the standard deviation of the mean)

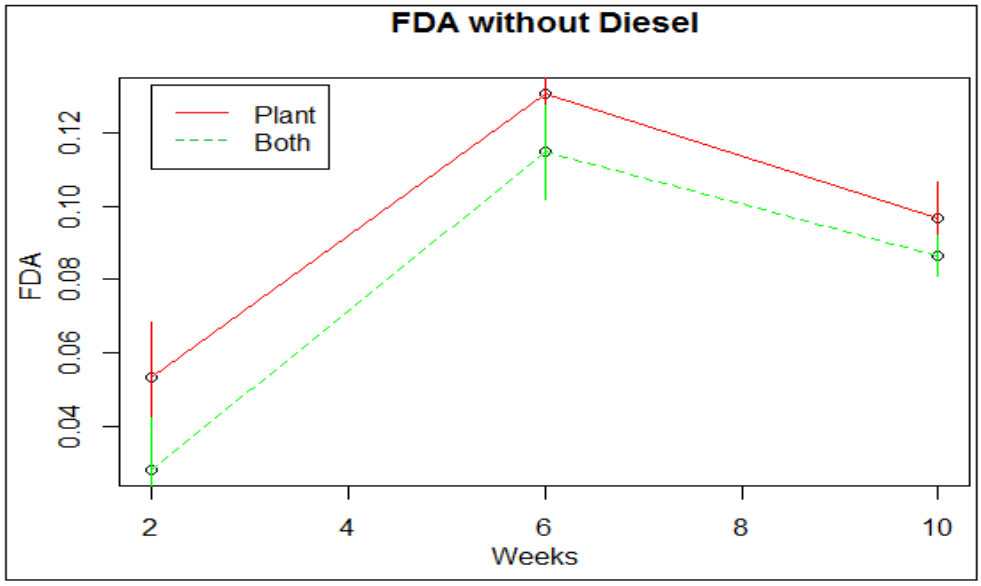

Figure 5.11 Microbial activity in the absence of diesel (Error bars represent the standard deviation of the mean).

5.5 Discussion

Generally, the interactions between bacteria and their host plants are renowneded for their complex natures, and when the organisms engaged in these interactions are under the selective pressure of a xenobiotic, the relationships often become more complicated in a genetic and biochemical sense. The idea of using plants alone (Gaskin and Bentham 2010; Lee et al. 2008; Rezek et al. 2008) or in combination with microorganisms (Becerra-Castro et al. 2013; Chaudhry et al. 2005; Zhang et al. 2010) to clean up environments contaminated with various organic contaminants is not new. Recently, in particular for petroleum hydrocarbons, it has been made evident that the combined use of plants and microorganisms with both strong degradation and plant growth promoting activities can accelerate hydrocarbon degradation and improve plants fitness (Khan et al. 2013a; Zhang et al. 2013). The latter finding is of great importance since the presence of petroleum hydrocarbons in soil deteriorates plant growth and, therefore, phytoremediation capacity (Andria et al. 2009; Yousaf et al. 2010).

Inoculation with *Gordonia* sp. S2RP-17 expressing ACC deaminase activity has been reported to enhance plant growth and hasten hydrocarbon degradation of maize (*Zea mays*) planted in diesel contaminated soil (Hong et al. 2011). Recently, the inoculation of carper grass (*Axonopus affinis*), planted in soil spiked with diesel, with *Pseudomonas* sp. ITRH25 (carrying the *alk*B gene for alkane degradation), *Pantoea* sp. BTRH79 (possessing alkane hydroxylase *CYP*153 genes and ACC deaminase activity) and *Burkholderia* sp. PsJN (showing ACC deaminase activity), demonstrated that the combined use of the three bacteria was more beneficial in terms of plant growth promotion and phytoremediation activity compared to inoculation with the bacterial strains individually (Tara et al. 2014).

The current study demonstrates the effects of applying two endophytic bacteria with a number of desirable traits ranging from diesel degradation capacity, production of biosurfactants and biofilms in the presence of diesel, and a suite of plant growth promoting peculiar traits such as the production of ACC deaminase and IAA, solubilization of inorganic P, mineralization of organic P, and production of siderophores, on phytoremediation of diesel.

Section 5

The results obtained in this study show that inoculation of *Populus deltoides* x (*trichocarpa* x *deltoides*) cv. Grimminge cuttings planted in sandy soil spiked with 5000 mg kg^{-1} diesel for a period of 10 weeks with a combination of *Acinetobacter oleivorans* PF1 and *Acinetobacter calcoaceticus* GK1 is a promising strategy to improve both plant growth and phytoremediation activity (Figure 5.12). In detail, throughout the experiment where plants were exposed to 5000 mg kg^{-1} diesel, plant growth biomass was consistantly higher for inoculated plants compared to the non-inoculated cuttings (Figure. 5.6, 5.7, 5.8). At 6 weeks (Figure. 5.4), the impact of inoculation was particularly strong and statistically significant (p-value=0,05) with the biomass of the inoculated cuttings increasing (59,95%) while the biomass of non-inoculated decreased (-15,33%). The extent of diesel degradation at 2 and 10 weeks showed that inoculation did not contribute to the diesel removal, but at 6 weeks the inoculated cuttings appeared to be slighly more effective, although not significantly (p-value=0.1). It is important to hihglight that non-inoculated poplars were able to increase diesel removal compared to both non-planted (control) pots and non-planted pots inoculated with bacteria. This lowering of the diesel concentration may be imputed either to the "rhizosphere effect", whereby the growth and metabolic activities of hydrocarbon degrading rhizospheric bacteria is enhanced (Leigh et al. 2006), or to the uptake and storage of the aromatic components of diesel by poplar roots as a number of studies indicate that PAHs with three or four aromatic rings can be taken up and stored by plant roots (Zhang and Zhu 2009), especially by poplar trees (Brentner et al. 2010). In order to explain the biodegradation pattern at this concentration (Figure 5.9), it is important to consider the bioavailability of diesel components in relation to bacterial utilization. Given the brief incubation period of 6 weeks, it is possible that the bacterial inocula were exhibiting an *r*-strategy behavior, whereby they were mostly in a resting phase with brief periods of activity stimulated by the appearance of an available substrate. It has previously been hypothesized that diesel degrading bacteria are likely to be *r*–strategists (Ciric et al. 2010) and generally, species belonging to the *Acinetobacter* genus are known to also be r-strategists (Yang and Lou 2011). On the contrary, strains belonging to high-G+C, mycolic-acid-containing actinomycetes (e.g. *Mycobacterium* and *Rhodococcus* spp.) are generally cnosidered K strategists

given their ability to thrive in enviroments with limited resources (Jacques et al. 2008; Margesin et al. 2003). Populations of K strategists are usually more stable or permanent in communities (Bordenave et al. 2007; Vinas et al. 2005).

Although, the presence of biosurfactants may be a precondition of effective bioremediation since the inoculum used here consists of strains that are positive for biosurfactant production (Section 3.5), care should be taken before exogenous biosurfactants are added. Indeed, increased biodegradation efficiency has been observed upon biosurfactant addition for slow-degrading consortia, while a notable decrease of biodegradation rate has been observed for fast degrading consortia (Owsianiak et al. 2009b; Owsianiak et al. 2009c). This may be explained by factors such as biosurfactant toxicity, micellar sequestration of hydrocarbon substrates, interference with oil-microbe hydrophobic interactions, or the preferential utilization of biosurfactants as a carbon and energy source (Chrzanowski et al. 2012; Van Hamme et al. 2006).

Exposure to diesel at 10000 mg kg^{-1} has been reported to cause phytotoxicity problems for various plant species (Kechavarzi et al. 2007; Merkl et al. 2005), including poplar trees (Tesar et al. 2002; Trapp et al. 2001). The results from this experiment are in agreement with these observations. In fact, at 6 and 10 weeks, the GI of the poplar cuttings deteriorated 18,86% and 33,86%, respectively. The observation that inoculation of the cuttings resulted in a more negative impact at 30,4% and 50,33% requires further investigation.

According to (Siciliano et al. 2001), the kind and amount of contaminant has a greater impact on the occurrence of catabolic rhizosphere bacteria as well as endophytes than the plant species. Moreover, the work of (Ciccillo et al. 2002) demonstrated that the effects of inoculation of *Burkholderia ambifaria* MCI 7 on maize can fluctuate from growth inhibition to growth promotion and that variability is hypothesized to be correlated with various abiotic and biotic factors. Indeed, is some cases endophytes, originally selected for beneficial effects due direct or indirect PGP traits, may depress growth detrimentally effecting host plants fitness (Kremer 2006; Oliveira et al. 2002). More recently, a weak phytoprotection effect of naphthalene-degrader strain *Pseudomonas putida* G7 lum towards pea plants exposed to naphthalene was correlated to the low root colonization of the strain at 10^1 CFU g^{-1}.

By comparing incubation times (Figure 5.5), it is obvious that the degradation rate, despite the inoculation of poplar cuttings, was not enhanced in the presence of 10000 mg kg^{-1} diesel oil. There are existing reports that point out that a critical factor for microbially enhanced phytoremediation is the capability of the inoculum to establish itself in the rhizosphere and effectively colonize endophytic compartments. The work of Tesar et al. (2002) showed that many culturable bacteria utilizing diesel fuel under laboratory conditions may not necessarily contribute to hydrocarbon remediation *in situ*, probably because of their inability to compete with authochthonous microorganisms. Likewise, in the work of Palmroth et al. (2002), it was concluded that the addition of microbial inocula did not enhance diesel fuel degradation, implying that the appropriate types and amounts of hydrocarbon degraders were already present in the soil. On the contrary, Andreolli et al. (2013) demonstrated that the ability of *Burkholderia fungorum* DBT1 to successfully infect the roots of poplar improved the phytoremediation efficiency of PAHs and decreasted PAH toxicity to plants as measured by root dry weight. The effectiveness of diesel bioremediation is often constrained by limitations of microbial ability due to the existence of numerous environmental and contaminant characteristics; thus, prior to fully implementing *in situ* bioremediation, the implications of soil matrix type, bioavailability, and TPH degradation activity should be evaluated in order to design the remediation approach in a realistic and evidence based way, therefore increasing the likelihood of success (Sutton et al. 2013).

Conclusively, the subject of research during this greenhouse experiment was to emphasize the interactions between a bacterial inoculum consisting of two endophytic strains, namely *Acinetobacter oleivorans* PF1 and *Acinetobacter calcoaceticus* GK1, known to posess various plant growth promoting and biodegradation traits, without any other interference known to stimulate the biodegradation process like fertilization, soil amendments, adjustment of pH, soil moisture etc. At the time of writing this thesis is not in my knowledge the application of a single strain or a consortium of strains holding peculiar traits of biodegradation as well as plant growth promotion based on shotgun-genome sequencing analysis, whereby a base reference about the causal agents of success or failure could be more easily deciphered.

Figure 5.12 Inoculated (back row) and non inoculated (front row) poplar cuttings at 5000 mg kg-1 at 6 weeks.

SECTION 6

Conclusions and future perspectives

Section 6

Introduction

The phenomenon of terrestrial ecosystems polluted by petroleum hydrocarbons along with the immense need for remediation has been widely reported (Banks et al. 2003; Glick 2010; Guo et al. 2012b; MacLeod et al. 2001; Stroud et al. 2007). Petroleum hydrocarbon (PHC) components can be biodegradable, recalcitrant or of intermediate biodegradability depending on their specific physico–chemical characteristics. Over the last years, extensive research has revealed that, among the different strategies to tackle hydrocarbon pollution, the application of soil, and plant–associated bacteria seems to useful as an eco–friendly, economically feasible and efficient restoration method (Al-Awadhi et al. 2012; Gerhardt et al. 2009; Ho et al. 2007; Khan et al. 2013a; McGuinness and Dowling 2009; Soleimani et al. 2010; Sorkhoh et al. 2011; Tang et al. 2010a).

Plant-associated bacteria include endophytic, phyllospheric and rhizospheric bacteria, and they have a variety of interactions with plants, ranging from being active pathogens, opportunist pathogens and bacteria that dwell within the plant for physical protection, to bacteria actively interacting with the host plant in mutually beneficial associations (Newman and Reynolds 2004; Weyens et al. 2009b).

The ability of bacteria to degrade petroleum hydrocarbons is attributed to the presence of catabolic genes and enzymes that allow them to utilize the complex chemicals found in petroleum mixtures as carbon and energy sources (Das and Chandran 2010; Rojo 2009). Many bacterial strains have been reported to encompass the metabolic pathways required for the degradation of the relevant hydrocarbons. Species of *Pseudomonas*, *Acinetobacter*, *Mycobacterium*, *Haemophilus*, *Rhodococcus*, *Paenibacillus* and *Ralstonia* belong to the most extensively studied bacteria (Tyagi et al. 2011). On the other hand, though a substantial number of hydrocarbons can be metabolized by bacteria, in the absence of plants this process is not always efficient enough due to the relatively low number of these microorganisms in bulk soil. Indeed, in the rhizosphere 10 to 1000 times higher microbial activity has been reported. Hence, the role of plants in remediation processes is important (Gaskin et al. 2008; Palmroth et al. 2002).

