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Tesis Doctoral

Isolation and characterization of a *Pseudomonas putida* with potential use as biofertilizer and its environmental applications

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A mi familia

A Germán

General Index

* Supplementary information is attached at the end of each chapter when corresponding.

Appendix

Abbreviations	**Meaning**
ACC	1-aminocyclopropane-1-carboxylate
ANOVA	Analysis of variance
BTEX	Benzene, toluene, ethylbenzene, and xylenes
C	Carbon
CAS	Chrome azurol S
CFU	Colony formig units
DAPG	Diacetylphloroglucinol
DHA	Dehydrogenase acyivity
DNA	Deoxyribonucleic acid
EPS	Exopolysaccharides
GFP	Green fluorescent protein
HCN	Hydrogen cyanide
HPLC	High-performance liquid chromatography
IAA	Indole-3-acetic
IAAld	Indole-3-acetaldeyde
IAM	Indole-3-acetamide
IAN	Indole-3-acetonitrile
INFOCA	Plan de Prevención y Extinción de Incendios de Andalucía
INT	2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenylteytrazolium chloride hydrate
INTF	Iodonitrotetrazolium violet-formazan
IPTG	Isopropyl-β-D-thiogalactopyranoside
IPyA	Indole-3-pyruvate
ISR	Induced systemic resistance
IVET	*In vivo* expression technology
LB	Luria-Bertani
N	Nitrogen
OTU	Operational taxonomic unit
P	Phosphorous
PAH	Polycyclic aromatic hydrocarbons

Abbreviations	**Meaning**
PCB	Polychlorinated biphenyls
PCE	Perchloroethylene
PCoA	Principal component analysis
PCR	Polymerase chain reaction
PGPR	Plant growth-promoting rhizobacteria
PNG	4-nitrophenyl-β-D-glucopiranoside
PNP	*p*-nitrophenol
PNPP	4-nitrophenyl phosphate-disodium
PSM	Phosphate-solubilising microorganism
RDP	Ribosomal data project
REP	Repetitive extragenic palindromic sequence
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
TAM	Tryptamine
TCE	Trichloroethylene
TPH	Total petroleum hydrocarbons

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Resumen

En los últimos años se ha producido un endurecimiento en la legislación para el uso de fertilizantes químicos inorgánicos, derivado de sus efectos adversos sobre el medio ambiente y a que este tipo de productos no tienen en cuenta otras posibles deficiencias de nutrientes en el suelo, como en el caso del fósforo y el hierro, debido a que los fertilizantes al uso no se componen de formas fácilmente biodisponibles para las plantas de estos elementos. En consecuencia, se ha dado lugar a la búsqueda y estudio de nuevas fuentes naturales de abonos, bioestimulantes y enmiendas para el suelo, unido a la creciente importancia que ha tomado la agricultura ecológica.

El conocimiento de las interacciones que se producen en la rizosfera entre plantas y microorganismos, es esencial para el desarrollo de nuevas herramientas biotecnológicas, basadas en microorganismos promotores del crecimiento vegetal (PGPR, Plant Growth-Promoting Rhizobacteria), que pueden aumentar el rendimiento de las cosechas, además de la disminución en el uso de agroquímicos y promover la restauración de ecosistemas afectados por perturbaciones, ya sean de origen natural y/o debidas a actividades antrópicas. El desarrollo de productos de origen biológico comprende el uso de microorganismos vivos que colonizan eficazmente la rizosfera, son capaces de mantenerse en ella y, además, de producir nutrientes asimilables por la planta hospedadora, influenciar positivamente de manera indirecta el crecimiento y desarrollo de la raíz y/o estimular el crecimiento de la planta mediante el control de patógenos.

Con dichos antecedentes, esta tesis se centró en la caracterización de una microorganismo rizosférico, *Pseudomonas putida* BIRD-1, que se aisló de suelo rizosférico y tiene capacidad para solubilizar nutrientes de baja disponibilidad y para producir fitohormonas que promueven el crecimiento vegetal. Como objetivo general esta tesis se propone superar las limitaciones en el conocimiento del efecto que ejercen distintas condiciones ambientales sobre los inoculantes microbianos, y mejorar la tecnología de aplicación de los mismos en condiciones que son adversas para el correcto desarrollo de especies vegetales de interés.

En el **capítulo 1** se realiza una caracterización de *Pseudomonas putida* BIRD-1, que es un rizobacteria promotora del crecimiento vegetal (PGPR) cuyo genoma tiene un tamaño de 5,7 Mpb. Esta cepa se adhiere eficientemente a las raíces de las plantas y coloniza la rizosfera a altas densidades celulares, incluso en suelos con sólo el 2% de humedad. Esta propiedad parece estar ligada a su capacidad de sintetizar trehalosa, como se deduce a partir de un mutante deficiente en la síntesis de trehalosa que exhibe menos tolerancia a la desecación de la cepa parental. El genoma de BIRD-1 codifica también una amplia gama de proteínas que ayudan a lidiar con el estrés provocado por especies reactivas de oxígeno generada en la rizosfera de las plantas. Las propiedades PGPR de *Pseudomonas putida* BIRD-1 se derivan de su capacidad para solubilizar fósforo y hierro, y producir fitohormonas. BIRD-1 es capaz de solubilizar formas insolubles de fosfato inorgánico a través de la producción de ácido. Además, el genoma de BIRD-1 codifica al

menos 5 fosfatasas relacionadas con la solubilización de fósforo; una de ellas es una fitasa que facilita la utilización de ácido fítico, la principal forma de almacenamiento de fósforo orgánico en las plantas. La pioverdina es el principal sideróforo producido por esta cepa; un mutante en la sideróforo sintasa FvpD no logró crecer en medio líquido sin suplemento de hierro, sin embargo, en suelo el mutante FvpD era tan competitivo como la cepa parental, ya que posee un gran número de receptores que captan los sideróforos producidos por otros microbios, lo que permite la adquisición de hierro y la supervivencia. BIRD-1 produce en exceso de ácido indol-3-acético a través de vías convergentes, lo que influye en su capacidad para estimular la germinación de semillas y crecimiento de las plantas.

En el **capítulo 2** se plantea el uso de *Pseudomonas putida* BIRD-1, en consorcio con otras bacterias, en un ensayo de biorremediación y rizorremediación a escala de campo, para recuperar una zona incendiada, que se enmarca dentro de un espacio natural protegido. Los incendios forestales constituyen una grave amenaza para los países de la cuenca mediterránea, que pueden arrasar grandes extensiones de bosques cada año. Después de los incendios, los suelos son más propensos a sufrir erosión y se inhibe su capacidad de recuperación, en parte, por los compuestos tóxicos producidos durante la combustión de la celulosa y la lignina. En este estudio se analizó el uso de técnicas de biorremediación y rhizoremediation para la restauración del suelo en un estudio de campo a gran escala en un ecosistema mediterráneo protegido después de un

incendio controlado. En la estrategias de biorremediación y rizorremediación planteadas, se combina el uso de cepas de *Pseudomonas putida*, microorganismos cultivables indígenas y gramíneas anuales. Después de ocho meses de seguimiento de los parámetros de calidad del suelo, incluida la eliminación de hidrocarburos monoaromáticos e hidrocarburos aromáticos policíclicos (HAPs), así como la evolución de la cubierta vegetal, se encontró los niveles en parámetros analizados eran equiparables a los previos al incendio. El análisis de la población microbiana reveló que los incendios indujeron cambios en la microbiota indígena, y que la estrategia de rizorremediación favorece la recuperación de la microbiota del suelo. La combinación de microorganismos y plantas analizada en este estudio representa una estrategia eficaz para la restauración de los suelos después de un incendio forestal.

En el **capítulo 3** se plantea el uso de *Pseudomonas putida* BIRD-1, en consorcio con otras bacterias, en un ensayo de biorremediación y rizorremediación a escala de campo, para recuperar una contaminada por hidrocarburos de petróleo. Los lodos de depuradora procedentes del refinado del petróleo son tóxicos y peligrosos, por lo tanto, los organismos de protección del medio ambiente han declarado prioritario el tratamiento para la inactivación de los residuos de petróleo debido a su toxicidad y peligrosidad. Los tratamientos físico-químicos son costosos y perjudiciales para el medioambiente, y los tratamientos biológicos alternativos son menos costosos, pero, en general, funcionan a un ritmo

más lento. Tratamientos de biorremediación y rizorremediación *in situ* y se realizaron en una zona contaminada con lodos de refinería de petróleo en condiciones de clima semiárido. Los tratamientos de biorremediación y rizorremediación incluyen el uso de un consorcio microbiano formado por rizobacterias promotoras del crecimiento vegetal (PGPRs) y bacterias degradadoras de hidrocarburos aromáticos policíclicos (HAPs), y el uso combinado del consorcio mencionado junto con especies vegetales pratenses, respectivamente. El tratamiento de rizorremediacioón reveló que el desarrollo de la vegetación favorece el incremento de las poblaciones microbianas con potencial para eliminar los desechos derivados del petróleo, lo que se traduce en la disminución de los hidrocarburos totales de petróleo (HTPs), siete meses después del establecimiento de dichos tratamientos biológicos.

Finalmente, se planteó establecer qué genes, de aquellos que están descritos como esenciales para la proliferación y adaptación de *Pseudomonas putida* en la rizosfera, se encuentran conservados específicamente en *Pseudomonas putida* BIRD-1 y, en general, en el resto de cepas de *P. Putida* cuyos genomas actualmente se encuentran secuenciados. Una búsqueda bibliográfica reveló que se han descrito 180 genes involucrados en la aptitud para sobrevivir en la rizosfera de *P. putida*, relacionados con diversas capacidades como la adhesión a superficies bióticas y la capacidad para formar biofilms, biosíntesis de flagelo, quimiotaxis, reguladores y sensores, adaptación a estreses, reparación del ADN, mecanismos de transporte y secreción y

metabolismo de diversos compuestos presentes en la rizosfera. Un estudio *in silico*, reveló que estos genes se encuentran conservados, en general, en las cepas de *P. Putida* secuenciadas y, en particular, en *Pseudomonas putida* BIRD-1 confirmando esta cepa tiene la maquinaria genética para sobrevivir y proliferar en la rizosfera. Además, se perfila como una excelente candidata para su uso en agricultura y como parte de tratamientos destinados a la remediación de ecosistemas incendiadas y suelos contaminados con hidrocarburos de petróleo.

General Introduction

1. Plant-microbes interactions in the rhizosphere: Plant growth-promoting rhizobacteria (PGPR)

The root is the organ that holds the plant to ground, providing for water and enabling the interchange of nutrients with the surrounding soil, creating a root-soil interface known as rhizosphere. The term rhizosphere, formed by the Greek words *rhiza* (root) and *sphere* (influence zone), was first defined by the plant-physiologist Hiltner as the narrow zone adjacent to the plant-roots supporting high levels of microbial activity (Hiltner, 1904). Nowadays, it has been redefined as the soil volume under the physical and biological influence of the root, including the root tissues colonized by microorganisms. Although the rhizosphere extent is difficult to define, in general, it is considered to reach a few millimetres (approximately 0 to 2 mm) from the root surface (Ahmad *et al.*, 2011), and can be divided into endorhizosphere (endodermis and cortical zone of the root), rhizoplane (root surface including strongly attached particles) and ectorhizosphere (soil immediately adjacent to the root) (Morgan *et al.*, 2005).

The rhizosphere is a region with high nutrients content and, therefore, of increased microbial activity. The 30 to 60% of the photosynthesis-fixed carbon can be transported to the root, and about 49% is loss in the form of root exudates or through root respiration (Kennedy, 1999). Root exudates are nutrient rich and comprise high- and low-molecular weight compounds like polysaccharides, proteins, mucilage, amino acids, organic acids, sugars, vitamins, fatty acids and sterols, nucleotides, enzymes and

other compounds like carbon dioxide, molecular hydrogen, protons, hydroxides, etc (Table 1) (Ahmad *et al.*, 2011).

Table 1. Compounds identified in plant-root exudates. Extracted from Ahmad *et al.*, 2011.

Class of compounds	Compounds
Amino acids	Alanine, α-aminoadipic acid, γ-aminobutyric acid, arginine, asparagine, aspartic acid, cysteine, cystine, glutamic acid, glutamine, glycine, histidine, homoserine, isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, proline, serine, therionine, tryptophan, tyrosine, valine
Organic acids	Acetic, acotinic, aldonic, butyric, citric, erythronic, formic, fumeric, glutaric, glycolic, lactic, malic, malonic, oxalic, piscidic, propionic, pyruvic, succinic, tartaric, tertronic, valeric acid
Sugars	Arabinose, deoxyribose, fructose, galactose, glucose, maltose, oligosaccharides, raffinose, rhamnose, ribose, sucrise, xylose
Vitamins	ρ-Aminobenzoic acid, biotin, choline, *n*-methionylnicotinic acid, niacin, panthothenate, pyridoxine, riboflavin, thiamine
Fatty acids and sterols	Palmitic, stearic, oleic, linoleic, linoleic acids, cholesterol, campestrol, stigmasterol, sitosterol
Nucleotides	Adenine, guanine, uridine, cytidine
Enzymes	Amylase, invertase, phosphatase, polygalactouranase, proteases
Miscellaneous	HCO_3^-, OH^-, H^+, CO_2, H_2, auxins, flavonones, glycosides, saponin, scopolotin

Rhizodeposition is unevenly distributed along the root: it is maximal in younger zones, like the apex, and areas were primary and secondary roots are emerging (Uren, 2007). Therefore, the root is a complex environment with longitudinal and radial gradient variations in its physic-chemical and biologic properties, influencing the development of the microbial community (Uren, 2007; Brimecombe *et al.*, 2007; Ahmad *et al.*, 2011). Due to providing a nutrient-rich environment, microbial densities can be two to three orders of magnitude higher than those of bulk soil (Molina *et al.*, 2000). Between the plant and bacterial population inhabiting the rhizosphere several kinds of relationships can be established, being mutualistic, symbiotic or harmful for the host if microbes are pathogenic. Nonetheless, the accomplishment of root colonization by bacteria is subjected to complex processes involving survivability, tolerance and competition with indigenous rhizospheric microorganisms (Somers and Vanderleyden, 2004).

Traits involved in the success of bacteria in the rhizosphere are getting to be understood by studying of genes and proteins involved in root colonization, reviewed in the case of *Pseudomonas* by Wu and colleagues (2011). The already identified features include proteins involved in attachment to biotic surfaces and biofilm formation (Yousef-Coronado *et al.*, 2008; Nielsen et al., 2011; Nilsson et al., 2011; Duque *et al.*, 2013), proteins related to flagella (Yousef-Coronado *et al.*, 2008; Duque *et al.*, 2013), proteins involved in chemotaxis processes (Matilla *et al.*, 2007), regulators and sensor proteins (Matilla *et al.*, 2007; Barret *et al.*, 2011),

proteins related to stress adaptation mechanisms (Reva *et al.*, 2006; Matilla *et al.*, 2007), proteins involved in DNA repair (Ramos-González *et al.*, 2005; Cheng *et al.*, 2009), proteins involved in transport processes, protein secretion (Barret *et al.*, 2011), metabolism related proteins (Ramos-González *et al.*, 2005; Matilla *et al.*, 2007; Cheng *et al.*, 2009) and specific protein synthesis (Cheng *et al.*, 2009), among others. Matilla and colleagues (2007) pointed out that strong selective pressures are acting in the vicinity of roots (Figure 1) that are related with i) the ability for uptaking and nourishing from particular compounds found in the rhizosphere, like amino acids and other aromatic compounds (Table 1), and ii) the ability to cope with different stresses, like oxidative stress.

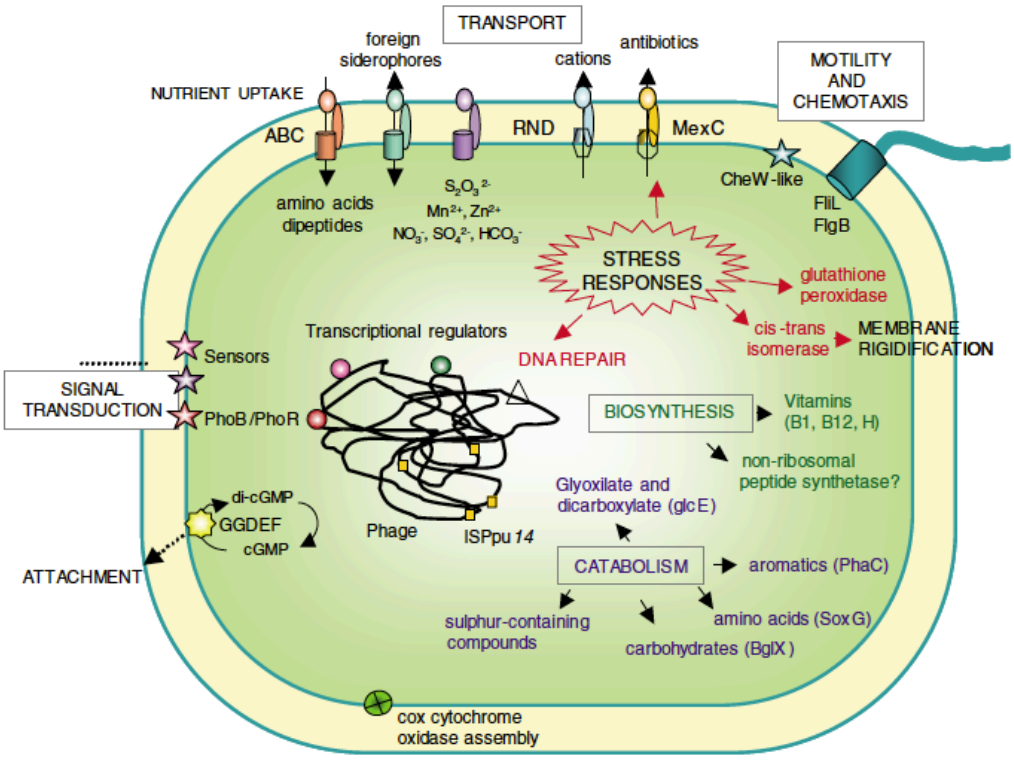


Figure 1. Scheme showing bacterial functions induced in the *Pseudomonas*-rhizosphere interaction. Extracted from Matilla *et al.*, 2007.

Rhizospheric mutualistic plant-microbe interactions are provided by plant growth-promoting rhizobacteria (PGPR), which were first defined as soil bacteria that colonize the root of plants following inoculation onto seeds and that enhance plant growth (Kloepper and Shroth, 1978). These microorganisms have common traits comprising i) ability for surviving after inoculation onto seeds, ii) multiplying in the spermosphere (area surrounding the seed) in response to root exudates, iii) attaching to the root surface and iv) colonizing the developing root system (Kloepper, 1994).

PGPRs efficiently colonize the rhizosphere of a wide range of plants of agricultural and environmental interest and stimulate plant development and/or reduce phytopathogens through direct or indirect mechanisms (biocontrol) (Glick, 1995; Lugtenberg and Kamilova, 2009; Matilla *et al.*, 2010; Barret *et al.*, 2011) (Figure 2). Microorganisms described as PGPR are bacteria belonging to *Pseudomonas*, *Bacillus*, *Azospirillum*, *Azotobacte*r and *Serratia,* among others (Somers and Vanderleyden, 2004).

Direct plant growth-promoting mechanisms comprise solubilisation of low-bioavailability soil nutrients, synthesis of phytohormones (Lugtenberg and Kamilova, 2009), volatile compounds that can stimulate plant development and synthesis of vitamins or amino acids (Simmons *et al.*, 1997; Compant *et al.*, 2010; Vial *et al.*, 2011). Plant growth can also be promoted by indirect effects that essentially are related to

phytopathogens suppression through the production of antifungal compunds, antibiotics, pesticides (Lugtenberg and Kamilova, 2009, Ahemad and Khan, 2012), general biocydes, enzymes (Zhang and Yuen, 2000; Haas and Keel, 2003), the competition for low-bioavailability nutrients like iron or through the suppression of plant diseases by eliciting induced systemic resistance (ISR) (van Loon and Bakker, 2005; Bakker *et al.*, 2007).

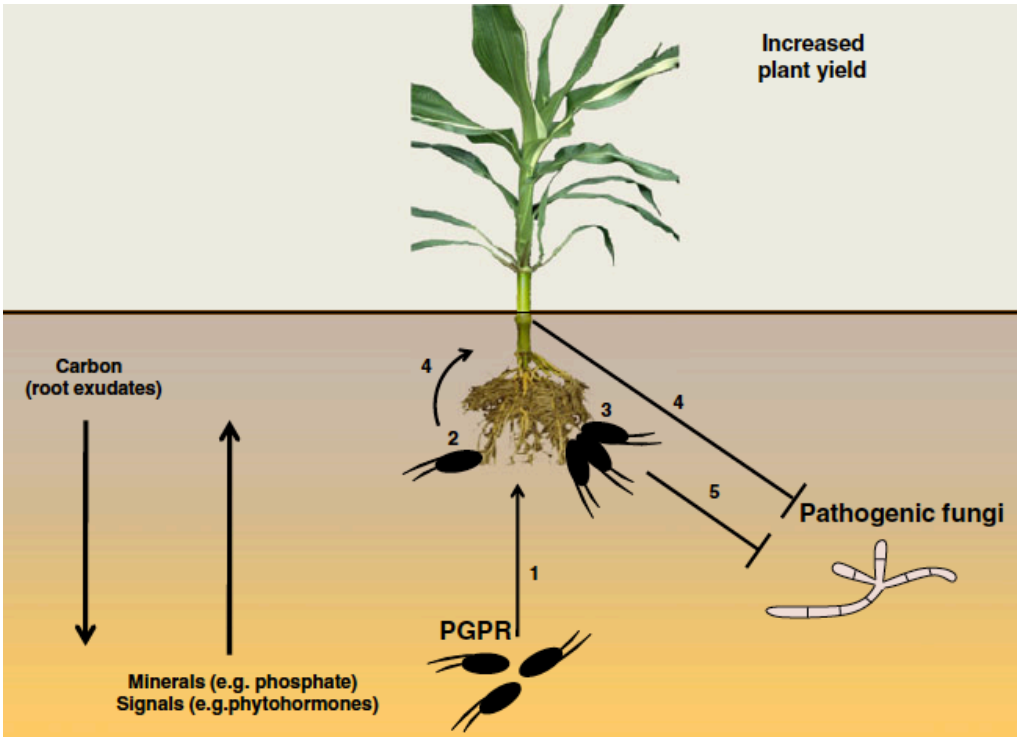


Figure 2. Beneficial plant-bacterial interactions in the rhizosphere. PGPR populations are selected by specific root exudates. Motility (1), adherence (2) and growth (3) are important steps of rhizosphere competence. PGPR strains could then improve the plant growth by releasing nutrients and phytohormones and/or inhibit root diseases caused by phytopathogenic fungi. Inhibition of the pathogen could be indirect through elicitation of plant ISR (4) or direct through the production of secondary metabolites (5). Extracted from Barret *et al.*, 2011.

Within the *Pseudomonas genus*, stains belonging to the species *P. putida* have been reported to have PGPR properties (Wu *et al.*, 2011).

2. The *Pseudomonas* genus

Members of the genus *Pseudomonas* are considered to be ubiquitous in the environment, since they can be found in a variety of milieus all along the globe. Migula first described the genus in the late 19th century as "rod-shaped and polar-flagella cells with some sporulating species", which was such a vague definition that made erroneous adscriptions of *Pseudomonas* to other genres. A century later, in 1986, Palleroni proposed five taxonomic groups (RNA-I – RNA-V) within the genus based on DNA-DNA or rRNA/DNA hybridization. Finally, sequencing the genes coding RNAr 16S allowed ascertaining the diversity inherent to *Pseudomonas*, locating the RNA-I group within the gammaproteobacteria (Bergey *et al.*, 2005).

Among the described species are *Pseudomonas putida*, *Pseudomas fluorescens*, *Pseudomonas aeruginosa*, etc. being up to 200 species conforming the group (Palleroni and Moore, 2004); the G+C genome content is 58% to 69%. Taxonomic classification of *Pseudomonas* sp. is as follows:

Domain: Bacteria
Phylum: Proteobacteria
Class: Gammaproteobacteria
Order: Pseudomonadales
Family: Pseudomonadaceae
Genus: *Pseudomonas*

Pseudomonas are gram-negative bacteria (0.5 – 0.8 μm x 1 – 3 μm) with one or more polar flagella, they are chemotrophic, aerobic with oxygen as terminal electrons acceptor in some cases anaerobic growth can be possible using nitrate as alternaive electron acceptor. They are oxidase positive and metabolise glucose via the Entner-Doudoroff pathway and the tricarboxylic acid cycle (del Castillo *et al.*, 2007). The genus comprises water and soil saprophytic bacteria with a high metabolic versatility, being capable of colonise a wide range of environments and assimilate a variety of carbon sources including recalcitrant compounds, which make them interesting candidates for biodegradation of pollutants (Ramos *et al.*, 1994; Segura *et al.,* 2009, Segura and Ramos, 2012).

Pseudomonas do not form spores. There are strains in the genus with the ability to synthesise siderophores, which are iron chelators, in iron-limiting conditions, and have a variety of receptors to internalize homologous and heterologous siderophores (Roca *et al.*, 2013). These green-yellow pigments are known as pyoverdines and pyocyanin and are fluorescent under short wavelength light (254 nm). These compounds are common in strains belonging to the species *Pseudomonas putida*, *Pseudomonas fluorescens* and *Pseudomonas aeruginosa*.

A characteristic trait among the genus is the presence of plasmids, which confers them the ability to cope with antibiotics, other antibacterial agents and catabolize toxic compunds; 13 plasmids groups have been

identified to be compatible with *Pseudomonas* (Greated *et al.*, 2002; Levchuck *et al.*, 2006).

Few strains have been described as human opportunistic pathogens, especially *Pseudomonas aeruginosa*, and there are plant pathogens as is known to be *Pseudomonas syringae*. Within the *Pseudomonas* genus there are members with potential uses and interest in the environmental and agronomical sectors, like pollutant degraders and plant growth-promoting bacteria, as *Pseudomonas putida* and *Pseudomonas fluorescens* (Lugtenberg and Dekkers, 1999), for example.

Pseudomonas are so nutritionally versatile that they are easily cultured under laboratory conditions, with a variety of carbon sources, neutral pH and mesophyll range of temperatures (Palleroni, 1986). Among the organic compounds that they use as energy sources, some *Pseudomonas* are able to use a variety of hidrocarbons, aromatic or linear, amines, amides, amino acids, alcohols and other aromatic compounds through an ample assortment of enzymes and catabolic pathways (Nelson *et al.*, 2002).

The relatively recent genome sequencing of several strains belonging to the *Pseudomonas* genus has shed light to the mechanisms that confer them the ample adaptation capacity that makes them able to colonize very different environments. Among the sequenced *Pseudomonas* there are opportunistic pathogens like *P. aeruginosa* PAO1 (Stover *et al.*, 2000),

plant pathogens like *Pseudomonas syringae* pv. tomato (Buell *et al.*, 2003) and innocuous strains like *Pseudomonas putida* KT2440 (Nelson *et al.*, 2002), *Pseudomonas fluorescens* Pf-5 (Paulsen *et al.*, 2005) and *Pseudomonas putida* BIRD-1 (Matilla *et al.*, 2011). Genomic comparison of pathogen and innocuous strains revealed that the later did not bear the genetic machinery encoding exotoxins, specific hydrolytic enzymes and type III secretion systems. Nevertheless, both kinds of strains share traits like adhesins, type IV pili, proteins involved in stress response, etc. (Nelson *et al.*, 2002).

Some *Pseudomonas* strains are able to synthesise phytohormones, iron chelators, to solubilise soil low-availability compounds, toxic substances to other microorganisms, which make them interesting candidates for agriculture as PGPR and/or biocontrol agents (Haas and Keel, 2003; Roca *et al.*, 2013). Other members of the genus are able to cope and/or catabolize environmental pollutants, which makes them suitable for their use in environmental processes like bioremediation and rhizoremediation treatments (Segura *et al.,* 2009; Segura and Ramos, 2012; Pizarro-Tobias *et al.*, unpublished results). Another interesting trait common to the genus is the ability to attach to surfaces, to produce adhesins and other proteins related to attachment, which are essential for the rhizospheric success of the soil strains (Yousef-Coronado *et al.*, 2008).

Thus, the *Pseudomonas* genus arouses much interest for its feasible uses in biotechnology, remediation of polluted sites and as biocontrol and plant growth-promoting agents in agriculture.

2.1. *Pseudomonas putida*

Pseudomonas putida belongs to the group of fluorescent *Pseudomonas*, due to their ability to produce fluorescence-emitting pigments. These are chemoorganothophic bacteria, which have a versatile metabolism that allows them to colonize different habitats, either pristine or polluted (Nelson *et al*, 2002; Wu *et al*., 2011). They have been isolated from agricultural, forest and industrial soils, and from continental waters. This species is able to colonize plants rhizosphere, and have a potential use in bioremediation of polluted soils (Segura *et al.,* 2009; Segura and Ramos, 2012; Pizarro-Tobias *et al*., unpublished results).

Among the *Pseudomonas putida* species it has not been found any animal or plant pathogen; this has been taken as positive for the development of biotechnological techniques, like the design of catabolic pathways aimed for pollutants degradation (Ramos *et al*., 1986; Ramos *et al*., 1987), production of intermediates in the chemical synthesis of complex molecules (Wubbolts and Timmis, 1990). Further, several strains have been proven being good colonizers of a variety of plants rhizosphere, like *Pseudomonas putida* KT2440 in maize rhizosphere (Espinosa-Urgel *et al*., 2002). Nowadays, some strains are being used as base of biological

fertilizers and remediation treatments (Segura *et al.*, 2009; Segura and Ramos, 2012; Roca *et al.*, 2013).

2.2.1. *Pseudomonas putida* BIRD-1

The strain used in this study is *Pseudomonas putida* BIRD-1, a recently sequenced PGPR strain (Matilla *et al.*, 2011), with genome sequence available at GenBank (http://www.ncbi.nlm.nih.gov/genbank/) under accession number CP002290. This strain's genome size is 5,731,541 bp without plasmid and has a 61.74 % G+C content. Its genome size is similar to that of *Pseudomonas putida* W619 (Wu *et al.*, 2011) and smaller than those of *Pseudomonas putida* KT2440 (Nelson *et al.*, 2002), *Pseudomonas putida* F1 and *Pseudomonas putida* GB-1. It comprises a series of genes encoding properties related to rhizosphere colonization and determinants for favouring plant growth. This strain is capable of solubilising inorganic phosphate through the production of gluconic acid, it can also solubilise iron through the synthesis of pyoverdines, it has the ability to synthesise plant hormones like indole-3-acetic acid (IAA) through two putative tryptophan-dependent pathways (Roca *et al.*, 2013) and it holds genes encoding proteins for the synthesis of salycilate.

Therefore, this strain has the genetic potential to promote plant growth through direct and indirect mechanisms, being object of interest for its potential use as part of biostimulants, biofertilizers and in other environmental treatments. Moreover, the use of Pseudomonas putida

BIRD-1 has been protected through an international patent (Pizarro – Tobías and Ramos-Duque, 2008).

2.2.2. *Pseudomonas putida* KT2440

Another strain used in this study is *Pseudomonas putida* KT2440, a plasmid-free derivative of the strain *P. putida* mt-2, which was first isolated in Japan in 1963 by Hosakawa. *Pseudomonas putida* mt-2 bears the TOL plasmid (*pWWO*) that codifies for enzymes involved in toluene and xylenes degradation (Worsey and Williams, 1975). Later on 1981, *Pseudomonas putida* KT2440 was constructed by curing mt-2 strain from the TOL plasmid (Bagdasarian et al., 1981). KT2440 is inefficient in foreign DNA restriction, a trait that has made this strain largely studied in the expansion of degradative catabolic pathways (Bagdasarian and Timmis, 1982; Ramos *et al.*, 1994), as host in the cloning and expression of heterologous genes to be used in biotransformation processes of chemical (Delgado *et al.*, 1992; Kraak *et al.*, 1997; Kellerhals *et al.*, 1999) or pharmacologic compounds (Tan *et al.*, 1997).

Pseudomonas putida KT2440 genome has been sequenced (Nelson *et al.*, 2002). This strain's genome size is 6,18 Mp and has a 61.6 % G+C content. Within its genome it has been identified 804 copies of a repetitive extragenic palindromic sequence, of 35 bp known as REP (Aranda-Olmedo *et al.*, 2002). It has 105 genomic islands with distinct G+C content, which are implied in the uptake and degradation of organic compounds, ion transport, synthesis and secretion of secondary

metabolites, being responsible of the metabolic versatility of *Pseudomonas putida*. This feature has also been attributed to plasmids, which bear genes that canalise substrates into central metabolism. Examples of these are the TOL plasmid involved in the catabolism of toluene and xylenes (Williams and Murray, 1974), the NAH7 plasmid involved in the catabolism of naphthalene (Dunn and Gunsalus, 1973) and the CAM plasmid involved in the catabolism of camphor (Rheinwald *et al.*, 1973).

This strain has the ability to cope with toxic compounds through the use of extrusion pumps, like the RND pumps and the MFS transporters (Ramos *et al.*, 2002). Is has also acquired mono- and di-oxygenase systems, oxidoreductases, ferredoxins and cytochromes, dehydrogenases, proteins involved in sulphur metabolism and glutation-S-transferases, alternative sigma factors and stress response regulators (Nelson *et al.*, 2002). It has also have genes involved in plant-bacteria interactions in the rhizosphere (Matilla *et al.*, 2007).

3. Solubilisation of low-availability nutrients mediated by *Pseudomonas* in the rhizosphere.

3.1. Solubilisation of phosphate

Phosphorous (P), as well as nitrogen, is an essential nutrient for plant development. Although abundant in soil, is usually in unavailable forms for plants, since soluble P concentrations in soil are under 1 mg/Kg (10 M $H_2PO_4^-$) (Rodríguez and Fraga, 1999). Therefore, it is often added to agricultural soils through phosphate fertilizers to promote high crop yields by increasing its bioavailability. Nevertheless, its bioavailability is highly dependent on soil type and pH, soon being immobilised in acid soils by oxides and hydroxides of aluminium and iron, and by calcium in alkaline soils (Rodríguez and Fraga, 1999), being in consequence inorganic phosphates commonly associated with aluminium and iron compounds in acid soils, whereas calcium phosphates are predominant in calcareous soils (Gyaneswar *et al.*, 2002). Thus, agricultural soils are an important sink of P due to low efficiency of chemical fertilizers, making the increase of P availability a target for new forms of sustainable agriculture.

Phosphate-solubilising microorganisms (PSMs) have the ability to solubilise immobilised P in soil into bioavailable forms for plant uptake, taking part in the soil phosphorous cycle (Richardson *et al.*, 2009). PSMs are ubiquitous in soil and their proportion vary in the case of bacteria from 1% to 50% and in the case of fungi from 0.1% to 0.5% (Gyaneswar *et*

al., 2002). A variety of microorganisms can solubilise different forms of phosphate in soil and in the rhizosphere of plants (Rodríguez and Fraga, 1999); most of them are able to solubilise calcium-phosphate complexes, being fewer the capable of solubilising aluminium or iron phosphate complexes (Gyaneswar *et al.*, 2002). The solubilisation of soil inorganic phosphate has commonly been attributed to fungi, *Penicillium* and *Aspergillus*, among others, which solubilise insoluble phosphate through the excretion of acids (Pea *et al.*, 1981; Vassileu *et al.*, 1995). This process has also been described in several bacteria either in symbiotic relationship with plants belonging to *Rhizobium* and *Bradyrhizobium* genera, among others (Halder *et al.*, 1990: Halder and Chakrabarty, 1993), or free-living like *Bacillus*, *Burkholderia*, *Flavobacterium*, *Erwinia*, etc. (Rodríguez and Fraga, 1999).

Strains belonging to the *Psedudomonas* genus have also been reported to be efficient phosphate solubilisers through the synthesis of organic acids (Rodríguez and Fraga, 1999; Vyas and Gulati, 2009), specifically gluconic and 2-ketogluconic acids. These are produced via the metabolism of glucose, which is a carbon source present in root exudates (Table 1) (Ahmad *et al.*, 2011), and is catalysed by the pyrroloquinoline-dependent glucose dehydrogenase enzyme (del Castillo et al., 2007). The releasing of acids to the cell external medium generates a decrease of the surrounding soil pH, making phosphorous available by mobilising phosphate from insoluble forms such as di- and tri- calcic phosphate and rock phosphate, among others (Figure 3) (Rodríguez and Fraga, 1999; Roca *et al.*, 2013).

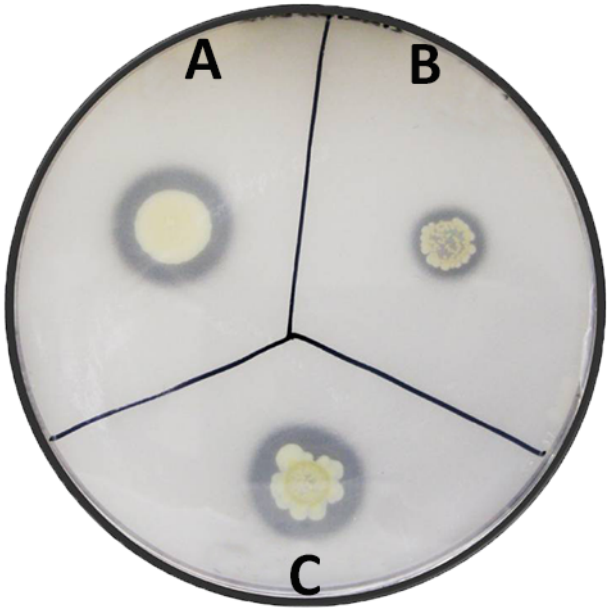


Figure 3: Pikovskaya test with *Pseudomonas putida* BIRD-1 (A) and mutants in the Entner-Doudoroff (B, C) pathway. Clear halos indicate solubilisation of tricalcium phosphate, extracted from Roca *et al.*, 2013.

Organic forms of P are also commonly found in soil; due to the accumulation of plants rests, in the form of organophosphonates, several forms of phosphoesters and phytates, the latter being the main form of P stored in plants (Gyaneswar *et al.*, 2002). Enzymes like phytases (Richardson and Hadobas, 1997), phosphatases (Burini *et al.*, 1994), phosphohydrolases (Rodríguez and Fraga, 1999), phosphocenate hydrolases (McGrath et al., 1998), D-alphaglycerophosphatases (Skrary and Cameron, 1998) and C-P lyases (Ohtake *et al.*, 1996), are responsible for insoluble organic phosphate solubilisation. As plants are poorly capable of obtaining P through soil phytate, the bacterial production of phytases is an interesting trait in PGPR strains, since it increases the available P to plants (Aeron *et al.*, 2011). *Pseudomonas putida* BIRD-1 has been reported use solubilise phytate (Figure 4) (Roca *et al.*, 2013).

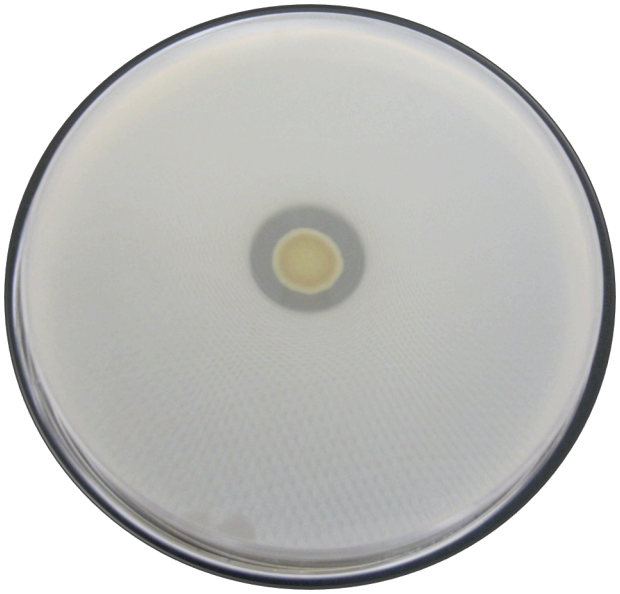


Figure 4: Phytase agar test with *Pseudomonas putida* BIRD-1. Clear halo indicate solubilisation of phytate.

3.2. Solubilisation of iron

Iron is also an essential nutrition element usually found in insoluble oxide hydrate complexes that are not bioavailable for microorganisms, and has been described to be limiting for their growth. It is involved in several enzymatic processes that comprise oxygen metabolism, electron transfer and RNA and DNA synthesis (Schalk *et al.*, 2011).

Some microorganisms have the ability for producing siderophores (Figure 5) that are molecules with a high affinity for iron (van Loon, 2007), capable of chelating Fe(III) and other metals with lower affinity (Schalk *et al.*, 2011). These complexing molecules can also be used by plants (Lemanceau *et al.*, 2009; Gamalero and Glick, 2011), making this feature an interesting trait commonly found in PGPR strains (Schalk *et al.*, 2011). These chelating agents are excreted to the cell external milieu

and form the siderophore-iron complex, which is then internalized by the cell through TonB-dependent transporters (Molina *et al.*, 2006). This process is also an indirect mechanism for suppressing plant pathogens growth, since siderophore-producing bacteria lower the availability if this essential nutrient for less efficient competing microorganisms (Visca *et al.*, 2007).

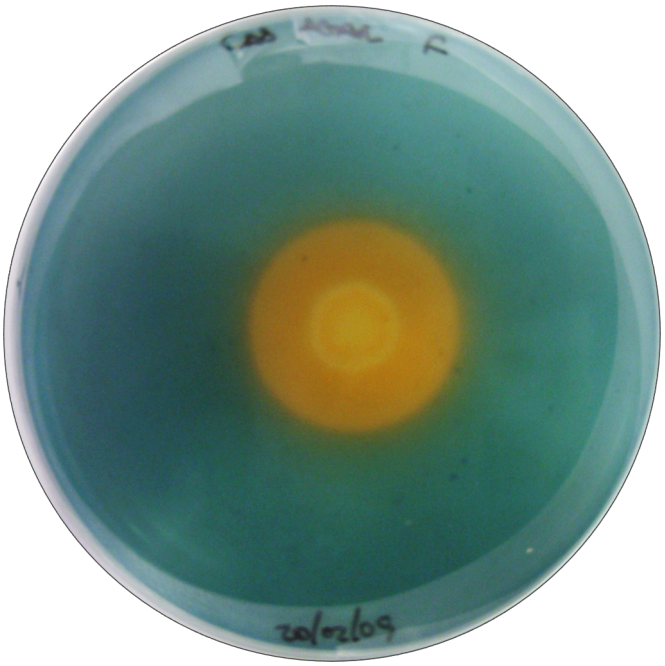


Figure 5: CAS agar test with *Pseudomonas putida* BIRD-1. Yellow halo indicate solubilisation of iron.

There are a wide variety of siderophores structures, being the catecholates most common in bacteria and the hydroxamates usually produced by fungi. Mixed siderophores with both, catecholates and hydroxamates groups, like pyoverdine and pseudobactin, are usually produced by *Pseudomonas* (Schalk *et al.*, 2011). It has been described that several *Pseudomonas putida* strains have outer membrane receptors that allows them to internalize heterologous siderophores (Martínez-Bueno *et al.*, 2000), which could be a key feature for rhizosphere success.

Pyoverdines (Figure 6) are green-fluorescent pigments that are produced by the *Pseudomonas* rRNA homology group I, which includes *Pseudomonas putida*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Pseudomonas chlororaphys* and *Pseudomonas syringae*, in low-iron conditions (Meyer, 2000). The study of different pyoverdines from various strains of *Pseudomonas* has showed that they share three structural components: i) a conserved fluorescent dihydroxyquinoline chromophore, ii) an acyl side chain bound to the amino group of the chromophore, and iii) a variable peptide chain linked by an amide group bound to the C1 carboxyl group of the chromophore, whereas the catecholate and hydroxamate groups provide high affinity binding sites for Fe(III) (Visca *et al.*, 2007).

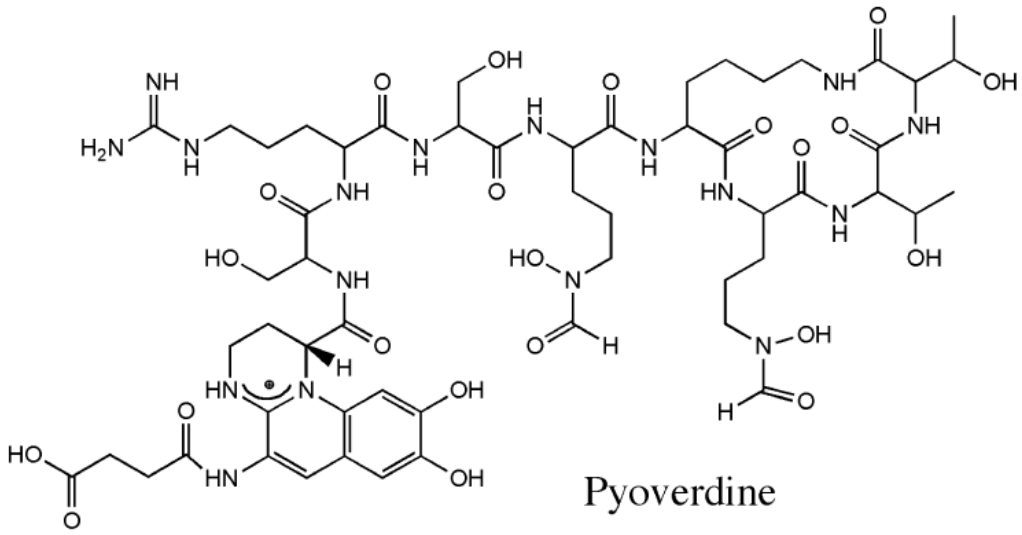


Figure 6. Example of pyoverdine structure. Adapted from Schalk *et al.*, 2011.

Studies to discern the ecological role and various functions of these compounds have been performed, revealing that, apart from iron acquisition and an indirect mechanism of biocontrol of pathogens, they could be related with helping bacteria to cope with heavy metals toxicity

(Schalk *et al.*, 2011). Thus, the versatile functions of these compounds contribute to the colonisation and survival efficiency of *Psedumonas* in the rhizosphere.

4. Production of compounds with plant growth-promoting properties

As said before, the biosynthesis of phytohormones, vitamins and other beneficial compounds for plant development is a feature of PGPR strains. Several microorganisms, like *Pseudomonas putida*, have been found to be efficient producers of phytohomones, like indole-3-acetic acid (IAA) (Dangar and Basu 1987; Patten and Glick 2002; Roca *et a*l., 2013) and capable of synthesising 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which promotes the correct development of plants by reducing levels of ethylene (Li *et al*., 2000; Penrose and Glick 2001).

4.1. Indole-3-acetic acid (IAA)

Indole-3-acetic acid (IAA) is an endogenous auxin present in plants and one of the best-characterized phytohormones. Auxins, from the Greek *auxein* meaning "to grow", are a group of compounds involved in plants-development, directly influencing growth, cellular division and root formation (Cleland, 1990; Spaepen *et al*., 2007). A variety of bacteria belonging to *Azospirillum*, *Azotobacter*, *Rhizobium*, *Pseudomonas*, etc. have been described to produce this auxin, especially in the presence of tryptophan (Patten and Glick, 1996). Tryptophan is a compound common found in root exudates (Table 1) (Ahmad *et al*., 2011), and is also released by microbial and plant decomposing tissues (Spaepen *et al*., 2007).

The study of the different IAA biosynthetic pathways has revealed that there is a high degree of similarity between bacterial and plant pathways. Six different IAA biosynthetic bacterial pathways have been identified, being five of them tryptophan-dependent (Figure 7) (Spaepen *et al.*, 2007).

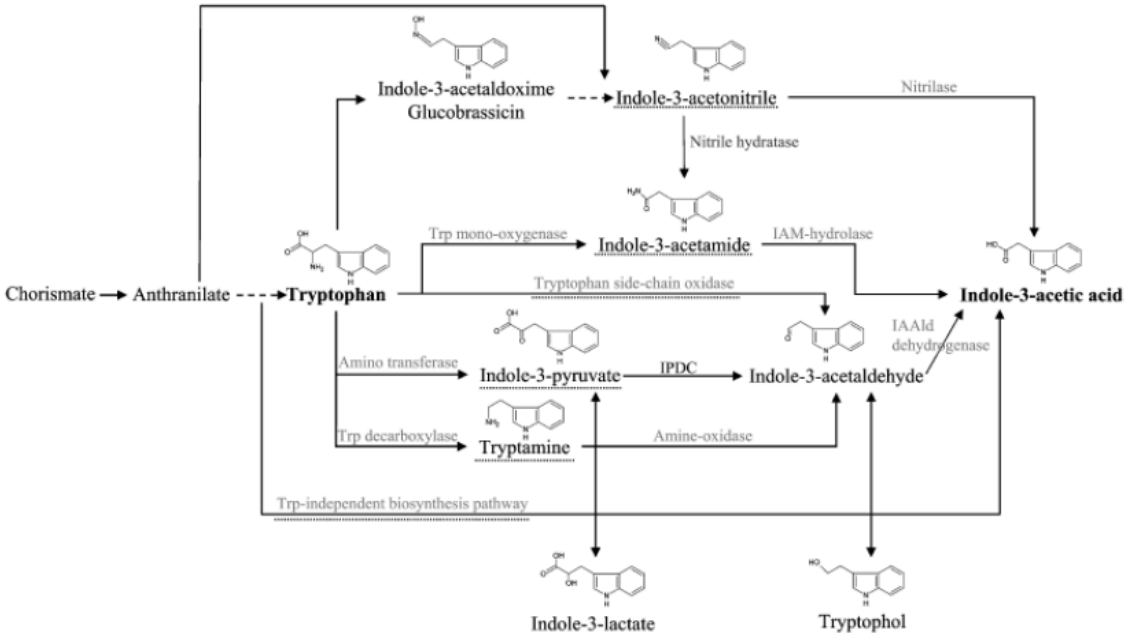


Figure 7. Different bacterial IAA biosynthetic pathways. The intermediate referring to the name of the pathway or the pathway itself is underlined with a dashed line. IAAld, indole-3-acetaldehyde; IAM, indole-3-acetamide; IPDC, indole-3-pyruvate decarboxylase; Trp, tryptophan. Extracted from Spaepen *et al.*, 2007.

i. The indole-3-acetamide pathway (IAM) is a two-step pathway in which tryptophan is converted into IAM through the action of a monooxygenase, and then into IAA via a hydrolase.

ii. The indole-3-pyruvate (IPyA) pathway is a thee-step pathway in which tryptophan is converted into IPyA through the action of an aminotransferase, then IPyA is decarboxylated into indole-3-acetaldeyde (IAAld), and finally IAAld is oxidised into IAA.

iii. The tryptamine (TAM) pathway is a three-step pathway in which tryptophan is converted in TAM through the action of a decarboxylase, then in IAAld through an amine-oxidase, and finally into IAA through the action of a dehydrogenase.

iv. The tryptophan side-chain oxidase pathway is a two-step pathway in which tryptophan is converted into IAAld, and the oxidised into IAA.

v. The indole-3-acetonitrile (IAN) pathway has been preferentially studied in plants, but some bacteria have been found to have enzymes with activity to convert IAN into IAA through IAM (Spaepen *et al.*, 2007).

vi. Tryptophan-independent pathways have been proposed, for both bacteria and plants, but have yet further to be studied and proven.

The study of biosynthesis of IAA has showed that bacteria may have more than one pathway, as has recently described for *Pseudomonas putida* BIRD-1 (Roca et al., 2013).

4.2. 1-aminocyclopropane-1-carboxylate (ACC) deaminase

1-aminocyclopropane-1-carboxylate (ACC) is found in plant root exudates (Reid, 1987) and is the biosynthetic precursor of ethylene in plants (Yang and Hoffman, 1984). Ethylene is a plant-signaling molecule implicated in seed germination, seedlings root-elongation, root nodulation, plant development, flower senescence, leaf abcision and fruit

ripening that in high concentrations has deleterious effects (Glick *et al.*, 1998; Steenhooudt and Vanderleyden 2000; Saleem *et al.*, 2007).

ACC is synthesised through a two-step process in which S-adenosyle methionine is cinverted into ACC, and then ACC is oxidised into ethylene (Arshad and Frankenberger, 2008). It has been reported that plants synthesise ACC and secrete it into the rhizosphete under different types of biotic and abiotic stresses, like pathogen infections, flood, cold, draught, etc.; then some of the ACC may be reabsorbed by the plant and converted into ethylene, promoting a negative feedback due to the accumulation of this plant-growth regulator (Yang *et al.*, 2008; Martínez-Viveros *et al*, 2010). Some bacteria have been reported to enhance plant growth by lowering the levels of ethylene through the activity of the ACC deaminase, which hydrolyses ACC intro ammonia and α-ketobutyrate. These compounds can be used by bacteria as nitrogen and carbon sources, respectively (Klee *et al.*, 1991).

It has been reported that IAA and ACC deaminase are directly related and collaborate in plant development. Exogenous IAA has been described to increase ACC synthase activity in plants, catalizing ACC production that stimulates ACC deaminase activity in bacteria (Patten and Glick, 2002). Although it has been described that PGPR bacteria mutants without the capacity of producing ACC deaminase aren't able to stimulate root elongation (Glick *et al.*, 1994; Li *et al.*, 2000), it has recently been reported a case in which *Pseudomonas putida* BIRD-1 ACC deaminase

minus mutants did not negatively influenced root development in maize (Roca *et al.*, 2013).

5. Uses and applications of PGPR microorganisms in the environment

The increased knowledge about rhizosphere management and the potential benefits of microorganisms gives biotechnology the capacity of reducing fertilizers and agrochemicals in agriculture and contribute to pollutants removal. PGPR microorganisms are becoming a common ingredient of biofertilizers, bioremediation and rhizoremediation treatments in agricultural practices and environmental treatments (Vessey *et al.*, 2003; Nakkeeran *et al.*, 2005; Berg, 2009; Lugtenberg and Kamilova, 2009; Segura *et al.*, 2009; Kroll *et al.*, 2010; Segura and Ramos, 2012).

5.1. PGPRs in agriculture

For enhancing crop yields there already are PGPR commercial formulations, implicating that a variety of PGPR strains have been characterised and described as suitable for their use in modern sustainable agriculture (Bhattacharyya and Jha, 2011). There are several categories of PGPRs based on their mechanism of action, which comprise i) biofertilizers, ii) phytostimulators, and iii) biopesticides (Table 2); moreover, PGPR may enhance plant development through the combination of more than one of the cited properties (Martínez-Viveros *et al*, 2010). Apart from these traits, an important feature for a potential PGPR inoculant is the ability for maintaining the sufficient population levels in the rhizosphere to exert its beneficial effect.

Table 2. PGPR forms and their mechanism of action stimulating plant growth. Adapted from Bhattacharyya and Jha, 2011.

PGPR forms	Definition	Mechanism of action
Biofertilizer	A substance that contains live microorganisms which, when applied on the seed, plant surface or soil, colonizes the rhizosphere and promote plant growth through increased supply of primary nutrientsfor the host plant	Biological nitrogen fixation Utilization of insoluble phosphorus
Phytostimulator	Microorganisms with the ability to produce phytohormones such as indole acetic acid, gibberellic acid, cytokinins and ethylene	Production of phytohormones
Biopesticide	Microorganisms that promote plant growth by controlling phytopathogenic agents	Production of antibiotics, siderophores, HCN Production of hydrolytic enzymes Acquired and Induced systemic resistance

As a critical step in PGPRs efficiency in the field is the establishment of an effective initial population, it is of great importance to assess inoculation doses. Inoculation methods vary depending on the PGPR strain used and the field conditions, from irrigation to low-cost carriers to achieve homogeneous mixing with soil, like peat or clay, among others (Martínez-Viveros *et al*, 2010). In general, bacterial suspensions for soil inoculation and/or root dipping are prepared with high cell densities between 10^8 to 10^9 UFC mL^{-1}, as initial population may decline by orders of magnitude due to competition with other microorganisms for certain time until it reaches an equilibrium with its environment. Thus, to maintain effective and viable PGPR populations under field conditions may be necessary to

reinoculate after different periods of time depending on the strains and the environmental influence towards them (Martínez-Viveros *et al*, 2010).

Further, specific monitoring strategies for the PGPR strains may be developed to assess how density of introduced cells fluctuates under the desired population levels. These methods can vary from classic culture on specific media for the target microorganism, which at times may difficult differentiate morphological characteristics, to other direct mechanism like the use of marker genes. The use of strains resistant to certain antibiotics like rifampicin is most suitable, as the background level of indigenous microbiota resistant to this antibiotic is low. Nonetheless, although this method has limitations, it is frequently used due to its simplicity and relatively low cost (Ahmad *et al*., 2011).

Direct monitoring methods comprise the use or marker genes encoding biocontrol traits, like the production of antibiotics, like 2,4-DAPG (diacetylphloroglucinol) or general biocides like hydrogen cyanide (Haas and Défago, 2005). Other PCR approaches comprise the use of DNA fingerprintings, which allow differentiating up to the strain level, as reported for *Pseudomonas putida* by Aranda-Olmedo and colleagues (2002). The use of reporter genes like green fluorescent protein (GFP), LacZ, Lux and Luciferase gene have been developed, as they allow to study PGPRs in theyr natural environment through the use of fluorescence microscopes (Ahmad *et al*., 2011). Nonetheless, these

techniques aren't economically viable monitoring methods to be used in a basis for field treatments.

In summary, biotechnology is already been used to improve the efficacy of PGPRs in agricultural uses, although the economical success of these potential industrial products will also depend on business management, product marketing, extension education and extensive research (Bhattacharyya and Jha, 2011).

5.2. PGPRs in bioremediation and rhizoremediation

The release of toxic compounds for the environment and human health is a direct consequence of the industrialization. Organic contaminants are persistent in the environment comprising compounds released by the consumption of fossil fuels, like polycyclic aromatic hydrocarbons (PAHs) and total petroleum hydrocarbons (TPHs), polychlorinated biphenyls (PCBs) among other chlorinated aromatics, halogenated compounds like perchloroethylene (PCE) and trichloroethylene (TCE), and pesticides; inorganic pollutants are usually heavy metals (Zhuang *et al.*, 2007). Assessing new techniques of remediation of polluted soils and waters have led to the development of physical, chemical and biological treatments. Biological treatments are beginning to gain importance due to several advantages over other methods, like cost-effectiveness, convenience, the complete degradation of organic pollutants and no collateral destruction of the site or its native flora and fauna (Timmis and Pieper, 1999).

As said before, PGPR have plenty of uses for enhancing plant yield of plants of agricultural interest, and also some may be used for biocontrol of pathogens; nevertheless, some of these strains are able to degrade and mineralize toxic compounds, pointing out their prospect usage in remediation techniques (Segura *et al.*, 2009). For this reason, they are interesting to complement phytoremediation techniques by enhancing plant growth, since the use of plants in remediation treatments has the main disadvantage of the low tolerance plants have to soil contaminants, limiting the efficiency of this technology (Huang *et al.*, 2004).

Nonetheless, understanding the mechanisms of how PGPR strains interact with indigenous microbiota, and their pollutant tolerance has to be assessed for each specific situation, since few field-scale studies have been performed. To this matter, proper plant development through establishing the best plant-PGPR combination is quite essential for it has been described that plant exudates promote the proliferation of native organic-pollutants degraders (Segura *et al.*, 2009; Segura and Ramos, 2012). Singer and colleagues (2003) reported that several aromatic compounds contained in root exudates, like flavonoids, terpenes or lignin-derived components (Table 1), have similar chemical structures as some pollutants and can act as inducers of catabolic pathways (FIGURE). Besides, the degradation of the aromatic compounds released by roots leads to the formation of intermediates like protocatechuate, which is an intermediate in the degradation of PAHs; salycilate, which apart from inducing systemic acquired resistance in plants, also induces PAHs

degradation patways (Figure 8). These compounds along with phloroglucinol phenylacetic acid, substituted cinnamic acids, and others, are mineralized through the β-ketoadipate pathway, which is active in the catabolism of several aromatic pollutants (Segura *et al.*, 2009).

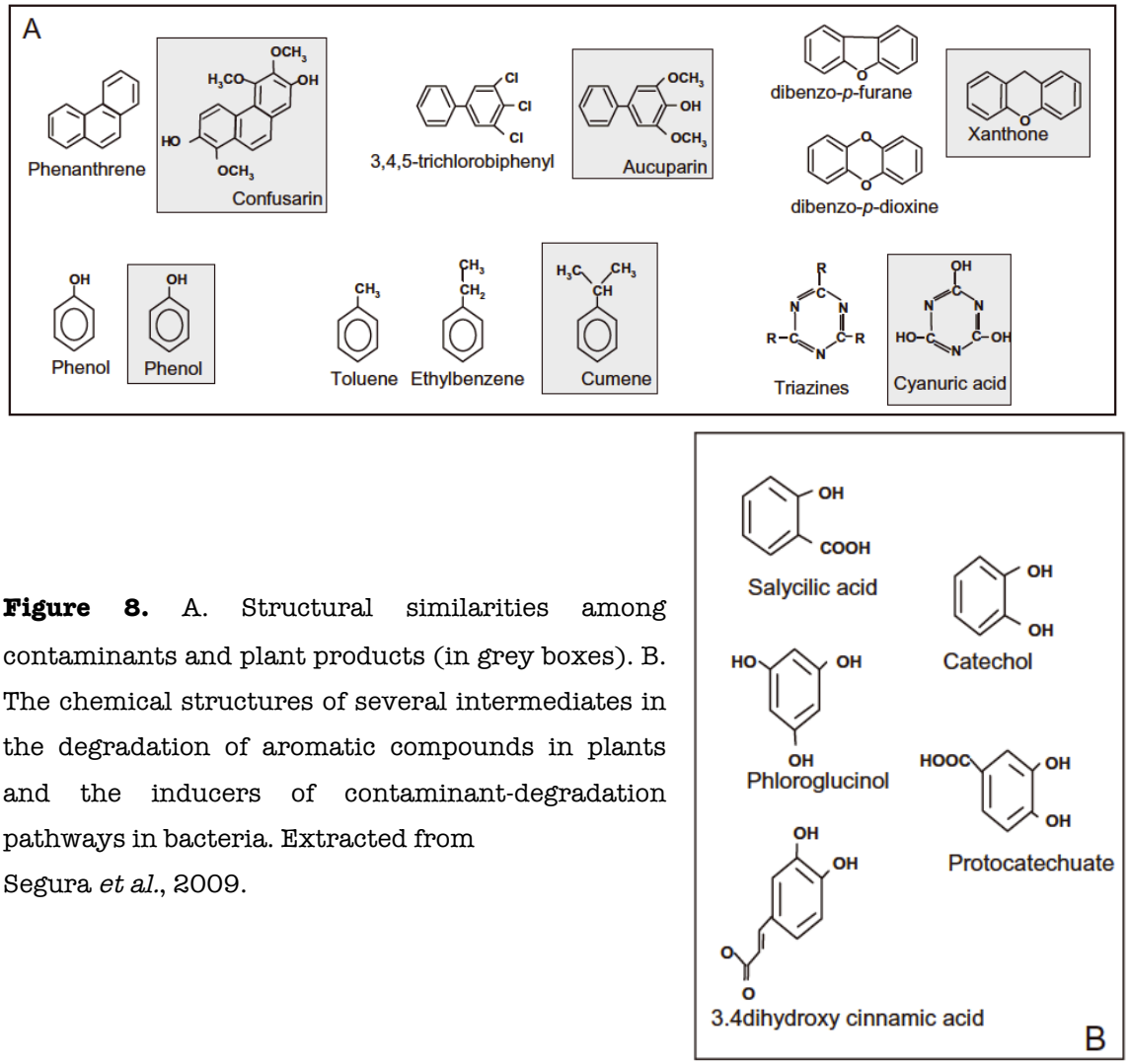


Figure 8. A. Structural similarities among contaminants and plant products (in grey boxes). B. The chemical structures of several intermediates in the degradation of aromatic compounds in plants and the inducers of contaminant-degradation pathways in bacteria. Extracted from Segura *et al.*, 2009.

In summary, it has to be taken into account that preliminary studies of the polluted area to design the correct strategy and plant-microbe combination have to be made. Nevertheless, rhizoremediation proposes itself as an efficient strategy in polluted scenarios where the need of action needs not to be urgent, for this technology works at slower pace than physic-chemical treatments.

Aims of the Thesis

Environmental application of microorganisms is limited by the lack of knowledge about the effects that the different environmental stressors exert over their survival and metabolic activity, which condition their ability for colonizing different niches. Moreover, it is unknown how microorganisms react to stress conditions when they are used in formulations for environmental applications.

This thesis is focused on the characterization of a rhizospheric microorganism, *Pseudomonas putida* BIRD-1, isolated from environmental samples, and that has the ability for solubilising soil unavailable nutrients and produce phytohormones that promote plant growth.

The general aim of this thesis is to overcome limitations in this knowledge and improve the technology of application of microbial inoculants in diverse environmental conditions, which are adverse for the correct development of vegetable species of interest.

Specific aims are:

1. Genotypic and phenotypic characterization of *Pseudomonas putida* BIRD-1, a strain with potential to be used in the development of agricultural and environmental microbial inoculants.

2. Categorize the effect of different environmental conditions over the performance this bacteria in the rhizosphere of plants, on its own and in consortium with other microorganisms of interest.

3. Assess the potential and effectiveness of this strain as plant growth-promoting agent in agricultural, bioremediation and rhizoremediation treatments.

4. Identify *Pseudomonas putida* BIRD-1 mechanisms involved in its ability for surviving, colonizing and proliferating in the rhizosphere.

Results

Chapter 1

Analysis of the plant growth-promoting properties encoded by the genome of the rhizobacterium *Pseudomonas putida* BIRD-1

This chapter was published as:

Roca A, Pizarro-Tobías P, Udaondo Z, Fernández M, Matilla MA, Molina-Henares MA, Molina L, Segura A, Duque A, Ramos JL (2012). Analysis of the plant growth-promoting properties encoded by the genome of the rhizobacterium *Pseudomonas putida* BIRD-1. Environ Microbiol 15: 780-794.

Abstract

Pseudomonas putida BIRD-1 is a plant growth-promoting rhizobacterium whose genome size is 5.7-Mbp. It efficiently adheres to plant roots and colonizes the rhizosphere of plants to high cell densities even in soils with only 2% moisture. This property seems to be linked to its ability to synthesise trehalose, as deduced from a mutant deficient in the synthesis of trehalose who exhibited less tolerance to desiccation than the parental strain. The genome of BIRD-1 also encodes a wide range of proteins that help it to deal with reactive oxygen stress generated in the plant rhizosphere. BIRD-1 PGPR properties derive from its ability to enhance phosphorous and iron solubilisation and to produce phytohormones. BIRD-1 is capable of solubilising insoluble inorganic phosphate forms through acid production. In addition, the genome of BIRD-1 encodes at least 5 phosphatases related to phosphorous solubilisation; one of them being a phytase that facilitates the utilization of phytic acid, the main storage form of phosphorous in plants. Pyoverdine is the main siderophore produced by this strain, a mutant in the FvpD siderophore synthase failed to grow on liquid medium without supplementary iron; however, in soils the FvpD mutant was as competitive as the parental strain because it has a number of receptors that capture the siderophores produced by other microbes, allowing iron acquisition and survival. BIRD-1 overproduces indole-3-acetic acid through convergent pathways, which influences its ability to stimulate seed germination and plant growth.

Introduction

The rhizosphere is a very dynamic environment in which complex interactions take place between plant roots and microorganisms (Molina *et al.*, 2000; Uroz *et al.*, 2010; Blom *et al.*, 2011). Management of the rhizosphere represents a genuine opportunity for biotechnology since it can increase the levels of crop production while reducing the use of fertilizers and agrochemicals in agriculture.

Plant Growth-Promoting Rhizobacteria (PGPR) efficiently colonise the rhizosphere of a wide range of plants and stimulate plant growth through direct or indirect mechanisms (Kloepper *et al.*, 1980; Glick, 1995; Somers and Vanderleyden, 2004; Lugtemberg and Kamilova 2009; Matilla *et al.*, 2010; Blom *et al.*, 2011). Direct plant growth promotion is often mediated by the synthesis of phytohormones or enhanced nutrition; e.g. by increasing the bioavailability of mineral nutrients such as phosphorous, nitrogen and iron (Lugtenberg and Kamilova, 2009), or by providing amino acids and other nutritional factors (Simmons *et al.*, 1997; Compant *et al.*, 2010; Vial *et al.*, 2011). Many PGPR produce siderophores, that are small metal-binding molecules which mainly chelate Fe(III) and other metals with lower affinity (Schalk *et al.*, 2011). The siderophore-metal complexes are preferentially used by microbes (van Loon, 2007), but they can also be taken up by plants (Lemanceau *et al.*, 2009; Gamalero and Glick, 2011). Plant growth promotion by PGPR can also be mediated by a number of indirect effects: (i) by avoiding the action of soil-borne pathogens through competition for iron, (ii) through the synthesis of

antibiotics, antifungals and pesticides (Hammer *et al.*, 1997; Lugtenberg and Kamilova, 2009; Pérez *et al.*, 2011; Ahemad and Khan, 2012), and (iii) through the production of biocides such as hydrogen cyanide and fungal cell wall degrading enzymes, e.g., chitinase and ß-1,3-glucanase (Zhang and Yuen, 2000; Haas and Keel, 2003; Meschke and Schrempf, 2010; Malfanova *et al.*, 2011). Some non-pathogenic rhizobacteria have been reported to suppress diseases in plants by triggering induced systemic resistance (ISR), a plant response that depends on jasmonic acid and on the plant's ethylene signalling pathways (van Loon and Bakker, 2005; Bakker *et al.*, 2007). Several PGPR are commercially available for application as crop inoculants and for biofertilization, phytostimulation, biocontrol and rhizoremediation (Nakkeeran *et al.*, 2005; Berg, 2009; Segura *et al.*, 2009; Kroll *et al.*, 2010).