In order for plants to survive petroleum hydrocarbon contaminated environment, they should possess some fundamental characteristics: (i) tolerance to one or

more components of petroleum mixtures, (ii) high competitiveness, (iii) fast growth, and (iv) production and subsequent secretion of enzymes able to degrade a wide range of hydrocarbons. Moreover, plants can be positively affected directly or indirectly by the presence of bacteria able to elicit drastic modifications in the health status of the plant via the synthesis of various plant hormones, such as, indole-3-acetic acid (IAA), gibberellins (GAs) and cytokinins (CKs), suppression of stress ethylene production by 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity, nitrogen fixation and the mobilization of unavailable nutrients such as phosphorus and other mineral nutrients which can affect plant growth and development (Hardoim et al. 2008).

Thus, emphasis has been given to the application of bacteria possessing both plant growth-promoting as well as biodegradative activity (Gurska et al. 2009a; Huang et al. 2005; Huang et al. 2004). Figure 6.1 depicts the mechanisms behind plant-microbes enhanced remediation of petroleum hydrocarbons.

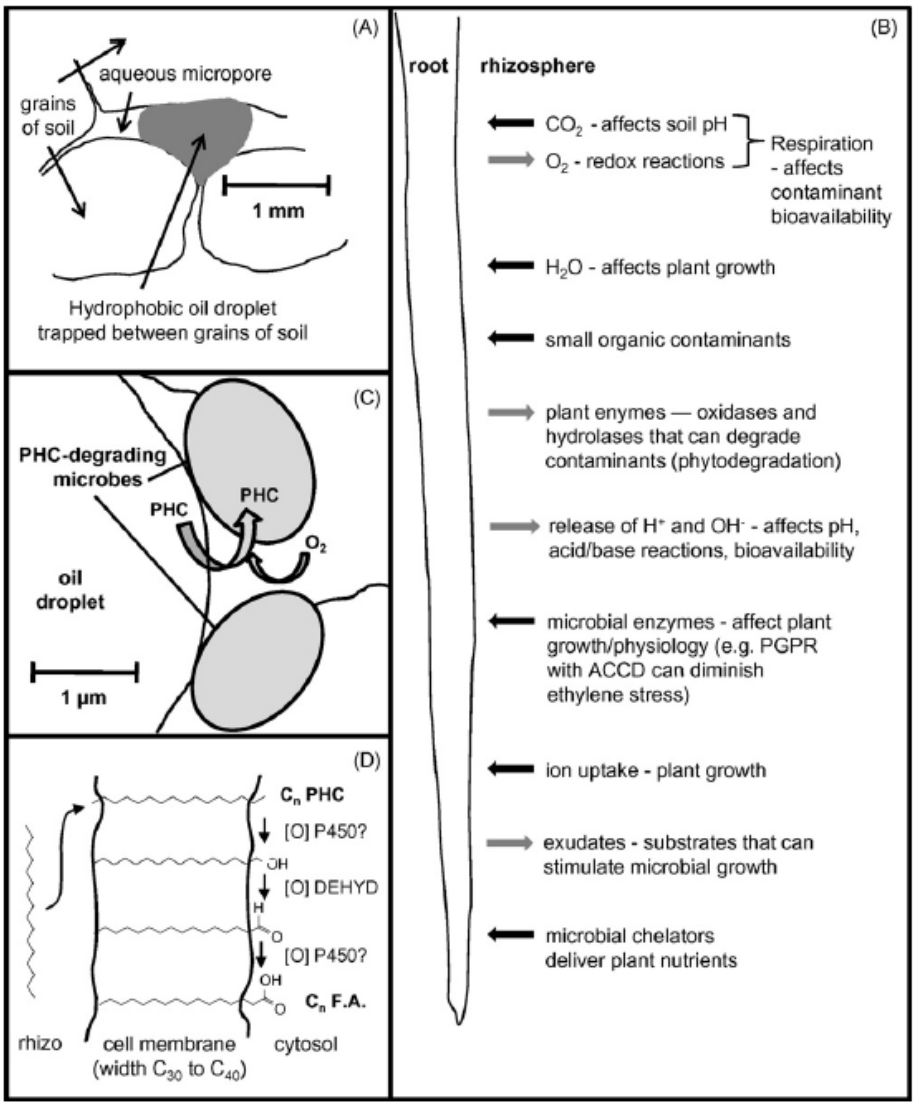

Figure 6.1 Mechanisms of plant-microbes involved in remediation of petroleum hydrocarbons: (A) Bioavailability of PHC, (B) General processes affecting rhizoremediation, (C) Aerobic PHC degradation, (D) Possible microbial oxygenation pathway of PHC (Gerhardt et al. 2009).

This thesis describes the design and application of bacterial consortia consisting of hydrocarbon degrading and plant growth promoting strains at sites contaminated by petroleum hydrocarbons. Information acquired by phenotypic characterization and single genome sequencing of the bacterial strains of concern was used for the design. As mentioned in the Introduction, in order for

the application of plant-bacteria associations as a method to tackle petroleum hydrocarbon contamination to be successful, the microorganisms selected must posses a number of traits including enzymatic degradation and plant growth promoting potential.

In Section 3 of this thesis, the soil, rhizospheric and endophytic bacteria isolated from poplar trees growing on a diesel-contaminated plume were characterized for their capacity to degrade diesel along and express a suite of plant growth promoting features. In order to evaluate the diesel degradation capacity of single isolates, the use of the 2,6-dichlorophenolindophenol indicator assay (see 3.3.3), revealed that among the 344 strains that were isolated, 30 strains were positive based on their ability to oxidize DCPIP from a blue to colourless form (Figure 3.10). These strains belong to the genera *Arthrobacter*, *Acinetobacter*, *Pseudomonas*, *Brevibacterium*, *Staphylococcus* and *Microbacterium*. Among these genera, *Pseudomonas*, *Arthrobacter* and *Acinetobacter* have been commonly reported as "key players" with respect to petroleum hydrocarbon degradation, while less information is available concerning *Staphylococcus*, *Brevibacterium* and *Microbacterium*. A thorough examination of the culturable hydrocarbon degrader isolated in this study revealed that many members have the potential to enhance host plant growth directly or indirectly, suggesting that diesel contamination has selected for a community enriched in plant growth promoters (Table 3.1). The poor survival of allochtonous degrading micro-organisms in a highly competitive environment has often been considered to be the most important reason for non successful performance of a bioremediation process (Yousaf et al. 2010). In fact, the activity of inoculated microorganisms can be inhibited by a variety of factors including the presence of toxic solvents and metals, the concentration and bioavailability of contaminants, the absence of key co-substrates and competition with the natural microbial flora. Therefore, for successful bioremediation, the selection of appropriate bacterial strains that, besides degrading pollutants in pure culture or in an axenic system, are also able to thrive under the often unfavorable conditions frequently found in natural soil for long periods of time, is crucial (Singer et al. 2005; Thompson et al. 2005). For these reasons, all members of the consortium isolated here were also examined with established protocols (see 3.3.5.1 - 3.3.5.9), for a number of traits (production of biosurfactants, formation of biofilms, tolerance to various

concentrations of solvents and metals, antibiotic sensitivity, quorum sensing signaling and chemotaxis), substantially involved in the foresaid processes. The results (see Appendix A) provided valuable information that was taken into consideration for the final selection of the most promising strains. Furthermore, statistical analyses suggested positive correlations between plant growth promoting capacity and solvent tolerance (Figure 3.2), plant growth promoting capacity and biofilm formation (Figure 3.3), biosurfactant production and quorum sensing (Figure 3.4), biosurfactant production and solvent tolerance (Figure 3.5), solvent tolerance and biofilm formation (Figure 3.6), solvent tolerance and heavy metal tolerance (Figure 3.7), and finally production of biosurfactants and biofilm formation (Figure 3.8).

Based on the results of the above-mentioned assays, two root endophytes (*Acinetobacter oleivorans* and *Acinetobacter calcoaceticus*) and one rhizospheric strain (*Staphylococcus aureus*), were selected for a more detailed examination of their biodegradation potential using GC-MS analysis of diesel fuel cultures (See 3.4). Among the selected strains, *Acinetobacter calcoaceticus* showed the best degradation properties and degraded about 41% of the initial hydrocarbon (1 g l^{-1}) after 10 days of incubation, followed by *Acinetobacter oleivorans* with 40%, while *Staphylococcus aureus* had weaker degradative potential since it degraded only 27% of the supplied hydrocarbons (Figure 3.10) during the same time period. Since monitoring of $OD_{600\ nm}$ is frequently used in studies characterizing the physiology of hydrocarbon utilization (Binazadeh et al. 2009; Bouchez-Naitali et al. 2008; Isaza and Daugulis 2009; Peng et al. 2007; Zeinali et al. 2007), the increase in biomass of *Acinetobacter calcoaceticus*, *Acinetobacter oleivorans*, and *Staphylococcus aureus* was quantified by measuring $OD_{600\ nm}$ at the start and the end of the experiment. In the case of *Acinetobacter calcoaceticus*, the $OD_{600\ nm}$ increased to 0.44, for *Acinetobacter oleivorans* $OD_{600\ nm}$ increased to 0.42, and for *Staphylococcus aureus*, to 0.81. The three selected strains *Acinetobacter oleivorans*, *Acinetobacter calcoaceticus* and *Staphylococcus aureus* also showed promising traits concerning the production of biosurfactants (Table 3.11), Quorum sensing, motility, biofilm formation and chemotaxis in the presence of diesel and hexadecane (Table 3.12), tolerance to various metals and solvents (Table 3.13), and antibiotic sensitivity (Table 3.14). Furthermore, *Acinetobacter oleivorans*, *Acinetobacter*

calcoaceticus and *Staphylococcus aureus* also showed promising plant growth promoting traits (Table 3.15).

Based on these promising results the next step was to perform whole genome shotgun sequencing of the two root endophytes, named from now on as *Acientobacter oleivorans* PF1 and *Acinetobacter calcoaceticus* PF2. Genomic DNA from both strains was extracted and a single 316v2-chip was used for sequencing on an IonTorrent PGM (Life Technologies Inc., Carlsbad, CA). In this work, the assembly and annotation data are presented for *Acinetobacter oleivorans* PF1, while for *Acinetobacter calcoaceticus* PF2 the work is ongoing.

For PF1, 2.8 million reads (mean length 303 bases) generated 853 Mb of data, of which 535,611 reads were assembled into 31 contigs, giving a consensus length of 3,766,014 bp at 43.5× coverage (see 4.2.1). Subsequently, the genomes of all fully sequenced and closely related bacterial strains on the NCBI database were downloaded and aligned to the *Acinetobacter oleivorans* PF1 data (see 4.2.2). In a next step, the gene context of gene clusters and the organization of specific operons was compared and for each gene and operon of interest the sequence of the genomic DNA fragment (”Query fragment”) was extracted, enlarged by two supplementary genes, one upstream and one downstream, prior to using BLAST to compare sequences to the NCBI bacterial genomes database. Matching sequences and gene coordinates of the best hits were downloaded to a GenBank file, and plotted against the ”Query fragments”. Although the generated data cover different physiological and biochemical aspects of the strain, my work focused on a list of 10 biochemical mechanisms involved in diesel degradation and in plant-growth promotion, which was developed by reviewing 24 published studies, carrying out keyword searches in the MetaCyc and NCBI databases (when no article was available), and through identification of 219 "query” genes (37 as single genes and 182 genes as a part of one of 38 operons) that have been shown to code for proteins that are key players for each of the mechanisms of interest (Supplementary Table 3).

The first investigated feature of *Acinetobacter oleivorans* PF1 was the basis of alkane degradation and revealed that in strain PF1 the *alk* operon is partially present, since among the 11 genes found in the genome of *Pseudomonas putida* Gpo1 (van Beilen et al. 2001), 9 are also present in the genome of *Acinetobacter oleivorans* PF1. However, it should be mentioned that they are not organized in

an operon, but are instead scattered throughout the genome (Figure 4.1). Besides this, another interesting feature of the *Acinetobacter oleivorans* PF1 genome is the presence of the flavin-binding monooxygenase gene, closely related to the *alm*A gene, encoding the first experimentally confirmed monooxygenase to be involved in the metabolism of long chain *n*-alkanes. With regard to degradation of aromatics, the main routes for degradation of aromatic compounds like naphthalene and phenanthrene were chosen for further investigation in *Acinetobacter oleivorans* PF1. The analysis pointed out that the lower naphthalene degradation pathway is present. In fact, all the genes related to the ortho-cleavage pathway are present, whilst the meta-cleavage pathway is incomplete. Notably, the ortho-cleavage pathway has been described in *Acinetobacter* sp. KS1 (Kim et al. 2003b), however, an analysis of the *Acinetobacter oleivorans* DR1 genome has revealed that the strain lacks naphthalene dioxygenase, the first enzyme involved in naphthalene degradation and is unable to grow on naphthalene. The gentisate pathway is also present, assuming that the first step (B1) can be performed by the proteins coding for by the *nahAaAbAcAd* genes found in the *Acinetobacter oleivorans* PF1 genome. A comparative genomic analysis between *Acinetobacter oleivorans* DR1 and *Acinetobacter baylyi* ADP1, *Acinetobacter calcoaceticus* PHEA-2, and *Acinetobacter baumannii* ATCC 17978 revealed that only strain DR1 possesses gentisate 1,2 diooxygenase and grows on gentisate (Jung et al. 2011). Moreover, genome annotation in *Acinetobacter oleivorans* PF1 indicated that the *wee* operon, which encodes the genes (*wza*, *wzb*, *wzc*, *wzx*, wzy) for emulsan biosynthesis is partially present. Another remarkable feature of this gene cluster is that there is apparently no transcription factor upstream of the operon (Figure 4.3). Interestingly, the phenotypic properties of strain *Acinetobacter oleivorans* PF1 such as biofilm formation, chemotaxis, motility and metal tolerance described in Section 3 were confirmed also by the annotation of the genome (see 4.3.4 and 4.3.5.6). These data highlight the metabolic capability of the strain to degrade aliphatic and aromatic components present in diesel oil, along with an affinity for and chemotaxis towards hydrocarbon substrates.

Since the plant growth promoting properties represent another pillar that can enhance bioremediation efficiency, it was important to analyze the genome for genes known to be involved in bacterial-plant interaction. With respect to

nutrient, availability the presence of phytate esterase in the *Acinetobacter oleivorans* PF1 genome (see 4.3.5.2) along with the positive phenotype (Table 3.15) implies that the strain indeed has phytase activity and, therefore, may effectuate the release of phosphorus via phytate degradation, which is the major component of organic P in soil. Indeed, strain *Acinetobacter oleivorans* PF1 possesses a gene responsible for the production of ACC-deaminase (Figure 4.5), and contains genes homologous to genes of the indole-3-pyruvic acid pathway (see 4.3.5.3). Next to these direct plant growth promoting mechanisms, the genome carries 2 operons putatively involved in the biosynthesis of 2 different siderophores, enterobactin and acinetobactin (see 4.3.5.5), together with the positive phenotype (Table 3.15), which indicates the potential for indirect plant growth promotion. The aforementioned characteristics prompted me to perform Average Nucleotide Identity (ANI) calculations (see 4.2.2) of *Acinetobacter oleivorans* PF1. Interestingly, the ANI calculation (see 4.2.2) delivered a similarity score of 91,83% (Figure 6.2) with *Acinetobacter oleivorans* DR1 (Jung et al. 2010). According to Chan et al. (2012), a combination of core genome phylogenetic analysis and ANI provides a reliable method for bacterial species delineation, whereby bacterial species are defined as monophyletic groups of isolates with genomes that exhibit at least 95% pair-wise ANI.

During this work, the genomes of thirteen *Acinetobacter* strains, including seven type strains, were sequenced to draft quality using 454 sequencing. Remarkably, the core genome phylogeny and ANI approach, revealed three misclassifications, one of which constitutes new species. Among these misclassifications, one refers to *Acinetobacter calcoaceticus* PHEA-2, since the core genome tree and comparisons of 16S rRNA gene has shown PHEA-2 to be closer to *Acinetobacter pittii* than to *Acinetobacter calcoaceticus*. It is worthwhile to mention, that strain PHEA-2 has been genome sequenced (Xu et al. 2003), and reported to convert benzoate to catechol, most likely via an ortho–cleavage pathway (Zhan et al. 2008).

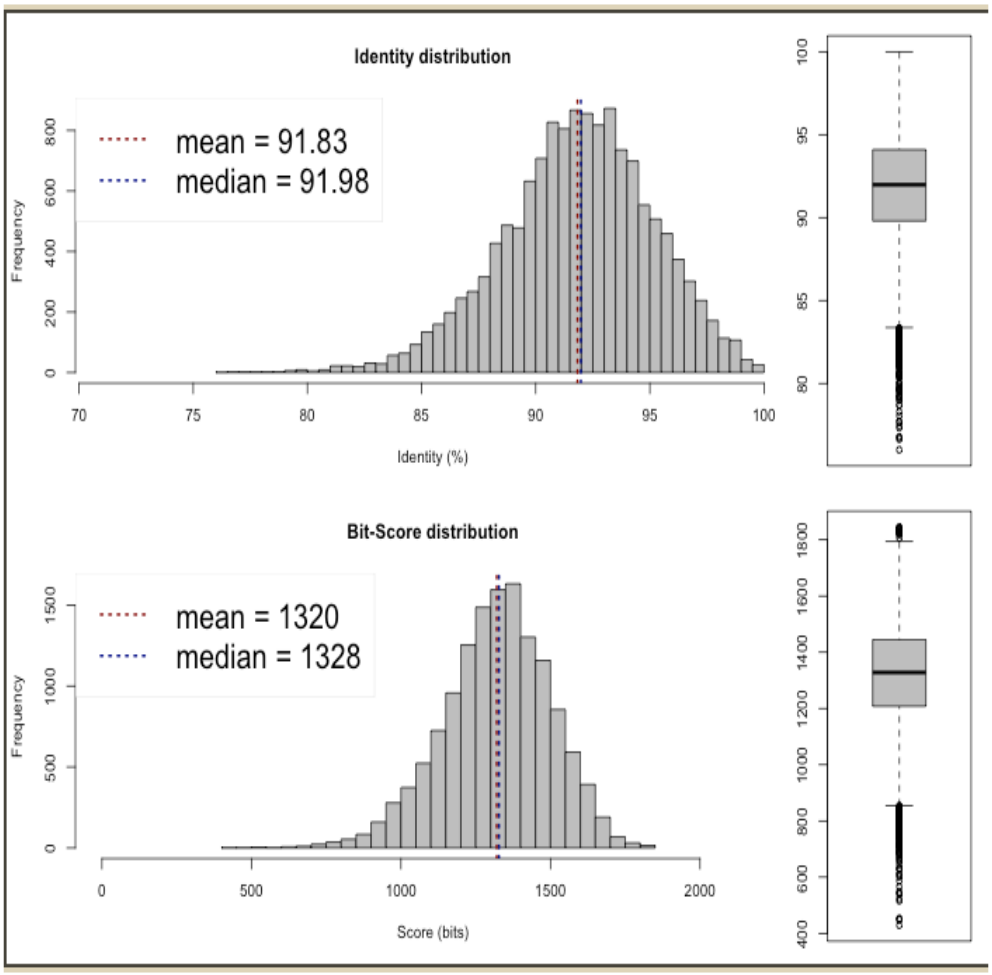

Figure 6.2 Average Nucleotide Identity (ANI) between *Acinetobacter oleivorans* PF1 and *Acinetobacter oleivorans* DR1.

In Section 5, a greenhouse experiment was conducted in order to investigate whether the inoculation of two endophytes, *Acinetobacter oleivorans* PF1 and *Acinetobacter calcoaceticus* PF2, may result in enhanced degradation of diesel oil along with improved biomass and fitness of poplar cuttings. With 5000 mg kg^{-1} diesel, a higher percentage of diesel was measured for the non-inoculated poplar cuttings at 2 and 10 weeks, while at 6 weeks the decrease tended to be higher for the inoculated poplar cuttings (43,5%), followed by 37,9% for the non-inoculated poplar cuttings (Figure 5.4); however this difference was not

statistically significant (p-value=0.1). At 10000 mg kg^{-1}, diesel removal was, at all measuring points higher, for the non-inoculated poplar cuttings compared to the inoculated ones (Figure 5.5). In parallel with monitoring the diesel removal, the growth indices (GI) of poplar cuttings were determined after 2, 6 and 10 weeks of growth without (control) or with 5000 mg kg^{-1} and 10000 mg kg^{-1} diesel added to the soil, both in the absence and in the presence of the bacterial inocula (see 5.4.3). In the absence of diesel at 2 weeks (Figure 5.6), the GI of inoculated cuttings (29,5%) seemed higher than the control (15%), however, this was not statistically significant (p-value=0.1). After 6 weeks (Figure 5.7), the GI of the control increased 49,84% and for the inoculated 85,3%, and that was statistically significant (p-value <0.01). A completely different result was observed at 10 weeks, where the control cuttings showed a significantly (p-value<0,01) higher GI than the inoculated ones (Figure 5.8). Throughout the experiment, in the presence of diesel at 5000 mg kg^{-1}, the GI of the inoculated cuttings is higher than the non-inoculated ones (Figures 5.6, 5.7, 5.8). Although at 2 weeks the difference is not statistically significant, at 6 weeks the GI for inoculated cuttings is about 60%, while in the non-inoculated diesel exposed cutting a negative GI of 15,3% (p-value=0.05); as such at 10 weeks the GI of inoculated cuttings (+24,2%) was significantly (p-value=0,02) higher than the non-inoculated ones (3,3%). The phenotypically (Table 3.15) and genotypically confirmed (Figure 4.5) potential of the selected bacteria to produce ACC-deaminase prompted me to suggest that diesel triggered the production of ACC deaminase, whereby the inoculated cuttings managed to ameliorate the induced by diesel toxicity. On the contrary, at 10000 mg kg^{-1}, the GI of the inoculated cuttings was constantly lower than the controls. Overall, the observed results are in agreement with the measured microbial activity in the soil at the corresponding time points (Figure 5.9).

Conclusions and future perspectives

The data presented in Section 3 of this thesis demonstrate the concept that key bacteria (*e.g*. *Acinetobacter*, *Pseudomonas*), known to be hydrocarbon degraders were the predominant ones isolated from poplars growing on a diesel contaminated location. Moreover, it is possible diesel oil has selected for a community rich in plant growth promoters. Besides this, the extensive screening carried out here revealed that the members of the community possess

numerous desirable properties such as production of biosurfactants, formation of biofilms, motility and chemotaxis towards hydrocarbons, tolerance to solvents and metals and resistance to various antibiotics. Although in literature it is widely acknowledged that the foresaid features substantially influence the fate of plant-bacteria synergies with respect to the degradation of petroleum hydrocarbons, the selection of promising candidates is being performed, without taking into account all of the involved parameters. Therefore, we propose a holistic selection protocol, in which the selection of bacteria for phytoremediation will take into account their biodegradation potential, their affinity and the tolerance to the contaminant, their resistance to other stress factors frequently found *in situ*, as well as their ability to promote, directly or indirectly, the growth of their host plant.

The advent of new genomics tools can help unravel the who, what, which and how: knowing who's provides important information on capabilities, knowing what key genes are present identifies the potential, while showing which genes are functional indicates who is active. Given this line of reasoning, Section 4 aimed to decipher the microbial "black box" of.Acinetobacter oleivorand PF1. Genome sequencing of the root endophyte *Acinetobacter oleivorans* PF1 provided interesting data not only about the ability of the strain to degrade various petroleum hydrocarbons, but about its ability to promote plant growth as well.

These peculiar characteristics together with the score from ANI calculations provides another piece of evidence that a phylogenetic analysis of the genus *Acinetobacter* based on the 16rRNA gene sequences does not provide reliable and informative results, while on the contrary a core genome phylogenetic tree provides concrete results that are in reverse compatible with established taxonomy. Taking into account all of these facts, together with the distinctive phenotypic traits observed, we can tentatively speculate that *Acinetobacter oleivorans* PF1 represents a new species. However, despite the availability of genome sequence data, bacterial taxonomy still remains a controversial topic (Coenye et al. 2005; Gevers et al. 2005; Jolley et al. 2012; Konstantinidis and Tiedje 2007; Rossello-Mora and Amann 2001), therefore, further experimentation is required including traditional approaches like phenotype and fatty acid profile, before an official announcement can be made.