Bacteria of the genus *Pseudomonas* are frequent rhizosphere colonizers and some strains of the species *P. fluorescens*, *P. putida*, *P. mendocina* and *P. stutzeri* have been shown to possess PGPR capabilities (reviewed by Wu *et al.*, 2010). Strains of the species *Pseudomonas putida* are acknowledged as one the most efficient phosphate-solubilising cultivable bacteria (Rodríguez and Fraga, 1999; Vyas and Gulati, 2009; Daniels *et al.*, 2010), and among them, the BIRD-1 strain is particularly efficient. This strain was isolated from a garden soil in a culture medium without iron addition and with insoluble inorganic phosphate as a source of phosphorous (Matilla *et al.*, 2011). The genome sequence of *P. putida* BIRD-1 was obtained using the *454* pyrosequencing technology (Matilla

et al., 2011). The complete genome sequence of *P. putida* BIRD-1 is made up of a 61.74%-GC circular chromosome of 5,731,541 bps with no plasmid. Its genome size is similar than that of *P. putida* strain W619 (5,774,330 bps) (Taghavi *et al.*, 2009; Wu *et al.*, 2010), but significantly smaller than that of *P. putida* strains KT2440 (6,181,860 bps) (Nelson *et al.*, 2002), F1 (5,959,964 bps) (GenBank accession CP000712), and GB-1 (6,078,430 bps) (GenBank accession CP000926). The genome of BIRD-1 shows high homology and synteny with that of KT2440 and that of the F1 strain. All three strains share around 80% of all of their genes.

In this study we analysed the genome of *P. putida* BIRD-1 in relation to its PGPR properties and the most relevant data are presented.

Materials and Methods

Strains, plasmids and culture media

The bacterial strains, cloning vectors, and plasmids used in this study have been described before (Matilla *et al.*, 2010; Molina-Henares *et al.*, 2009). *Pseudomonas putida* BIRD-1 and its mutant derivatives were routinely grown in M9 minimal medium supplemented with glucose (0.5% [w/v]) as the carbon source (Abril *et al.*, 1989). Cultures were incubated at 30°C and shaken on an orbital platform operating at 200 strokes per minute. When required, antibiotics were used at the following final concentrations (in μg per mL): ampicillin, 100; chloramphenicol, 30; kanamycin, 50; and tetracycline, 20. *Escherichia coli* strains were grown at 37°C in LB broth (Sambrook *et al.*, 1989) with shaking.

When indicated *P. putida* cells were grown on modified A medium (Nautilaya *et al.*, 1999) that lacks a phosphorous source and whose specific composition per liter was NH_4Cl, 267 mg; $MgSO_4 \times 7H_2O$, 410 mg; KCl, 300 mg; NaCl, 200 mg, 1 mL of an aqueous solution of iron citrate [6 g/L] and 0.5% (w/v) glucose, or 10 mM sodium benzoate as the carbon sources. As a phosphorous source we added either phosphate rock [5 g/L] or a mixture of insoluble inorganic phosphates made up of CaHPO4 × $2H_2O$ and $Ca_5(OH)(PO_4)_3$.

To screen the ability of *Pseudomonas putida* strains to use inorganic and organic insoluble phosphate as a phosphorous source Pikovskaya agar

plates (Naik *et al.*, 2008), and PSM (Phytate Screening Medium) agar solid medium were used (Jorquera *et al.*, 2008). The reaction was positive when a clear halo surrounding the bacterial colonies was formed after 3-7 days of incubation at 30ºC.

CAS agar solid medium was used to screen siderophore production (Alexander and Zuberer, 1991). The reaction was considered positive when an orange halo surrounding the bacterial colony was formed after 3-7 days of incubation at 30ºC.

To screen the ability of *Pseudomonas putida* strains to produce indole-3-acetic acid (IAA) on solid medium, amended LB and the Salkowski's reagent were used (Naik *et al.*, 2008). The composition of the amended LB per liter was 10 g bactotryptone, 5 g yeast extract, 10 g NaCl, 5 mM *L*-tryptophan, 600 mg sodium dodecyl sulfate (SDS), 10 mL glycerol and 15 g agar. The composition of the Salkowski's reagent per liter was 2% v/v of 0.5M $FeCl_3$ dissolved in 35% perchloric acid. Quantification of IAA in liquid medium was performed after growth of the strains for 24 h at 30ºC with different concentrations of *L*-tryptophan (0, 100, 250, 500 and 1,000 mg/L, as described) (Pattem and Glick, 2002).

Quantification of soluble phosphate in culture medium

Determination of soluble phosphate in solution was performed as described by Murphy and Riley (1958). Supernatants at 0, 2, 4, 8, 24, 48 and 72h from *P. putida* BIRD-1 cultures grown in phosphate rock (5 g/L)

were mixed with a reagent whose composition per 250 mL was 125 mL H_2SO_4 (5N), 37.5 mL of 4% ammonium molybdate solution and 75 mL of 0.1 M ascorbic acid. Forty milliliters of each supernatant were mixed with 8 mL of the reagent solution and incubated for 30 minutes in a 60ºC water bath. After cooling, absorbance at 620 nm was measured. The amount of soluble phosphate (mg/L) present in the culture supernatants was determined by comparing absorbance measures to a PO_4^{3-} standard curve.

Siderophore production

Strains were inoculated in M9 minimal medium supplemented with glucose (25 mM). Iron citrate (6 mg l^{-1}) was added to the control cultures (plus iron) but not to the iron-deprived ones. When the cultures reached an OD at 660 nm of around 1, they were illuminated with ultraviolet light in a LKB 2011-002 MacroVue transilluminator (LKB Bromma, Sweden) and photographed (GelDoc 2000, Bio-Rad). To record the UV-visible spectrum (220–700 nm) of the culture supernatant, 1 ml of culture was withdrawn, cells removed by centrifugation (14 000 *g* ×5 min) and the supernatant used to measure the absorption spectrum.

Toxic compound resistance assays

Individual colonies of *P. putida* BIRD-1 and mutant strains were picked from freshly cultured LB plates, streaked onto LB medium plates, supplemented with the suitable antibiotic, and grown overnight at 30°C. The biomass of this overnight plate was recovered from the plate surface

and resuspended in 15 ml of LB liquid medium to an OD_{660} of 0.1. Micro-well plate wells were filled with 180 µl of the above cell suspension and with 20 µl of the different 10× concentrated solutions of the stressors. Samples were incubated and data recordings were processed using a Bioscreen C MBR analyzer as described before (Daniels *et al.*, 2010). Toxic compounds tested were: $K_2Cr_2O_7$ (12.5 µg ml^{-1}), H_2O_2 (0.004%), Methyl viologen (100 µM), Tert-butyl hydroperoxide (0.00078%), KCN (0.325 mg ml^{-1}), ampicillin (100 µg ml^{-1}).

Surface sterilization, germination of seeds and root colonization assay

These assays were performed as described by Ramos-González *et al* (2005). Corn seeds were surface-sterilized by rinsing with sterile deionized water, washing for 10 minutes with 70% (v/v) ethanol, then for 15 minutes with 10% (v/v) bleach, and followed thorough rinsing with sterile deionized water. Surface sterilized seeds were pre-germinated on water-agar (2.25% agar [w/v]) at 30°C in the dark for 48 h.

For root colonization assays seeds were inoculated with approximately 5 × 10^6 CFU/mL from an LB medium overnight culture and suspended in M9 salts medium (Sambrook *et al.*, 1989). After incubation without shaking for 30 minutes at 30°C, seeds were washed in sterile distilled water and planted in 50 mL Falcon tubes containing 35 mL of a mixture of sterile peat and washed sand (3:1 v/v) and were then maintained in a controlled chamber at 24°C and 55-65% humidity with a daily light period of 16 h. At the indicate times plants were collected, shoots discarded and the roots placed in 50 mL Sterilin tubes containing 10 mL

of M9 minimal salts medium (Sambrook *et al.*, 1989) and 4 g of glass beads (0.3 mm diameter). Tubes were vortexed for 1 minute and the number of CFU attached to the surface of the root were estimated by drop-plating serial dilutions of the supernatant in selective media.

Corn seeds adhesion assays

Strains were cultured overnight at 30ºC in LB broth and culture turbidity was adjusted to OD_{660}=1 in a final volume of 1 mL M9 minimal salts and the assays done as described by Espinosa-Urgel *et al.* (2004).

Corn seeds growth on agar plates

Seeds were sterilized, as described above, and submerged for 30 minutes without shaking on overnight cultures (10^8 CFU/mL) of the different strains under test. Seeds were rinsed with sterile distilled water to wash out non-attached cells, and then placed on water-agar plates (agar 2.25% w/v). Growth was checked after 7 days of incubation in the dark at 28ºC. Roots were then weighed, for fresh weight, and stored in a stove at 90ºC for 48 hours.

Competitive root colonization assays

Surface sterilization, germination of seeds and bacterial inoculation were performed as described above, except that seedlings were inoculated with a mixture of a Sm^R BIRD-1 strain, as the wild type, and the mutant strain

in the specified gene. Inocula size differences between the wild-type and mutant strains were less than 2%. At the indicated times, bacterial cells were recovered from the rhizosphere, as specified above. LB-agar supplied with rifampin and streptomycin (or kanamycin) was used to select BIRD-1 or the mutant strains, respectively.

Microcosm Assays

Sterile maize seedlings were either inoculated or not with approximately 10^6 *P. putida* BIRD-1 cells per entire seedling (Espinosa-Urgel *et al.*, 2000). The seeds were then planted in sterilized potting material (Compo Sana Universal) and grown for 10 days. Then the plantlets were transplanted into plant pots containing 600 g of sterilized loamy silt soil classified as Humic Haploxerept with the following characteristics: 38% sand, 43% silt, and 19% clay; pH 7.9, the organic matter content was 2.1%, and its $CaCO_3$ content was 8%. To determine drought tolerance of wild type and mutant strains, soil water content was adjusted to different degrees. Viable microorganisms in the rhizosphere soil (soil closely associated to roots) and bulk soil from four selected plants were determined, as described above.

Germination of vegetable seeds

Non-sterile vegetable seeds (>30 per treatment) were sown in seedbeds previously filled with a mixture of non-sterile peat and washed sand (3:1 v/v) for control treatments. For the treated seeds, a 10^9 CFU/mL culture

of the assayed strain was diluted (1:10 v/v), and mixed (1:250 v/wt) with the peat-sand mixture; vegetable seeds were then sown and watered with distilled water to achieve appropriate substrate moisture. Seedbeds were covered to allow germination in the dark. Approximately 72 hours after, when germination was accomplished and germination rate was calculated, seedbeds were uncovered to allow stem development, and watered, with tap water, when necessary.

Greenhouse assays

Vegetable seeds of horticultural importance in the Mediterranean area (corn, cucumber, zucchini, lettuce, chard, pepper, melon, onion, tomato and bean) were chosen for greenhouse assays. Seeds were germinated as described above, and plants were watered with tap water when necessary along the assay. Monitoring of growth was performed every month by measuring 30 plants of each treatment; stem length and gauge, when necessary. The root length was measured after 3 months, and dry weight was calculated for each treatment. Survival of inoculated cells was screened every 15 days, by drop-plating serial dilutions of rhizosphere soil (soil closely associated to roots) in selective media, as described before.

Measurement of phosphatase activity in soil

Phosphatase activity was determined as described by Antolín *et al.* (2005). The amount of *p*-nitrophenol (PNP) released from 0.5 g soil from

each treatment (bulk soil and non- inoculated corn rhizosphere soil taken as controls *versus* BIRD-1 inoculated corn rhizosphere soil) was measured after incubation at 37ºC for 120 min with 0.115 M 4-nitrophenyl phosphate-disodium (PNPP) as substrate for the enzymatic reaction, in 2 ml of maleate buffer (0.1 M, pH 6.5). Then, samples were cooled at 2ºC for 15 minutes to stop enzymatic reaction, and 0.5 mL of 0.5 M $CaCl_2$ and 2 mL of 0.5 M NaOH were added and well-mixed. Each sample was centrifuged at 2000×g for 10 minutes. A blank experiment was performed for each assay, in which the substrate was added to the soil sample after incubation and before stopping the reaction. The amount of PNP per hour released from each soil sample (μg PNP g^{-1} h^{-1}) was determined by comparing absorbance measures to a PNP standard curve.

In vitro nucleic acid techniques

Total DNA extraction was performed as described before (Rodríguez-Herva *et al.*, 2007). Plasmid DNA was isolated using the Qiagen spin miniprep kit. For DNA digestion, the manufacturer's instructions were followed (Roche and New England Biolabs). Ligation of DNA and Southern blots were performed following the standard protocols (Sambrook *et al.*, 1989). Highly electro-competent cells were prepared as previously reported (Choi *et al.*, 2006) and transformed using an EC100 electroporator according to the manufacturer's instructions. Expand high fidelity Taq polymerase was used in the amplification of PCR fragments for cloning.

Construction of mutants

For the construction of mutants, gene fragments between 400 and 1000 bp were amplified with the appropriate oligonucleotides and cloned into pMBL1-T plasmid (Dominion MBL). Plasmids containing the corresponding inserts were then cut with BamHI (that cuts into the poly-linker of the vector, but not iton the insert sequence), and the ΩKm resistance cassette of plasmid pHP45Ω-Km (Prentki and Kirsch, 1984), previously excised with BamHI, was ligated into the BamHI restriction site. Approximately 600 ng of the resulting plasmid was electroporated into *P. putida* BIRD-1 (Enderle and Farwell, 1998) to allow a single recombination event between the cloned DNA fragment and its homologous sequence. The resulting kanamycin (25 μg ml^{-1}) resistant clones were selected and analyzed by Southern blot. Clones that contained the insertion in the appropriate location were kept for further analysis.

Nucleotide sequence accession number

The complete genome sequence of *P. putida* BIRD-1 is available in GenBank under accession number CP002290.

Results and Discussion

Adhesion and colonisation of plant roots

An early event in the process of plant root colonization by bacteria is their ability to adhere to biotic surfaces - either to the seed surface or to the root. With BIRD-1 our assays showed that approximately 10^4 to 10^5 CFU of *P. putida* BIRD-1 cells adhered per corn seed, a similar amount to that reported for the KT2440 strain (Espinosa-Urgel *et al.*, 2000; Weinel *et al.*, 2002; Molina-Henares *et al.*, 2006; Yousef-Coronado *et al.*, 2008). Eleven genes found to be involved in adhesion to abiotic and biotic surfaces in *P. putida* KT2440 (Duque *et al.*, 2012) were identified in BIRD-1 (Suppl. Table 1); their degree of identity was in the range of 40 to 100%. Mutants of BIRD-1 in LapA were 10-fold less efficient in adhesion to seeds than the parental strain, and exhibited decreased ability in rhizosphere colonization in competition with the wild-type strain.

Proliferation of BIRD-1 in the plant rhizosphere is favoured by the ability of the strain to use compounds secreted by the plant, among which are the twenty proteinogenic amino acids, organic acids (citric, lactic), sugars (glucose, fructose) and flavonoids. Phenomic assays with a wide battery of carbon sources revealed that *Pseudomonas putida* BIRD-1 is able to use a wide range of amino acids as carbon or nitrogen sources and has the complete set of genes required for the metabolism of proline, glutamate, glutamine, asparagine, tyrosine, valine, branched amino acids, serine and alanine among the amino acids. The strain can use citrate and succinate,

chemicals also present in root exudates, as well as a wide range of lignin-derived compounds including hydroxylated aromatic acids and methoxylated compounds. All of these pathways are common to KT2440 and gene analysis showed sequence and gene order conservation; an issue revised before by Jiménez *et al.* (2002) and Bielecki *et al.* (2011).

PGPR properties are particularly relevant in marginal soils or when plants are exposed to abiotic or biotic stresses. Water scarcity is a common problem in soils in the Mediterranean basin. We tested the survival of *Pseudomonas putida* BIRD-1 in soils with different degrees of humidity using *Pseudomonas putida* KT2440 as a reference strain for these studies. We introduced approximately 5×10^7 CFU BIRD-1 or KT2440/g soil and soil moisture was kept at 2%, 10%, 25% and 50%. At a moisture level of >25% survival of both strains over a 16-day period was similar with a slight increase in CFU/g reaching levels in the order of 2 to 4×10^8 CFU/g soil. When the soil moisture was kept at 2%, BIRD-1 survived at a higher density than KT2440 (Figure 1). Therefore, BIRD-1 is more tolerant to desiccation than *P. putida* KT2440 (Muñoz-Rojas *et al.*, 2006). Trehalose has been suggested to be important for the survival of *P. putida* at low humidity rates. Biosynthesis of trehalose in *Pseudomonas* can take place through two pathways that involve the conversion of maltose into trehalose via TreS (trehalose synthases (PPUBIRD1_1765) and PPUBIRD1_2817 or from maltodextrine catalysed by the TreY (PPUBIRD1_1772) / TreZ (PPUBIRD1_1774) proteins). To test the role of

trehalose production in resistance to desiccation in BIRD-1 we generated mutants in *treZ* and *trezS*.

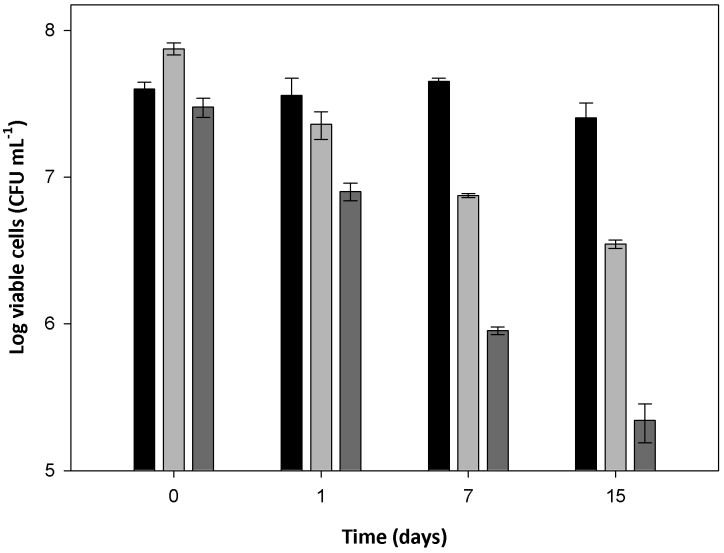


Figure 1. Viable *P. putida* KT2440, BIRD-1 and a TreZ mutant in rhizosphere soil. Soils were inoculated with ~5×10^7 CFU/g and sterile pre-germinated corn seeds sown. At the indicated time CFU/g of rhizosphere soil was determined using 3 different pots. Black bars (BIRD-1), light grey (KT2440), dark grey (*treZ* BIRD1 mutant). Other conditions as described in Materials and Methods.

In the low humidity soil the initial survival of the *treZ* mutant was clearly lower than that of the parental strain or that of mutants in the *treS* gene (Figure 1), suggesting that the maltodextrine pathway for the production of trehalose is relevant for colonization of soils by *P. putida*.

Matilla *et al.* (2007) showed that reactive oxygen species are produced in the plant rhizosphere as a consequence of plant root cell respiration, and that root colonization by bacteria requires the ability to overcome oxidative stress. To test resistance to oxidative stress in BIRD-1, a series of phenomic analyses were carried out in *P. putida* BIRD-1 with methylviologen, H_2O_2, chromate, arseniate and antibiotics such as β-

lactams that induce the formation of reactive oxygen species (Sandermann, 2004). The results showed that *P. putida* BIRD-1 reached similarly high cell densities in cultures without the stressor or with 1 mM methylviologen, 4 mM H_2O_2, 1 mM Cr_2O_7 and 100 μg/ml ampicillin. The genome of BIRD-1 was then analyzed in relation to the set of genes involved in oxidative stress responses, and it was found to encode 2 iron-manganese SodA superoxide dismutases (PPUBIRD1_0966 and PPUBIRD1_0999), and 4 catalases (PPUBIRD1_0143, 0518, 2060 and 2848). In addition we searched for the presence of alkylhydroperoxidases, cytochrome-c peroxidase and glutathione peroxidase. We found 6 homologs for the first group (PPUBIRD1_1273, 2010, 2150, 2706, 2708, 3265 and 3243), a single cytochrome peroxidase (PPUBIRD1_2732) and 2 glutathione peroxidases (PPUBIRD1_3741, and 3931). The redundancy of the genes encoding different isoenzymes related to oxidative stress in the genome of *P. putida* BIRD-1 probably points towards the importance of these proteins and their likely synergic action in the removal of active oxygen species.

Evidence of plant-growth promoting activities encoded by BIRD-1

In another set of assays we tested the stimulatory effect of BIRD-1 on growth of maize roots on agar plates as a model. To this end sterilized corn seeds were deposited on the surface of a water-agar plate either inoculated or not with *P. putida* BIRD-1 (10^6 CFU/cm^2 of surface plate). Germination of corn seeds was monitored over time and the size of the primary root was observed to be larger for seeds on agar surfaces with

bacteria than in the absence of microbes at any incubation time (Figure 2), and that secondary roots also developed more regularly in seeds germinated with bacteria than without.

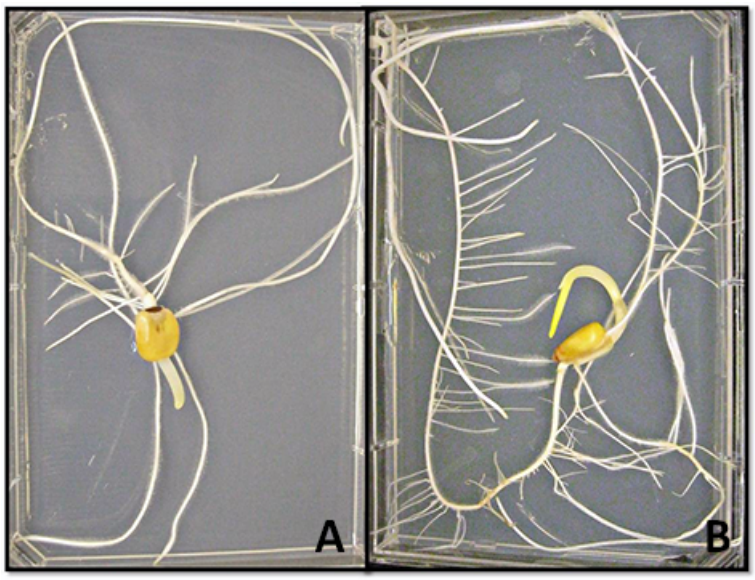


Figure 2. Root development of corn seeds germinated on agar plates. Water-agar plates were prepared, one plate was kept as a control (A) and 0.1 ml of a suspension containing 10^8 CFU/ml was spread on top of the other plate (B). Once the surfaces were dry, a single sterile corn seed was deposited per agar plate and root development monitored over time. The picture corresponds to a typical situation after 1 week incubation at 22°C.

To further confirm these results different plant seeds were also submerged in water with 10^6 CFU of BIRD-1 per ml and after 1 h, 100 seeds of each treatment were sown in seedbeds. We found that the germination rate of all tested plants was higher than in the untreated control (Figure 3A). In addition, we also determined the growth of the plants' aerial part by measuring the size of the plants growing in pots with and without >10^6 CFU BIRD-1/g soil. We observed that the plants growing in soils inoculated with *P. putida* were larger than those growing in non-inoculated soils (Figure 3B).

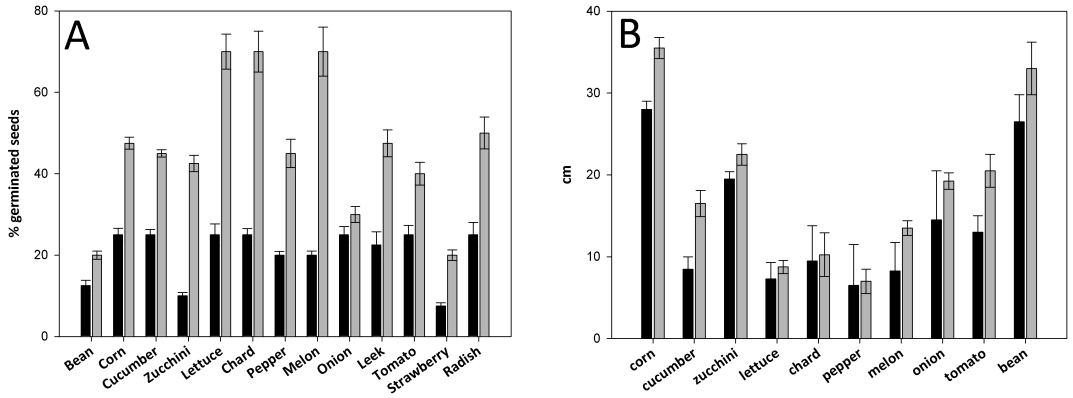


Figure 3. A: Germination rate of seeds of plants of agronomical interest. The assay was performed as described in Matherials and Methods. We used 100 seeds of each plant per assay.. Germination took from 3 days for corn seeds to 10 days for strawberry seeds. The assay was repeated three times and the standard error was in the range of 5 to 15% of the mean values. **B:** Height of plants after 30 days. The seeds of plants were sown in a mixture of pit: sand and a fluvisol soil (1:1:1) and watered twice a week to keep soil water capacity in the range of 30 to 50%. Plants were incubated for 30 days under light-dark (12h:12h) conditions at 22°C in a plant growth chamber.

This series of assays supported that BIRD-1 exhibited PGPR properties, and based on the reported PGPR traits for *Pseudomonas*, one or several of the following explanations is possible: (i) increased solubilisation and mineralization of nutrients, particularly insoluble phosphates (de Freitas *et al.*, 1997; Richardson 2001; Miller *et al.*, 2010); (ii) facilitation of iron uptake by plants by producing higher amounts of siderophores (Cattelan *et al.*, 1999; Pal *et al.*, 2001; Glick and Pasternak 2003; Bakker *et al.*, 2007; Fernández-Piñar *et al.*, 2011); (iii) the ability to produce higher levels of indole acetic acid (IAA) (Dangar and Basu 1987; Patten and Glick 2002; Dobbelaere *et al.* 2003; Dey *et al.* 2004; Viruel *et al.*, 2011); (iv) the ability to produce, 1-aminocyclopropane-1-carboxylate (ACC) deaminase to reduce the level of ethylene in the root of developing plants

thereby increasing the root length and growth (Li *et al.* 2000; Penrose and Glick 2001); (v) the ability to cope with oxidative stress generated in the rhizosphere of plants (Stajner *et al.*, 1995, 1997; Matilla *et al.*, 2010). We then designed specific experiments to test these alternative mechanisms.

Solubilisation of inorganic phosphate

The phosphate solubilisation trait of *Pseudomonas putida* has been attributed to the production of organic acids (Jones and Darrah, 1994; Rodríguez and Fraga, 1999; Shekar and Nautiyal, 1999; Miller *et al.*, 2010). To test BIRD-1's ability to solubilise different phosphorous sources we cultured the strains on modified medium A with various insoluble inorganic phosphate sources (tricalcium phosphate, dicalcium phosphate and phosphate rock) in the presence of different C-sources and monitored growth as CFU/mL. We found that growth occurred with the three P sources with glucose, fructose, glycerol and aromatic acids such as benzoate, although growth with glucose led to higher cell densities (Suppl. Figure 1). Figure 4 shows the results with phosphate rock as a source of phosphorous, and 5 mM sodium benzoate as a C-source. We found that cells can grow exponentially and that phosphate in solution reached a concentration of 0.5 mg/L, what indicated that the strain solubilized phosphate (Figure 4); the pH throughout the experiment was in the range of 5.8 to 7.0.

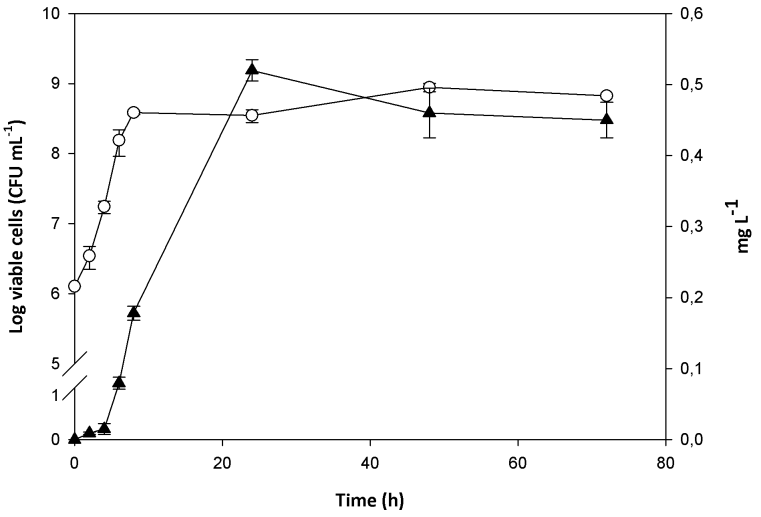


Figure 4. Solubilisation of phosphate and growth of *P. putida* BIRD-1. We used A-medium with 5 g phosphate rock per 100 mL as a source of inorganic phosphorous, and inoculated cultures with an initial cell density of 2×10^6 CFU/ml. At the indicated times phosphate in solution (O) and CFU/ml (▲) were determined as described in Materials and Methods.

The production of organic acids especially that of gluconic acid and 2-ketogluconic acid appears to be the most frequent agent of mineral phosphate solubilisation by bacteria such as *Pseudomonas* sp., *Erwinia herbicola*, *Burkholderia* sp., *Rhizobium leguminosarum, Bacillus firmus*, and other soil bacteria (Duff and Webley 1959; Banik and Dey 1982; Halder *et al.*, 1990; Halder and Chakrabarty 1993; Rodríguez and Fraga, 1999). *Pseudomonas* BIRD-1 produces gluconic acid in the periplasmic space after the oxidation of glucose. In this study we have identified a BIRD-1 mutant that fail to use glucose as a C-source and that exhibits a knockout in Entner-Doudoroff pathway Δ *eda* (2-dehydro-3-deoxyphosphogluconate aldolase). This mutant converted most glucose to 2-ketogluconate (50% of glucose was converted into the acid), and as a consequence, in the Pikovskaya's assay the mutant strain exhibited

increased ability to solubilise insoluble tricalcium phosphate (Suppl. Figure 2).

Several enzymes have also been shown to be involved in making insoluble phosphorous compounds available for cell growth. These processes are achieved via the action of phosphatases, such as phosphohydrolases (Gügi *et al.*, 1991; Rodríguez and Fraga 1999), phytases (Richardson and Hadobas 1997), phosphonoacetate hydrolases (McGrath *et al.*, 1998), D-α-glycerophosphatases (Skrary and Cameron 1998) and C-P lyases (Ohtake *et al.*, 1996). We searched the genome of *P. putida* BIRD-1 to identify genes encoding these activities. We found that 5 potential phosphatases involved in making phosphorous available are encoded by the genome of BIRD-1, namely a 4-phytase family member (PPUBIRD1_5077), a phosphonoacetate hydrolase of the PhnA family (PPUBIRD1_0727), two PAP2 acid phosphatases (PPUBIRD1_2395 and PPUBIRD1_0951), an alkaline phosphatase (PPUBIRD1_0932), and an exopolyphosphatase (PPUBIRD1_5012). In agreement with *P. putida* BIRD-1 encoding a phytase is the fact that the strain is able to grow reaching high cell densities with phytate, the main form of phosphorous stored in plants, as a source of phosphorous (Suppl. Figure 3). We have also determined the total phosphatase activity of BIRD-1 in the rhizosphere of corn plants, and in bulk soil following the protocol described by Antolín *et al.* (2005). At all time points assayed the phosphatase activity was higher in cells recovered from the rhizosphere than in cells from bulk soil (Figure 5), which suggests that plant root

exudates may enhance the expression of one or more of the indicated enzymes. These results are in agreement with an earlier observation by Ramos-González *et al.*, (2005), using IVET technology, which revealed that phosphorous uptake and phosphorous solubilisation genes were induced in *P. putida* KT2440 growing in the rhizosphere of plants.

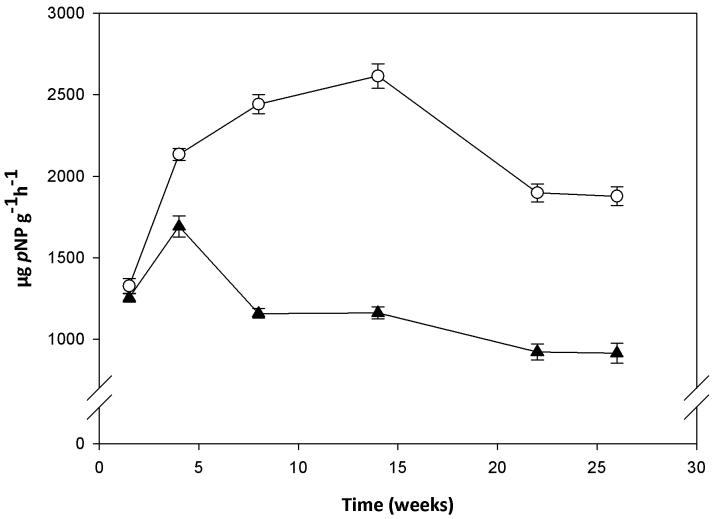


Figure 5. Phosphatase activity in bulk soil and rhizosphere soil. Plants were grown for 25 days in pots with 1 kg soil. At the indicated time 3 plants were removed, and the soil attached to roots was sieved through a 2 mm mesh, this was considered the rhizosphere soil. The rest of soil (bulk) was also sieved in the same way. Triplicate assays were run using one *g* soil per assay to determine total phosphatase activity, as described in Materials and Methods. O, Rhizosphere soil; ▲, bulk soil.

Production of siderophores

Pseudomonas putida BIRD-1 secretes pyoverdine when grown under iron-deficient conditions. As a step towards understanding the molecular events involved in pyoverdine synthesis we screened the BIRD-1 mini-Tn*5* mutant library to identify genes involved in pyoverdine biosynthesis by selecting clones unable to grow on liquid minimal medium without supplemented iron. A single clone with an insertion in the *pvdD* gene

(PPUBIRD1_1630) was found. Some genes related to pyoverdine synthesis were grouped (i.e. PPUBIRD1_1630 through to 1634) while other *pvd* genes for pyoverdine synthesis were found to be scattered throughout the *P. putida* BIRD-1 genome (e.g. *pvdA* which corresponded to PPUBIRD1_1983).

The genome of BIRD-1 encodes a TonB-dependent pyoverdine-iron receptor, FpvA, which is highly specific for the pyoverdine produced by this microorganism. The *fvpA* gene is in an operon with *pvdS* which encodes a sigma-24 family factor and its corresponding anti-sigma (Matilla *et al.*, 2011). Expression of this cluster is known to be under the control of the PvdS and FpvR genes both of which are present in the genome of BIRD-1 and which guarantee the regulated expression of siderophore production. Transport of iron from the periplasm to the cytoplasm is most likely mediated by a three-component ABC transporter made up of the FecBCE proteins (PPUBIRD1_3269/3271) whose genes form an operon, although we cannot rule out the possibility of other ABC transporters playing this role.