Section 6

In Section 5, the main objective was to use a greenhouse experiment (Figure 6.3) to create a base reference about the effects of inoculation of a consortium consisting of sequenced bacterial strains. For that reason, a "worst case' scenario where poplar inoculated cuttings were planted into plastic pots filled with an acid washed sandy soil without nutrients was chosen. The development of such a system should help us to understand in detail the exact role of the factors involved to the success or failure of plant-bacteria remediation schemes. In fact, the exploration of poplar cutting–*Acinetobacter oleivorans* PF1 and *Acinetobacter calcoaceticus* PF2 associations provided promising data at the lower concentration of diesel (5000 mg kg^{-1}), especially concerning the level of plant stress alleviation (Figure 6.4). On the contrary, at the high diesel concentration neither degradation rate, nor plant growth were improved by poplar-*Acinetobacter* associations. For the remediation of sites contaminated by petroleum hydrocarbons through the use of plant-bacteria synergies, knowledge of both plants and bacteria is of utmost importance if good results are to be obtained. Although references in the literature about the implementation of metagenomic approaches in bioremediation (Fang et al. 2013; Iwai et al. 2010; Suenaga et al. 2007) are not rare, there still is a scarcity of information about the application of -omics technologies for understanding plant-bacteria interactions. A compilation of traditional and more modern technologies (the so-called –omics technologies) can provide valuable information about the interactions between plants and bacteria thriving in various unfavorable environments. Relatively recent reviews report that cultivation-independent molecular techniques have extended capabilities in this regard, and describe the potential for the integration of multiple molecular or computational techniques for a more effective evaluation of links between particular microbial populations, plants and ecosystem processes *in situ* (Cheng et al. 2010; Morales and Holben 2011; Suenaga 2012). Despite the consensus about the pivotal role of bacteria in the degradation of petroleum hydrocarbons, another perspective that should be envisaged is the involvement of fungi. The most toxic fraction of these petroleum hydrocarbons consist of Polycyclic Aromatic Hydrocarbons (PAH), and share structural similarities with lignin, a plant polyaromatic polymer. Forest saprophytic fungi, especially white-rot fungi, are known to produce powerful enzymes (peroxidases, laccases and mono- and di-oxygenases) that decompose

these compounds into metabolizable molecules (Kanaly and Hur 2006). Hence, white-rot fungi have been used in field trials of bioremediation for hydrocarbons, but with variable outcomes (Pointing 2001).

However, most of the work has been performed with three model species that are adapted to grow on woody material, but not in polluted soil. Deeper in the forest soil profile (in the humic layer and below) are found other fungi, called ectomycorrhizal (ECM), that live in symbiosis with trees. At this depth, most of these nutrients are embedded in a very recalcitrant matrix, and some of these fungi are able to take up N from complex humified soil extracts. They can do so using a combination of enzymes (different than white-rot fungi) and Fenton's reaction (Rineau et al. 2012), an enzyme-free mechanism that has been applied in the past to remediate soils contaminated with petroleum. ECM fungi have been found to degrade PAH in pure culture, even though in a lesser extent than saprophytic ones, but similar experiments in fungus-plant systems are very scarce (Meharg and Cairney 2000).

Soil bacteria, saprophytic and symbiotic fungi are all capable of degrading hydrocarbons in soil. Moreover, *in vivo*, they are associated, and form interdependent consortia with specific properties (de Boer et al. 2005; Tarkka et al. 2009). Several studies suggest that fungi and bacteria can have complementary activities for hydrocarbon degradation, directly (cooperative degradation) or indirectly (Frey-Klett et al. 2011).

Until now, there have been very few attempts yet to test fungal-bacterial co-cultures for hydrocarbon degradation. ECM-bacteria or saprophytic fungi-bacteria combinations yielded very variable results such as improved mineralization (Boonchan et al. 2000) and decreased mineralization (Gramss et al. 1999Gramss et al. 1999; Joner et al. 2006). Therefore, I strongly believe that phytodegradation of petroleum hydrocarbons using a bacteria/ECM/saprophytic fungus consortium, can be a promising strategy.

In such a complicated interaction the role of the above-mentioned –omics technologies, in terms of shedding light on the extremely complicated interactions, is expected to be of high importance.

SECTION 7

Literature

Section 7

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Section 7

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Section 7

SECTION 8

List of Publications and Scientific Manifestations

Section 8

Book Chapters

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Section 8

APPENDIX A Plant growth promoting activities and other traits of the 2,6 DCPIP positive strains.

	Genus	ACC Deaminase	IAA	Acetoin	Org. Acids	Inorganic P	Phytase	SID	Pectinase
Soil	*Arthrobacter* sp.	2+	+	-	-	-	+	+	+
Root	*Acinetobacter calcoaceticus*	2+	-	-	-	+	+	+	+
Root	*Acinetobacter calcoaceticus*	2+	-	-	-	+	+	+	+
Root	*Acinetobacter calcoaceticus*	2+	-	-	-	+	+	+	+
Root	*Staphylococcus aureus*	+	+	+	-	+	+	+	+
Root	*Acinetobacter oleovorans*	2+	+	-	+	+	+	+	+
Root	*Acinetobacter venetianus*	2+	-	-	-	+	+	+	+
Root	*Acinetobacter rhizosphaerae*	2+	-	-	-	+	+	+	+
Root	*Acinetobacter calcoaceticus*	2+	-	-	-	+	+	+	+
Root	*Pseudomonas* sp.	+	-	-	+	+	+	+	+
Root	*Pseudomonas putida*	+	-	-	-	-	+	+	+
Root	*Pseudomonas putida*	2+	-	-	-	+	+	+	+
Root	*Pseudomonas reinekei*	+	-	-	-	-	+	+	+
Root	*Pseudomonas fluorescens*	2+	-	-	-	-	+	+	+

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	Genus	ACC Deaminase	IAA	Acetoin	Org. Acids	Inorganic P	Phytase	SID	Pectinase
Root	*Staphylococcus aureus*	+	+	-	+	+	-	-	-
Root	*Pseudomonas* sp.	+	+	-	-	-	+	+	+
Root	*Pseudomonas koreensis*	+	+	-	-	+	+	+	+
Root	*Staphylococcus aureus*	+	+	-	-	+	+	+	+
Soil	*Staphylococcus aureus*	-	+	-	-	-	+	+	+
Soil	*Staphylococcus aureus*	+	-	-	-	+	+	+	+
Soil	*Acinetobacter calcoaceticus*	2+	-	-	-	+	-	+	-
Rhizosphere	*Pseudomonas brassicacearum*	3+	-	+	-	-	+	+	+
Rhizosphere	*Staphylococcus aureus*	+	-	-	+	-	+	+	+
Rhizosphere	*Pseudomonas* sp.	3+	-	-	-	-	+	+	+
Rhizosphere	*Acinetoabacter calcoacticus*	3+	-	-	-	+	+	+	-
Rhizosphere	*Acinetobacter* sp.	+	-	-	-	+	+	+	-
Rhizosphere	*Acinetobacter* sp.	+	-	-	-	-	+	+	-
Rhizosphere	*Brevibacterium* sp.	+	-	-	-	-	-	+	-
Rhizopshere	*Microbacterium hydrocarbonoxydans*	+	+	-	+	+	-	-	-

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	Genus	ACC Deaminase	IAA	Acetoin	Org. Acids	Inorganic P	Phytase	SID	Pectinase
Rhizopshere	*Microbacterium hydrocarbonoxydans*	+	+	-		+	-	-	-

APPENDIX A Plant growth promoting activities and other traits of the 2,6 DCPIP positive strains (continued).

	Genus	Lux screen assay			TLC overlay	T-Streak
		pSB401	**pSB536**	**pSB1075**		
Soil	*Arthrobacter* sp.	-	+	-	+	+
Root	*Acinetobacter calcoaceticus*	-	+	-	-	-
Root	*Acinetobacter calcoaceticus*	-	+	-	-	-
Root	*Acinetobacter calcoaceticus*	-	+	-	-	-
Root	*Staphylococcus aureus*	-	-	-	-	-
Root	*Acinetobacter oleovorans*	-	+	-	-	+
Root	*Acinetobacter venetianus*	-	+	-	+	+
Root	*Acinetobacter rhizosphaerae*	-	+	-	+	+
Root	*Acinetobacter calcoaceticus*	-	+	-	+	+
Root	*Pseudomonas* sp.	-	+	+	+	+
Root	*Pseudomonas putida*	-	+	+	+	+
Root	*Pseudomonas putida*	+	+	+	+	+

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	Genus	Lux screen assay			TLC overlay	T-Streak
		pSB401	pSB536	pSB1075		
Root	*Pseudomonas reinekei*	+	+	+	+	+
Root	*Pseudomonas fluorescens*	+	+	+	+	+
Root	*Staphylococcus aureus*	-	-	-	-	-
Root	*Pseudomonas* sp.	-	+	+	+	+
Root	*Pseudomonas koreensis*	-	+	+	+	+
Root	*Staphylococcus aureus*	-	-	-	+	-
Soil	*Staphylococcus aureus*	-	-	-	+	-
Soil	*Staphylococcus aureus*	-	-	-	+	-
Soil	*Acinetobacter calcoaceticus*	-	+	+	+	-
Rhizosphere	*Pseudomonas brassicacearum*	-	+	+	+	+
Rhizosphere	*Staphylococcus aureus*	-	-	-	+	-
Rhizosphere	*Pseudomonas* sp.	-	-	-	-	-
Rhizosphere	*Acinetoabacter calcoacticus*	+	+	-	+	+

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	Genus	Lux screen assay			TLC overlay	T-Streak
		pSB401	**pSB536**	**pSB1075**		
Rhizosphere	*Acinetobacter* sp.	-	+	-	-	+
Rhizosphere	*Acinetobacter* sp.	-	+	-	+	+
Rhizosphere	*Brevibacterium* sp.	+	+	-	-	-
Rhizopshere	*Microbacterium hydrocarbonoxydans*	-	-	-	-	-
Rhizopshere	*Microbacterium hydrocarbonoxydans*	-	-	-	-	-

APPENDIX A Plant growth promoting activities and other traits of the 2,6 DCPIP positive strains (continued).

	Genus	Motility assays			Solvent tolerance assays			
		Swimming	**Swarming**	**Twitching**	**n-hexane**	**octane**	**dodecane**	**n-hexane : cyclohexane**
Soil	*Arthrobacter* sp.	0 mm	8,3 mm	10,8 mm	10^{-1}	NG	10^{-2}	NG
Root	*Acinetobacter calcoaceticus*	7,4 mm	7 mm	10,6 mm	NG	10^{-6}	10^{-6}	NG
Root	*Acinetobacter calcoaceticus*	6,2 mm	7,2 mm	11,4 mm	NG	10^{-6}	10^{-6}	NG
Root	*Acinetobacter calcoaceticus*	7,2 mm	6,4 mm	12,2 mm	NG	10^{-6}	10^{-6}	NG
Root	*Staphylococcus aureus*	0 mm	0 mm	0 mm	10^{-4}	10^{-4}	10^{-4}	10^{-4}
Root	*Acinetobacter oleovorans*	26,6 mm	7,8 mm	11,6 mm	10^{-2}	10^{-6}	10^{-6}	10^{-3}
Root	*Acinetobacter venetianus*	7,4 mm	10 mm	10 mm	NG	10^{-6}	10^{-6}	NG
Root	*Acinetobacter rhizosphaerae*	6,8 mm	7,8 mm	10,8 mm	NG	10^{-6}	10^{-6}	NG
Root	*Acinetobacter calcoaceticus*	7,4 mm	5,2 mm	11 mm	NG	10^{-6}	10^{-6}	NG
Root	*Pseudomonas* sp.	0 mm	0 mm	0 mm	10^{-1}	10^{-3}	10^{-6}	10^{-3}
Root	*Pseudomonas putida*	12,3 mm	8 mm	11,7 mm	10^{-2}	10^{-2}	10^{-2}	10^{-2}
Root	*Pseudomonas putida*	12,3 mm	8 mm	11,7 mm	10^{-2}	10^{-2}	10^{-2}	10^{-2}
Root	*Pseudomonas putida*	24,3 mm	0 mm	0 mm	10^{-2}	10^{-2}	10^{-2}	10^{-2}

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	Genus	Motility assays			Solvent tolerance assays			
		Swimming	**Swarming**	**Twitching**	**n-hexane**	**octane**	**dodecane**	**n-hexane : cyclohexane**
Root	*Pseudomonas reinekei*	19 mm	0 mm	0 mm	10^{-5}	10^{-4}	10^{-5}	10^{-4}
Root	*Pseudomonas fluorescens*	32,7 mm	6,7 mm	11,3 mm	10^{-5}	10^{-5}	10^{-5}	10^{-5}
Root	*Staphylococcus epidermis*	22,3 mm	5,3 mm	9,7 mm	10^{-3}	10^{-3}	10^{-3}	10^{-3}
Root	*Pseudomonas* sp.	25,7 mm	13,4 mm	10,9 mm	10^{-6}	10^{-5}	10^{-6}	10^{-6}
Root	*Pseudomonas koreensis*	0 mm	0 mm	0 mm	NG	10^{-2}	10^{-2}	NG
Root	*Staphylococcus aureus*	0 mm	14,4 mm	0 mm	10^{-2}	10^{-3}	10^{-3}	10^{-1}
Soil	*Staphylococcus aureus*	0 mm	0 mm	0 mm	NG	10^{-3}	10^{-3}	NG
Soil	*Staphylococcus aureus*	0 mm	0 mm	0 mm	10^{-1}	10^{-3}	10^{-3}	10^{-1}
Soil	*Acinetobacter calcoaceticus*	7,2 mm	7,8 mm	12 mm	NG	10^{-6}	10^{-6}	NG
Rhizosphere	*Pseudomonas brassicacearum*	19,7 mm	15,4 mm	11,3 mm	10^{-5}	10^{-5}	10^{-5}	10^{-5}
Rhizosphere	*Staphylococcus aureus*	8,3 mm	0 mm	0 mm	NG	10^{-3}	10^{-4}	NG
Rhizosphere	*Staphylococcus aureus*	8,3 mm	0 mm	0 mm	NG	10^{-3}	10^{-4}	NG
Rhizosphere	*Pseudomonas* sp.	26,7 mm	14,8 mm	11,3 mm	10^{-6}	10^{-6}	10^{-6}	10^{-6}
Rhizosphere	*Acinetoabacter calcoacticus*	24,2 mm	9,6 mm	9,6 mm	10^{-2}	10^{-6}	10^{-6}	10^{-3}

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	Genus	Motility assays			Solvent tolerance assays			
		Swimming	**Swarming**	**Twitching**	**n-hexane**	**octane**	**dodecane**	**n-hexane : cyclohexane**
Rhizosphere	*Acinetobacter* sp.	21,4 mm	10,6 mm	10 mm	NG	10^{-6}	10^{-6}	NG
Rhizosphere	*Acinetobacter* sp.	20,6 mm	7,2 mm	10,2 mm	NG	10^{-6}	10^{-6}	NG
Rhizosphere	*Brevibacterium* sp.	7,4 mm	8,6 mm	10,6 mm	NG	10^{-6}	10^{-6}	NG
Rhizopshere	*Microbacterium hydrocarbonoxydans*	8mm	9mm	6mm	NG	10^{-3}	10^{-5}	NG
Rhizopshere	*Microbacterium hydrocarbonoxydans*	7mm	7mm	7mm	NG	NG	10^{-5}	NG

APPENDIX A Plant growth promoting activities and other traits of the 2,6 DCPIP positive strains (continued), where DC: Drop Collapse, Oil D:Oil Displacement, Hex: Hexadecane.