Many *Pseudomonas putida* strains are capable of using a wide range of structurally unrelated exogenous siderophores by means of multiple receptors that are located in the outer membrane. In the genome of the KT2440 strain Martínez-Bueno *et al.* (2002) identified 10 TonB-dependent siderophore receptors (FecA-like) for which the strain lacked the corresponding biosynthetic genes for the siderophore, although the

strain had its corresponding adjacent FecI/FecR control system (Table 1). This was taken as evidence that KT2440 was an efficient iron sequestering strain. We carried out a similar bioinformatics analysis with the BIRD-1 genome and found that BIRD-1 also possesses these 10 TonB-like iron receptors (Table 1) as well as 15 other FecA-like proteins that were not linked to *fecI*/*fecR*-like genes (Table 1). Eleven of these 15 FecA-like proteins have a best hit in BLAST analysis with the corresponding protein system of KT2440 while the others exhibited >95% identity with iron-receptors of *P. putida* F1.

The unusual systems of iron acquisition probably contribute to broaden the ecological competence of *Pseudomonas putida* in terms of colonization and persistence in the rhizosphere. Since BIRD-1 has multiple iron-receptors we hypothesized that the FvpD mutant will grow and survive in soils through the acquisition of iron via the series of alternative receptors encoded in its genome. We established competition assays with the parental strain and the FvpD mutant in the rhizosphere of corn plants and, as expected, we found that both colonised and grew at equivalent cell densities.

Table 1. FecA-like receptors in the genome of *P. putida* BIRD-1

Receptor	Gene ID *P. putida* BIRD-1	SWISSPROT/TrEMBL blast hit	Identity %	Organism (*P. putida*)
FecA-like associated to FecI/FecR				
Pp-rec-5	PPUBIRD1_4301	Probable TonB-dependent receptor	89	KT2440
Pp-rec-7	PPUBIRD1_0385	TonB-dependent siderophore receptor	95	KT2440
Pp-rec-8	PPUBIRD1_0190	TonB-dependent siderophore receptor	98	KT2440
Pp-rec-14	PPUBIRD1_1056	TonB-dependent haemoglobin/ transferrin/lactoferrin family receptor	95	KT2440
Pp-rec-17	PPUBIRD1_2217	TonB-dependent siderophore receptor	98	KT2440
Pp-rec-18	PPUBIRD1_0918	FecA-like outer membrane receptor	99	KT2440
Pp-rec-19	PPUBIRD1_3458	TonB-dependent siderophore receptor	99	KT2440
Pp-rec-20	PPUBIRD1_3580	Ferric-pseudobactin M114 receptor pbuA	97	F1
Pp-rec-21	PPUBIRD1_3262	TonB-dependent siderophore receptor	89	GB1
Pp-rec-22	PPUBIRD1_0868	TonB-dependent siderophore receptor	99	KT2440
FecA solo				
Pp-rec-23	PPUBIRD1_1698	TonB-dependent siderophore receptor	99	F1
Pp-rec-24	PPUBIRD1_2432	Outer membrane ferric siderophore receptor, putative	99	KT2440
Pp-rec-25	PPUBIRD1_2577	Outer membrane ferric siderophore receptor, putative	98	KT2440
Pp-rec-26	PPUBIRD1_3090	Outer membrane ferric siderophore receptor	99	KT2440
Pp-rec-27	PPUBIRD1_3412	Ferric enterobactin receptor	98	KT2440
Pp-rec-28	PPUBIRD1_2426	TonB-dependent siderophore receptor	97	KT2440
Pp-rec-29	PPUBIRD1_0575	TonB-dependent siderophore receptor	98	KT2440
Pp-rec-30	PPUBIRD1_3267	TonB-dependent siderophore receptor	97	KT2440
Pp-rec-31	PPUBIRD1_0912	TonB-dependent siderophore receptor	99	KT2440
Pp-rec-32	PPUBIRD1_0300	TonB-dependent siderophore receptor	98	KT2440
Pp-rec-33	PPUBIRD1_0294	TonB-dependent siderophore receptor	99	KT2440
Pp-rec-34	PPUBIRD1_3765	TonB-dependent siderophore receptor	99	KT2440
Pp-rec-35	PPUBIRD1_2177	TonB-dependent siderophore receptor	99	KT2440
Pp-rec-36	PPUBIRD1_2178	TonB-dependent siderophore receptor	99	KT2440
Pp-rec-37	PPUBIRD1_3161	TonB-dependent siderophore receptor	98	F1
Pp-rec-38	PPUBIRD1_4461	TonB-dependent siderophore receptor	95	F1

The top box shows *fecA* genes physically linked to *fecI/fecR* regulatory systems. The bottom box shows non-linked FecA-like. The PPUBIRD1_xxxx number locates the corresponding receptor in the genome of BIRD-1 strain. The annotation refers to the best blast hit with sequences deposited in the SWISSPROT/TrEMBL database and we indicate the degree of identity and the microorganism source that gave the best hit.

Biosynthesis of IAA

The most physiologically active auxin in plants is indole-3-acetic acid (IAA), which is known to stimulate both rapid (e.g. increases in cell elongation) and long-term (e.g. cell division and differentiation) responses in plants (Cleland 1990; Spaepen *et al.*, 2007). IAA is the most common and best characterized phytohormone. It has been estimated that 80% of bacteria isolated from the rhizosphere can produce IAA, particularly in medium with tryptophan (Patten and Glick 1996). We carried out an assay to test IAA production in DF minimal salt medium based on the Salkowski reagent, as described by Patten and Glick (2002). We found that *P. putida* BIRD-1 produced and excreted IAA to the outer medium (up to 120 ppm/unit of DO_{660}) and that production was at least 2-fold higher when the medium was supplemented with 3 mM tryptophan (Suppl. Figure 4).

In BIRD-1, indole-3-acetic acid (IAA) is produced through at least two putative tryptophan-dependent IAA-biosynthetic pathways that are also present in other *P. putida* strains (Wu et al., 2010) (Figure 6). In one of the IAA biosynthesis pathways BIRD-1 possesses two copies of tryptophan 2-monooxygenase which is involved in the conversion of tryptophan into indole-3-acetamide (PPUBIRD1_0418 and PPUBIRD1_1202), while in the other pathway, a single gene is present in the initial step that involves the conversion of tryptophan into tryptamine (PPUBIRD1_3125). Regarding the secretion of IAA it should be noted that BIRD-1 possesses three genes encoding putative auxin

efflux carriers (PPUBIRD1_2233, PPUBIRD1_2634 and PPUBIRD1_0977), similar to those which have been shown to facilitate the excretion of the plant hormone to the surrounding medium or soil when the plant/microbe interaction is studied in sown plants.

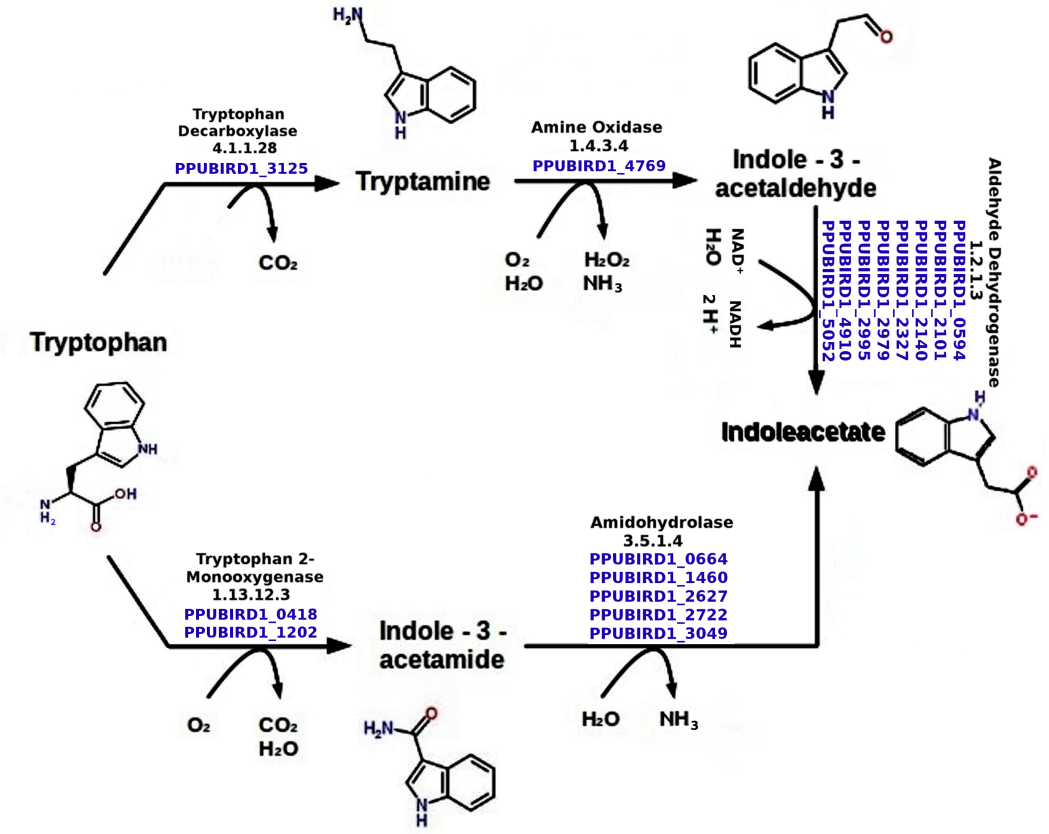


Figure 6. Synthesis of IAA based on the annotation of the BIRD-1 genome.

Since two potential IAA pathways were present in the genome of BIRD-1 we decided to individually inactivate each of the pathways through the generation of mutants by site-directed mutagenesis. For the tryptophan 2-monoxygenase single and double mutants were generated using pCHESI-Km and pCHESI-Gm. A single mutant in tryptophan decarboxylase was also constructed using pCHESI-Km and a double mutant inactivating tryptophan decarboxylase and one of the tryptophan

2-monooxygenases was constructed. We then measured the capacity of the mutants to produce IAA using the quantitative assay. The results showed that after 24 h incubation in DF minimal medium the single and double mutants produced amounts that were about a half of that produced by the wild-type strain. These IAA levels were still high enough to allow for a faster growth of maize roots than in the absence of bacteria (See Suppl. Figure 5). These results are intriguing and thus we are further analysing IAA biosynthesis and IAA metabolism in this strain.

Inactivation of the ACC deaminase

Ethylene is a plant-growth regulator that affects plant growth, development and senescence. AAC is present in plant root exudates (Reid 1987) and some PGPR have been proposed to promote plant growth by lowering the levels of ethylene through the activity of the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which hydrolyzes ACC, the immediate biosynthetic precursor of ethylene in plants (Yang and Hoffman 1984). The products of this hydrolysis, ammonia and α-ketobutyrate, can be used by the bacterium as a source of nitrogen and carbon for growth (Klee *et al.* 1991). In this way, the bacterium act as a sink for ACC and thus lower ethylene levels in plants, preventing some of the potentially deleterious consequences of high ethylene concentrations (Glick *et al.*, 1998; Steenhoudt and Vanderleyden 2000; Saleem *et al.*, 2007). We found that BIRD-1 has an ORF (PPU_BIRD_3642) that exhibits high homology to the ACC deaminase of different microorganisms. We inactivated the PPU_BIRD_3642 allele using site-directed mutagenesis

and found that its absence did not influence plant root development under standard growth conditions in soil in controlled chambers (Suppl. Figure 6), what indicated that ACC deaminase did not play a significant role in the development of maize roots.

Concluding remarks

Several mechanisms by which PGPR promote plant growth have been described in the literature. In the genome of *Pseudomonas putida* BIRD-1 we have identified many of the genes that are involved in this phenotype. Concordantly, BIRD-1 is able to solubilise organic and inorganic phosphate, to synthesize plant hormones, to produce siderophores and to survive the oxidative stress imposed by the rhizosphere. Furthermore, the BIRD-1 genome also encodes all of the functions required to adhere to seeds and to colonize roots, we have been shown that it is a good colonizer of maize, tomato, pepper, zucchini and strawberry roots. BIRD-1 possesses a complete set of chemotaxis genes (our unpublished results) and responds to amino acids in root exudates as a signal (our unpublished results). The combination of all of these properties makes it an excellent microbe for utilization in agriculture. BIRD-1 promotes the growth of the plant by synthesizing IAA and other chemicals. Furthermore, its ability to survive dessicating conditions, grants this strain an additional property of exceptional utility for its use in non-irrigated farming. We view the *Pseudomonas*-plant pair as a mutualistic interaction in which the microorganism solubilises P and Fe and also produces plant growth hormones that favour plant growth; while the plants release up to 20% of

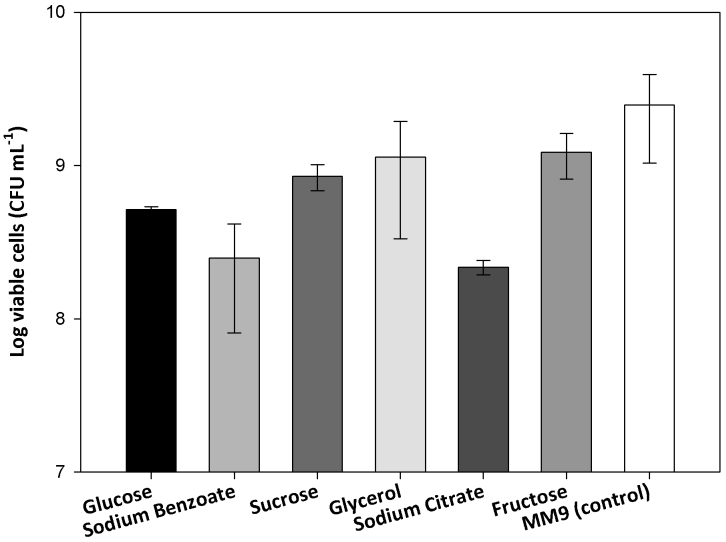
fixed CO_2 as root exudates that can be used by *Pseudomonas* to proliferate, so that at the end a beneficial interaction is established due to the concomitant high microbial cell densities in the plant rhizosphere.

P. putida BIRD-1 possesses an exceptional arsenal of mechanisms to flourish in the rhizosphere of plants and to promote plant growth. More studies are necessary to firmly establish these abilities in field tests and to determine the PGPR efficiency in a less controlled environment.

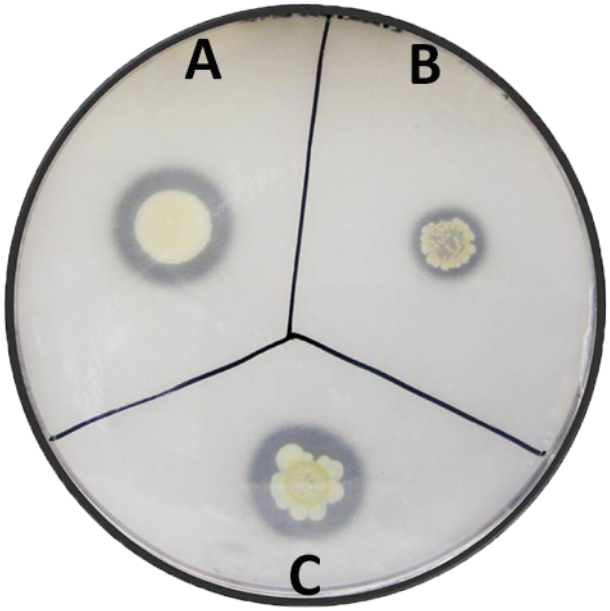
Acknowledgments

Work was supported by Fondo Social Europeo and FEDER Funds through Programa Campus of Junta de Andalucía (Project SV40), awarded by Agencia IDEA to Bio-Iliberis R&D, and by project BIO2010-17227 from the Ministry of Science and Innovation awarded to CSIC.

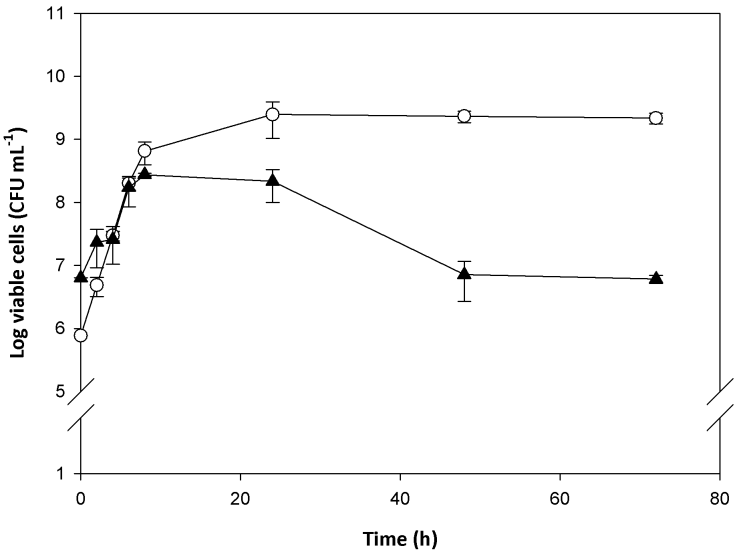
Supplementary information



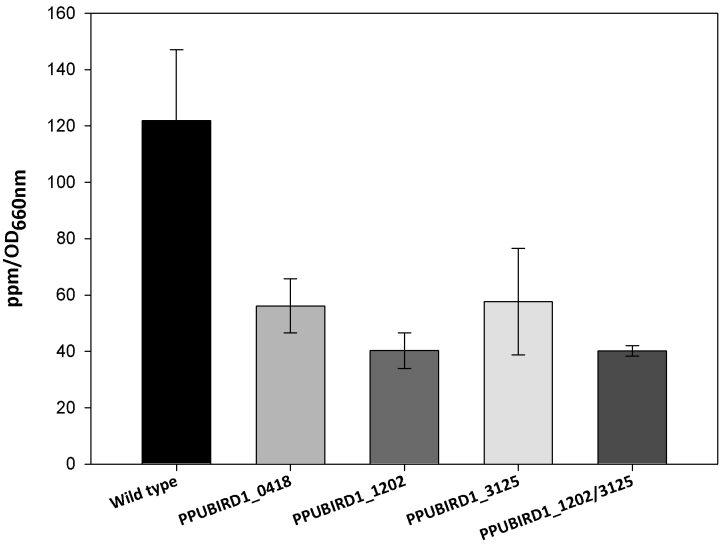
Supplementary Figure 1. Growth of *Pseudomonas putida* BIRD-1 with different sources of inorganic phosphorous. We used A-medium with the indicated C source and insoluble tricalcium phosphate (500 mg L^{-1}) as a source of phosphate. Viable cells at the beginning of the assay were 2 to 3 × 107 CFU mL^{-1}. After 24 h incubation at 30°C the CFU mL^{-1} was determined after spreading serial dilutions on LB medium. The control is M9 medium with 50 mM soluble phosphate.



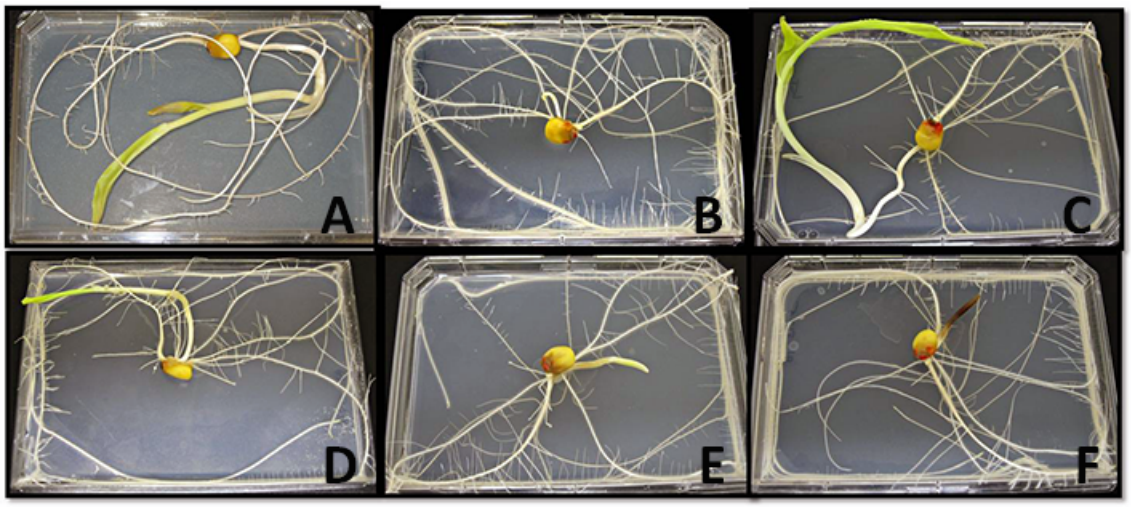
Supplementary Figure 2. Pikovskaya test with BIRD-1 and mutants in the Entner–Doudoroff pathway. Plates were prepared as described in Matherials and methods. We inoculated cells using a toothpick in a single point on the agar surface. Plates were incubated for 48 h at 30°C and then the solubilization halo was visualized. (A) Wild-type; (B) *zwf* mutant and (C) *eda* mutant.



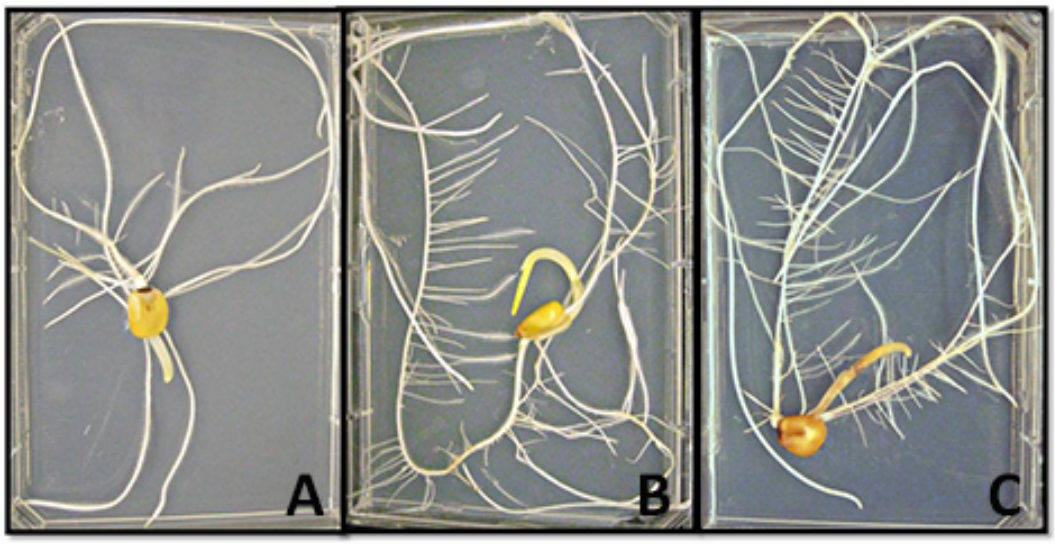
Supplementary Figure 3. Growth of *P. putida* BIRD-1 with phytate. Assays were performed with A-medium containing 1 g l^{-1} phytate. At the indicated times cfu ml^{-1} were determined. As a control, cells growing on M9 minimal medium were used. ○, growth in M9 medium; ▲, growth with phytate.



Supplementary Figure 4. Production of IAA by wild-type BIRD-1 and different isogenic mutants. IAA concentration was determined as described in Materials and methods. The solid back bar represents the wild-type strains and the other bars the indicated mutants.



Supplementary Figure 5. Corn root development with mutants in the IAA biosynthetic pathways. The picture shows controls without bacteria (A), with wild-type BIRD-1 (B) and KT2440 (C) strains, and three isogenic mutants of BIRD-1 with knockouts in PPUBIRD1_0418, 1202 and 3125 genes (D, E and F respectively).



Supplementary Figure 6. Corn root development in a control seed without bacteria (A), and inoculated seeds with BIRD-1 (B) and a mutant deficient in the ACC deaminase gene (C). Conditions were as described for Figure 2 except that a knockout (PPUBIRD1_3642) ACC mutant was used.

Supplementary Table 1. Genes involved in adhesion to biotic and abiotic surfaces in *Pseudomonas putida* strain BIRD-1 deduced from the identification of reciprocal genes in *P. putida* KT2440.

	***P. putida* KT2440**			***P. putida* BIRD1**		
Group	**Locus (PP_)**	**Gene Name**	**Product Name**	**Locus (PPUBIRD1_)**	**e-value**	**Identity %**
Large adhesion proteins	0168	*lapA*	Surface adhesion protein	0199	0	91
	0806	*lapF*	Surface adhesion protein	0852	0	87
	0805	*lapH*	Component of an ABC transport system for LapF	0851	0	90
Regulators	0952	*rpoN*	RNA polymerase factor sigma-54	1005	0	98
	1650	*gacS*	Global sensor kinase GacS	3966	0	90
Flagella	4352	*flhB*	Flagella protein FlhB	1506	$1e^{-180}$	84
	4369	*fliF*	Flagella MS-ring protein	1490	0	88
	4376	*fliD*	Flagellar hook-associated protein	1467	$2e^{-86}$	40
Others	4378	*fliC*	Flagellin	1465	$2e^{-55}$	65
	1633	--	Hypothetical secreted protein	3983	$2e^{-66}$	100
	5329	*pstS*	PstS	5121	$1e^{-170}$	89

Duque *et al.* (2013) using different approaches identified 11 genes whose products were involved in attachment to biotic and abiotic surfaces. Reciprocal genes were identified in the *P. putida* genome by BLAST search. The corresponding PP numbers in the genome of KT2440 and BIRD-1 are given together with the identity of the corresponding proteins in the two strains. The genes are named as annoted in the KT2440 genome.

Chapter 2

Restoration of a Mediterranean forest after a fire: bioremediation and rhizoremediation field-scale trial

This chapter will be submitted as:

Pizarro-Tobías P, Roca A, Fernández M, Niqui JL, Solano J, Duque A, Ramos JL. Restoration of a Mediterranean forest after a fire: bioremediation and rhizoremediation field-scale trial

Abstract

Forest fires pose a serious threat to countries in the Mediterranean basin, often razing large areas of land each year. After fires, soils are more likely to erode and resilience is inhibited in part by the toxic aromatic hydrocarbons produced during the combustion of cellulose and lignings. In this study we explored the use of bioremediation and rhizoremediation techniques for soil restoration in a field-scale trial in a protected Mediterranean ecosystem after a controlled fire. Our bioremediation strategy combined the use of *Pseudomonas putida* strains, indigenous culturable microbes and annual grasses. After eight months of monitoring soil quality parameters, including the removal of monoaromatic and polycyclic aromatic hydrocarbons (PAHs) as well as vegetation cover, we found that the site had returned to pre-fire status. Microbial population analysis revealed that fires induced changes in the indigenous microbiota, and that rhizoremediation favours the recovery of soil microbiota in time. The plant-microbe combination analysed in this study represents an effective strategy for the restoration of soils after a forest fire.

Introduction

During high temperatures and lack of rainfall, forest fires represent the most frequent perturbation within Mediterranean ecosystems (Vila-Escalé *et al.*, 2007; Hernández *et al.*, 1997). Loss of forest mass is an extended concern throughout the Mediterranean basin; in 2012 almost 200,000 hectares of forests were affected by fire in Spain. This amount was three times the land area affected in 2011, making it one of the most devastating years for forest biomass in the Iberian Peninsula (http://www.magrama.gob.es). While drought and heat are natural causes of wildfires, many occur due to incorrect agricultural management, negligence or as the result of economic interests (Olivella *et al.*, 2006; Vergnoux *et al.*, 2011).

Fire-induced perturbations comprise changes in the physical, mineralogical, chemical and biological properties of soil (Certini, 2005), with levels of severity depending on the intensity and duration of combustion (Campbell *et al.*, 1994; Franklin *et al.*, 1997; DeBano *et al.*, 1998). The immediate effects of fire on soil include: (i) the incineration of associated vegetation cover, which changes nutrient availability and surface organic matter content (Vázquez *et al.*, 1993); (ii) a significant decrease in microbial cell density per gram of soil (DeBano, 1998; Certini, 2005); (iii) compositional changes in soil microbial populations (Torres *et al.*, 1997; Smith *et al.*, 2008); (iv) reduction of water infiltration and rainfall retention, which is required to support plants and, thus, important for resisting erosion (DeBano, 2000; González-Pérez *et al.*,

2004); and (v) the release of several pyrolytic substances as PAHs, which are toxic and have a tendency to bioaccumulate (Vila-Escalé *et al.*, 2007). Soil dynamics depend not only on physicochemical properties, but also on microbiological health since the return of vegetation after a fire is directly impacted by the metabolic activity of microorganisms, which facilitate nutrient cycling (Certini, 2005).

Technology for the remediation of PAH-polluted sites has traditionally been centred on physicochemical treatments (Fernández *et al.*, 2012); however, more recently, the use of microorganisms for *in situ* degradation of pollutants has gained popularity as a bioremediation process (Kuiper *et al.*, 2004; Segura *et al.*, 2009). In these processes either native degraders or exogenous microorganisms with appropriate metabolic traits are used. When indigenous microbes are used, the process is known as bioaugmentation (Andreoni *et al.*, 2004; Segura *et al.*, 2009).

Due to the slow natural recovery of soil after a fire, bioremediation techniques have been developed combining microorganisms with plants to accelerate the recovery of soil properties, increase microbial biomass and accelerate plant recolonisation. The general process is referred to as phytoremediation, whereas the process is known as rhizoremediation when plants with root-associated microorganisms are used (Kuiper *et al.*, 2001; Kuiper *et al.*, 2004, Wood, 2008, Segura *et al.*, 2009, Segura and Ramos, 2012). Hence, in a broad sense, the term bioremediation

encompasses rhizoremediation; however, in this article we will refer to the use of microorganisms alone (without plants) as “bioremediation” to distinguish it from rhizoremediation (joint plant-microbe processes).

There have been few studies that tackle field-scale bioremediation and/or rhizoremediation assays without using physicochemical treatments (Huang *et al.*, 2004; Bamforth and Singleton, 2005); in addition, these technologies had not been tested in ecosystems affected by fire, emphasizing the importance of results of the current study. The aims of this current field-scale study were to assess bioremediation and rhizoremediation techniques for the recovery of soil health to pre-fire levels. The chosen methods involved the use of rapidly-growing pasture seeds to curb erosion; the addition of exogenous growth-promoting (PGPR) microbes and degradative bacteria and/or indigenous culturable microbiota to restore pristine soil properties, induce the degradation of PAHs, and to promote seed germination, plant recolonisation and vegetation growth. The results suggest that Mediterranean ecosystems affected by fire can be restored to pre-fire conditions within months via rhizoremediation technology.

Materials and Methods

Field experiment site description

The protected Parque Natural de los Montes de Málaga (www.juntadeandalucia.es/medioambiente) served as the Mediterranean ecosystem in this study. Located in the south of Spain in the province of Málaga, the park occupies an area close to 5,000 Ha (Figure 1).

Figure 1. Location of the Parque Natural de los Montes de Málaga (green region) at the south of Spain. The red spot in the map corresponds to the location of the burnt site object of this study.

This ecosystem was declared protected in 1989 and comprises *Pinus Halepensis*, *Quercus ilex*, *Quercus suber* and *Quercus faginea* forests. The park is also home of endangered species, such as the *Chamaeleo chamaeleon* (chameleon). The southern Mediterranean region of Spain is heavily affected by fire (http://www.magrama.gob.es), so fireguard

training and testing of new materials for use in fire extinction is considered to be of great importance (Pausas, 2012). An experimental fire, reaching 450 ºC, was induced under the strict supervision of the Andalusian Forest Fire Brigade (INFOCA, http://www.juntadeandalucia.es}) in April 2008, allowing us to use it afterwards for this study.

The burnt plot (N 36º 52.804' - W 004º 21.013') was located on a hill with a high slope that was subdivided into 15 terraces (subplots), each with an area of approximately 100 m^2, which were cleared of calcined vegetation before setting the assay. Different treatments were tested on different terraces to ease the leaching effect from adjacent conditions. To avoid contamination between inoculated subplots, non-inoculated ones were set up at the top part of the hill. A non-burnt plot was established nearby, which was also divided into terraces (comprising four subplots), for control treatments in pristine soil to be applied in parallel. A preliminary soil analysis was performed and monoaromatic and PAHs were measured. The assay was initiated on October 2008.

Bioremediation treatments were designed to analyse *in situ* the role of indigenous microbiota and introduced microbes with or without plants on hydrocarbon removal and soil restoration. The exogenous microbes chosen were two wild-type strains of *Pseudomonas putida* harbouring the catabolic plasmid pWW0 (Ramos *et al.*, 1991), the KT2440 (Bagdasarian *et al.*, 1981) and BIRD-1 strains (Matilla *et al.*, 2011, Roca *et al.*, 2012).

Rhizoremediation assays were run with two kinds of plants, white clover (*Trifolium repens*) and Avex III, which is a commercial pasture seed mixture, composed of annual ryegrass, annual legumes, *Avena strigosa* and annual vetches. The applied treatments are summarized in Table 1.

Table 1. Composition of treatments and strains applied to burnt and pristine soil.

Burnt Soil			
Treatment	**Composition**	**Microorganisms applied**	**Plant seeds mixture**
Control	Untreated bare soil	None	None
Plants control	Non-inoculated plants	None	Peat AVEXIII (*Avena strigosa*, vetches, ryegrass, annual legumes) *Trifolium repens*
Bioremediation	Microbial consortium	*P. putida* BIRD-1 (pWW0) *P. putida* KT2440 (pWW0) Indigenous bacterial consortium	None
Rhizoremediation	Plants and microbial consortium	*P. putida* BIRD-1 (pWW0) *P. putida* KT2440 (pWW0) Indigenous bacterial consortium	Peat AVEXIII (*Avena strigosa*, vetches, ryegrass, annual legumes) *Trifolium repens*
Pristine Soil			
Treatment	**Composition**	**Microorganisms applied**	**Plant seeds mixture**
Control	Untreated	None	None
Treated	Plants and microbial consortium	*P. putida* BIRD-1 (pWW0) *P. putida* KT2440 (pWW0) Indigenous bacterial consortium	Peat AVEXIII (*Avena strigosa*, vetches, ryegrass, annual legumes) *Trifolium repens*

Characteristics that were assayed include bacterial survival, impact on indigenous microbial populations, soil restoration parameters, hydrocarbon analysis and evolution of the landscape.

Treatments procedure

An organic solid vegetable support was used (commercial peat) as a carrier for microorganisms.. Treatments applied are summarized in Table 1.

i) *Control:* The control bulk soil subplots remained untreated.

ii) *Control plants:* Avex III seeds (10 g/m^2) and *Trifolium repens* (5 g/m^2) were spread over the soil surface, then, a slight topsoil work was made to promote seed germination.

iii) *Bioremediation:* 1L of each microorganism/consortium was mixed with 10L of tap water and then mixed with 80L of peat. After spreading the mixture over the soil surface, topsoil was slightly worked to achieve a homogeneous mixture of the peat with the soil.

iv) *Rhizoremediation:* For each treated plot, 80 L of peat was homogeneously mixed with Avex III (10 g/m^2) or clover (5 g/m^2), then, 1L of each microorganism in 10L of tap water was added to the peat-seed mixture. After spreading the mixture over the soil surface, the topsoil was slightly worked to promote seed germination.

During the study, three samples of topsoil (10 cm) of each subplot were collected.

Strains and culture media

The bacterial strains and plasmids used in this study are shown in Table 1. *Pseudomonas putida* BIRD-1 was grown in M9 minimal medium supplemented with sodium benzoate (10 mM) as the carbon source (Abril *et al.*, 1989). The catabolic plasmid pWW0 was transferred to *Pseudomonas putida* BIRD-1 by conjugation, as described by Ramos *et al.* (1991). *P. putida* BIRD-1 harbouring the catabolic plasmid pWW0 was grown in M9 minimal medium supplemented with methyl benzoate as carbon source, and supplemented with spectinomycin (100μg/mL) and rifampicin (20 μg/mL). *Pseudomonas putida* KT2440 (pWW0) was grown in M9 minimal medium supplemented with toluene and rifampicin (10 μg/mL). Cultures were incubated at 30°C and shaken on an orbital platform operating at 200 strokes per minute. Monitoring of survival of each strain in soil was performed by drop-plating dilution series in solid M9 minimal medium supplemented with the required carbon sources and antibiotics, as described previously.

Indigenous culturable microorganisms were isolated by enrichment from the superficial layer of burnt soil (5 cm depth) using M9 minimal medium supplemented with diesel fuel as a carbon source. Monitoring of survival of soil microorganisms was performed by drop-plating dilution series in solid M9 minimal medium supplemented with diesel fuel, as described above.

Identity verification, in soil, of the introduced *P. putida* strains was performed using REPc fingerprinting, as described by Aranda-Olmedo *et al.* (2002), combined with colony hybridization, as described by Sambrook *et al.* (1989) using DNA probes corresponding to the *xylS* gene (to identify the pWW0 catabolic plasmid) or the PP_0314 gene (to identify *P. putida* KT2440).

Library construction and biodiversity analysis

Total DNA was extracted from approximately 0.5 g of each soil sample using the FastDNA kit (Qbiogene Inc CA, USA) and purified on agarose gels. The universal Eubacterial primers GM3F (5'-AGAGTTTGATCMTGGC-3') and GM4R (5'-TACCTTGTTACGACTT-3') were used for amplifying the 16S rDNA gene (Muyzer *et al.*, 1993). Each PCR reaction was performed in 50µl reaction volume containing 5 µ l of reaction buffer, 0.2 mM of primers, 2 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, and 2.5 U DNA polymerase. The PCR conditions was as follows: 5 min of denaturation at 95°C, followed by 35 cycles of 1 min at 95°C, 1 min for primer annealing, 2 min at 72°C for primer extension, and a final cycle at 72°C for 10 min. The products of two consecutive PCRs were then pooled and purified through extraction from agarose gels prior to cloning into pGEM-T vectors. The resulting plasmids were transformed in competent *Escherichia coli* DH5α cells and positive transformants were color-screened on LB plates supplemented with ampicillin (100µg/ml), X-Gal (80 µ g/ml), and isopropyl-β-D-thiogalactopyranoside (IPTG;20 mM). Clones with the correct insert were

sequenced using the vector primers M13 F (5′-GGAAACAGCTATGACCATG-3′) and M13 R (5′-GTTGTAAAACGACGGCCAGT-3′).

The quality of the obtained sequences was manually checked using DNA Baser (http://www.dnabaser.com/download/download.html) and verified with Bellerophon (Huber *et al.*, 2004) and CHECK_CHIMERA (Maidak *et al.*, 1996), and all chimeric sequences were discarded. These sequences were then compared with those in the GenBank database using the BLASTn tool and the RDP database with classifier tool and aligned using ClustalW (Thompson *et al.*, 1994). DNA aligned of 16S rDNA gene sequences were used to construct a DNA distance matrix and rarefaction matrices with DOTUR package (Schloss and Handelsman, 2005). FASTUNIFRAC (Hamady *et al.*, 2010) was used to produce Principal Coordinate Analyses (PCoA) comparing all samples.

Hydrocarbons measurement

To perform the PAH analysis, soil samples were dried at 40ºC and frozen. Defrosted samples were dried completely in a second step using an equivalent weight of mortar-ground anhydrous sodium sulfate. For PAH extraction, approximately 45 g of soil was placed inside a cellulose extraction thimble (Filtros ANOIA, Barcelona, Spain) and extracted with a mixture of dichloromethane:acetone (1:1) for 15 hours. Once the extraction was completed, the organic solvents were evaporated and the remaining residue was re-dissolved in a small volume of dichloromethane

(4-5 mL). To remove polar compounds, clean up of the organic extract was performed using Sep-Pak® Plus Florisil cartridges (WATERS Corp., Milford, MASS, USA), previously conditioned with 10 mL of dichloromethane. For the next step, dichloromethane was evaporated and the residue was resuspended in 2 mL of acetone. Finally, samples were filtered through a nylon Minisart syringe filter of NY (0,45 µm, 13 mm Ø, Sartorius Stedim Biotech, GmbH, Goettingen, Germany). Analysis of PAH was carried out using an Agilent Technologies HPLC system 1200 Series, equipped with a photodiode array detector (DAD, G1315D) and a scanning fluorescence detector (FLD, G1321A). The column used was a ZORBAX Eclipse PAH (Agilent Technologies, 5 µm, 4.6 I.D. x 150 mm). The mobile phase used was an acetonitrile-milli Q water gradient comprising 40% (v/v) acetonitrile from 0 to 1.25 minutes, programmed 100 % (v/v) acetonitrile between 1.25 to 18 minutes. The initial solvent composition (60% milli Q water, 40% acetonitrile) was then maintained for further 3.5 minutes.

Measurement of soil quality parameters

Measurement of pH in soil

The pH values were measured in air-dried soil, sieved through 2 mm, using a glass combination electrode (soil: water ratio, 1:2.5 w:v), as described by Acosta-Martínez *et al.* (2003).

Measurement of phosphatase activity in soil

Phosphatase activity was determined as described by Antolín *et al.* (2005). The amount of *p*-nitrophenol (PNP) released from 0.5 g soil from three samples from each subplot, by triplicate, was measured after incubation, in the dark at 37 ºC for 2 hours with 0.115 M 4-nitrophenyl phosphate-disodium (PNPP) as substrate for the enzymatic reaction, in 2 ml of maleate buffer (0.1 M, pH 6.5). Samples were cooled at 2 ºC for 15 minutes to stop the enzymatic reaction and 0.5 mL of 0.5 M $CaCl_2$ and 2 ml of 0.5 M NaOH were added and well-mixed. Each sample was centrifuged at 2000 × g for 10 minutes. A blank experiment by duplicate was performed for each assay, in which the substrate was added to the soil sample after incubation and before stopping the reaction. The amount of PNP per hour released from each soil sample (μg PNP/gh) was determined by comparing absorbance measures to a PNP standard curve. Rhizosphere soil was considered to be soil closely attached to roots, sieved through a 2 mm mesh.

Measurement of β-glucosidase activity in soil

β-glucosidase activity was determined as described by García *et al.* (1994). The amount of 4-nitrophenol (PNP) released from 0.5 g of soil from three samples from each subplot, by triplicate, was measured after incubation in the dark, at 37ºC for 2 hours with 0.5 mL of 50 mM 4-nitrophenyl-β-D-glucopiranoside (PNG) as substrate for the enzymatic reaction, in 2 ml of maleate buffer (0.1 M, pH 6.5). Then, samples were cooled at 2 ºC for 15 minutes to stop the enzymatic reaction, and 0.5 mL

of 0.5 M $CaCl_2$ and 2 ml of 0.5 M NaOH were added and mixed well. Each sample was centrifuged at 3,500 × g for 10 minutes. A blank experiment, by duplicate, was performed for each assay, in which the substrate was added to the soil sample after incubation and before stopping the reaction. The amount of PNP per hour released from each soil sample (µg PNP/gh) was determined by comparing absorbance values to a PNP standard curve.

Measurement of dehydrogenase activity in soil

Dehydrogenase activity was determined as described by García *et al.* (1994). The amount of iodonitrotetrazolium violet-formazan (INTF) released from 1 g soil from three samples from each subplot, by triplicate, was measured after incubation in the dark, at 37 ºC for 20 hours with 0.2 mL of 0.4% 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenylteytrazolium chloride hydrate (INT) as substrate for the enzymatic reaction, and 2 ml of distilled water. Then, 5 mL of an extracting mixture (tetrachloroethylene: acetone (1:1.5 v:v) was added and well-mixed for 2 min. Each sample was centrifuged at 1,000 × g for 10 min. A blank experiment, by duplicate, was performed for each assay, without substrate, in which the extracting mixture was added after incubation. The amount of INTF per hour released from each soil sample (µg INTF/gh) was determined by comparing absorbance measures to an INTF standard curve.

Plant biomass monitoring

Five samples of clover and pasture plants were harvested from each plot at 4, 8 and 14 weeks sampling times. Plants were the manually separated into shoots and roots; fresh weight and length were recorded, and samples were dried in a stove at 90 ºC for 48 hours. Samples were then allowed to cool to room temperature and dry weight was measured.

The visual aspect of the area and each plot was photographed at each sampling time.

Results

Bacterial survival

The survival of exogenous *P. putida* strains was determined by plate counting on selective medium and verified by either PCR or colony hybridization. Both strains, *P. putida* BIRD-1 and KT2440, were able to survive and maintained a population size around 10^6 CFU/g soil for about six months, and then dropped below detection limits during the aestival season (July 2008) (Figure 2a,b).

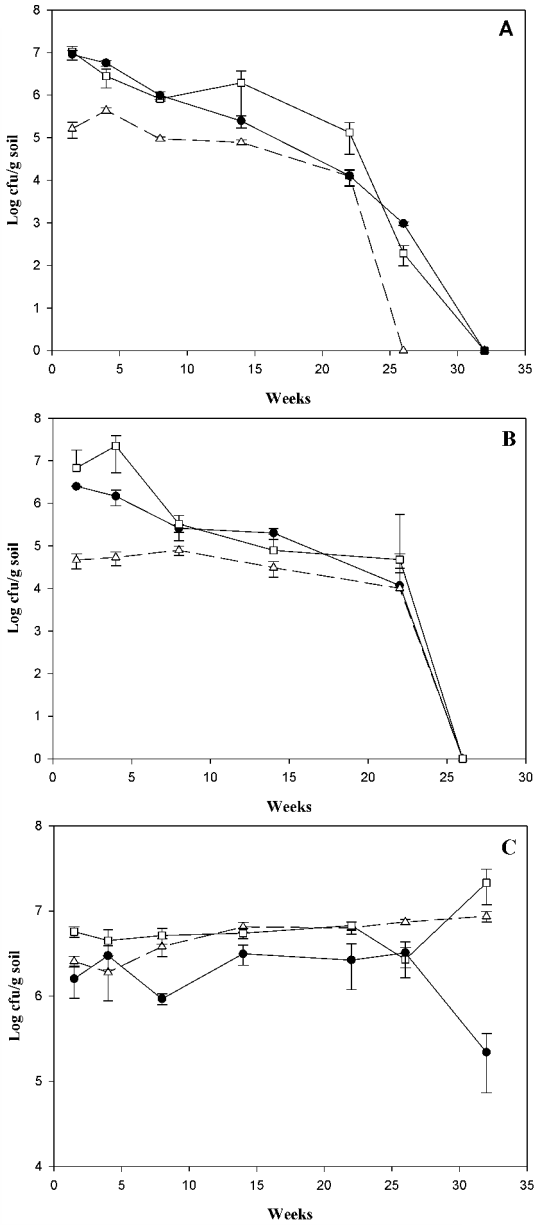


Figure 2. Viable *P. putida* BIRD-1 (A), *P. putida* KT2440 (B) and indigenous culturable bacteria (C) in rhizosphere of introduced plants in treated pristine soil (filled circle) and rhizoremediation (square), *versus* bioremediation (triangle).

No significant differences were detected in the survival of these strains in burnt soil compared to non-burnt pristine soil. However, survival improved when the strains, especially *P. putida* BIRD-1, were associated to plants. Cell densities were two orders of magnitude higher in the rhizosphere than in bulk soil. As expected, the mixture of culturable indigenous microbiota, monitored by plate counting with diesel fuel as a sole carbon source, revealed a population size of around $5{\cdot}10^6$ CFU/g soil. These levels remained quite constant until the end of the study regardless of whether the soil was unburnt or burnt or whether exogenous *P. putida* strains and/or plants were present (Figure 2c).

Metagenomic analysis of soil microbial population

In order to determine the consequences of fire on soil microbiota, as well as to study the effect of rhizoremediation treatments over the spectrum of indigenous microbial populations, a metagenomic analysis of 16S RNA for bacterial biodiversity was carried out at month 1 (autumn, November 2008) and 6 months after the introduction of microorganisms (spring, April 2008).

Rarefaction analyses were performed to compare bacterial richness between pristine, burnt bulk soil and soil undergoing rhizoremediation. Analyses were based on a minimum of 125 sequences and the number of operational taxonomic units (OTUs) was estimated using a cut-off of 97%

for sequence similarity, a generally accepted level for comparative analysis of whole and partial 16S rRNA sequences (Konstantinidis *et al.*, 2006). The rarefaction curve (Figure 3) showed a similar number of OTUs in all soil samples, indicating similar bacterial richness.

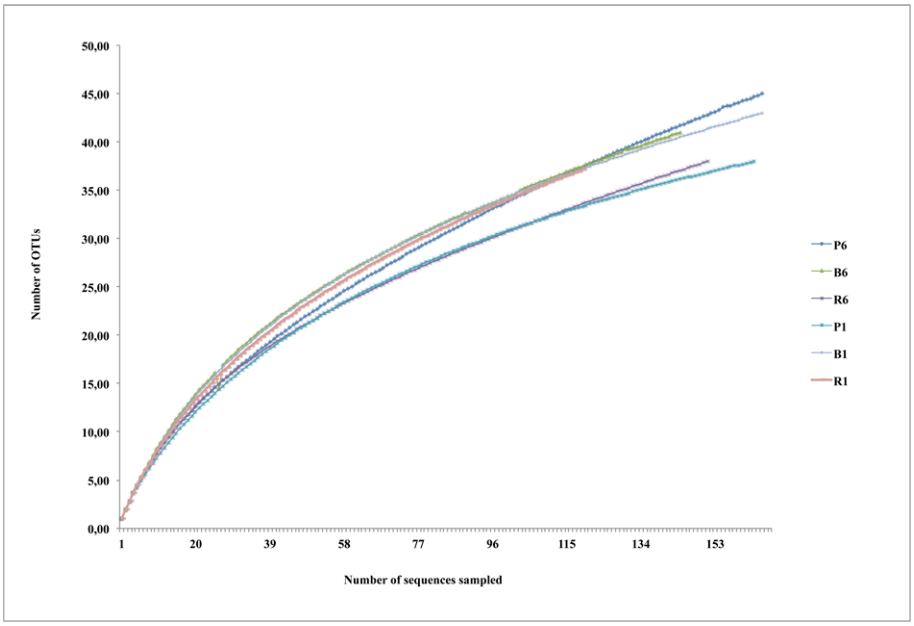


Figure 3. Rarefaction analysis for pristine soil (P), bulk burnt soil (B) and rhizoremediation treatment (R) at two different times of the assay 1 month (1: autumn) and 6 months (6: spring). Rarefaction curves were constructed with DOTUR software.

The analysis of relative abundance at phylum level (Figure 4) showed changes in the bacterial community distribution and proportion. Proteobacteria, Acidobacteria and Bacteriodetes were the predominant phyla in all the cases we studied; combined, these three phyla constituted 80% of the total. Specifically Acidobacteria, which was the prevailing phylum in pristine soil and 46% of the total, experienced a remarkable population reduction to 29% of the total in burnt soil. In contrast to Acidobacteria, an increase in the proportion of Proteobacteria and Bacteriodetes was observed in burnt soil.

Less common phyla exhibited significant changes in their relative abundance: Verrucomicrobia, which made up 5.3% of the total in pristine soil, dropped to under detection limits in untreated burnt soil. On the other hand, Actinobacteria was more abundant in burnt (8.6%) than in pristine samples (3.8%) at the first sampling time point; similarly, Gemmatimonadetes was also more predominant in burnt (4.2%) than in pristine (undetectable) soil at the last sampling time point. No bacterial phylum was reduced exclusively in soil under rhizoremediation treatment.

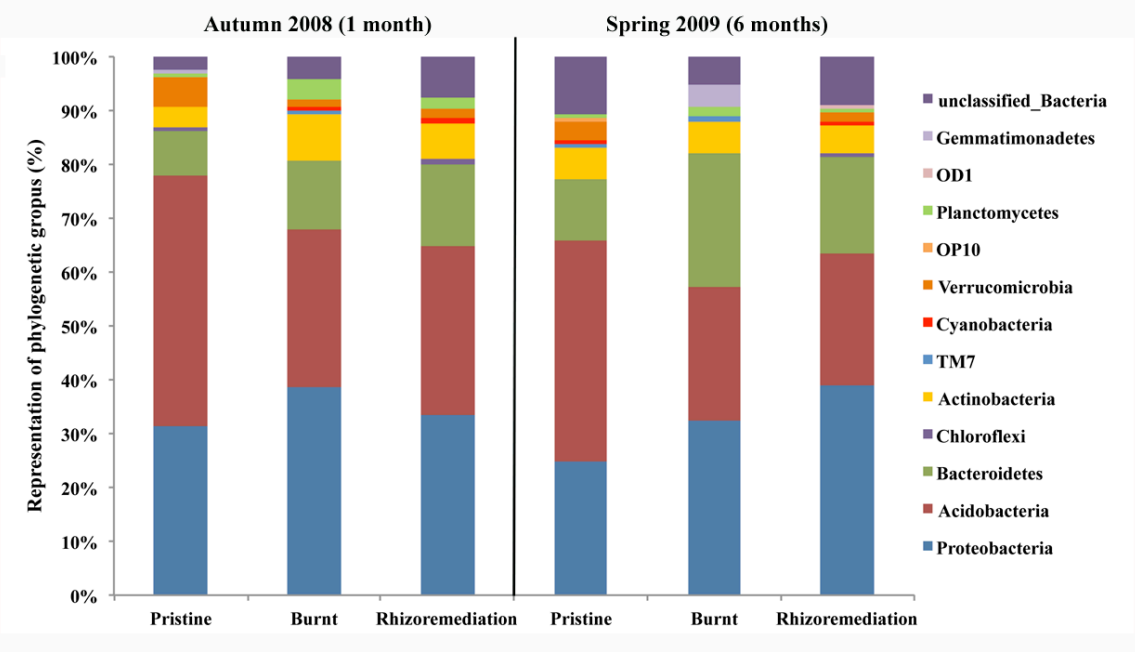


Figure 4. Relative abundance of phylogenetic groups at phylum level based on 16S rRNA genes. Pristine soil, bulk burnt soil and rhizoremediation treatment at two different times of the assay 1 month (autumn) and 6 months (spring).

A principal component analysis was performed to compare genetic distance matrix between groups (Figure 5). Statistical differences were observed in the phylogenetic composition of microbial populations

between burnt and pristine soil, with intermediate values observed for sites that were treated using rhizoremediation. Moreover, burnt and pristine soil microbial populations showed season-induced variations while those for rhizoremediation were not affected by climate.

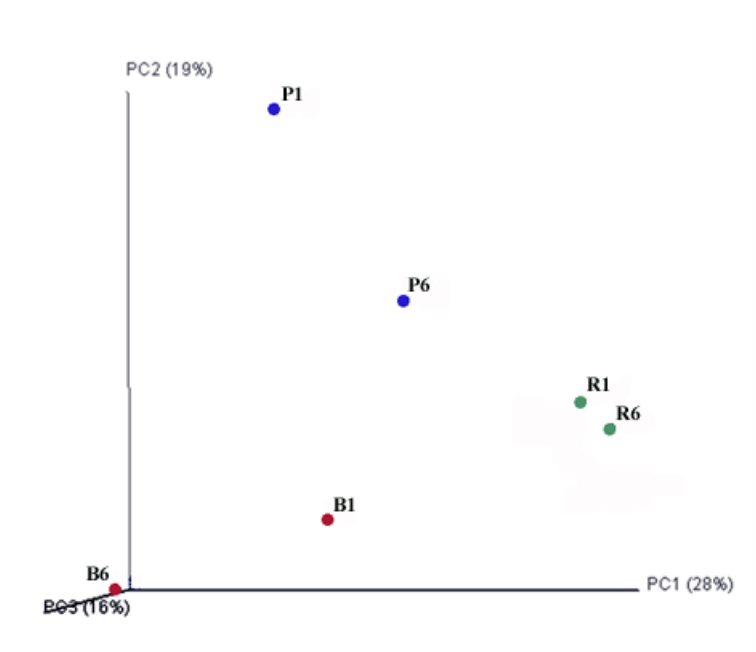


Figure 5. Principal component analysis (PCoA) performed to compare sample groups from phylogenetic distance-matrix, determining similarity between or differences among pristine soil (P), bulk burnt soil (B) and rhizoremediation treatment (R) at two different times of the assay 1 month (1: autumn) and 6 months (6: spring).

Monitoring of pyrolytic substances in soil

At the beginning of the assay a number of monoaromatic hydrocarbons (BTEX: benzene, toluene, ethylbenzene and xylene) and PAHs that had been generated during the fire were detected (Table 2); accordingly, these compounds were below detection limits in pristine soil. An average concentration of 149 μg/kg soil for BTEX and 387 μg/kg soil for PAHs were measured in burnt plots. After 2 months of treatment, BTEX concentrations in the burnt plot dropped below detection limits.

Table 2. Concentration of pyrolytic hydrocarbons (µg per Kg of soil) generated after fire, *versus* the concentration of the same substances measured after 2 months of treatment (Control burnt, bioremediation, untreated plants and rhizoremediation treatments). ND: below detection limits.

Pyrolitic substances	Rings	Compounds	Initial concentration	% of total	Two months after the outset of the study			
					Control burnt	Bioremediation	Plants Control	Rhizoremediation
BTEX	1	Benzene	38	25.5	ND	ND	ND	ND
		Toluene	62	41.6	ND	ND	ND	ND
		Ethylbenzene	17	11.4	ND	ND	ND	ND
		Xylene	32	21.5	ND	ND	ND	ND
Σ BTEX			**149**	**100**	**ND**	**ND**	**ND**	**ND**
PAHs	2	Naphthalene	118	30.5	31.6	43.2	34.0	26.8
	3	Acenaphthene	ND	0	ND	ND	ND	ND
		Fluorene	48.3	12.5	1.9	1.7	1.7	ND
		Phenanthrene	121	31.3	26.9	11.5	15	9.46
		Anthracene	1.6	0.4	1.6	ND	ND	ND
	4	Fluoranthene	26	6.7	6.4	ND	ND	ND
		Pyrene	29.6	7.6	8.4	0.97	1.7	0.5
		Benzo(a)Anthracene	12.5	0.4	1.2	ND	1.5	ND
		Crysene	8.6	2.2	6.9	ND	0.3	ND
	5	Benzo(b)Fluoranthene	1.5	0.4	2.0	ND	ND	ND
		Benzo(k)Fluoranthene	0.8	0.2	0.9	ND	ND	ND
		Benzo(a)Pyrene	17	4.4	1.9	ND	0.6	ND
	6	Dibenzo(a,h)Anthracene	8,1	2.1	ND	ND	ND	ND
		Benzo(g,h,i)Perylene	2.0	0.5	3.9	ND	ND	ND
		Indene	3.0	0.8	ND	ND	ND	ND
Σ PAHs			**387**	**100**	**94**	**56.4**	**54.7**	**37**

Low molecular PAHs, comprising 2 to 4 carbon rings, made up the main fraction of the total PAHs (≈ 95%) in burnt plots. The dominant PAH compounds were naphthalene and phenanthrene, which each made up ≈ 30% of the total. Two months after the beginning the assay, the concentration of total PAHs on the treated plots was at least 40% lower than in non-treated burnt soil.

Soil quality indicators

Soil quality indicators, such as soil pH and enzyme activity, can be used as an indirect measure of soil quality changes or as indexes of soil disturbance or restoration (Karaca *et al.*, 2011).

pH

Soil alkalynization (from pH 6.5 to 7.5) was observed in burnt samples versus control soil (Figure 6). Untreated burnt soil showed no significant change in pH (over 7.5) throughout the study, whereas plots treated with rhizoremediation experienced a pH decrease after about four months. Nevertheless both soils treated with bioremediation and rhizoremediation treatments experienced a decrease in pH to 6.5, reaching pristine soil pH levels after five months.

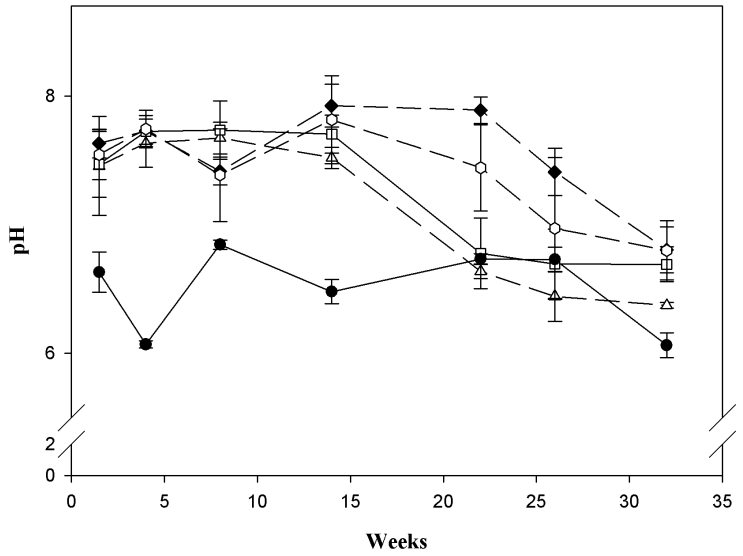


Figure 6. pH measurements performed along the study during 8 months, which are described in material and methods section. The results are the average of three independent assays, assessed on pristine soil (P) (filled circle), burnt bulk soil (C) (filled diamond), bioremediation (B) (triangle) and rhizoremediation (R) (square), and plants control (CP) (circle).

Enzymatic activity assays

In soil ecotoxicology, soil enzyme activities are used as indexes of soil disturbance or restoration due to their sensitivity to natural and anthropogenic induced stresses. These indexes are easy to measure and can detect the impact of microbial activities on nutrient cycling (Nannipieri *et al.*, 2002; Gianfreda *et al.*, 2005, Karaca *et al.*, 2011). In this study, three different enzymatic activities were monitored (Figure 7): (i) dehydrogenases, which take part in reactions involved in energy transfer in microbial metabolism (Karaca *et al.*, 2011); (ii) phosphatases, which hydrolyse organic phosphorous compounds into different forms of inorganic phosphorous (Karaca *et al.*, 2011); and (iii) β -glucosidases, which are involved in the saccharification of cellulose. β-glucosidases and

phosphatases are directly involved in C and P cycles, respectively (Bandick and Dick, 1999).

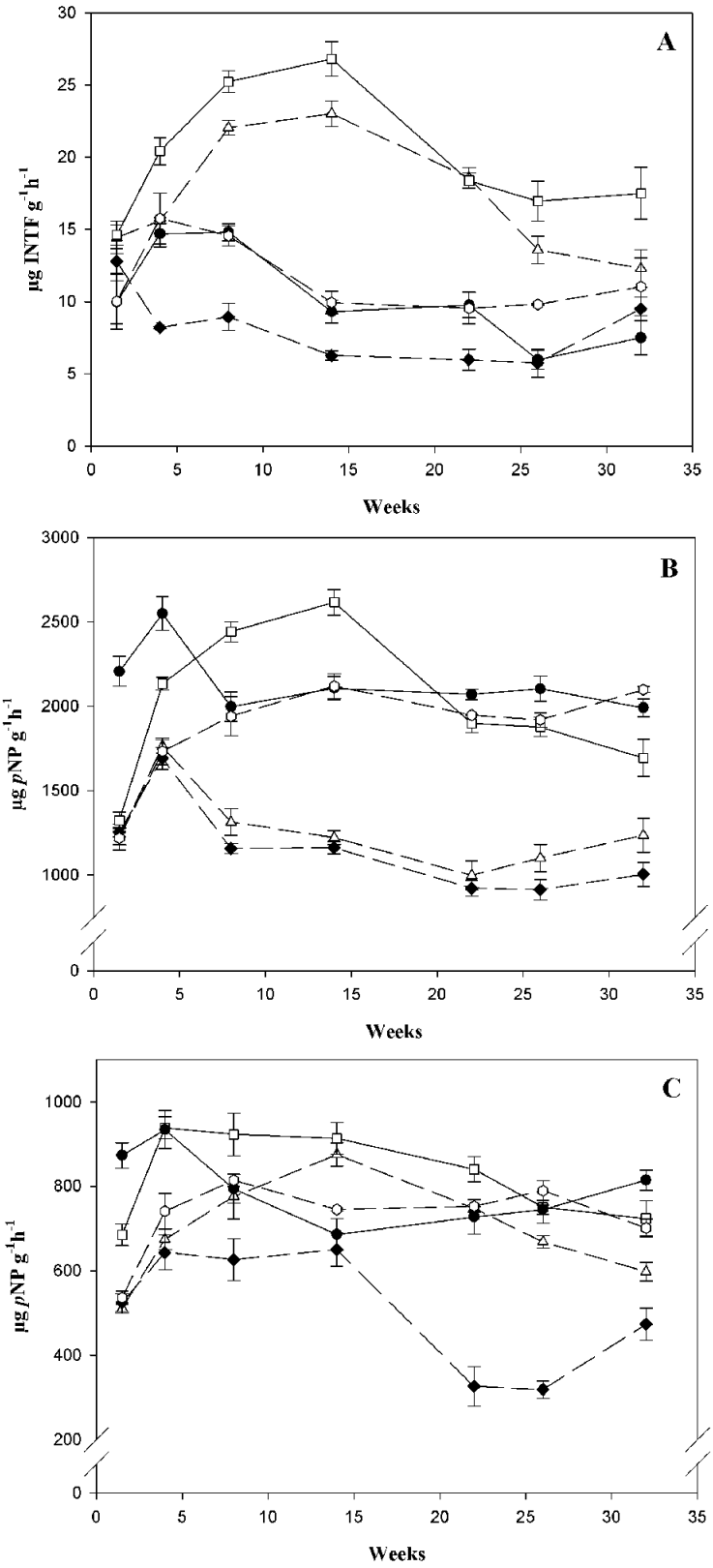


Figure 7. Enzymatic activities measurements performed along the study during 8 months, which are described in material and methods section. The results are the average of three independent assays performed by duplicate. Figure 5a shows dehydrogenase activity, figure 6b shows phosphatase activity and figure 6c shows β-glucosidase activity assessed on pristine soil (filled circle), burnt bulk soil (filled diamond), bioremediation (triangle) and rhizoremediation (square), and plants control (circle).

Dehydrogenase activity (DHA) (Figure 7a) showed that levels were around 10 to 15 μg INTF$g^{-1}h^{-1}$, and unchanged between burnt and pristine soil at the beginning of the assay. Bioremediation and rhizoremediation treatments steadily raised DHA levels for about 15 weeks, leading to total increases of 3- and 4-fold, respectively, over burnt soil, reaching 27 μg INTF $g^{-1}h^{-1}$ in rhizoremediation plots.

Phosphatase activity (Figure 7b) was found to be 2,000 μg ρNP $g^{-1}h^{-1}$ in pristine soil. Fire led to a decrease in activity by about 50%. These values returned to levels found in pristine soil after four weeks with rhizoremediation treatment; two months with non-inoculated plants; and remained unaltered at 1,000 μg ρNP $g^{-1}h^{-1}$ with bioremediation, showing only a slight increase after five months of treatment.

β–glucosidase activity (Figure 7c) was also affected by fire, decreasing by 30 to 45% after a fire (from 800 down to 500 μg ρNP $g^{-1}h^{-1}$). These levels were restored after four weeks with rhizoremediation (fluctuating between 700 to 900 μg ρNP $g^{-1}h^{-1}$), whereas bioremediation required 3 months to achieve restoration parameters.

Plant fitness and effect on the landscape

To evaluate plant fitness, we measured weight, length of roots and aerial parts of plants between the various conditions. Both plants used on this study showed increased size and dry weight when inoculated with bacteria (Table 1). Length increases were 25% for clover and 58% for

Avex III plants; average dry weight increases were 43% for clover and 67% for Avex III, after fourteen weeks of treatment. It should be noted that 4 weeks after the beginning of the assay the plant growth-promoting effect of the artificial consortium over introduced vegetation development and soil coverage could be perceived in rhizoremediation *versus* non-inoculated plant subplots (Figure 8).

Figure 8. Introduced plants growth and soil coverage after 4 weeks of treatment on burnt soil. A) shows non-inoculated plants and B) shows plants on rhizoremediation treatment.

Finally, the visual impact of bioremediation and rhizoremediation processes on the landscape was documented by a series of photographs (Figure 9).