Genus		Biosurfactants Assays									
		DC SN	DC PELLET	Oil D Test SN	Oil D Test Pellet	EA Index SN		EA Index Pellet		Lipase	Lipase
						Hex	Diesel	Hex	Diesel		
Soil	*Arthrobacter* sp.	-	-	2,3 cm	0.5 cm	0%	<20%	<20%	<20%	-	-
Root	*Acinetobacter calcoaceticus*	-	2+	1.3 cm	-	55,56%	<20%	<20%	<20%	+	-
Root	*Acinetobacter calcoaceticus*	-	-	1 cm	-	55,56%	<20%	<20%	<20%	+	-
Root	*Acinetobacter calcoaceticus*	-	-	0.9 cm	0,3 cm	55,56%	<20%	<20%	<20%	+	-
Root	*Staphylococcus aureus*	2+	2+	1.1 cm	0,4 cm	66,67%	<20%	<20%	<20%	+	-
Root	*Acinetobacter oleovorans*	2+	-	1.7 cm	-	77,78%	<20%	<20%	<20%	2+	-
Root	*Acinetobacter venetianus*	-	-	0.9 cm	-	55,56%	<20%	<20%	<20%	+	-
Root	*Acinetobacter rhizosphaerae*	+	-	0.9 cm	-	55,56%	<20%	<20%	<20%	-	-
Root	*Acinetobacter calcoaceticus*	-	+	0.7 cm	-	44,44%	<20%	<20%	35%	-	-

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Genus		Biosurfactants Assays									
		DC SN	DC PELLET	Oil D Test SN	Oil D Test Pellet	EA Index SN		EA Index Pellet		Lipase	Lipase
						Hex	Diesel	Hex	Diesel		
Root	*Pseudomonas* sp.	-	+	1.2 cm	0,6 cm	0%	<20%	<20%	20%	+	-
Root	*Pseudomonas putida*	-	+	1.2 cm	-	66,67%	<20%	<20%	<20%	+	-
Root	*Pseudomonas putida*	2+	2+	1.1 cm	-	61,11%	<20%	<20%	<20%	-	-
Root	*Pseudomonas reinekei*	-	-	0.4 cm	-	0%	<20%	<20%	<20%	-	-
Root	*Pseudomonas fluorescens*	-	+	0.3 cm	0,3 cm	0%	<20%	<20%	<20%	-	-
Root	*Staphylococcus epidermis*	-	-	1.7 cm	0,3 cm	33,33%	<20%	<20%	<20%	-	-
Root	*Pseudomonas* sp.	-	-	O cm	0,4 cm	0%	<20%	<20%	<20%	-	-
Root	*Pseudomonas koreensis*	-	-	O cm	0.5 cm	0%	<20%	<20%	<20%	-	-
Root	*Staphylococcus aureus*	-	-	1.5 cm	-	16,67%	<20%	<20%	<20%	-	-
Soil	*Staphylococcus aureus*	-	-	0 cm	-	0%	<20%	<20%	<20%	-	-
Soil	*Staphylococcus aureus*	-	-	0 cm	-	0%	20%	<20%	<20%	-	-

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Genus		Biosurfactants Assays									
		DC SN	DC PELLET	Oil D Test SN	Oil D Test Pellet	EA Index SN		EA Index Pellet		Lipase	Lipase
						Hex	Diesel	Hex	Diesel		
Soil	*Acinetobacter calcoaceticus*	-	-	1.2 cm	-	61,11%	<20%	<20%	45%	+	-
Rhizosphere	*Pseudomonas brassicacearum*	2+	-	0 cm	0.5 cm	0%	<20%	<20%	<20%	-	-
Rhizosphere	*Staphylococcus aureus*	2+	-	1.3 cm	-	66,67%	<20%	< 20%	90%	-	-
Rhizosphere	*Pseudomonas* sp.	2+	-	0 cm	0,4 cm	0%	<20%	<20%	20%	-	-
Rhizosphere	*Acinetoabacter calcoacticus*	-	-	0.6 cm	-	55,56%	<20%	<20%	<20%	+	-
Rhizosphere	*Acinetobacter* sp.	2+	-	0.5 cm	0,4 cm	44,44%	<20%	<20%	<20%	-	-
Rhizosphere	*Acinetobacter* sp.	-	-	0.7 cm	-	16,67%	<20%	<20%	<20%	-	-
Rhizosphere	*Brevibacterium* sp.	-	-	1.4 cm	-	11,11%	<20%	<20%	<20%	2+	-
Rhizopshere	*Microbacterium hydrocarbonoxydans*	-	-	0.4 cm	-	66,67%	50%	<20%	<20%	-	-
Rhizopshere	*Microbacterium hydrocarbonoxydans*	-	-	0.4 cm	0,3 cm	50%	<20%	<20%	<20%	+	-

APPENDIX A Plant growth promoting activities and other traits of the 2,6 DCPIP positive strains (continued).

	Genus	Chemotaxis		Heavy Metal tolerence agar diffusion method				
		Diesel	Hexadecane	Pb	Cu	Zn	Ni	Cd
Soil	*Arthrobacter* sp.	-	-	2.5mM	2mM	1mM	0.5mM	0mM
Root	*Acinetobacter calcoaceticus*	-	-	1mM	1mM	1mM	1mM	0mM
Root	*Acinetobacter calcoaceticus*	+	-	1mM	1mM	1,5 mM	1mM	0mM
Root	*Acinetobacter calcoaceticus*	+	-	1mM	1mM	1,5 mM	1mM	0mM
Root	*Staphylococcus aureus*	-	-	3mM	2.5mM	2mM	1.5mM	0mM
Root	*Acinetobacter oleovorans*	+	-	1mM	1mM	1,5 mM	1mM	0mM
Root	*Acinetobacter venetianus*	+	-	1mM	1mM	1,5 mM	1mM	0mM
Root	*Acinetobacter rhizosphaerae*	-	+	1mM	1mM	1mM	0.5mM	0mM
Root	*Acinetobacter calcoaceticus*	-	+	1mM	1mM	1,5 mM	0.5mM	0mM
Root	*Pseudomonas* sp.	-	+	1mM	2mM	1mM	1mM	0mM
Root	*Pseudomonas putida*	+	+	1mM	0.5mM	0.5mM	0.5mM	0mM

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	Genus	Chemotaxis		Heavy Metal tolerence agar diffusion method				
		Diesel	Hexadecane	Pb	Cu	Zn	Ni	Cd
Root	*Pseudomonas putida*	-	-	1mM	0.5mM	1mM	0.5mM	0mM
Root	*Pseudomonas reinekei*	+	-	0.5mM	0.5mM	0.5mM	0.5mM	0mM
Root	*Pseudomonas fluorescens*	-	-	2.5mM	2mM	3.5mM	2mM	0.5mM
Root	*Staphylococcus aureus*	-	-	1mM	1.5mM	0.5mM	1mM	0mM
Root	*Pseudomonas* sp.	-	-	1.5mM	1.5mM	1.5mM	1.5mM	0mM
Root	*Pseudomonas koreensis*	-	-	2.5mM	1mM	1mM	1mM	0mM
Root	*Staphylococcus aureus*	-	-	1mM	1mM	1mM	0.5mM	0mM
Soil	*Staphylococcus aureus*	-	-	3mM	2mM	2mM	1.5mM	0mM
Soil	*Staphylococcus aureus*	-	-	2.5mM	2mM	2mM	1.5mM	0mM
Soil	*Acinetobacter calcoaceticus*	-	-	1mM	1mM	1mM	1mM	0mM
Rhizosphere	*Pseudomonas brassicacearum*	-	-	1mM	2mM	1.5mM	1mM	0mM
Rhizosphere	*Staphylococcus aureus*	+	-	2mM	2mM	2mM	1.5mM	0mM

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	Genus	Chemotaxis		Heavy Metal tolerence agar diffusion method				
		Diesel	**Hexadecane**	**Pb**	**Cu**	**Zn**	**Ni**	**Cd**
Rhizosphere	*Pseudomonas* sp.	-	-	2mM	2.5mM	3mM	1mM	0mM
Rhizosphere	*Acinetoabacter calcoacticus*	-	-	1mM	1mM	1,5mM	1mM	0mM
Rhizosphere	*Acinetobacter* sp.	-	-	1mM	1,5mM	1,5mM	0,5mM	0mM
Rhizosphere	*Acinetobacter* sp.	-	-	1mM	1mM	1mM	1mM	0mM
Rhizosphere	*Brevibacterium* sp.	-	-	1mM	1mM	4mM	1,5mM	0mM
Rhizopshere	*Microbacterium hydrocarbonoxydans*	-	-	1mM	1mM	1mM	1mM	0mM
Rhizopshere	*Microbacterium hydrocarbonoxydans*	-	-	1mM	1mM	1mM	1mM	0mM

APPENDIX B Genome Summary of Acinetobacter oleivorans PF1.

Mechanism	Enzyme	Pathway	Operon	Gene name	PFAM	Closest relative	Found by
Alkane degradation	Alkane monooxygenase	Alkane hydroxylase system	Alk	AlkB	Alkane hydroxylase	*A. baumanii*	Annotation
Alkane degradation	Rubredoxin 1	Alkane hydroxylase system	Alk	AlkF	-	-	Blast (*P.putida* GPo1 as a ref)
Alkane degradation	Rubredoxin 2	Alkane hydroxylase system	Alk	AlkG	Rubredoxin	*A. baumanii*	Blast (*P.putida* GPo1 as a ref)
Alkane degradation	Coniferyl-Aldehyde dehydrogenase=Aldehyde dehydrogenase	Alkane hydroxylase system	Alk	AlkH	ADH_CALDH_CalB	*A. baumanii*	Annotation
Alkane degradation	Aldehyde dehydrogenase	Alkane hydroxylase system	Alk	AlkH	ADH_CALDH_CalB	*A. calcoaceticus*	Blast (*P.putida* GPo1 as a ref)
Alkane degradation	Choline dehydrogenase=Alcohol dehydrogenase	Alkane hydroxylase system	Alk	AlkJ	GMC_OXRED_C	*A. calcoaceticus*	Blast (*P.putida* GPo1 as a ref)
Alkane degradation	3-methylmercaptopropionyl-CoA ligase (DmdB) =Acyl CoA synthetase	Alkane hydroxylase system	Alk	AlkK	ttLC_FACS_AEE21_like	*A. calcoaceticus*	Blast (*P.putida* GPo1 as a ref)
Alkane degradation	Outer membrane protein W precursor	Alkane hydroxylase system	Alk	AlkL	OmpW superfamily	*A. calcoaceticus*	Blast (*P.putida* GPo1 as a ref)
Alkane degradation	type IV pilus biogenesis protein PilJ =Methyl accepting trasnducer protein	Alkane hydroxylase system	Alk	AlkN	MCP_Signal	*A.* NIPH	Blast (*P.putida* GPo1 as a ref)
Alkane degradation	Transcriptional regulator, LuxR family	Alkane hydroxylase system	Alk	AlkS	REC/LuxR_C_Like	*A. calcoaceticus*	Blast (*P.putida* GPo1 as a ref)
Alkane degradation	Rubredoxin reductase (NAD+)	Alkane hydroxylase system	Alk	AlkT	Pyr_redox	*A. baumanii*	Blast (*P.putida* GPo1 as a ref)
Alkane degradation	benzoate dioxygenase, ferredoxin reductase component; Anthranilate dioxygenase reductase =Methane monooxygenase	Methane monooxygenase	mmo	mmo	fer2/BenDO_FAD_NAD	*A. calcoaceticus*	Blast (*M. capsulatus* as a ref)
Alkane degradation	Particulate methane monooxygenase	Methane monooxygenase	-	-	-	-	-