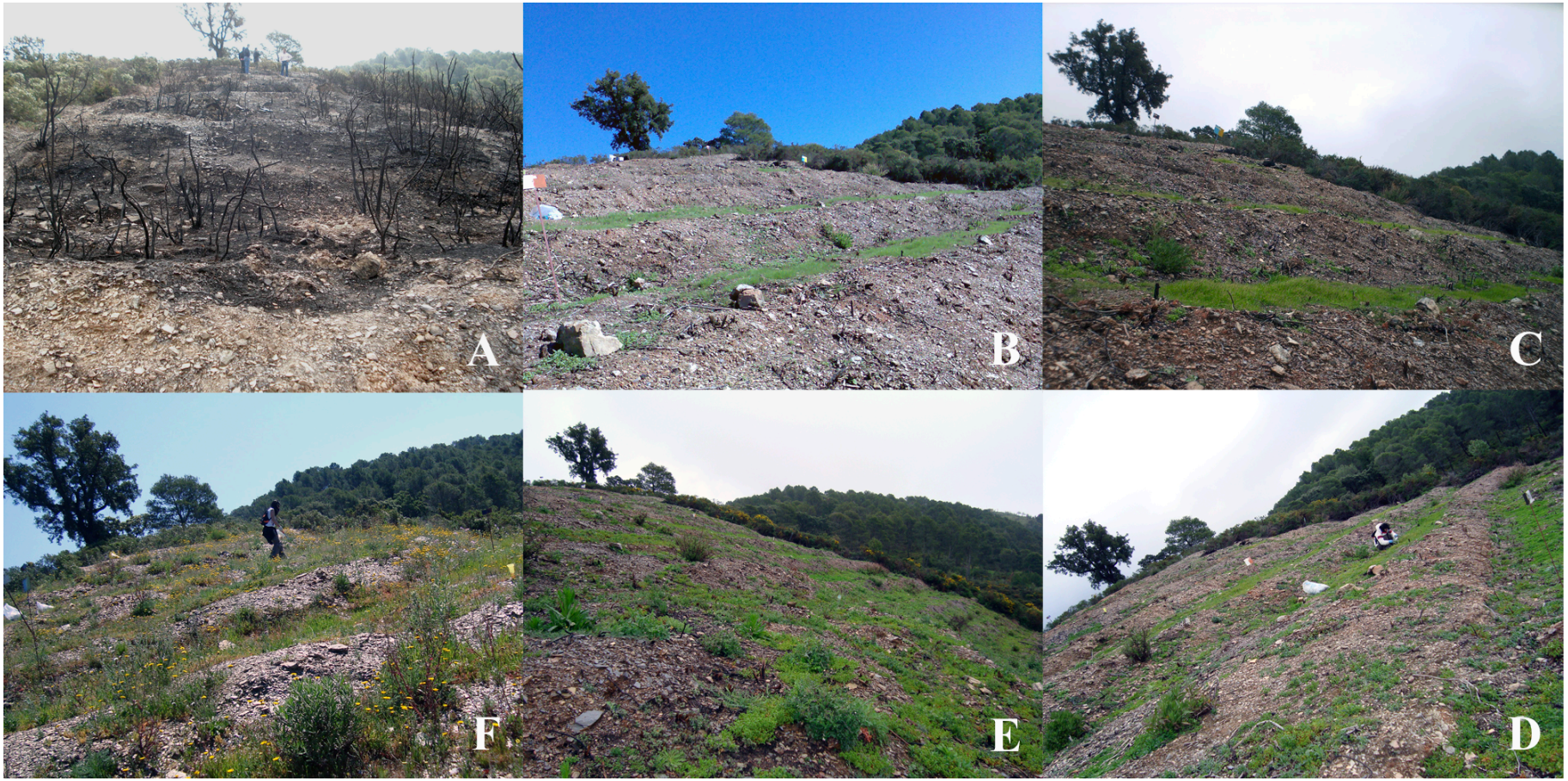


Figure 9. General evolution of the treated burnt parcel along the study (8 months, from October 2008 to June 2009). Clockwise: before treatment (A), four weeks after treatment (B), eight weeks (C), fourteen weeks (D), twenty-two weeks (E), thirty-three weeks (F).

Discussion

Bacterial performance in bioremediation and rhizoremediation technologies

The ability to survive in harsh, polluted environments is one of the key requirements of the microorganisms selected for bioremediation processes. For survival, microorganisms need to show evidence of: a) enhanced adaptation to the particular environment undergoing remediation and b) mechanisms to overcome the deleterious effects caused by the pollutant(s).

Pseudomonas putida strains are excellent candidates for soil restoration, especially in rhizoremediation processes because they are good rhizosphere colonizers (Molina *et al.*, 2000; Lugtenberg *et al.*, 2001; Ramos-González *et al.*, 2005; Matilla *et al.*, 2011; Wu *et al.*, 2011; Roca *et al.*, 2012) and metabolically versatile (Palleroni 1992, 2010). Apart from these important traits, our results revealed that the *Pseudomonas* strains used in this study can overcome the toxic effects of hydrocarbon compounds produced during the combustion of organic matter. Microbial tolerance to hydrocarbons has been linked to the strains' ability to degrade these compounds (Park *et al.*, 2004); nevertheless, tolerance is a relatively complex process involving the activation of extrusion mechanisms, the establishment of oxidative stress responses and an overall fitness programme (Silby *et al.*, 2011; Krell *et al.*, 2012). *Pseudomonas putida* KT2440 and BIRD-1 lack the metabolic potential for

hydrocarbon degradation; however, the transfer of the pWW0 plasmid endows them with the ability to degrade some BTEX compounds.

From the multiple parameters presented in this field assay, only drought conditions showed a clear negative effect on introduced *P. putida* strains; in fact, the decrease in exogenous *Pseudomonas* populations concurred with the wilting of plants during the summer season when lack of precipitation and high temperatures converge.

As Mediterranean ecosystems are constantly affected by fires (Vila-Escalé *et al.*, 2007; Hernández *et al.*, 1997), indigenous microbial adaptation to this condition is expected (Fonturbel *et al.*, 1995; Choromanska and DeLuca, 2001; Smith *et al.*, 2008). Monitoring of native culturable hydrocarbon degraders showed that these populations remained unaltered through fires and landscape and climate variations, whereas introduced strains failed to survive through climate variations.

Impact of fire and rhizoremediation treatments on soil microbial populations

Fires have been studied for their ability to change soil properties (Vázquez *et al.*, 1993; Certini, 2005) and disrupt indigenous microbial populations (Torres *et al.*, 1997; DeBano, 1998; Certini, 2005; Smith *et al.*, 2008). We carried out biodiversity analysis, which corroborated the resilience of native Mediterranean microbiota since only changes in the bacterial community distribution were observed, with no population loss

(Fig. 3). These findings reinforce the hypothesis that indigenous microorganisms in Mediterranean ecosystems exhibit a high level of adaptation to fires (Fonturbel *et al.*, 1995; Choromanska and DeLuca, 2001; Smith *et al.*, 2008; Vila-Escalé *et al.*, 2007; Hernández *et al.*, 1997). Nevertheless, changes in the composition of microbial populations had been previously observed in connection with soil deterioration/pollution generated by fire: a) increasing soil pH values due to soil organic matter denaturation lead to a decrease in Acidobacteria and to an increase in Bacteroidetes populations (Certini, 2005; Smith *et al.*, 2008; Lauber *et al.*, 2009); b) the ratio of Proteobacteria increased in burnt plots, a phylum where strains with enhanced abilities for PAH metabolism can be found (Mueller *et al.*, 1997; Watanabe, 2001), whereas microbial populations that lack this ability do not proliferate in these soils due to the selective pressure these compounds exert (Martínez *et al.*, 2000); c) the relative abundance of Gemmatimonadetes is increased in burnt soils and is also modulated by soil aridness (DeBruyn *et al.*, 2011), pH (Lauber *et al.*, 2009) and the presence of pyrogenic carbon (Khodadad *et al.*, 2011).

The rhizoremediation treatment may introduce factors that can affect the structure of soil microbial communities, including the introduction of exogenous bacteria, the introduction of exogenous vegetal species and enrichment in indigenous culturable bacteria. Nevertheless, as we mentioned above, the relative abundance of most of the bacterial groups in soils undergoing rhizoremediation was intermediate between burnt and pristine forest soil, which suggests that the main perturbation on

indigenous microbial populations was fire. This also suggests that the rhizoremediation treatment tended to restore the original structure of microbial communities, probably due to the observed restoration of soil characteristics such as pH, vegetal cover and decreases in PAHs.

Rhizoremediation enhances hydrocarbon degradation

One of the main consequences observed after a fire is the generation of new, toxic and recalcitrant forms of carbon (González-Pérez *et al.*, 2004), such as PAHs (Vila-Escalé *et al.*, 2007), as well as associated volatile hydrocarbons such as BTEX (Bamforth and Singleton, 2005). The PAH profiles observed in the current study comprised 60% of naphthalene and phenanthrene, which is consistent with reported profiles corresponding to wood combustion (Xu *et al.*, 2006; Kim *et al.*, 2011) and, in particular, to pine needles and wood (Conde *et al.*, 2005).

The lower complexity of these monoaromatic chemical structures (Bamforth and Singleton, 2005), as well as the increase in the proportion of bacterial populations with degrading potential, rapidly cleared these compounds from burnt soils, regardless of applied treatment. Furthermore, bio-attenuation mediated decreases in PAHs in untreated soil due to the presence of native degraders.

Nevertheless, rhizoremediation treatments promoted the almost complete removal of heavier pyrolytic hydrocarbons in a relatively short time, which indicates that the combination of microorganisms, introduced

PGPR, native degraders and plants was the most effective method for remediation. Since introduced *P. putida* strains were not PAHs degraders, native microorganisms played a central role in PAH elimination. Because native populations were not significantly increased, it appears that the rhizosphere exerts a direct effect on stimulating the expression and/or activity of bacterial catabolic pathways, as was proved recently for naphthalene degradation by *P. putida* (Fernández *et al.*, 2012).

Soil quality parameters indicated best restoration through rhizoremediation

The first, and most noticeable, consequence of a forest fire is the black ash coat (Knicker, 2007) generated by the combustion of the vegetation layer, which leads to the release of cations (Certini, 2005; Smith *et al.*, 2008). This explains the increase in soil pH observed after the burning. Since changes in pH can negatively affect microbial populations and their ability to degrade toxic compounds (Leahy and Colwell, 1990), the stabilization of pH to pre-fire levels is vital in remediation strategies. Our study revealed that bioremediation and rhizoremediation treatments were equally effective at restoring pH levels to pre-fire levels. This is also apparent, as non-inoculated plants were less capable of restoring pH levels *versus* inoculated plants.

Our results support the notion that soil enzymatic activities are reliable indicators of the health and functionality of microorganisms in response to fire stress (Fioretto *et al.* 2005; Cetin *et al.* 2009) and that

hydrocarbon levels can exert negative effects, to varying degrees, on these activities (Kiss *et al.*, 1998). Dehydrogenase activity measurements showed that soil microbiota was not severely affected by fire, as it is a direct indicator of respiration of viable cells (García *et al.*, 1997). This could be due to the positive "fertilizing effect" that nutrients from charred necromass provide (Baath and Arnebrant, 1994), as well as to the previously discussed adaptations to fire of native strains. Nevertheless, bioremediation and rhizoremediation treatments showed improvements over untreated plots due to the bioaugmentation of native and exogenous microorganisms, added organic substrates and the development of a vegetation cover.

Phosphatase and β-glucosidase activities were, as expected, severely affected by fire (Saa *et al.*, 1993; Eivasi and Bayan, 1996; Boerner *et al.*, 2000; Boerner and Brinkman, 2003). During the study period, phosphatase activity was clearly related to the presence of vegetation and only reached pristine levels with the rhizoremediation treatment—as a result of the secretion of this enzyme by root exudates (Tarafdar and Claasen, 1988). Changes in phosphatase and β-glucosidase activities were similar, remediation hastened recovery to levels found in pristine soils due to the positive effect of the rhizosphere on microbial activity (Valé *et al.*, 2005) and the provision of substrates from rhizodeposition (Morgan and Whipps, 2001) leading to improved enzyme synthesis (Turner *et al.*, 2002).

In all cases, the use of bioremediation processes enhanced soil quality parameters. Burnt soils reached pristine parameters faster with rhizoremediation providing the most remarkable benefits due to the greater microbial activity provided by the release of enzymes and substrates in root exudates (Badalucco and Kuikman, 2001).

Landscape restoration

Germination and growth of inoculated plants was rapid in comparison to non-inoculated plants, which were negatively affected by the presence of pyrolytic pollutants. Furthermore, higher degradation rates and microbial activities were observed for inoculated plants. These results emphasize the important link between pollutant removal and the generation of adequate niches for native degraders (Aprill and Sims, 1990; Segura *et al.*, 2009; Segura and Ramos, 2012). Furthermore, the use of plant growth-promoting rhizobacteria has shown to alleviate pollutant-induced plant stress (Qiu *et al.*, 1994; Kuiper *et al.*, 2001; Zhuang *et al.*, 2007). On this issue, *Pseudomonas putida* BIRD-1 presents itself as an interesting strain in rhizoremediation due to its robust PGP properties (Matilla *et al.*, 2011; Roca *et al.*, 2012) and proved tolerance to soil hydrocarbons.

The use of PGPR- and native degraders-inoculated pasture plants in this study provided remediation advantages due to (a) the rapid ease of the visual impact through rapidly growing aerial plants; (b) enhanced soil coverage; (c) the added substrate and reduced erosion provided by large

root surfaces with extensive soil penetration; (d) the establishment of suitable niches for enhanced degradation processes based on the establishment of microbial consortia; (e) the increase of microbial activity.

Conclusions

The use of bioremediation and rhizoremediation strategies in the current trial did not harm indigenous microbiota, and the release of non-native microbes only remained detectable in the soil for about six months after inoculation. Rhizoremediation treatments improved ecosystem resilience, accelerating its natural ability to return to the initial pre-fire state. The strains used in this study have proved their ability to survive in burnt soils, while exhibiting and a strong capacity to promote plant growth and development, making them suitable candidates for future use in the restoration of ecosystems affected by fires.

Acknowledgments

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Chapter 3

Field trial on removal of petroleum-hydrocarbon pollutants using a microbial consortium for bioremediation and rhizoremediation

This chapter will be submitted as:

Pizarro-Tobías P, Niqui JL, Roca A, Solano J, Fernández M, Bastida F, García C, Ramos JL. Field trial on removal of petroleum-hydrocarbon pollutants using a microbial consortium for bioremediation and rhizoremediation

Abstract

Petroleum waste sludges are toxic and dangerous which is why Environmental Protection Agencies have declared their treatment top priority. Physico-chemical treatments are expensive and environmentally unfriendly, while alternative biological treatments are less costly but, in general, work at a slower pace. An *in situ* bioremediation and rhizoremediation field-scale trial was performed in an area contaminated with oil refinery sludge under semiarid climate. The bioremediation and rhizoremediation treatments included the use of an artificial consortium made up of plant growth-promoting rhizobacteria (PGPR) and polycyclic aromatic hydrocarbons (PAHs) degrading bacteria; and the combined use of the mentioned consortium along with pasture plants, respectively. Rhizoremediation revealed that the development of vegetation favoured the evolution of indigenous microbiota with potential to remove petroleum wastes. This was inferred as the decline of total petroleum hydrocarbons (TPHs) seven months after the biological treatment.

Introduction

Petroleum-derived hydrocarbons are a widespread concern due to their deleterious effect on the environment and human health (Hutcheson *et al.*, 1996; Samanta *et al.*, 2002). Oil refinery sludges are complex wastes generated by petrochemical industries during petroleum refining processes. These wastes include different length chains aliphatic hydrocarbons along with volatile compounds (BTEX) and polycyclic aromatic hydrocarbons (PAHs) (Overcash and Pal, 1979),that contain a wide range of harmful substances. These compounds are persistent in the environment and bring about changes in soil properties (Fine *et al.*, 1997) altering indigenous microbial community (Aislabie *et al.*, 2004; Katsivela *et al.*, 2004; Militon *et al.*, 2010). Therefore, more suitable treatments are necessary (Khan *et al.*, 2004) beyond the currently used more expensive and environmentally deleterious *ex situ* techniques (i.e. burning, dumping, etc.).

Several studies have pointed towards microorganisms being an important mean of removing hydrocarbons (Leahy and Colwell, 1990; Harayama *et al.*, 1999; Mishra *et al.*, 2001; Röling *et al.*, 2002; Ros *et al.*, 2010). Bacteria from different phyla are known for their capacity to degrade a wide range of chemicals derived from petrochemical activities (Prince *et al.*, 2010). Single species, however, are only able to degrade a limited number of petroleum-derived compounds and the true potential of biodegradation derives from the coordinated action of microbes in consortia, making it possible to deal with a wider range of pollutants at

one go. *In situ* degradation rates and the extent of removal depend on nutrient availability, physico-chemical characteristics and relative soil humidity (Atlas and Bartha, 1972); therefore, enhancement of bacterial biodegradative activity through the management of suitable soil conditions is vital when applying bioremediation treatments.

The association between plants and microorganisms makes an effective feasible clean-up technology (van der Lelie *et al.*, 2001, Segura *et al.*, 2009) called rhizoremediation (Yee *et al.*, 1998) since it takes advantage of the nutrients exudated by plants which are used by the microorganisms to proliferate in the rhizosphere (Morgan and Whipps, 2001), and of the development of adequate "*in situ*" reactors for the degradation of pollutants (Aprill and Sims, 1990; Gaskin and Bentham, 2010). Bioremediation and rhizoremediation are also great options for hydrocarbon remediation because of their relatively low-cost, energy efficiency, prospect usage on a wide range of pollutants and the minimal or close to null generation of secondary wastes (Khan *et al.*, 2004). The effectiveness of indigenous pollutant-degrading microbial populations (Leahy and Colwell, 1990; Militon *et al.*, 2010; Ros *et al.*, 2010) together with the enhancement of degradation processes and the subsequent appearance of plants (Shaw and Burns, 2005), contribute to the resulting visual impact of environmental improvement (Kamath *et al*, 2004); nonetheless, remediation is to be prospected in the long term.

There are several reports on the use of bioremediation techniques for the removal of *in situ* field-scale petroleum-derived hydrocarbons using microorganisms combined with organic amendments, nutrients and/or added soil (Jørgensen et al., 2000; Mishra *et al.*, 2001; Katsivela *et al.*, 2005; Marin *et al.*, 2005; Ros *et al.*, 2010). One of the most frequently used processes for remediating petroleum sewages *in situ* is landfarming (Kuyukina *et al.*, 2003; McCarthy *et al.*, 2004; Marin *et al.*, 2005; Ros *et al.*, 2010), which is an efficient-cost option compared to other remediation technologies; nonetheless, large amounts of soil are needed along with the maintenance of soil nutrients, oxygen levels, degree of moisture and pH (Khan *et al.*, 2004).

Rhizoremediation provides a yet further step in bioremediation processes through the introduction of plants in the equation; plants which improve soil texture, aeration, and support the development of soil microorganisms while enhancing their catabolic metabolism (Segura *et al.*, 2009; Segura and Ramos, 2012). Besides the use and stimulation of degrading microbiota, the use of plant growth-promoting rhizobacteria (PGPR) complement this technology for the alleviation of pollutant-induced plant stress favouring the development of vegetation (Zhuang *et al.*, 2007). There are few examples of *in situ* field-scale trials using this technology (Gurska *et al.*, 2009), and which stress the need to further develop this strategy in the field, particularly under semiarid conditions.

The objective of this study is to assess bioremediation and rhizoremediation technologies using plant growth-promoting (PGPR) and degradative bacteria combined with a mixture of leguminous plants and pastures at a field-scale under semiarid climate conditions. We aim at evaluating the survival of microbial consortia, the success of plant restoration, and variations in microbial communities and activity in a soil that has been affected by hydrocarbon contamination for years.

Materials and Methods

Field experiment and soil sampling

The experimental area (900 m^2) was located in Murcia (N37°34'38" – W10°53'46"), Spain. The area was divided into several plots that had been used for years for the nonspecific deposition of hydrocarbons and different wastes from petrochemical processes. The studied terrain was rectangular shaped with a total area of 900 m^2 (15 m x 60 m). Preliminary data provided by the refinery management, indicated that the average concentration of total petroleum hydrocarbons (TPHs) in the plot reached a 3% (w:w), which is equivalent to 30,000 mg Kg^{-1}.

The experimental area was divided in 16 plots (3.50 m x 8 m). Each side of each plot was separated from the adjacent one by 0.5 m to minimize cross-contamination from adjacent treatments. Four replicates of four different treatments were randomly established (Table 1, Figure 2). Before setting the treatments, the entire plot was cleared from vegetation rests and lightly ploughed to facilitate seeding. The soil was classified as loam, Aridic calcisol (FAO-ISRIC-ISSS, 1998) with an average of 15 g Kg^{-1} of total organic C and 1.8 mg Kg^{-1} of total N. Soil samples were taken during the 33 weeks of experiment, starting in March 2011.

For each sampling, five subsamples per plot were collected from the upper 10 cm of soil and then were sieved at < 2mm.

Table 1. Summary of treatments applied in the field study.

Treatment	Composition	Microorganisms applied	Plants seeds mixture
Control	Untreated bare soil	None	None
Plants control	Non-inoculated plants	None	Peat AVEXIII (*Avena strigosa*, vetches, ryegrass, annual legumes) *Trifolium repens*
Rhizoremediation	Plants and microbial consortium	BIRD-1, DOT-T1E, EAF11-2, RNM-2 LH128	Peat AVEXIII (*Avena strigosa*, vetches, ryegrass, annual legumes) *Trifolium repens*
Bioremediation	Microbial consortium	BIRD-1, DOT-T1E, EAF11-2, RNM-2 LH128	None

Treatments were set as follows:

i) *Control treatment:* The control bulk soil, which remained untreated.

ii) *Control plants treatment:* For the Avex III plus Clover plant control treatment 1 Kg of the seeds mixture was spread over the soil surface, then, topsoil work was performed to allow seed germination in the dark.

iii) *Inoculated peat-microorganisms treatments:* For each treated plot, 1 L of each microorganism was mixed with 120 L of peat. After spreading the mixture over the soil surface, topsoil work was performed to achieve a homogeneous mixture of the peat with the soil.

iv) *Inoculated seed-peat-microorganisms treatments:* For each treated plot, 120 L of peat were homogeneously mixed with Avex III (1 Kg) or

Clover (500 g), then, 1 L of each microorganism was mixed with the peat-seeds mixture. After spreading the mixture over the soil surface, topsoil work was performed to allow seed germination in the dark.

Organic carrier for microorganisms and seeds.

An organic solid vegetable support was used as carrier for microorganisms (commercial peat). The selected seeds to be used in the rhizoremediation procedure were *Trifolium repens* (white clover) and a commercial pasture seed mixture called Avex III that consisted of annual ray grass, legumes and vetches, and *Avena strigosa*. These plants are commonly growing under semiarid climate. Treatments applied are summarized in Table 1.

Strains and culture media

A consortium of hydrocarbons degrading and PGPR bacteria were used for different remediation strategies in a field area contaminated with oil refinery sludges. The bacterial strains used in this study are shown in Table 2. *Pseudomonas putida* BIRD-1 (Matilla *et al.*, 2011; Roca *et al.*, 2012) was grown in MM9 minimal medium supplemented with sodium benzoate (10 mM) as the sole carbon source (Abril *et al.*, 1989); *Pseudomonas putida* DOT-T1E (Ramos *et al.*, 1995) was grown in M9 minimal medium supplemented with toluene as the carbon source; *Pseudomonas putida* RNM2 was isolated from burnt forest soil and was grown in LB broth (Maniatis *et al.*, 1982) supplemented with

naphthalene crystals; *Pseudomonas putida* EAF11-2 was isolated from burnt forest soil and was grown in LB broth (Maniatis *et al.*, 1982) supplemented with phenanthrene crystals; *Sphingomonas* sp. LH128 (Bastiaens *et al.* 2000) was grown in LB broth (Maniatis *et al.*, 1982) supplemented with phenanthrene crystals.

Table 2. Strains used in this study and their properties.

Introduced strains	Properties	Selective medium	References / source
Pseudomonas putida BIRD-1	PGPR	MM9 agar Sodium benzoate Cm_{30} Irgasan	Matilla *et al.*, 2011 Roca *et al.*, 2013
Pseudomonas putida DOT-T1E	BTEX degrading	MM9 agar Toluene Rif_{10}	Ramos *et al.*, 1995
Pseudomonas putida EAF11-2	Naphthalene and Phenanthrene degrading	LB agar Indole crystals	Isolated within Bio-Ilíberis R&D premises from a burnt forest soil
Pseudomonas putida RNM-2	Naphthalene degrading	LB agar Catechol (0.1M)	Isolated within Bio-Ilíberis R&D premises from a PAHs polluted soil
Sphingomonas sp. LH128	Phenanthrene degrading	LB agar Rif_{50}	Bastiaens *et al.*, 2000

Cultures were incubated at 30°C and shaken on an orbital platform operating at 200 strokes per minute. Monitoring of survival of *P. putida* BIRD-1, DOT-T1E and *Sphingomonas* sp. LH128 strains in soil was performed by drop-plating of dilution series on solid selective media supplemented with the required carbon sources and antibiotics, as described in Table 2.

For monitoring, *P. putida* EAF11-2 drop plating of dilution series (from soil/rhizosphere samples) in LB agar medium (Maniatis *et al.*, 1982) were incubated at 30 °C for 12-16 hours. Then, indole crystals were added to the lid of Petri plates and incubated for 15 days, monitoring the emergence of blue colonies every day. This procedure was performed to identify dioxygenase activity, which converts indole into blue indigo (Ensley *et al.* 1983; Ahn *et al*, 1999).

For assessing RNM-2 cell density changes in soil, drop plating of dilution series (from soil/rhizosphere samples) in LB agar medium (Maniatis *et al.*, 1982) were incubated at 30 °C for 4 days. Then, colonies were transferred to Whatman paper nº1 and moistened with a catechol solution (0.1M) and incubated at room temperature for 30 minutes. A yellow halo surrounding the colonies indicated a positive reaction. This procedure was performed to identify catechol-2,3 dioxygenase activity through the formation of hydroxymuconic semialdehyde from catechol (Pankhurst *et al.*, 1965; Sandhu *et al.*, 2009).

Chemical and biochemical analysis

Total Petroleum Hydrocarbons (TPH) measurement.

Previously to TPH analysis, soil samples were air-dried and sieved (2 mm mesh). Approximately 2.5 g of dry soil was placed, by triplicate, in 50 mL Falcon tube and mixed with 4 g of anhydrous sodium sulphate, previously dried at 105°C to remove residual water from the salt. A second

subsample of soil was used to determine soil moisture. Then, 15 mL of dichloromethane was added to a Falcon tube and the mixture was vigorously shaken for 1 minute using a vortex apparatus. The extract was then centrifuged for ten minutes at 8000 rpm and the supernatant was decanted and saved for ulterior analysis. To perform a new extraction cycle, a second 15 mL aliquot of clean solvent was added to the tube and the extraction process was repeated three more times. The successive extraction solutions were combined with the first one in a 250 mL spherical flask. Finally, the solvent, around 60 mL of dichloromethane, was evaporated to dryness using a rotary evaporator, and the 250 mL flask was totally dried and weighed until constant weight. TPH content was expressed as *g* TPH Kg^{-1} dry soil.

Measurement of dehydrogenase activity (DHA) in soil

Dehydrogenase activity was determined as described by García *et al.* (1997). The amount of iodonitrotetrazolium violet-formazan (INTF) released from 1 *g* soil from each treatment was measured after incubation in the dark at 22 ºC for 20 hours with 0.2 mL of 0.4% 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenylteytrazolium chloride hydrate (INT) as substrate for the enzymatic reaction, and 2 ml of distilled water. Then, 5 mL of an extracting mixture (ethylene: chloride acetone (1:1.5 v:v)) were added and well-mixed for 1 minute and filtered through a Whatman No. 5 filter paper. A blank experiment was performed for each assay, without substrate, in which the extracting mixture was added after incubation. The amount of INTF per hour released from each soil sample (μg INTF/gh) was determined by comparing absorbance measures at 490 nm to an

INTF standard curve. Rhizosphere soil is considered soil closely attached to roots, sieved through a 2 mm mesh.

Measurement of soil pH

The pH values were measured in air-dried soil, sieved through a 2 mm sieve, using a glass combination electrode (soil: water ratio, 1:2.5 w:v), as described by Acosta-Martínez *et al.* (2003).

Plant biomass measurement

Five replicates of plants were picked from every subplot. Shoots and roots were weighted, for fresh weight, and stored in a stove at 90 ºC for 48 hours. Then, roots were allowed to cool down to room temperature and weighed again for dry weight.

Bacterial Community analysis

Genomic DNA was extracted from approximately 0.5 g, of a composed soil sample from each plot, using the FastDNA kit (Qbiogene Inc CA, USA), according to instructions from the manufacturer. The DNA was extracted immediately after sampling. The DNA concentration was determined using NanoDrop 2000 Spectrophotometer and confirmed by electrophoresis on a 0.8% (w/v) agarose gel. Partial sequences of the 16S rRNA gene including the variable V3 region the 570 bp region of 16S rDNA genes, were amplified from the DNA using the universal Eubacterial primers BSF8, 5'-

NNNNNNNNNNNNNTCAGAGTTTGATCCTGGCTCAG-3' and the USR515 5'-NNNNNNNNNNNNNNCACCGCCGCKGCTGGCA-. Each PCR reaction was performed in 50 μL reaction mixture containing 25 ng of DNA, 0.2 μM of dNTP, 0,2 μM of each primer, 1 X High Fidelity PCR buffer (Invitrogen, Carlsbad CA), 2.5 U Platinum Taq DNA Polymerase High Fidelity (invitrogen) and using the following condition: 94 ºC for 5 min followed by 30 cycles of 94 ºC for 30 s 56 ºC for 30 s and 72 ºC for 90 s and final elongation step 72 ºC for 5 min. Each PCR product was visualized on 1% agarose gel to confirm DNA amplicone size and purified by gel electrophoresis and QIAquicK Gel extraction kit (Qiagen CA USA). Amplicon pyrosequencing was performed with a 454/Roche Titanium instrument (Roche NJ USA) according to standard protocols. All of the sequences from the pyrosequencing reads were processed using QIIME (Quantitative insights Into Microbial Ecology) software pipeline. Sequences were sorted based on sample specific barcode and primers. Low quality sequences were filtered out to remove sequences shorter that 200 bp and with an average quality score lower than 25. After quality checking, these sequences were used to identify the most likely match of each sequence using Riosomal Data Project (RDP). The taxonomic sequence identification was also crosschecked against BLASTn database. QIIME was used also to produce the rarefaction plots and the taxa assignments chart.

Statistical analyses

A descriptive statistical analysis (the mean and absolute error) was calculated for each parameter. Also, we performed some inferential statistical analyses, such as analysis of variance (one-way ANOVA) within treatments, assuming a normal distribution of the data and homoscedasticity. For *post-hoc* analysis we used the Tukey test ($p<0.05$) to determine changes in the analysed parameters for each treatment.

Results and Discussion

The study focused on the analysis of the effects of bioremediation and rhizoremediation treatments in a polluted site with oil refinery sludges. Combinations of pasture seeds and/or a microbial consortium were arranged in order to assess the survival and efficiency of the introduced microbes (Table 2), and to compare the effect of bioremediation without plants and that of the rhizoremediation process. Along seven months, the general appearance of the landscape, the evolution of the introduced plants, the survival of introduced bacteria, the impact on indigenous microbial populations, TPHs analysis, pH, and dehydrogenase activity in soil, were measured.

Proliferation of introduced consortium population

Several *Pseudomonas putida* strains with the ability to degrade different chemicals or exhibiting PGPR properties, as well as a *Sphingomonas* sp. strain (Table 2) were selected to create an artificial consortium to be used in this study. *P. putida* DOT-T1E degrades several aromatic compounds such as BTEX (benzene, toluene, ethylbenzene and xylenes) (Ramos *et al.*, 1995); PAHs (polycyclic aromatic hydrocarbons) are degraded by *P. putida* EAF11-2, *P. putida* RNM-2 and *Sphingomonas* sp. LH128 (Bastiaens *et al.*, 2000), and *P. putida* BIRD-1 enhances plant growth (Matilla *et al.*, 2011; Roca *et al.*, 2013).

Survival of each introduced strain, both in soil and in the rhizosphere, was determined by plate counting on selective media (Table 2). Monitoring of each strain revealed that in general survival of the introduced strains was highly dependent on their initial association to the plants (Figure 1). Most strains inoculated in rhizoremediation treatments increased their initial cell density and remained at high levels during the first twenty weeks of the treatment following a steady decrease of cell numbers at the beginning of the dry season (Figure 1a). Thereafter, some strains lost viability and only *P. putida* BIRD-1 and DOT-T1E remained at high cell densities 33 weeks after their introduction. *Pseudomonas putida* RNM-2 was detected in the rhizosphere for only 13 weeks, before dropping below detection limits. In bare soil (bioremediation treatments) (Figure 1b) no increase in the initial population of any of the strains was found, and in the case of *P. putida* RNM-2 and *Sphingomonas* sp. LH128 both remained undetected 24h after the inoculation. Therefore, the establishment of microbiota in soil seems to be influenced by rhizodeposition of nutrients by plants that provide microenvironments with different chemical, physical and biological conditions than bare soil, these results are in agreement with those by Foster (1998) and Lambers *et al.*, (2009).

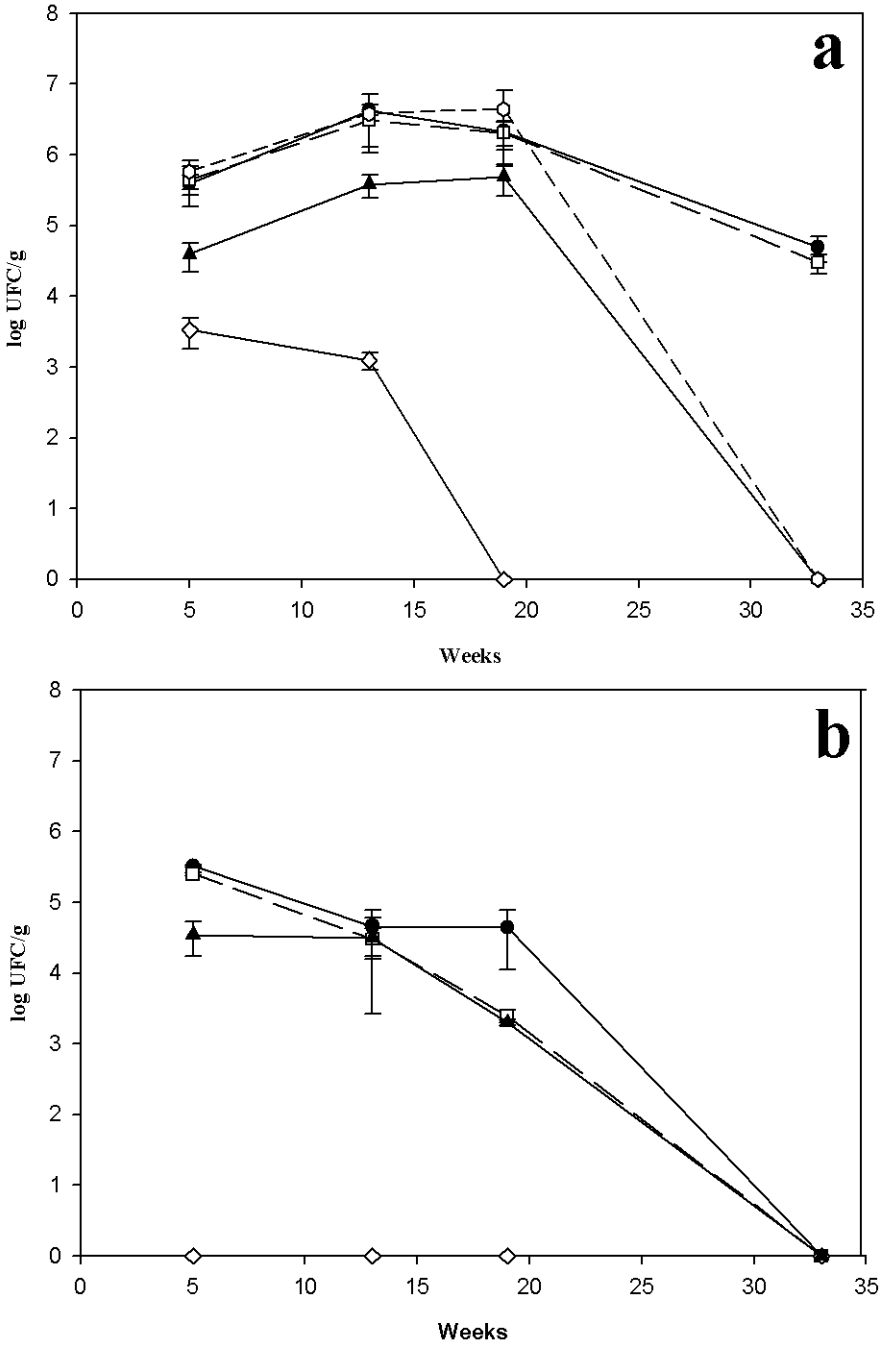

Figure 1. Survival of introduced strains. Viable *P. putida* BIRD-1 (filled circle), *P. putida* DOT-T1E (square), *P. putida* RNM-2 (diamond), *P. putida* EAF11-2 (filled triangle) and *Sphingomonas* sp. LH128 (circle) in rhizosphere of introduced plants (rhizoremediation treatments) (1a) and bulk soil (bioremediation treatments) (1b). Data showed as mean (n=5) and error bars refer to standard deviation.

It should be mentioned that in all cases (Figure 1a), cell density of most of the introduced strains, excepting *P. putida* BIRD-1 and DOT-T1E, decreased below detection limits during the aestival season because of the lack of precipitations, high temperatures and the wilting of planted pasture in rhizoremediation treatments. The *P. putida* strains BIRD-1 and DOT-T1E showed higher survival capability as evidenced by the population levels of both bacteria remaining at about 10^5 CFU/g soil at the end of the assay. In the case of *P. putida* BIRD-1, this result confirms its ability to survive at low soil moisture, as reported by Roca *et al.* (2013). *Pseudomonas putida* DOT-T1E bears the pGRT1 plasmid that carries the *ttgGHI* operon, which encodes an efflux pump for the extrusion of aromatic hydrocarbons, which confers this strain the ability to proliferate in the presence of these toxic chemicals (Rodríguez- Herva *et al.*, 2007).

Total Petroleum Hydrocarbons (TPHs)

For years the plot used for this study received deposits from hydrocarbon wastes, composed mainly by alkanes, asphaltenes, resins, bitumens, etc., which are sludges derived from petroleum refining processes (Overcash and Pal, 1979). These kinds of compounds are considered toxic, mutagenic, carcinogenic, persistent, pernicious and can be accumulated through the trophic chain (Kamath et al., 2004). Due to the complex nature of these wastes it is not practical to monitor compounds separately. Therefore to assess the potential variations in the total concentration of petroleum wastes Total Petroleum Hydrocarbons (TPHs)

were measured along the assay. Initial TPH concentrations before the treatment, that the pollutants were unevenly distributed along the plot, in a range between 20 g Kg^{-1} to 125 mg Kg^{-1} of soil, as shown in Figure 2.

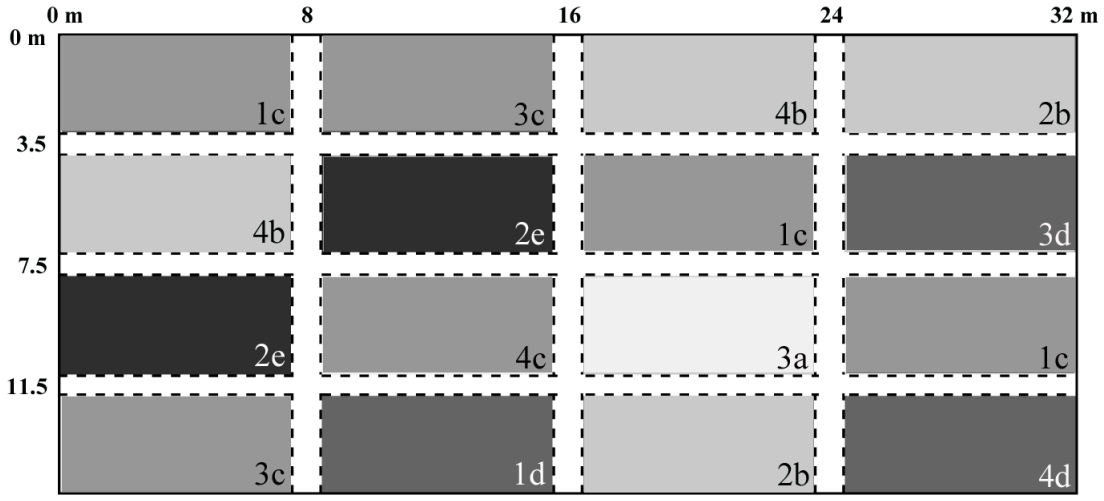

Figure 2. Distribution treatments and total petroleum hydrocarbons (TPHs) in the plots at the beginning of the assay. Numbers correspond to treatments: control/untreated (1), plants control (2), rhizoremediation (3) and bioremediation (4). Letters correspond to average TPHs concentration (n=5) in plots: <25 g/Kg (a), 25 – 50 g/Kg (b), 50 – 75 g/Kg (c), 75 – 100 g/Kg (d), 100 – 125 g/Kg (e).

In spite of the complexity observed in TPHs distribution in the studied parcels, four months after the outset of the assay a decrease trend was observed in the parcels were plants have been introduced, whether inoculated or not (Figure 3). This could be explained by variations on indigenous microbial populations related to the generation of ecological niches and the exudation of nutrients in the rhizosphere, also supporting the establishment and survival of the inoculated consortium in soils polluted with petroleum hydrocarbons, as previously reported by Kozdrój and van Elsas (2000). At the end of the trial, we observed that the TPHs

decrease in rhizoremediation treatments was of about a 15%, when compared to the control treatments.

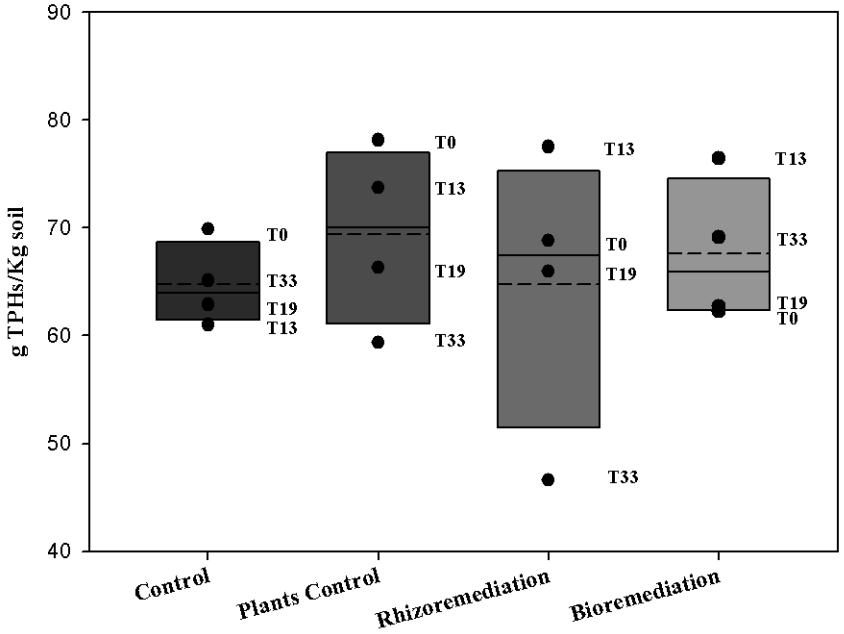

Figure 3. Total petroleum hydrocarbons (TPHs) measured along the study. Vertical boxes show the median (solid line), mean (dash line) and the 5th/95th percentiles (n=5). Sampling times: T0) beginning of the assay (March 2011), T13) 13 weeks (June 2011), T9) 19 weeks (August 2011) and T33) 33 weeks (October 2011).

Microbial activity

Microorganisms influence ecosystems because they are responsible of the biochemical cycles of carbon, nitrogen, phosphorous and sulphur through soil microbial activities (Karaca *et al.*, 2011) and soil structure (Harris and Birch, 1989). The study of enzymatic activities in soils is considered a good indicator of soil quality since enzymatic activities are responsive to natural and anthropogenic induced stresses and soil degradation (Nannipieri *et al.*, 2002; Gianfreda *et al.*, 2005, Karaca *et al.*, 2011).

Therefore, monitoring the evolution of soil enzymatic activities helps to evaluate soil recuperation.

Dehydrogenase activity (DHA) is used as an indicator of oxidative metabolism linked to respiration of viable cells (Skujins, 1973; García *et al.*, 1994; Quilchano and Marañón, 2002; Makoi and Ndakidemi, 2008) and it is considered a good index of soil status in semiarid Mediterranean areas (García *et al.*, 1997). Dehydrogenase activity was monitored to assess the effect of soil pollutants and remediation treatments on microbial activity. DHA measurements revealed that after the first 13 weeks of treatment activity had increased in soils undergoing rhizoremediation (Figure 4) indicating the enhancement of microbial activity derived from this treatment, whereas no differences were observed between control and bioremediation treatments.

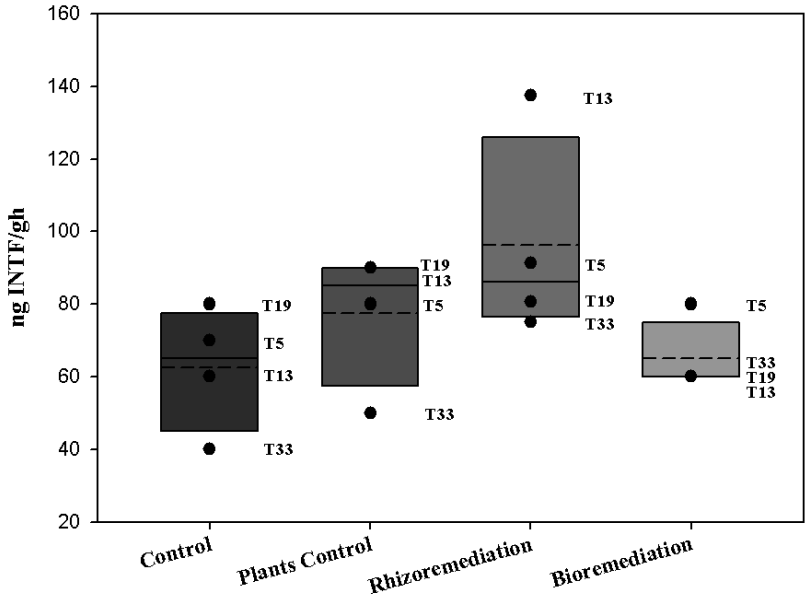

Figure 4. Dehydrogenase activity (DHA) measured along the study. Vertical boxes show the median (solid line), mean (dash line) and the 5th/95th percentiles (n=5). Sampling times: T5) 5 weeks (April 2011), T13) 13 weeks (June 2011), T9) 19 weeks (August 2011) and T33) 33 weeks (October 2011).

After 8 months of treatment, DHA activity in soils under rhizoremediation was 40% higher than in other treatments. Overall, control soil tended to show lower values of DHA than the rest of treatments. These assays suggest that the combined effect of the introduction of plants and microbes with PGPR and biodegradation properties had a beneficial effect on soil enzymatic activities.

In parallel with DHA evolution, monitoring of pH in soil was performed along the trial (Figure 5), as changes in this parameter can have a negative effect on microbial populations and, therefore, in their ability for degrading recalcitrant compounds (Leahy and Colwell, 1990). Most adequate condition for bacteria and fungi metabolism is neutrality, being the latter more tolerant of acidic conditions (Atlas, 1988). An optimal pH range from 7.4 to 7.8 has previously been reported for the mineralization of oily sludge in soil (Verstraete *et al.*, 1976; Dibble and Bartha, 1979).

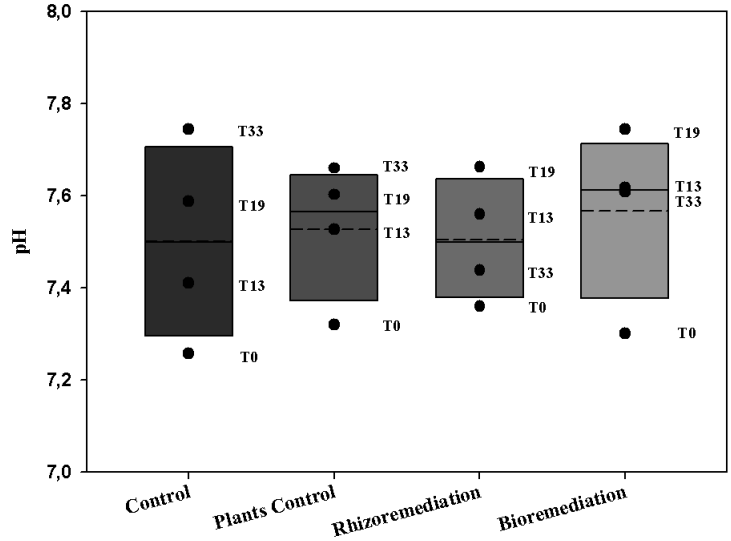

Figure 5. pH measured along the study. Vertical boxes show the median (solid line), mean (dash line) and the 5th/95th percentiles (n=5). Sampling times: T0) beginning of the assay (March 2011), T13) 13 weeks (June 2011), T9) 19 weeks (August 2011) and T33) 33 weeks (October 2011).

At the beginning of the trial, pH was around neutrality, between 7.3 and 7.8, in all the treatments (Figure 5). Similar values maintained along the assay, no statistical differences ($p>0.05$) were observed among plots, which made an optimal environment for degradation of hydrocarbons pollutants as described before.

Microbial diversity and community composition

To assess the effects of bioremediation and rhizoremediation treatments on soil microbiota in soil polluted with petroleum hydrocarbons, barcoded amplicon pyrosequencing of the hypervariable V3 region of the 16S rDNA gene was performed. Microbial diversity was studied before setting the treatments and during the remediation process at 30, 90 and 150 days in control, non-inoculated plants, rhizoremediation and bioremediation treatments. The raw sequence data obtained from 454 pyrosequencing were analyzed using QIIME software after sorting trimming and processing through chimeric analysis.

Rarefaction analysis (Figure 6) showed that 90 days from the start of the assay, treatments where plants had been introduced (non-inoculated and rhizoremediation) exhibited an increase in the number of OTUs.

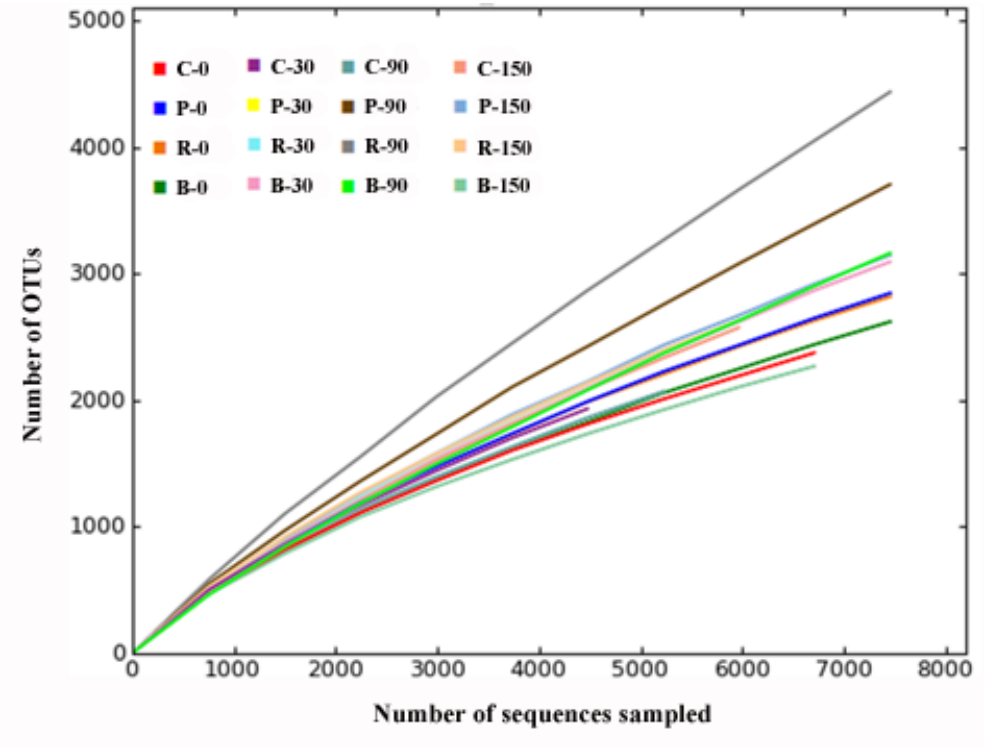

Figure 6. Rarefaction analysis. Control soil (C), plants-control (P), rhizoremediation (R) and bioremediation (B) at different times in the assay: at the beginning of the treatment (0), 30 days (30), 90 days (90) and 150 days (150).

However, it was previously reported that diversity in the rhizosphere differs significantly from that of bulk soil, being higher in the latter case (Marilley *et al.*, 1999; Weisskopf *et al.*, 2005; García-Salamanca *et al.*, 2012). Thus, this could be explained by the deleterious effect that hydrocarbons pollution exerts over the indigenous microbial population of a formerly pristine soil, and the selection of certain microbial groups in the rhizosphere of plants, as recently reported by Solano and colleagues (submitted). The increase of biodiversity associated to introduced plants, could be related to the generation of ecological niches and the exudation of nutrients in the rhizosphere, promoting variations on indigenous microbial population and supporting the establishment and survival of

the inoculated consortium in soils polluted with petroleum hydrocarbons, as previously reported by Kozdrój and van Elsas (2000).

The prevailing bacterial *phyla* in pre-treated polluted soil were Actinobacteria and Proteobacteria, along with Choroflexi Gemmatimonadetes, Bacteroidetes and Planctomycetes; however, changes in bacterial community distribution were noticed along the remediation assay (Figure 7).

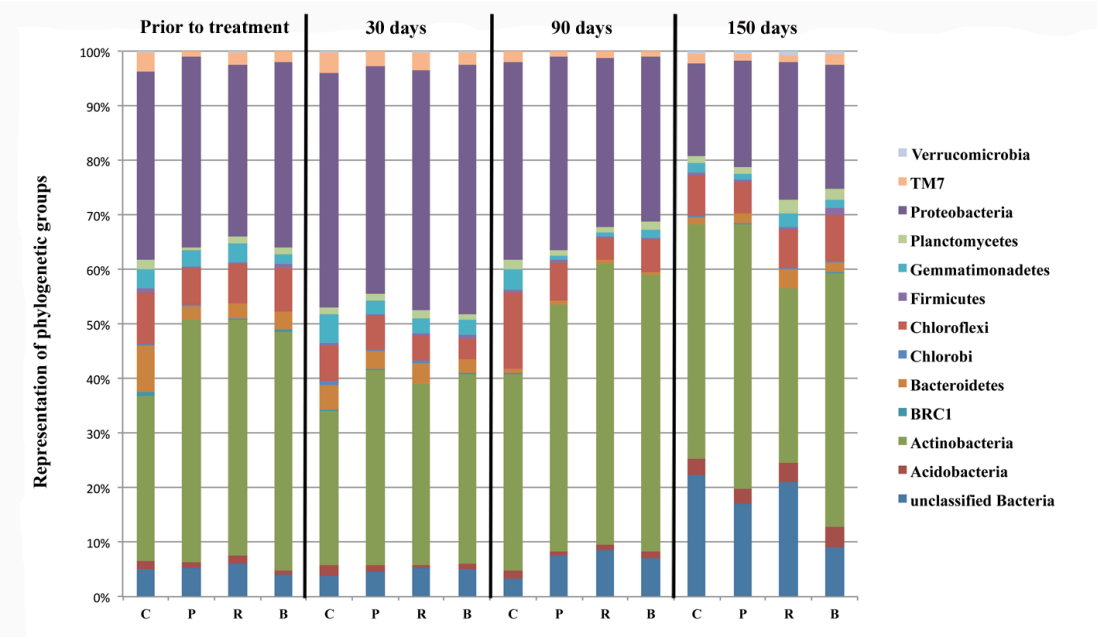

Figure 7. Relative abundance of phylogenetic groups at the phylum level based on 16S rDNA genes. Control (C), plants-control (P), rhizoremediation (R) and bioremediation (B) at four different times in the assay: before treatment, 30 days, 90 days and 150 days.

Actinobacteria have been described as an important group in soils under hard environmental conditions (Fierer *et al.*, 2012; Wang *et al.*, 2012). After 30 days of treatment, the Proteobacteria *phylum* increased in all plots while the relative abundance of Actinobacteria decreased; the change in the population distribution of these predominant *phyla* could

come as consequence of soil tillage performed before setting the assay, as had already been reported in semiarid conditions by Acosta-Martínez and colleagues (2010). Nevertheless, the increase of Proteobacteria in bioremediation and rhizoremediation treatments is more remarkable (over 30% in both cases), compared to non-inoculated treatments (around 20% in both cases). This could be due to the addition, and therefore enrichment, of bacteria belonging to this *phylum* contained in the applied remediation treatments.

After 90 days of treatment an increase in Actinobacteria, specially in the rhizoremediation treatment, and that of unclassified bacteria was recorded, as well as a significant decrease in Bacteroidetes in the inoculated plots (bioremediation and rhizoremediation) (Figure 7), which is consistent with results by Bouchez-Naitali and colleagues (1999) who reported the prevalence of Actinobacteria in hydrocarbon polluted soils from different geographical locations. This *phylum* comprises the Actinomycetales order, which consists of several species belonging to the *Mycobacterium*, *Microbacterium* and *Nocardia* genera, which have been reported to act as petroleum hydrocarbons degraders (Margesin *et al.*, 2003; Kloos *et al.*, 2006; Ros *et al.*, 2010), and the *Georgenia* genus that has been related to degradation processes in soils contaminated with petroleum hydrocarbons (Militon *et al.*, 2010, Woo *et al.*, 2011). These genera are among the main 16 genera (with a relative abundance ≥ 1%) identified in our assays (Table 3). Therefore, the increase in degradative microbial population belonging to Actinobacteria could be related to

rhizoremediation treatments favouring the activation of degradation processes and leading to a decrease in TPHs, as described before, through the creation of adequate niches for the proliferation of indigenous bacteria, as previously reported for ryegrass species by Kirk and colleagues (2005).

After 150 days of treatment (Figure 7), a significant change in bacterial community distribution occurred, leading to a strong reduction of the relative abundance of several *phyla*, which was especially notorious (around 50%, compared to the beginning of the assay) for the Proteobacteria *phylum* in non-inoculated plots (untreated and non-inoculated plants), whereas reduction of this *phylum* in inoculated plots (bioremediation and rhizoremediation) was around 30% which could due to the persistence at high population levels of introduced microbiota (Figure 1), specially when associated to plants; whereas the unclassified bacteria and Acidobacteria *phyla* increased. This considerable change within the microbial population could be ascribed to the inception of the aestival season, where the lack of precipitations and higher temperatures concur, as recently reported for Mediterranean ecosystems by Pizarro-Tobías and colleagues (unpublished reults).

Table 3. Bacterial relative abundance at genus level (≥1%) along the assay. Control (C), plants control (P), rhizoremediation (R) and bioremediation (B), at four different times in the assay: before treatment, 30 days, 90 days and 150 days.

Phylum	**Genus**	**C 0**	**P 0**	**R 0**	**B 0**	**C 30**	**P 30**	**R 30**	**B 30**	**C 90**	**P 90**	**R 90**	**B 90**	**C 150**	**P 150**	**R 150**	**B 150**
Actinobacteria	*Georgenia*	1.6	1.9	1.7	1.7	1.0	2.0	1.3	1.8	2.4	1.7	5.2	4.4	2.6	3.3	1.1	2.8
	Gordonia	0.1	1.8	1.3	1.6	0.8	0.9	2.3	1.7	0.8	1.0	1.8	0.4	0.5	0.7	1.0	0.8
	Agromyces	0.6	1.1	0.6	1.1	0.2	0.5	0.2	0.6	0.4	0.5	0.2	0.3	0.6	0.9	0.3	0.9
	Microbacterium	2.3	2.4	3.1	1.3	3.0	2.5	6.6	2.3	1.2	1.4	1.0	0.8	1.3	1.9	2.6	1.6
	Mycobacterium	4.5	4.5	8.5	4.0	2.9	3.2	3.5	3.0	2.5	5.4	2.6	2.8	6.6	6.5	5.7	5.2
	Nocardia	0.6	0.7	0.7	0.7	0.6	0.3	0.2	0.3	0.5	0.8	1.5	0.4	0.6	1.5	0.5	1.2
	Williamsia	0.3	1.2	0.7	0.3	0.3	0.2	0.1	0.3	0.2	0.6	0.7	0.6	0.6	0.2	0.0	0.2
Bacteroidetes	*Flavobacterium*	0.9	0.3	0.3	0.3	0.4	0.5	1.0	0.2	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.1
	Cytophaga	5.3	1.2	1.8	1.9	2.1	1.3	0.9	1.2	0.3	0.3	0.1	0.2	0.7	0.9	2.2	0.5
Gemmatimonadetes	*Gemmatimonas*	0.4	1.1	0.3	0.5	0.5	0.3	0.7	0.4	0.0	0.2	0.0	0.0	0.1	0.1	0.2	0.0
Proteobacteria	*Balneimonas*	0.1	0.0	0.1	0.1	0.0	0.1	0.1	0.2	0.4	0.1	0.7	0.2	0.1	0.2	0.1	0.1
	Hyphomicrobium	0.2	0.3	0.7	0.4	0.5	0.2	1.1	0.5	0.0	0.1	0.0	0.0	0.2	0.0	0.3	0.1
	Phaeospirillum	1.7	0.6	0.8	0.8	1.2	0.8	1.3	1.0	0.4	0.3	0.1	0.2	0.0	0.1	0.2	0.1
	Skermanella	0.3	0.6	0.5	1.0	1.0	1.2	1.1	0.7	2.5	1.5	5.2	1.8	0.7	1.6	0.6	1.5
	Massilia	0.0	0.1	0.1	0.1	0.0	0.2	0.0	0.1	0.0	0.0	0.0	0.0	0.4	0.2	0.1	1.9
	Methyloversatilis	0.1	0.1	0.1	0.1	1.6	0.6	0.5	0.6	0.1	0.1	0.0	0.0	0.0	0.0	0.1	0.0

As described above, Actinobacteria and Proteobacteria were the predominant *phyla*, and had already been described as key in the bioremediation of alkane-polluted semiarid soils (Pucci *et al.*, 2000), and reported to be an important part of microbial population in several oil-polluted scenarios (Watanabe, 2001), also pointing out the selective pressure of pollutants over indigenous microbial populations. Bioremediation and rhizoremediation treatments showed a tendency towards the stimulation of petroleum-degrading bacterial communities, aimed at accelerating the recovery of the polluted soil in terms of biodiversity.

Introduced plant development and landscape evolution

To assess and compare the evolution of plant biomass generation along the assay in the different treatments, dry weight of shoots and roots in each plot were measured. Three months after the beginning of the assay, plants in the plots undergoing rhizoremediation treatments exhibited an increase of about a 30% in shoot dry weight (Figure 8a and 8b) comparing to plants-control treatments; no significant differences were found in terms of percentage of root dry weight (data not shown) among them. Since the introduced bacterial consortium contained *Pseudomonas putida* BIRD-1, we ascribed the plant-growth promoting effects to BIRD-1, which is a PGPR strain that efficiently colonizes plants roots while solubilising iron, organic and inorganic phosphate, and synthesising phytohormones (Matilla *et al.*, 2011; Roca *et al.*, 2012).

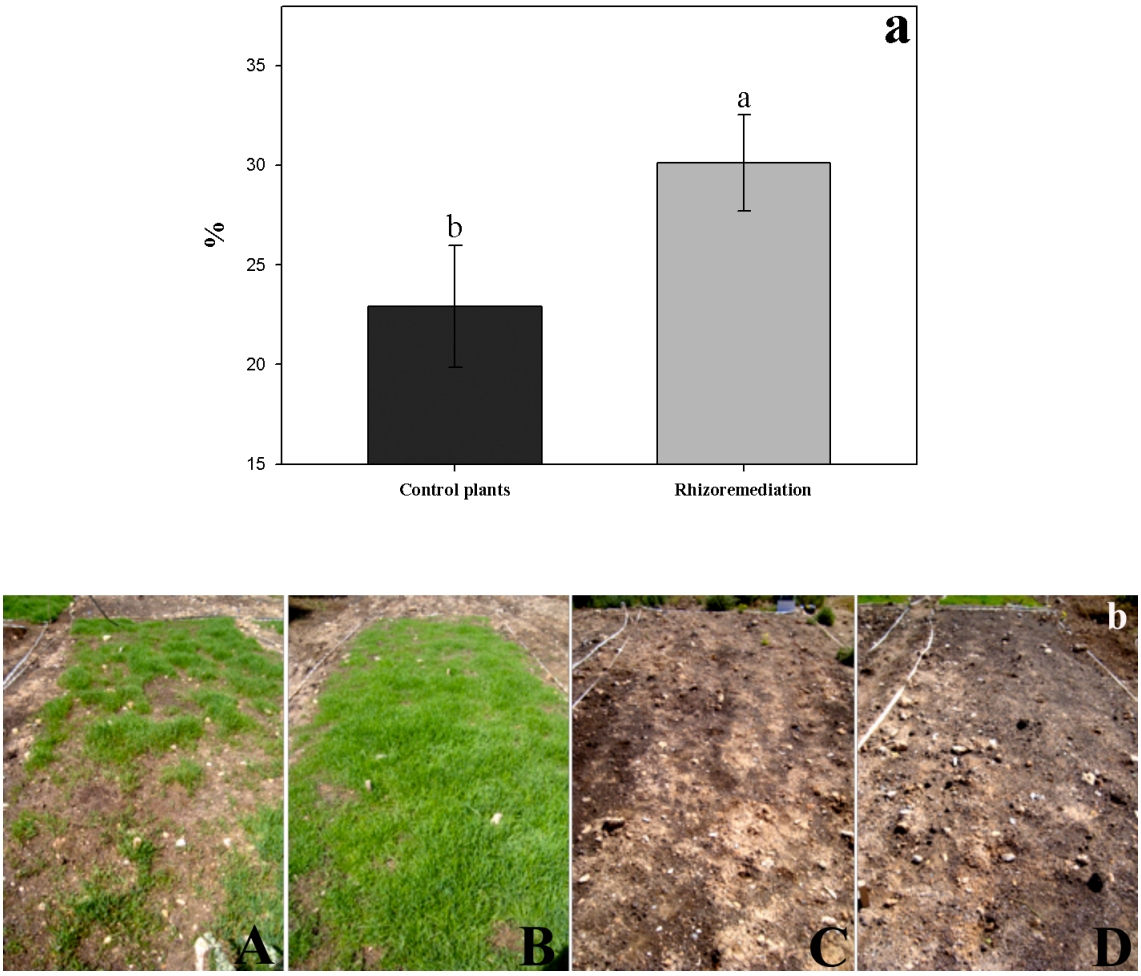

Figure 8. Introduced plant fitness at different times in the study. Figure 8a shows percentage of shoot dry weight (n=5) of inoculated plants in rhizoremediation treatments compared to non-inoculated plants in plant-control treatments three months after the beginning of the trial. Different letters mean statistical differences according to the Tukey test ($p<0.05$). Figure 8b shows growth of introduced plants and vegetation coverage after 1 month from the beginning of the trial on plant control treatment (A), introduced plants in rhizoremediation treatments (B), control treatment/untreated (C) and bioremediation treatment (D).

The effect of introduced plants on the landscape, in general, and on each plot in particular, was recorded photographically (Figure 9). Visually, the positive impact of the rhizoremediation processes could be appreciated from the first month after the beginning of the assay: higher germination and plant coverage could be observed in plots were plants had been introduced in association with microorganisms. No germination was observed for *Trifolium repens* in any of the plots, which could be due to this specific sensitivity of plants to the concentration of petroleum hydrocarbons, a phenomenon recently reported in soil contaminated with 12,000 ppm of diesel (Barrutia *et al.*, 2011). Therefore, the choice of plants in rhizoremediation is highly relevant and in the case of removal of hydrocarbons, pasture grasses are good candidates for rhizoremediation treatments since they are not affected by hydrocarbons when associated to PGPR and degradative bacteria consortium.

Four months after the beginning of the assay, in the aestival season (july 2011), introduced flora had completed its phenological cycle, and total wilt of the plants was observed. Bioremediation plots presented no visual differences with those that remained untreated along the assay, and no vegetation germination was recorded in any of these plots.

Figure 8. General evolution of the treated plots along the study (8 months): before the treatment (A, March 2011), one month of treatment (B, April 2011), three months of treatment (C, June 2011), end of trial (D, October 2011).

Concluding remarks

We found out that none of three of the introduced *P. putida* strains (BIRD-1, DOT-T1E and EAF11-2) and, *Sphingomonas* sp. LH128 were affected by pollutant concentrations in soil as high as 30,000 mg Kg^{-1} soil and had the ability to survive, particularly associated to plants, for at least, 20 weeks. This made the established artificial consortium a good candidate to be used in rhizoremediation treatments. Plant growth-promoting effects were observed in plots where pasture plants had been associated to the introduced artificial consortium, favouring also the growth of indigenous microbiota with the ability to degrade hydrocarbons, in particular Actinobacteria, which comprise genera related to petroleum-hydrocarbons degradation processes. As expected from the complex chemical composition of pollutants, the decrease in total TPH concentrations was modest in the period under study; however longer assays should provide solid support on the effectiveness of the approach adopted for removal of recalcitrant compounds.

Acknowledgments

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General Discussion

The rhizosphere, as first defined as the area influenced by the root system (Hiltner, 1904), is a complex environment with longitudinal and radial gradient variations in its physico-chemical and biological properties (Brimecombe *et al.*, 2007; Ahmad *et al.*, 2011); where exudation, nutrient and water uptake take place and microbial population and activity have been reported to be higher than in bulk soil (Molina *et al.*, 2000; Morgan *et al.*, 2005). Root exudates comprise amino acids, organic acids, sugars, vitamins, fatty acids and sterols, nucleotides, proteins and volatile compounds such as carbon dioxide and molecular hydrogen, etc. (Ahmad *et al.*, 2011). Although nutrient-rich, the rhizosphere is a changing environment where its inhabitants are exposed to multiple carbon sources, decomposing aromatic compounds derived from lignins, secondary metabolites derived from plants and microbes and signalling molecules among others, all of which exert a selective pressure over them (Matilla *et al.*, 2007; Attila *et al.*, 2008). Thus, diversity of microbial populations colonizing the root vicinity has been reported to differ not only significantly from that of bulk soil (Marilley *et al.*, 1999; Weisskopf *et al.*, 2005; García-Salamanca *et al.*, 2012), but also its composition is affected by soil characteristics, plant species and root area (Chen *et al.*, 2006). Nonetheless, microorganisms inhabiting the rhizosphere can act as beneficial or detrimental to plant growth.

Plant growth-promoting rhizobacteria (PGPR) constitute a fraction of bacterial populations that favour seed germination and, then, plant growth and development by increasing the availability or producing

specific nutrients and direct or indirect antagonism of plant deleterious microbes. One of the most important traits for PGPR strains to be effective, once they are released in the environment, is the capacity to colonize and survive in the rhizosphere.