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Mechanism	Enzyme	Pathway	Operon	Gene name	PFAM	Closest relative	Found by
Alkane degradation	Organic solvent tolerance protein	-	ostA/imp	ostA	OstA/OstA_C	*A. calcoaceticus*	*E. coli*
Alkane degradation	Monooxygenase, flavin-binding family	Long chain alkane degradtion	-	almA	Pyr_redox_3	*A. calcoaceticus*	Blast (*A. baumanii* as a ref)
Alkane degradation	Aldehyde dehydrogenase	Long chain alkane degradtion		ald1	Aldedh	*A. calcoaceticus*	A. sp. M1
Biosurfactant production	Emulsan	putative galactoside acetyltransferase	wee	weeC	Hexapep	*A. oleivorans*	*A. lwofii*
Biosurfactant production	Emulsan	Peptidyl-prolyl cis-trans isomerase	wee	mip	FKBP_C/FKBP_N	*A. calcoaceticus*	*A. lwofii*
Biosurfactant production	Emulsan	Protein Tyrosine kinase	wee	wzc	Wzz/WNGR/AAA_31	*A. baumanii*	*A. lwofii*
Biosurfactant production	Emulsan	Putative protein tyrosine phosphatase	wee	wzb	LMWPc	*A. calcoaceticus*	*A. lwofii*
Biosurfactant production	Emulsan	Putative outer membrane protein	wee	wza	Poly_export	*A. pittii*	*A. lwofii*
Biosurfactant production	Emulsan	putative NDP-N-acetyl-D-galactosaminuronic acid dehydrogenase	wee	weeB	UGCP_MGDP_dh_N/UGCP_MGDP_dh/UGCP_MGDP_dh_C	*A. pittii*	
Biosurfactant production	Emulsan	putative NDP-N-acetyl-D-galactosaminuronic acid dehydrogenase	wee	weeB	UGCP_MGDP_dh_N/UGCP_MGDP_dh/UGCP_MGDP_dh_C	*A. pittii*	*A. lwofii*

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Mechanism	Enzyme	Pathway	Operon	Gene name	PFAM	Closest relative	Found by
Biosurfactant production	Emulsan	putative dTDP-glucose-4,6-dehydratase	wee	weeK	CoA_binding_3 /Polysacc_synt_2	*A. baumanii*	*A. lwofii*
Biosurfactant production	Emulsan	putative aminotransferase	wee	weeJ	DegT_DnrJ_EryC 1	*A. pittii*	*A. lwofii*
Biosurfactant production	Emulsan	putative emulsan repeating unit flippase	wee	wzx	Polysacc_synt	*A. baumanii*	*A. lwofii*
Biosurfactant production	Emulsan	putative galactose UDP transferase	wee	weeH	Bac_transf	*A. calcoaceticus*	*A. lwofii*
Biosurfactant production	Emulsan	UTP Glucose 1 phosphate	wee	galU	NTP_transferase	*A. calcoaceticus*	*A. lwofii*
Biosurfactant production	Emulsan	putative UDP-glucose dehydrogenase	wee	ugd	UGCP_MGDP_dh_ N /UGCP_MGDP_dh /UGCP_MGDP_dh _C	*A. calcoaceticus*	*A. lwofii*
Biosurfactant production	Emulsan	phosphoglucose epimerase	wee	pgi	PGI	*A. calcoaceticus*	*A. lwofii*
Biosurfactant production	Emulsan	putative glucose-4 epimerase	wee	galE	Epimerase /Epimerase_Csub	*A. calcoaceticus*	*A. lwofii*
Biosurfactant production	Emulsan	putative phosphoglucomutase	wee	pgm	PGM_PMM_II /PGM_PMM_III /PGM_PMM_IV		*A. lwofii*
Biosurfactant production	Emulsan	Putative UDP N Acetylglucosamine N epimerase	wee	weeA	epimerase_2	*A. calcoaceticus*	*A. lwofii*
Biosurfactant production	Emulsan	putative emulsan repeating unit polymerase	wee	wzy	O_Ag_pol_Wzy	-	*A. lwofii*
Biosurfactant production	Emulsan	putative glycosyl transferase	wee	weeD	(Glyco_transf_8 /Glyco_transf_1_4)	-	*A. lwofii*
Biosurfactant production	Emulsan	unknown protein	wee	weeE	ADH_N/ADH_zinc_ N/GFO_IDH_MocA / GFO_IDH_MocA_C	-	*A. lwofii*

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Mechanism	Enzyme	Pathway	Operon	Gene name	PFAM	Closest relative	Found by
Biosurfactant production	Emulsan	unknown protein	wee	weeF	Hepar_II_III	-	*A. lwofii*
Biosurfactant production	Emulsan	putative glycosyl transferase	wee	weeG	Glyco_transf_4 /Glyco_transf_1_ 4	-	*A. lwofii*
Biosurfactant production	Emulsan	putative acetyltrasnferase	wee	weeI	Hexapep	-	*A. lwofii*
PAH degradation	putative 4-hydroxybenzoyl-CoA thioesterase	catechol degradation	-	-	4HBT	*A. calcoaceticus*	Blast (A. *baumanii* as a ref)
PAH degradation	putative 4-hydroxybenzoyl-CoA thioesterase	catechol degradation	-	-	4HBT	*A. calcoaceticus*	Blast (*A. baumanii* as a ref)
PAH degradation	Fumarylacetoacetate hydrolase family protein	Gentisate degradation	-	-	FAA_Hydrolas superfamily	*A. calcoaceticus*	Blast (*P.putida* as a ref)
PAH degradation	Salicylate hydroxylase (Salicylate pathway)	Salicylate degradation	nah	nahG,n ahW	FAD_Binding_3	*A. calcoaceticus*	*Pseudomonas* spp.
PAH degradation	Maleylacetoacetate isomerase (EC 5.2.1.2) @ Glutathione S-transferase, zeta (EC 2.5.1.18)	Gentisate degradation	-	-	GST_N_Zeta /GST_C_Zeta	*A. calcoaceticus*	Blast (*P.putida* as a ref)
PAH degradation	Catechol 2,3 dioxygenase (Salicylate pathway)	Salicylate degradation	nah	nahH	Glyoxalase	*A. calcoaceticus*	*Pseudomonas putida*
PAH degradation	2-Hydroxybenzalpyruvate aldolase (naphthalene pathway) [Dihydrodipicolinate synthase]	Naphthalene degradation	nah	nahE	DHDPS	*A. oleivorans* DR1	*Pseudomonas putida*
PAH degradation	2-Hydroxymuconic semialdehyde dehydrogenase (Salicylate pathway)	Salicylate degradation	nah	nahN	Abhydrolase_6	*A. calcoaceticus*	*Pseudomonas putida*
PAH degradation	Iron-Sulfur protein small subunit (naphthalene pathway) [Anthranilate dioxygenase small subunit]	Naphthalene degradation	nah	nahAd	Ring_Hydroxyl_B	*A. oleivorans* DR1	*Pseudomonas putida*

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Mechanism	Enzyme	Pathway	Operon	Gene name	PFAM	Closest relative	Found by
PAH degradation	cis-Naphthalene dihydrodiol dehydrogenase (naphthalene pathway)	Many potential genes, display only the 2 most liekly because of operon	nah	nahB,doxB, phnB	ADH_Short(no_C2)	*A. baumanii*	*Pseudomonas* spp. /*Burholderia* spp.
PAH degradation		catechol degradation	phe	pheB	Dioxygenase_N /Dioxygenase_C	*A. calcoaceticus*	Blast (*P. putida* aa a ref)
PAH degradation	Fumarylacetoacetate hydrolase family protein	Gentisate degradation	nah	nagK	FAA_Hydrolas superfamily	*A. nosocomialis*	-
PAH degradation	cis-Naphthalene dihydrodiol dehydrogenase (naphthalene pathway)	Many potential genes, display only the 2 most liekly because of operon	nah	nahB,doxB,phnB	ADH_Short(no_C2)	*A. calcoaceticus*	*Pseudomonas* spp. /*Burholderia* spp.
PAH degradation	Iron-Sulfur protein large subunit (naphthalene pathway) [benzoate 1,2 dioxygenase]	Naphthalene degradation	nah	nahAc	Rieske /Ring_Hydroxyl_A	*A. baumanii*	*Pseudomonas putida*
PAH degradation	nah gene regulator	Salicylate degradation	nah	nahR	HTH_1 /LysR_substrate	*A. oleivorans* DR1	*Pseudomonas putida*
PAH degradation		catechol degradation	phe	pheA	CM_2/PDT/ACT	*A. oleivorans* DR1	Blast (*P. putida* aa a ref)
PAH degradation	salicylaldehyde dehydrogenase (naphthalene pathway)	Naphthalene degradation	nah	nahF,doxF	Aldedh	*A. calcoaceticus*	*Pseudomonas* spp.
PAH degradation	Chloroplast-type ferredoxin (Salicylate pathway)	Salicylate degradation	nah	nahT	Fer2	*A. baumanii*	*Pseudomonas putida*
PAH degradation	Reductase (naphtalene pathway)	Naphthalene degradation	nah	nahAa, doxA	fer2/FAD_Binding_6 /NAD_Binding_1	A. baumanii	*Pseudomonas* spp.
PAH degradation	cis-Naphthalene dihydrodiol dehydrogenase (naphthalene pathway)	Many potential genes, display only the 2 most liekly because of operon	nah	nahB,doxB,phnB	ADH_Short(no_C2)	A. calcoaceticus	*Pseudomonas* spp. /*Burholderia* spp.
PAH degradation	Iron-Sulfur protein small subunit (naphthalene pathway) [Benzoate 1,2 dioxygenase beta subunit]		nah	nahAd	Ring_Hydroxyl_B	*A. calcoaceticus*	*Pseudomonas putida*

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Mechanism	Enzyme	Pathway	Operon	Gene name	PFAM	Closest relative	Found by
PAH degradation	Anthranilate 1,2 dioxygenase [Iron-Sulfur protein large subunit (naphthalene pathway)]	Naphthalene degradation	nah	nahAc	Rieske /Ring_Hydroxyl_A	*A. calcoaceticus*	*Pseudomonas putida*
PAH degradation	Rhamnosyl transferase 1 (biosurfactant synthesis)	Surfactant synthesis	Rhl	RhlA	Abhydrolase_6	*A. calcoaceticus*	*Pseudomonas aeruginosa*
PAH degradation	Ferredoxin (naphthalene pathway)	Naphthalene degradation	nah	nahAb	Rieske	*A. calcoaceticus*	*Pseudomonas putida*
PAH degradation	Laccase domain protein	Salicylate degradation	Pa	PA4543	Cu-oxidase_4	*A. baumanii*	*Pseudomonas aeruginosa*
PAH degradation	Gentisate 1,2 dioxygenase	Gentisate degradation	-	-	Cupin_2	-	Blast (*A. oleivorans* or *baumanii* as a ref)
PAH degradation	Rhamnosyl transferase 2 (biosurfactant synthesis)	Surfactant synthesis	Rhl	RhlB	Glyco_transf_28	-	*Pseudomonas aeruginosa*
PAH degradation	Rhamnosyl transferase 2 (biosurfactant synthesis)	Surfactant synthesis	Rhl	RhlC	Glyco_transf_2_3	-	*Pseudomonas aeruginosa*
PAH degradation	1.2 Dihydroxynaphthalene oxygenase (naphthalene pathway)	Naphthalene degradation	nah	nahC	Glyoxalase	-	*Pseudomonas putida*
PAH degradation	2-Hydroxychromene-2-carboxylate isomerase (naphthalene pathway)	Naphthalene degradation	nah	nahD,doxD	DSBA	-	*Pseudomonas* spp.
PAH degradation	2-oxo-4-pentanoate hydratase (Salicylate pathway)	Salicylate degradation	nah	nahL	FAA_hydrolase	-	*Pseudomonas putida*
PAH degradation	Acetaldehyde dehydrogenase (Salicylate pathway)	Salicylate degradation	nah	nahO	Semialdehyde_dh /AcetDehyd-dimer	-	*Pseudomonas putida*
PAH degradation	4-Hydroxy-2-oxovalerate hydrolase (Salicylate pathway)	Salicylate degradation	nah	nahM	HMGL-like /DmpG_comm	-	*Pseudomonas putida*
PAH degradation	4-Oxalocrotonate decarboxylase (Salicylate pathway)	Salicylate degradation	nah	nahK	FAA_hydrolase	-	*Pseudomonas putida*
PAH degradation	4-Oxalocrotonate tautomerase (Salicylate pathway)	Salicylate degradation	nah	nahJ	Tautomerase	-	*Pseudomonas putida*
PAH	Extradiol dioxygenase/ring fission	Salicylate degradation	phn	phnC/p	LigB	-	*Burkholderia sartisoli*

degradation	dioxygenase			bhA			*/Sphingomonas paucimobilis*
Mechanism	**Enzyme**	**Pathway**	**Operon**	**Gene name**	**PFAM**	**Closest relative**	**Found by**
PAH degradation	2-Hydroxymuconic semialdehyde dehydrogenase (Salicylate pathway)	Salicylate degradation	nah	nahI	Aldedh		*Pseudomonas putida*
PAH degradation	Dyp-type peroxidase	Aromatic compounds degradation	dyp	-	Dyp_Perox	*A. calcoaceticus*	-
ACC degradation	ACC deaminase	-	-	-	PALP	*A. calcoaceticus*	*Acinetobacter oleivorans*
Phosphorus uptake	Enzyme	Phytase esterase	-	-	Phytase-like	*A. calcoaceticus*	*Acinetobacter oleivorans*
Phosphorus uptake	Enzyme	Inorganic pyrophosphatase (EC 3.6.1.1)	-	-		*A. calcoaceticus*	Annotation
Phosphorus uptake	Enzyme	Alkaline phosphatase (EC 3.1.3.1)	-	-			Annotation
Phosphorus uptake	Enzyme	Exopolyphosphatase (EC 3.6.1.11)	-	-			Annotation
Phosphorus uptake	Phosphate transporter, high affinity	Phosphate transport system regulatory protein PhoU	-	-			Annotation
Phosphorus uptake	Phosphate transporter, low affinity	Sodium-dependent phosphate transporter	-	-			Annotation
Phosphorus uptake	Regulatory element	Phosphate regulon transcriptional regulatory protein PhoB (SphR)	-	-			Annotation
Phosphorus uptake	Regulatory element	Phosphate regulon sensor protein PhoR (SphS) (EC 2.7.13.3)	-	-			Annotation
Phosphorus uptake	Regulatory element	Soluble pyridine nucleotide transhydrogenase (EC 1.6.1.1)	-	-			Annotation

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Mechanism	**Enzyme**	**Pathway**	**Operon**	**Gene name**	**PFAM**	**Closest relative**	**Found by**
Phosphorus uptake	Regulatory element	Phosphate regulon transcriptional regulatory protein PhoB (SphR)	-	-			Annotation
Phosphorus uptake	Enzyme	Alkaline phosphatase	-	-			Annotation
Phosphorus uptake	Phosphate transporter, high affinity	Phosphate transport ATP-binding protein PstB (TC 3.A.1.7.1)	-	-			Annotation
Phosphorus uptake	Phosphate transporter, high affinity	Phosphate transport system permease protein PstA (TC 3.A.1.7.1)	-	-			Annotation
Phosphorus uptake	Phosphate transporter, high affinity	Phosphate transport system permease protein PstC (TC 3.A.1.7.1)	-	-			Annotation
Phosphorus uptake	Phosphate transporter, high affinity	Phosphate ABC transporter, periplasmic phosphate-binding protein PstS (TC 3.A.1.7.1)	-	-			Annotation
Phosphorus uptake	Phosphate transporter, low affinity	-	-	-	PHO4	*A. baumanii*	*Acinetobacter oleivorans*
Phosphorus uptake	Phosphate transporter, low affinity	Probable low-affinity inorganic phosphate transporter	-	-			
Chemotaxis	Chemotaxis	Signal transduction histidine kinase CheA	che	cheW	Hpt/cheY-binding /H-kinase_dim /HATPase_C/cheW	*A. calcoaceticus*	*E. coli* O157
Chemotaxis	Chemotaxis	Chemotaxis regulator	che	cheY	-	-	*E. coli* O157

		transmitting signal to flagellar motor component					
Mechanism	**Enzyme**	**Pathway**	**Operon**	**Gene name**	**PFAM**	**Closest relative**	**Found by**
Chemotaxis	Chemotaxis	cheZ	che	cheZ	cheZ	-	*E. coli* O157
Chemotaxis	Chemotaxis	chemotaxis methyltransferase	che	cheR	cheR_N/cheR	-	*E. coli* O157
Chemotaxis	Chemotaxis	fused chemotactic sensory histidine kinase (soluble) in two-component regulatory system with CheB and CheY	che	cheA	Hpt/cheY-binding /H-kinase_dim /HATPase_C/che W	*A. calcoaceticus*	*E. coli* O157
Chemotaxis	Chemotaxis	chemotaxis response regulator protein-glutamate methylesterase	che	cheB	Response_reg /cheB_methylest	-	*E. coli* O157
Chemotaxis	Chemotaxis	flagellar motor protein	mot	motA	motA_expB	*A. calcoaceticus*	*P. putida DOT-T1E*
Chemotaxis	Chemotaxis	flagellar motor protein	mot	motB	motB_plug/OmpA	-	*P. putida DOT-T1E*
Chemotaxis	Chemotaxis	Regulator or pca operon	pca	pcaR	HTH_lclR/lclR	-	*P. putida*
Chemotaxis	Chemotaxis	4-hydroxybenzoate transporter	pca	pcaK	MFS_1	*A. calcoaceticus*	*P. putida*
Chemotaxis	Chemotaxis	Beta-ketoadipyl CoA thiolase	pca	pcaF	Thiolase_N/Thiols e_C	*Acineotbacter* sp. NIPH 817	*P. putida*
Chemotaxis	Chemotaxis	Protein pilJ	che/pil	pilJ	MCPSignal	*A. oleivorans* DR1	*A. oleivorans*
Chemotaxis	Chemotaxis	tar methyl accepting chemotaxis protein II	tar	tar/tap	tarH	-	*E. coli*
Chemotaxis	Chemotaxis	tap methyl accepting chemotaxis protein IV	tap	tar/tap	tarH/HAMP /MCPSignal	-	*E. coli*
Chemotaxis	Chemotaxis	Twitching motility protein pilG	pil	pilG	Response_reg	*A. baumanii*	*A. oleivorans*

Chemotaxis	Chemotaxis	Twitching motility protein pilH	pil	pilH	Response_reg	*A. baumanii*	*A. oleivorans*
Mechanism	**Enzyme**	**Pathway**	**Operon**	**Gene name**	**PFAM**	**Closest relative**	**Found by**
Chemotaxis	Chemotaxis	Pilus assembly protein pilI	pil	pilI	PapD_N/PapD_C	*A. calcoaceticus*	*A. oleivorans*
Chemotaxis	Chemotaxis	Pilus assembly protein pilI	pil	pilI	PapD_N/PapD_C	*A. oleivorans*	*A. oleivorans*
Chemotaxis	Chemotaxis	Methyl-accepting chemotaxis assembly protein	pil	pilJ	MCPSignal	*A. oleivorans* DR1	*A. oleivorans*
Chemotaxis	Chemotaxis	Methyltransferase pilK	pil	pilK	cheR_N/cheR	-	*P. aeruginosa PAO1*
Chemotaxis	Chemotaxis		chp	chpA	Hpt/Hpt/Hpt/Hpt /H-kinase_dim /HATPase_c/chew /Response_reg	-	*A. calcoaceticus PHEA-2*
Chemotaxis	Chemotaxis	chemosensory pili system protein	chp	chpB	Response_reg /cheB_methylest	-	*P. aeruginosa PAO1*
Chemotaxis	Chemotaxis		chp	chpC	cheW	-	*P. aeruginosa PAO1*
Chemotaxis	Chemotaxis	toluene o-xylene monooxygenase component	tou	touA	Phenol_Hydrox/YHL	-	*P. sp OX1*
Chemotaxis	Chemotaxis	toluene o-xylene monooxygenase component	tou	touB	TmoB	-	*P. sp OX1*
Chemotaxis	Chemotaxis	ferredoxin	tou	touC	Rieske	-	*P. sp OX1*
Chemotaxis	Chemotaxis	toluene o-xylene monooxygenase component	tou	touD	MmoB_DmpM	-	*P. sp OX1*
Chemotaxis	Chemotaxis	toluene o-xylene monooxygenase component	tou	touE	Phenol_Hydrox	-	*P. sp OX1*
Chemotaxis	Chemotaxis	toluene o-xylene monooxygenase component	tou	touF	fer2/FAD_Binding_6/NAD_Binding_1	-	*P. sp OX1*

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Mechanism	**Enzyme**	**Pathway**	**Operon**	**Gene name**	**PFAM**	**Closest relative**	**Found by**
Chemotaxis	Chemotaxis	toluene o-xylene monooxygenase, regulatory component	tou	touR		-	No reference gene
Biofilm formation	Homoserine lactone	N-acylhomoserine lactone synthase, autoinducer synthesis protein	sol	solI	Autoind_synth	*A. calcoaceticus*	*A. oleivorans DR1*
Biofilm formation	Homoserine lactone	homoserine/homoserine lactone efflux protein	-	-	LysE	*A. oleivorans*	*A. oleivorans DR1*
Biofilm formation	Attachment polymers	pelA	pel	pelA	(Glyco_Hydro_114 /Polysacc_deac_1)	-	*P. aeruginosa PAO1*
Biofilm formation	Attachment polymers	pelB	pel	pelB	TPR_19/TPR_15	-	*P. aeruginosa PAO1*
Biofilm formation	Attachment polymers	pelC	pel	pelC	-	-	*P. aeruginosa PAO1*
Biofilm formation	Attachment polymers	pelD	pel	pelD	DUF4118/GAF_3	-	*P. aeruginosa PAO1*
Biofilm formation	Attachment polymers	pelE	pel	pelE	(Tetraspannin /HEAT_2/TPR_16)	-	*P. aeruginosa PAO1*
Biofilm formation	Attachment polymers	pelF	pel	pelF	DUF3492 /Glyco_Transf_1	-	*P. aeruginosa PAO1*
Biofilm formation	Attachment polymers	pelG	pel	pelG	-	-	*P. aeruginosa PAO1*
Biofilm formation	Attachment polymers	pslA	psl	pslA	CoA_binding_3 /Bact_transf	-	*P. aeruginosa PAO1*
Biofilm formation	Attachment polymers	pslB	psl	pslB	NTP_transferase /Mannose_P_Isomer	-	*P. aeruginosa PAO1*
Biofilm formation	Attachment polymers	pslC	psl	pslC	Glyco_transf_2_3	-	*P. aeruginosa PAO1*

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Mechanism	**Enzyme**	**Pathway**	**Operon**	**Gene name**	**PFAM**	**Closest relative**	**Found by**
Biofilm formation	Attachment polymers	pslD	psl	pslD	Poly_export	-	*P. aeruginosa PAO1*
Biofilm formation	Attachment polymers	pslE	psl	pslE	Wzz	-	*P. aeruginosa PAO1*
Biofilm formation	Attachment polymers	pslF	psl	pslF	Glyco_Transf_1	-	*P. aeruginosa PAO1*
Biofilm formation	Attachment polymers	pslG	psl	pslG	Cellulase	-	*P. aeruginosa PAO1*
Biofilm formation	Attachment polymers	pslH	psl	pslH	Glyco_trans_4_4 /Glyco_trans_1_4	-	*P. aeruginosa PAO1*
Biofilm formation	Attachment polymers	pslO	psl	pslN	Topoisom_I	-	*P. aeruginosa PAO1*
Biofilm formation	Attachment polymers	VPS	vps	vpsR	-	-	-
Biofilm formation	Fimbrial protein	F17 fimbrial protein	cup	cupA1	Fimbrial	*A. oleivorans*	*A. oleivorans DR1*
Biofilm formation	Fimbrial protein	P pilus assembly protein, chaperone PapD	pap	papD (cupA2)	PapD_N/PapD_C	*A. baumanii*	-
Biofilm formation	Fimbrial protein	type 1 fimbriae anchoring protein FimD	pap	fimD/papC (cupA3)	PapC_N/Usher /PapC_D	*A. oleivorans*	-
Biofilm formation	Fimbrial protein	fimbrial family protein (fimbrial adhesin precursor)	cup	cupA4	Fimbrial	*A.* sp GG2	*A. oleivorans DR1*
Biofilm formation	Fimbrial protein	putative fimbrial protein precursor (pilin)(=F17 fimbrial protein precursor)	cup	cupA1	Fimbrial	*A. baumanii*	*A. oleivorans DR1*
Biofilm formation	Fimbrial protein	P pilus assembly protein, chaperone PapD	pap	papD (cupA2)	PapD_N/PapD_C	*A. calcoaceticus*	-

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Biofilm formation	Fimbrial protein	type 1 fimbriae anchoring protein FimD	pap	fimD/papC (cupA3)	PapC_N/Usher /PapC_D	*A. oleivorans*	-
Mechanism	**Enzyme**	**Pathway**	**Operon**	**Gene name**	**PFAM**	**Closest relative**	**Found by**
Biofilm formation	Fimbrial protein	fimbrial family protein (fimbrial adhesin precursor)	cup	cupA4	Fimbrial	*A. calcoaceticus*	*A. oleivorans DR1*
Biofilm formation	Attachment polymers	YafK	yaf	yafK	-	-	-
Biofilm formation	Fimbrial protein	adhesive protein CupB5	cup	cupB5	-	-	-
Biofilm formation	Fimbrial protein	Cellulose synthase operon	wss	wssA	Yhjq	-	*P. fluorescens*
Biofilm formation	Fimbrial protein	Cellulose synthase operon	wss	wssB	Glyco_tranf_2_3/ PilZ	-	*P. fluorescens*
Biofilm formation	Fimbrial protein	Cellulose synthase operon	wss	wssC	BcsB	-	*P. fluorescens*
Biofilm formation	Fimbrial protein	Adhesin LapA	lap	lapA	HemolysinCabind /VWA /HemolysinCabind /HemolysinCabind /HemolysinCabind /HemolysinCabind	-	*P. putida*
Plant growth promotion	Tryptophan monooxygenase	IAA synthesis/indole-3-Acetamide			Amidase	-	*P. syringae*
Plant growth promotion	indoleacetamide hydrolase	IAA synthesis/indole-3-Acetamide			Amino_oxidase	-	*A. baumanii*
Plant growth promotion	Diaminobutyrate--2-oxoglutarate aminotransferase	IAA synthesis/(indole-3-pyruvic acid)			Aminotran_III	*A. calcoaceticus*	*Arabidopsis thaliana*
Plant growth promotion	indole-3-pyruvate decarboxylase	IAA synthesis/(indole-3-pyruvic acid)			TPP_enzyme_N /TPP_enzyme_M /TPP_enzyme_C	*A.* sp. NIPH817	-
Plant growth promotion	Aldehyde dehydrogenase	IAA synthesis/(indole-3-pyruvic acid)			Aldedh	*A. calcoaceticus*	-
Plant growth	tryptophan 2,3 dioxygenase	IAA			-	-	-

promotion		synthesis/tryptophan side chain pathway					
Mechanism	**Enzyme**	**Pathway**	**Operon**	**Gene name**	**PFAM**	**Closest relative**	**Found by**
Plant growth promotion	DOPA decarboxylase (2, diaminobutyrate decarboxylase)	IAA synthesis/tryptamine			Pyridoxal_deC	*A. calcoaceticus*	*P. putida*
Plant growth promotion	Amino oxidase	IAA synthesis/tryptamine			Amino_oxidase	*A. calcoaceticus*	*A. baumanii*
Plant growth promotion	indole-3-acetaldehyde oxidase	IAA synthesis/tryptamine			-	-	-
Plant growth promotion	nitrilase	IAA synthesis/indole-3-acetonitrile (A)			CN_hydrolase	-	-
Plant growth promotion	nitrile hydratase	IAA synthesis/indole-3-acetonitrile (B)			Nhase_beta	-	*A. calcoaceticus*
Plant growth promotion	acetamidase	IAA synthesis/indole-3-acetonitrile (B)			FmdA_AmdA /FmdA_AmdA		*A. baumanii*
Plant growth promotion	2,3-butanediol dehydrogenase	Acetoin & butanediol biosynthesis	aco	adh	ADH_N_assoc /ADH_N/ADH_zinc	*A. baumanii*	*P. putida*
Plant growth promotion	Dihydrolipoamide acetyltransferase component (E2) of acetoin dehydrogenase complex (EC 2.3.1.-)	Acetoin & butanediol biosynthesis	aco	acoC	Biotin_lipoyl /Abhydrolase_6	*A. baumanii*	*P. putida*
Plant growth promotion	Acetoin dehydrogenase E1 component beta-subunit (EC 1.2.4.-)	Acetoin & butanediol biosynthesis	aco	acoB	Transket_pyr /Transketolase_C	*A. calcoaceticus*	*P. putida*
Plant growth promotion	Acetoin dehydrogenase E1 component alpha-subunit (EC 1.2.4.-)	Acetoin & butanediol biosynthesis	aco	acoA	E1_DH	*A. baumanii*	*P. putida*
Plant growth promotion	Transcriptional regulator, LysR family	Acetoin & butanediol biosynthesis	aco	acoX	NAD_Kinase	-	*P. putida*
Plant growth promotion	Transcriptional activator of acetoin dehydrogenase operon AcoR	321918	323189	3			Annotation
Plant growth	probable membrane protein	323259	324170	3			Annotation

promotion	STY1534						
Plant growth promotion	2,3-butanediol dehydrogenase, S-alcohol forming, (S)-acetoin-specific (EC 1.1.1.76)	326092	325307	-1			Annotation
Mechanism	**Enzyme**	**Pathway**	**Operon**	**Gene name**	**PFAM**	**Closest relative**	**Found by**
Plant growth promotion	Dihydrolipoamide dehydrogenase of acetoin dehydrogenase (EC 1.8.1.4)	327522	326119	-3			Annotation
Siderophore biosynthesis		acinetobactin biosynthesis	bau	bauD	FecCD	*A.calcoaceticus*	*A. baumanii*
Siderophore biosynthesis		acinetobactin biosynthesis	bau	bauC	FecCD	*A.calcoaceticus*	*A. baumanii*
Siderophore biosynthesis		acinetobactin biosynthesis	bau	bauE	ABC_Tran	*A. baumanii*	*A. baumanii*
Siderophore biosynthesis		acinetobactin biosynthesis	bau	bauB	Peripla_BP_2	*A.calcoaceticus*	*A. baumanii*
Siderophore biosynthesis		acinetobactin biosynthesis	bau	bauA	Plug	*A.calcoaceticus*	*A. baumanii*
Siderophore biosynthesis		acinetobactin biosynthesis	bau	basC	K_oxygenase	-	*A. baumanii*
Siderophore biosynthesis		acinetobactin biosynthesis	bau	basD	Condensation/NRPS/Condensation/NRPS	-	*A. baumanii*
Siderophore biosynthesis		staphylobactin biosynthesis	sbn	sbnA	PALP	*A.calcoaceticus*	*S. aureus*
Siderophore biosynthesis		staphylobactin biosynthesis	sbn	sbnB	OCD_Mu_crystall	*A.* sp	*S. aureus*
Siderophore biosynthesis		staphylobactin biosynthesis	sbn	sbnC	IucA_IucC/FhuF	*A.calcoaceticus*	*S. aureus*
Siderophore biosynthesis		staphylobactin biosynthesis	sbn	sbnD	MFS_1/Sugar_tr	*A.calcoaceticus*	*S. aureus*
Siderophore biosynthesis		staphylobactin biosynthesis	sbn	sbnE	IucA_IucC/FhuF	*A.calcoaceticus*	*S. aureus*
Siderophore biosynthesis		staphylobactin biosynthesis	sbn	sbnF	IucA_IucC/FhuF	*A.calcoaceticus*	*S. aureus*

Siderophore biosynthesis		staphylobactin biosynthesis	sbn	sbnG	HpcH_HpaI	*A.calcoaceticus*	*S. aureus*
Siderophore biosynthesis		staphylobactin biosynthesis	sbn	sbnH	Orn_Arg_deC_N /Orn_DAP_Arg_DeC	*A.calcoaceticus*	*S. aureus*
Mechanism	**Enzyme**	**Pathway**	**Operon**	**Gene name**	**PFAM**	**Closest relative**	**Found by**
Siderophore biosynthesis		staphylobactin biosynthesis	sbn	sbnI	ParBc		*S. aureus*
Siderophore biosynthesis		enterobactin biosynthesis	ent	entA	adh_short_C2	*A.calcoaceticus*	*A. baumanii*
Siderophore biosynthesis		enterobactin biosynthesis	ent	entB	Isochorismatase /PP-binding	*A.calcoaceticus*	*A. baumanii*
Heavy metal tolerance	Metallothioneins	metal chelation					*P. putida KT2440*
Heavy metal tolerance	RND domains	Heavy metal extrusion				*A.baumanii*	Annotation
Heavy metal tolerance	RND domains	Heavy metal extrusion				*A.calcoaceticus*	Annotation
Heavy metal tolerance	RND domains	Heavy metal extrusion				*A.calcoaceticus*	Annotation
Heavy metal tolerance	RND domains	Heavy metal extrusion				*A.calcoaceticus*	Annotation
Heavy metal tolerance	RND domains	Heavy metal extrusion				*A.calcoaceticus*	Annotation
Heavy metal tolerance	RND domains	Heavy metal extrusion				*A.calcoaceticus*	Annotation
Heavy metal tolerance	RND domains	Heavy metal extrusion				*A.oleivorans* DR1	Annotation
Heavy metal tolerance	RND domains	Heavy metal extrusion				*A.calcoaceticus*	Annotation
Heavy metal tolerance	RND domains	Heavy metal extrusion				*A.calcoaceticus*	Annotation
Heavy metal tolerance	RND domains	Heavy metal extrusion				*A.calcoaceticus*	Annotation
Heavy metal tolerance	RND domains	Heavy metal extrusion				*A.brisouii*	Annotation

Heavy metal tolerance	RND domains	Heavy metal extrusion				*A.calcoaceticus*	Annotation
Heavy metal tolerance	RND domains	Heavy metal extrusion				*A.nosocomialis*	Annotation
Mechanism	**Enzyme**	**Pathway**	**Operon**	**Gene name**	**PFAM**	**Closest relative**	**Found by**
Heavy metal tolerance	RND domains	Heavy metal extrusion				*A*.sp NIPH817	Annotation
Heavy metal tolerance	Cr efflux	Heavy metal extrusion	chr	chrA		*A.calcoaceticus*	Annotation
Heavy metal tolerance	Cr efflux	Heavy metal extrusion	chr	chrA		*A.calcoaceticus*	Annotation
Heavy metal tolerance	Cr efflux	Heavy metal extrusion	chr	chrA		*A.baumanii*	Annotation
Heavy metal tolerance	Cr efflux	Heavy metal extrusion	chr	chrB		*A.calcoaceticus*	Annotation
Heavy metal tolerance	As efflux	Heavy metal extrusion	ars	arsR		*A.calcoaceticus*	Annotation
Heavy metal tolerance	As efflux	Heavy metal extrusion	ars	arsR		*A.pittii*	Annotation
Heavy metal tolerance	As efflux	Heavy metal extrusion	ars	arsR		*A.calcoaceticus*	Annotation
Heavy metal tolerance	As efflux	Heavy metal extrusion	ars	arsR		*A.calcoaceticus*	Annotation
Heavy metal tolerance	As efflux	Heavy metal extrusion	ars	arsB (ACR3)		*A.calcoaceticus*	Annotation
Heavy metal tolerance	As efflux	Heavy metal extrusion	ars	arsC		*A.calcoaceticus*	Annotation
Heavy metal tolerance	As efflux	Heavy metal extrusion	ars	arsC		*A.calcoaceticus*	Annotation
Heavy metal tolerance	As efflux	Heavy metal extrusion	ars	arsH		*A.calcoaceticus*	Annotation
Heavy metal tolerance	Co-Zn-Cd efflux	Heavy metal extrusion	czc	czcA		*A.calcoaceticus*	Annotation
Heavy metal tolerance	Co-Zn-Cd efflux	Heavy metal extrusion	czc	czcB		*A.calcoaceticus*	Annotation

Heavy metal tolerance	Co-Zn-Cd efflux	Heavy metal extrusion	czc	czcC		*A.calcoaceticus*	Annotation
Heavy metal tolerance	Co-Zn-Cd efflux	Heavy metal extrusion	czc	czcD		*A.calcoaceticus*	Annotation
Mechanism	**Enzyme**	**Pathway**	**Operon**	**Gene name**	**PFAM**	**Closest relative**	**Found by**
Heavy metal tolerance	Co-Zn-Cd efflux	Heavy metal extrusion	czc	czcD		*A.calcoaceticus*	Annotation
Heavy metal tolerance	Co-Zn-Cd efflux	Heavy metal extrusion	czc	czcD		*A.pittii*	Annotation
Heavy metal tolerance	Co-Zn-Cd efflux	Heavy metal extrusion	czc	czcD		*A.calcoaceticus*	Annotation
Heavy metal tolerance	Co-Ni resistance	Heavy metal extrusion	cnr	cnrCBA			
Heavy metal tolerance	Ni, Co, low Cd resistance	Heavy metal extrusion	ncc	nccCBA			
Heavy metal tolerance	Ag, Cu resistance	Heavy metal extrusion	cus	cusCBA			
Heavy metal tolerance	Cd, Zn resistance	Heavy metal extrusion	czr	czrCBA			
Heavy metal tolerance	Pb, Cd, Zn, Hg transporter ATPase	Heavy metal extrusion				*A.calcoaceticus*	