Pseudomonas, which are considered ubiquitous inhabitants of the environment, have been reported as the most effective root-colonizing bacteria (Lugtenberg *et al.*, 2001) as members of this genus often live in mutualistic association with plants in the rhizosphere, where rhizodeposits are exchanged for phytohormones precursors, solubilisation of low-available nutrients like iron and phosphate, compounds with antifungal activity and chemicals that promote induced systemic resistance. Not all *Pseudomonas* are PGPR strains, but those with these properties are suitable for agriculture and environmental uses.

To assess genes for competence, proliferation and stress adaptation of *Pseudomonas putida* in the rhizosphere we carried out a literature search, that gave as a result 180 genes involved in rhizosphere fitness (Table 1). Identified traits included 25 proteins related with the ability to attach to biotic surfaces and biofilm genesis, 13 related with flagella biosynthesis, 2 involved in chemotaxis responses, 14 regulators and sensor proteins, 64 proteins involved in stress adaptation and detoxification, 8 proteins involved in DNA repair mechanisms, 8 proteins involved in transport and secretion processes, 30 related to metabolism,

7 involved in protein synthesis and 9 hypothetical proteins with unknown functions.

1. Attachment, biofilm formation and fagella

A critical issue in root colonization is the ability of *Pseudomonas putida* to recognize plant-root exudates. Espinosa-Urgel and colleagues (2002), in a video accompanying the referred article, showed how *Pseudomonas* cells recognize corn seeds and move towards the seeds. Later, a research article by Lacal and colleagues (2010) revealed that all TCA cycle intermediates present in root exudates are recognized by the McpS chemoreceptor, and these are among others signalling molecules that promote motility towards the source of chemicals. Different approaches have been used to identify these features, with the initial and most highlighting technique, which is the generation of random mini-Tn5 mutants to assess attachment to seed and root surfaces. Espinosa-Urgel and colleagues (2000) obtained eight *Pseudomonas putida* KT2440 random transposon mutants with diminished ability in attachment to corn seeds. Sequencing of the DNA adjacent to the insertions and later characterization of mutants suggested that initial steps in attachment and biofilm formation in *Pseudomonas putida* share certain similarities with pathogenic microorganisms infecting host tissues.

The analysis of these mutants (Yousef-Coronado *et al.*, 2008; Martínez-Gil *et al.*, 2010; Duque *et al.*, 2013), revealed that two functions are essential in attachment and biofilm formation that involves two large

surface adhesion proteins influencing plant colonization: i) LapA (Table 1), and genes related to its synthesis and transport (LapB, LapC, LapD), which is the most essential trait in the initial steps of attachment to plants surfaces and biofilm formation, and therefore success in the rhizosphere; and ii) LapF, which plays a significant role at later stages than LapA in the maintenance of the biofilm structure at the sessile stages of the colonizing *Pseudomonas*. Further analysis of mutants revealed that flagella itself and sigma factors that control its biogenesis, FliA and RpoN, were also affected in the early steps of attachment. These results are in accordance with a study on early biofilm formation by *Escherichia coli* (Domka *et al.*, 2007), and a more recent visual analysis of flagella role and surface colonization published recently (Wood, 2013). Reciprocal adhesion genes (Table 1) with a high degree of identity (≈ 90%) were also identified in the PGPR strain *Pseudomonas putida* BIRD-1 (Roca *et al.*, 2013), which has showed to be efficient colonizer of several plants of agronomical and environmental interest (Roca *et al.*, 2013; Pizarro-Tobías and colleagues, unpublished results).

Moreover, Reva and colleagues (2006) when assessing important features for *Pseudomonas putida* to cope with environmental stresses through transcriptome analysis of random transposon mutants found that LapA (Table 1) was involved in coping with urea-induced stress (chaotropic solute), by generating a hydrophilic micromilieu suitable for the incorporation of urea into its mesh, avoiding the water stress.

An important trait described for PGPR bacteria is their ability to produce siderophores, which increase iron uptake by the microbes and even by the host plants (Gamalero and Glick, 2011); besides, iron constitutes an important element involved in attachment and biofilm formation (O'Toole and Kolter, 1998). It has been described that *Pseudomonas putida* strains have multiple iron acquisition systems, which enables them to acquire complexed iron through self-produced and exogenous siderophores, aiding in being efficient rhizosphere colonizers (Martínez-Bueno *et al.*, 2002). To elucidate to what extent is iron involved in seed attachment, colonization and, therefore, in *Pseudomonas putida* KT2440 rhizosphere fitness, Molina and colleagues (2005) performed corn-seeds attachment assays. As corn seeds are rich in ferrous iron the assay was carried out with iron chelators-pretreated seeds, resulting in a diminish in the number of seed-attached cells, which could indicate that iron could play a role as chemo-attractant and/or the establishment of bacteria on the surface of the seed. To further assess which is seed-borne iron part in attachment and biofilm formation, mutants in the TonB system gene cluster (*exbB/exbD/tonB*, PP_5306-5308) (Table 1), which is essential for siderophore iron uptake, were assayed for corn seed adhesion, resulting in an increased unattachment of bacterial cells. Competition assays in unsterile corn rhizospheric soil of the mutant *versus* the wild type strain resulted in the displacement of the mutant, pointing out the importance of the TonB system, which is relevant for bacterial fitness in the rhizosphere. An *in silico* analysis performed on the *Pseudomonas putida* BIRD-1 genome revealed that it has 25 iron receptors with over

95% identity to their reciprocal *Pseudomonas putida* KT2440 gene products (Roca *et al.*, 2013), which is consistent with the capacity of this strain to attach to seeds and survive and colonize the rhizosphere of maize and several plants of agronomical and environmental importance (Roca *et al.*, 2013, Pizarro-Tobías *et al.*, unpublished results). The TonB/ExbD/ExbB cluster was found to be highly conserved in all *Pseudomonas putida* whose genome has been sequenced, with >73% identity (Table 2).

Exopolysaccharides (EPS) like alginate, are molecules involved in biofilm formation and stability, and have been found to play an important role in the ability of *Pseudomonas putida* to cope with desiccation, which is an often-adverse agent to cope with in the environment (Ramos *et al.*, 2001). A gene related to alginate biosynthesis, *algD* (PP_1288), was identified being induced in the rhizosphere by Ramos-González and colleagues (2005), which also had previously been related to water stress (van de Mortel and Halverson, 2004) and involved in root colonization success. An orthologue gene was found in *Pseudomonas putida* BIRD-1 (Table 1), with a degree of identity of 99.77%; further, this strain showed an enhanced capacity to overcome water stress in soil, when compared to *P. putida* KT2440 (Roca *et al.*, 2013). This gene was also found to be highly conserved in all sequenced *Pseudomonas putida* strains, with >85% identity (Table 2). Chang and colleagues (2007) reported that alginate takes part in biofilm structure maintenance in water scarcity conditions, increasing survival capacity of *P. putida* cells within the matrix, pointing

out the importance of exopolysaccharides in *Pseudomonas* environmental fitness and stress tolerance. Nielsen and colleagues (2011) studied the role of two other putative exopolysaccharides found in *Pseudomonas putida* mt2, namely, the Pea exopolysaccharide (*pea*) and a cellulose-like polymer (*bcs*) (Table 1), and they studied their role(s) in biofilm formation and environmental stress tolerance through directed mutagenesis and subsequent phenotype assays. They reported the importance of Bcs and alginate in maize rhizosphere colonization; also that both, Bcs and Pea, promote the retention of water within the biofilm matrix during water scarcity. In a parallel study using analogous techniques, Nilsson and colleagues (2011) reported the existence of an additional putida exopolysaccharide (peb) gene cluster (PP_1789 – PP_1791, PP_1792 – PP_1795) (Table 1), which is also involved in biofilm stability in addition to the large cell surface proteins LapA and LapF, which are key factors in biofilm formation as described before. The *bcs* and *pea* clusters are conserved with a degree of identity 95% in *Pseudomonas putida* BIRD-1 (Table 1). Regarding to the rest of sequenced *Pseudomonas putida*, we found that the *bcs* cluster was less conserved in UW4, W619, S16 and PC9 strains (<45% identity) (Table 2); the *pea* cluster was also found to be less conserved in UW4 (<50% identity) (Table 2). In the case of *peb* cluster, the orthologue genes showed a low degree of identity (<30%), and within the cluster only the ORF PP_1792 was predicted as orthologue for *P. putida* BIRD-1 (Table 1). This cluster was also found being relatively unpreserved among the sequenced *Pseudomonas putida*, except for *P. putida* S16 (>74% identity) (Table 2).

Matilla and colleagues (2011a) identified two gene clusters coding for surface polysaccharides, through the insertion of a plasmid harbouring *rup4959* (locus PP_4959) in miniTn5 derivatives of *P. putida* KT2440: , i) putida exopolysaccharide (pea, PP_3133-3141) (Nielsen *et al.*, 2011; Nilsson *et al.*, 2011), and ii) putida exopolysaccharide (peb, PP_1789-1791) (Nilsson *et al.*, 2011), related to increased levels of cyclic diguanylate; and LapA was also found to be essential for biofilm formation (Yousef-Coronado *et al.*, 2008), as described before.

2. Regulators and sensor proteins

Regulator proteins involved in fitness in the rhizosphere have been identified through the analysis of mutants with enhanced sensitivity of stress conditions as well as through transcriptomic analysis. The work of Reva and colleagues (2006) identified the CbrAB two-component response regulator system to be involved in coping with abiotic stresses; they found that *cbrAB* mutants were more sensitive than the parental strain to cold stress (4ºC). Amador and colleagues (2010) have also reported the importance of CbrB for *Pseudomonas putida* rhizosphere fitness. They have identified a variety of functions unrelated to carbon metabolism subjected to CbrB regulation, such as chemotaxis, stress tolerance and biofilm formation. The CbrAB two-component response regulator system is well conserved in all sequenced *P. putida* strains (Table 2), for which *P. putida* BIRD-1 has reciprocal genes, with a percentage of identity over 99% (Table 1).

Some global stress response genes were identified during the study of Reva and colleagues (2006) (PP_0063, PP_0816, PP_4646, PP_5322) (Table 1), for which orthologues were found in *P. putida* BIRD-1 genome with a percentage of identity over 98%; we also found that these are also highly conserved in the rest of sequenced Pseudomonas (>72% identity) (Table 2). Besides, several genes were found to be redundant in coping with one of more of the stresses assessed, pointing out that the stress response machinery of *Pseudomonas putida* is relatively small but efficient, being the maintenance of intracellular pH, redox status and metabolism key factors in environmental fitness.

A gene coding for a response regulator (PP_4959; Table 1), which is implicated in the regulation of the second messenger c-di-GMP (cyclic diaguanylate), was found being induced in the rhizosphere by Matilla and colleagues (2007). Cyclic diguanylate has been reported to be involved in bacterial shifting from planktonic to sessile state (Römling and Amikam, 2006). Matilla and colleagues (2011a) used transcriptional fusions to reporter genes to confirm the activation of this gene (PP_4959) in maize rhizosphere. They also found that is activated in microaerobiosis conditions and in the combination of maize root exudates, conditions often found in the vicinity of roots. An orthologue of PP_4959 for *Pseudomonas putida* BIRD-1 was found (PPUBIRD1_4743) with a 99.87% of identity (Table 1). It should be noted that ORF PP_4959 is co-transcribed with 4958 and 4957, and these are also conserved in BIRD-1 (PPUBIRD1_4742 and PPUBIRD1_4741, with 97.77% and 98.98%

identity, respectively). We found that the c-di-GMP response regulator (PP_4959) is also highly conserved in all sequenced *Pseudomonas putida* strains (>70% identity) (Table 2).

3. Metabolism

Another method used for identifying bacteria promoters induced in the rhizosphere is the *in vivo* expression technology (IVET), which allows identifying upregulated genes in particular conditions by generating transcriptional fusions of genomic sequences to a reporter gene encoding an enzymatic activity. Ramos-González and colleagues (2005) found 28 genes of *Pseudomonas putida* in a non-saturating screen that were induced in maize rhizosphere, related to the biosynthesis of cell envelope components, nutrient acquisition, energy metabolism, DNA metabolism and defense mechanisms, stress and detoxification, chemotaxis and motility, among others (Table 1). Several genes related to amino acid acquisition where induced, including *putP* a proline permease previously reported to be induced in the presence of maize exudates (PP_4946) (Vílchez *et al.*, 2000), a gamma-aminobutyric acid (GABA) permease (PP_2543), and an amino oxidase involved in lysine catabolism (PP_0383) (Revelles *et al.*, 2004) (Table 1). Reciprocal genes for these three KT2440 proteins were found in *Pseudomonas putida* BIRD-1 genome, with over 98% of identity, confirming that the ability for amino acid metabolism is conserved in this strain; as well as highly conserved for the rest of the sequenced *P. putida* (>79% identity), with the exception of the GABA permease in *P. putida* UW4 (55% identity) (Table 2). As was

expected, an essential gene in central metabolism, *aceE* (PP_0339) encoding for a pyruvate dehydrogenase, was induced; as presumed, this gene was found in BIRD-1 genome with a 99.77% identity (Table 1), and in the rest of sequenced *P. putida* (>93% identity) (Table 2).

The study of protein expression profiles of bacteria in the presence of root exudates constitutes an interesting approach for identifying essential traits in rhizosphere colonization and fitness. Cheng and colleagues (2009) performed functional studies with *Pseudomonas putida* UW4, comparing the wild-type strain with an ACC deaminase mutant, in the presence of *Brassica napus* (canola) root exudates to identify key proteins implicated in the interactions of the bacteria root-influenced environment. They identified that a large proportion of proteins expressed were related to the utilization of nutrients present in root exudates (Table 1), as previously reported by Mark and colleagues (2005) for *Pseudomonas aeruginosa* PA01; also, several proteins involved in cell envelope components biosynthesis, like exopolisaccharides, chaperones, proteins involved in DNA repair or modification, etc. (Table 1). Nonetheless, it was found that some essential proteins involved in the mutualistic bacteria-plant interaction were down regulated, which could suggest that in the presence of root exudates bacteria have enough nutrients to avoid the protein-expression machinery needed when absent in free-living conditions.

4. Stress adaptation

One of the most realistic and complete approaches for unravelling gene expression of rhizospheric bacteria was accomplished with *Pseudomonas putida* KT2440 in maize rhizosphere by performing a genomic-wide analysis using microarrays, later validated by the performance of real time RT-PCR (Matilla *et al.*, 2007). To obtain a better picture of specific induced genes in the rhizosphere, controls in rich medium with planktonic cells growing exponentially and planktonic cells in stationary phase, and sessile cells in sand microcosms were used. Over 90 genes were found to be differentially induced in the rhizosphere, noting that most of them were related to the uptake of specific carbon and nitrogen sources, such as amino acids, catabolic pathways for the degradation of aromatic compounds, stress response and detoxification, signal transduction sensors and response regulators. It is worth mentioning the up-regulation of the sensor histidine kinase PhoR (PP_5321) (Table 1), which is involved in the response to inorganic phosphate limitation; an orthologue for this gene was found in *P. putida* BIRD-1 (PPUBIRD1_5113) with a 99.08% identity (Table 1); we found that *phoR* is highly conserved in all sequenced strains of *Pseudomonas putida* (>81% identity) (Table 2). The induction of this Pho system had previously been reported being modulated by the cell redox state (Schau *et al.*, 2004), which implies that terminal oxidases are required for this system induction. As expected, two genes involved in cytochrome biosynthesis were induced in this study (PP_0109 and PP_0110). Reciprocal genes in *P. putida* BIRD-1 were found for both (PPUBIRD1_0137 and PPUBIRD1_0138) with ≈98%

of identity; further, we found that both genes were highly conserved among the sequenced *P. putida* strains (>72% identity), except for UW4 (<67% identity) (Table 2).

The search in the genome of *Pseudomonas putida* BIRD-1 for genes involved in oxidative stress led to the identification of 16 related genes, including several superoxide dismutases, catalases, alkylhydroperoxidases, cytochrome peroxidases and glutathione peroxidases (Roca *et al.*, 2013). The redundancy of genes to deal with oxygen active species points out the importance of these enzymes for rhizosphere fitness. Two of the genes involved in coping with oxidative stress found BIRD-1 genome (PPUBIRD1_3741 and PPUBIRD1_2060) were reported to be induced in the rhizosphere in Matilla and colleagues (2007) (PP_1874) and Cheng and colleagues (2009) (PP_3668) studies, respectively (Table 1). These genes were also found being highly conserved among sequenced *P. putida* strains, with >71% identity and >76% identity, respectively.

Matilla and colleagues (2007) also constructed *Pseudomonas putida* KT2440 mutants for 9 genes from the over 90 upregulated genes they found being induced in the rhizosphere, which were representing every group of rhizosphere expressed traits. When assayed in competition with the wild type strain in maize rhizosphere, mutants were less efficient colonizers than the wild type to different extents. Results indicated that

essential traits for rhizosphere fitness are stress adaptation mechanisms and ability for metabolize a variety of nutrients composing root exudates.

As presented above, numerous studies with varied approaches have been performed to elucidate the molecular basis of essential traits involved in rhizosphere fitness of bacterial strains when in mutualistic relationship with different plant species. Barret and colleagues (2011) reviewed the topic, pointing out that, in the case of the *Pseudomonas* genus, the essential features induced in different mutualistic interactions within the rhizosphere comprise stress response and secretion systems.

Degree of conservation of rhizosphere competence features among sequenced *Pseudomonas putida*

Overall, we found orthologues in *Pseudomonas putida* BIRD-1 genome for most of the described genes involved in rhizosphere fitness; with an exception of 18 genes, which comprise 10% of the described traits (Table 1). Thus, it can be said that BIRD-1 has the genomic machinery to be a successful and a competent strain to be used with plants of agronomical and environmental interest, as previously indicated an ample study performed on this strain (Roca *et al.*, 2013), which revealed the genome of this strain encodes a number plant growth-promoting traits (Matilla *et al.*, 2011b). Field-scale trials performed in a burnt protected Mediterranean environment (Pizarro-Tobías *et al.*, unpublished results) and in a pretroleum-hydrocarbon polluted site (Pizarro-Tobías *et al.*,

unpublished results) using *Pseudomonas putida* BIRD-1 as a PGPR strain, in consortium with other bacterial strains, showed the ability of this strain to adapt and survive to harsh and polluted environments for a sufficient period of time to exert its beneficial effects. To assess whether these traits are equally conserved in other strains of *Pseudomonas putida*, an *in silico* analysis was performed to find the presence of these genes in the genome of 8 other sequenced *P. putida* strains, being: F1, UW4, W619, DOT-T1E, S16, PC9 and Idaho (Table 2).

Of the 25 genes identified as being involved in adhesion, attachment and biofilm formation processes, *lapA*, *lapF* and *lapH* have been identified as key determinants of attachment in *P. putida* (Yousef-Coronado *et al.*, 2008; Martínez-Gil *et al.*, 2010; Duque *et al.*, 2013). We found that these three genes are highly conserved in most of the sequenced *P. putida* strains with >70% of identity, except for UW4 and PC9 (<48%). The LapA and LapF proteins were found being worse conserved in W619 (36% and 65% identity, respectively) when comparing to LapH (80% identity) (Table 2). LapBC cluster was found being conserved in all 10 strains, with over a 78% identity in all the cases. The *algD*, *galU*, *hemN* and PP_4934 genes were also found being conserved in all the strains (>85%, >91%, >83% and >89% identity, respectively) (Table 2). The KT2440 gene clusters related to the synthesis of surface exopolysaccharides, Pea and Bcs, were found to be reciprocal in BIRD-1, F1, DOT-T1E, GB-1 and Idaho strains (Table 2). All of the KT2440 adhesion determinants that were not found to be reciprocal in BIRD-1 (PP_1789, PP_1790, PP_1791, PP_1793

and PP_1795), as said before (Table 1), were only found being conserved in the S16 strain, a microbe isolated from plant roots with the ability to degrade nicotine (Wang *et al.*, 2007) Nonetheless, a gene identified in *Pseudomonas putida* W619 to be involved in attachment of endophytes to higher plants (Wu *et al.*, 2011), was found to have an orthologue only in BIRD-1 (73% of identity) (Table 1, 2). Further research would have to be performed to assess whether this strain behaves as an endophyte, though it is unlikely that a single gene is determinant for such a complex process as endophytism.

Regarding to the 13 genes related to flagella biosynthesis and assembly, which have been above described as determinant for attachment and rhizosphere colonization in *Pseudomonas putida* (Yousef-Coronado *et al.*, 2008; Duque *et al.*, 2013), overall it was found that all of them have a relatively good degree of conservation in the sequenced strains (Table 2); except for the *fliD* gene, with <50% of identity in the 9 *P. putida* strains compared to KT2440.

The two genes found to be involved in rhizosphere chemotaxis processes (PP_4331 and PP_4988) (Table 1) were found to be highly conserved in most of the strains screened (75% to 92% identity), except for UW4 (66% and 58% identity, respectively) and Idaho (38% for PP_4331) (Table 2).

With regards to the 14 regulators and sensor proteins found to be involved in rhizosphere fitness (Table 1), Duque and colleagues (2013)

remarked the importance of the RNA polymerase sigma factor RpoN, along with the transcriptional regulator FleQ, for the expression of *fliD*, encoding for a flagellar cap protein. In other microorganisms such as *Pseudomonas aeruginosa rpoN* mutants were shown being hampered in adhesion. The RpoN was found being conserved in all sequenced *P. putida* strains (>89% identity) (Table 2).

The sensor histidine kinases PhoR, involved in the response to inorganic phosphate limitation as described above (Matilla *et al.,* 2007), ColS (Ramos-González *et al*., 2005; Barret *et al*., 2011), and GacS (Duque *et al*., 2013), were found being relatively conserved in all 9 sequenced *P. putida* strains (Table 2). The transcriptional regulators encoded by PP_2070, PP_4424 (Ramos-González *et al*., 2005), and PP_3640 (Matilla et al., 2007), were found to be less conserved among the *P. putida* strains (Table 2).

One third of the described proteins involved in rhizosphere competence are related to stress adaptation and detoxification mechanisms (Table 1), which is consistent with the results showed by Matilla and colleagues (2007), who described nutrient acquisition and stress response as the major opposite forces acting on bacterial adaptation in the rhizosphere. Only 4 of the identified genes in *Pseudomonas putida* KT2440 didn't have orthologues in BIRD-1 genome (Table 1), which shows that these strains have similar machinery to overcome rhizosphere stress. It can also be

said for the rest of the *Pseudomonas putida* strains (Table 2), since most of the described genes were relatively conserved among them.

Eight proteins were found to be involved in DNA replication, recombination, repair and defense mechanisms (Table 1). Among them, it is interesting to note that a putative gene named *traX* encoding a protein involved in conjugal DNA transfer/uptake of *Pseudomonas putida* W619, which was induced in the rhizosphere (Wu *et al.*, 2011), is relatively well conserved (69% identity) only in BIRD-1, S16 and PC9 strains (Table 2). *hsdM* gene encoding a protein with endodeoxyribonulease activity was found being well conserved only in BIRD-1 and F1 strains, with 100% and 89% of identity, respectively (Table 2).

Eight transport and secretion proteins were described to be involved in rhizosphere fitness of *Pseudomonas putida* (Table 1). It is worth metioning that the *tonB* gene cluster (Table 1) involved in iron uptake and adhesion to seeds in *Pseudomonas putida* KT2440 as described above (Molina *et al.*, 2005), is found being highly conserved in all the sequenced *P. putida* strains (Table 2). The rest of genes are well conserved in most of the strains, except for UW4 in which the *hlpAB* cluster and PP_4541 encoding an esterase has <33% of identity (Table 2).

A significant proportion of the genes involved in rhizospheric competence (17%), is related to metabolism (Table 1), as described by Matilla and colleagues (2007). When comparing to *Pseudomonas putida* KT2440,

BIRD-1 has reciprocal genes for all the described proteins, except for an arylsulfatase (PP_3352), an aminotransferase (PP_3786) and an endolysin (PP_3854) (Table 1). It is worth noting that the aminotransferase was not conserved in any of the strains apart from KT2440 (Table 2). The rest of the metabolism-related genes were well conserved in all of the strains, which is consistent with the metabolic versatility commonly attributed to the *Pseudomonas* genus (Wu *et al.*, 2011).

Seven genes were found to be induced in the rhizosphere related to protein synthesis, folding and degradation (Table 1), which are highly conserved among the sequenced *P. putida* strains (Table 2).

Of the 9 hypothetical proteins, with unknown function, described as putatively involved rhizosphere competence by *Pseudomonas putida* (Table 1), only 3 didn't have an orthologue in BIRD-1 (PP_2076, PP_3855 and PP_5390).

Overall, it can be said that the genomic machinery necessary for *Pseudomonas putida* to accomplish survival and maintenance in the rhizosphere when a plant-bacteria mutualistic relationship is ongoing, is well conserved within the species *P. putida*. This is consistent with *Pseudomonas putida* being a frequent rhizosphere colonizer and, in some cases, plant growth-promoting bacteria (Wu *et al*, 2011; Roca *et al.*, 2013).

In summary, *Pseudomonas putida* BIRD-1 has been found to be an excellent candidate for agronomical uses (Roca *et al.*, 2013) and as part of rhizoremediation treatments for burned ecosystems (Pizarro-Tobías *et al.*, unpublished results) and petroleum-hydrocarbons polluted sites (Pizarro-Tobías *et al.*, unpublished results), for it has the genomic machinery to overcome the different stressors and use nutrients present in the rhizosphere and the ability to survive in soil and rhizosphere in consortium and in competence with other indigenous and introduced strains, as well as in the presence of a variety of toxic compounds.

Table 1. *Pseudomonas putida* genes encoding traits involved in rhizosphere competence. *In silico* pairing with reciprocal genes found in *P. putida* BIRD-1 genome.

Group	Gene	Ref. strain ORF ID	Operon[a]	BIRD-1 ORF ID (PPUBIRD1)[b]	Product[c]	Protein Identity (%)	E-value	Reference[d]
Adhesion	*lapD*	PP_0165	PP_0164-PP_0165	0195	Diguanylate cyclase	98.92	0.0	20
Biofilm formation	*lapC*	PP_0166	PP_0166-PP_0167	0196	HlyD family type I secretion membrane fusion protein	99.78	0.0	20
	lapB	PP_0167		0197	Secretion ATP-binding protein	99.86	0.0	20
	lapA	PP_0168	-	0199	Surface adhesion protein	99.97	0.0	3, 8, 14, 16, 20
	lapH	PP_0805	PP_0803-PP_0806	0851	TolC family type I secretion outer membrane protein	99.13	0.0	3, 16
	lapF	PP_0806		0852	Putative surface protein	96.67	0.0	3, 5, 16
	algD	PP_1288	PP_1281-PP_1288	4273	GDP-mannose 6-dehydrogenase	99.77	0.0	14
	-	PP_1789	PP_1789-PP_1791	0454*	HAD superfamily Hydrolase	22.17	0.009	8, 12
	-	PP_1790		3811*	Acylneuraminate cytidylyltransferase	43.48	1.1	8, 12
	-	PP_1791		0016*	Aldolase	20.81	1e-04	8, 12
	-	PP_1792	PP_1792-PP_1795	1485	Glycosyl transferase family protein	30.18	2e-23	8,12
	-	PP_1793		2597*	Glycosyl transferase family protein	26.55	3e-08	8, 12
	-	PP_1794		-	Hypothetical protein	-	-	8, 12
	-	PP_1795		0194*	Hypothetical protein	22.78	0.21	8, 12
	bcsQ	PP_2634	PP_2629-PP_2638	3057	Cellulose synthase containing putative MinD/ParA-binding domain	98.72	1e-132	8, 12
	bcsA	PP_2635		3056	Cellulose synthase catalytic subunit	98.21	0.0	8, 11, 12
	bcsB	PP_2636		3054	Cellulose synthase regulator protein BscB	98.55	0.0	8, 11, 12
	bscC	PP_2637		3053	Endo-1,4-D-glucanase/cellulase	99.19	0.0	8, 11, 12
	peaG	PP_3139	PP_3133-PP_3141	2593	Glycoside hydrolase family protein	96.50	0.0	8, 11, 12
	peaH	PP_3140		2592	Glycosyl transferase family protein	98.73	0.0	8, 11, 12
	peaI	PP_3141		2591	WecB/TagA/CpsF family glycosyl transferase	95.82	7e-14	8, 11. 12
	galU	PP_3821	-	1959	UTP-glucose-1-phosphate uridylyltransferase	99.64	0.0	20
	hemN	PP_4264	-	1592	Coproporphyrinogen III oxidase	99.78	0.0	20
	-	PP_4934	-	4717	Bifunctional heptose 7-phosphate kinase/heptose 1-phosphate adenyltransferase	100	0.0	2
	ndvB	PputW619_2133	PputW619_2132 - PputW619_2133	3043	Glycosyltransferase - Cellobiose phosphorylase	79.49	0.0	19

Table 1. Continued

Group	Gene	Ref. strain ORF ID	Operon[a]	BIRD-1 ORF ID (PPUBIRD1)[b]	Product[c]	Protein Identity (%)	E-value	Reference[d]
Flagella	*fliA*	PP_4341	PP_4341-PP_4344	1510	DNA-directed RNA polymerase specialized sigma subunit	100	5e-164	20
	flhB	PP_4352	PP_4352-PP_4361	1506	Flagellar biosynthesis protein FlhB	99.74	0.0	3, 16
	fliO	PP_4356		1502	Flagellar assembly protein FliO	100	2e-64	13
	fliN	PP_4357		1501	Flagellar motor switch protein	99.38	6e-87	20
	fliL	PP_4359		1499	Flagellar basal body protein FliL	100	1e-84	6
	fliI	PP_4366	PP_4365-PP_4367	1493	Flagellum-specific ATP synthase	99.78	0.0	20
	fliF	PP_4369	PP_4368-PP_4370	1490	Flagellar MS-ring protein – flagellar hook	99.49	0.0	3, 16
	fleQ	PP_4373	-	1470	Fis family transcriptional regulator	100	0.0	20
	fliD	PP_4376	-	1467	Flagellar cap protein FliD	46	5e-96	3, 16
	fliC	PP_4378	PP_4377-PP_4378	1465	Flagellin FliC	65.09	1e-57	3, 16
	flgL	PP_4380	PP_4380-PP_4386	1462	Flagellar hook-associated protein FlgL	67.95	0.0	20
	flgD	PP_4389	PP_4387-PP_4391	1453	Flagellar basal body rod modification protein	99.57	2e-132	20
	flgB	PP_4391		1451	Flagellar basal-body rod protein FlgB	93.43	2e-81	6
Chemotaxis	-	PP_4331	PP_4331-PP_4334	1520	Hypothetical protein	99.24	9e-73	6
	-	PP_4988	PP_4987-PP_4992	4775	Chemotaxis protein CheA	98.91	0.0	6
Regulators	-	PP_0700	-	0741	FecR anti-FecI sigma factor	99.39	0.0	6
Sensor proteins	*colS*	PP_0902	PP_0901-PP_0902	0953	Integral membrane sensor signal transduction histidine kinase	99.33	0.0	1, 13
	rpoN	PP_0952	PP_0948-PP_0952	1005	RNA polymerase factor sigma 54	99.80	0.0	3, 16
	-	PP_1066	PP_1065-PP_1070	1116	Fis family transcriptional regulator	100	0.0	6
	gacS	PP_1650	PP_1647-PP_1650	3966	Sensor protein GacS - multi-sensor hybrid histidine kinase	99.35	0.0	3, 16
	-	PP_2070	-	3583	AraC family transcriptional regulator	90.25	2e-141	13
	-	PP_2127	PP_2126-PP_2127	3526	Sensor histidine kinase (signal transduction)	97.44	0.0	6
	-	PP_3640	-	2149	AraC family transcriptional regulator	92.08	0.0	6
	-	PP_4295	-	1561	TetR family transcriptional regulator	99.51	1e-137	6
	pfrI	PP_4424	-	4559*	AsnC family transcriptional regulator	34.21	1e-25	13
	-	PP_4508	-	2668	AraC family transcriptional regulator	99.71	0.0	6
	-	PP_4959	PP_4957-PP_4959	4743	Response regulator receiver modulated diguanylate cyclase/phosphodiesterase	98.87	0.0	6, 8

Table 1. Continued

Group	Gene	Ref. strain ORF ID	Operon[a]	BIRD-1 ORF ID (PPUBIRD1)[b]	Product[c]	Protein Identity (%)	E-value	Reference[d]
Regulators	-	PP_4966	PP_4966-PP_4967	4750	ArsR family transcriptional regulator	100	0.0	13
Sensor proteins	*phoR*	PP_5321	PP_5320-PP_5321	5113	PAS/PAC sensor signal transduction histidine kinase	98.85	0.0	6
Stress adaptation	-	PP_0063	PP_0062-PP_0063	0092	Lipid A biosynthesis lauroyl acyltransferase	99.32	0.0	14
Detoxification	-	PP_0109	PP_0103-PP_0111	0137	Cytochrome oxidase assembly	98.33	1e-175	6, 20
	cyoE-1	PP_0110		0138	Protoheme IX farnesyltransferase – polyprenyltransferase	98.57	2e-131	6, 20
Transporters	-	PP_0196	PP_0196-PP_0197	0225	ABC transporter ATP-binding protein	99.37	0.0	6
	-	PP_0296	-	0325	Glycine/betaine ABC transporter substrate-binding protein	99.31	5e-172	2
	-	PP_0368	-	0405	Acyl-CoA dehydrogenase	100	0.0	14
	-	PP_0373	-	0401	Hypothetical protein	100	3e-19	6
	-	PP_0383	PP_0382-PP_0383	0418	Amine oxidase	99.82	0.0	13
	-	PP_0486	-	0523	Transcriptional regulator, GntR family	98.52	0.0	14
	-	PP_0670	-	0710	Bile acid/Na+ symporter family protein	95.58	3e-166	6
	-	PP_0721	PP_0719-PP_0725	0767	50S ribosomal protein L25	92.31	0.0	13
	cyoE-2	PP_0816	PP_0809-PP_0816	0860	Protoheme IX farnesyltransferase	100	5e-177	14
	-	PP_0906	PP_0906-PP_0907	0957	Multidrug efflux protein	99.50	0.0	6
	phoP	PP_1186	PP_1186-PP_1187	1227	Winged helix family two component transcriptional regulator	98.67	1e-115	14
	oprD	PP_1206	-	1242	Porin	82.20	0.0	2
	-	PP_1271	PP_1271-PP_1273	4290	Major facilitator superfamily transporter	98.93	0.0	6, 14
	aapJ	PP_1297	PP_1297-PP_1300	4263	Amino acid ABC transporter substrate-binding protein	99.68	0.0	2
	fpr	PP_1638	-	3978	oxidoreductase FAD/NAD(P)-binding domain-containing protein	100	5e-177	2
	relA	PP_1656	PP_1654-PP_1656	3961	(p)ppGpp synthetase I SpoT/RelA	99.73	0.0	14
	-	PP_1874	PP_1873-PP_1874	3741	Glutathione peroxidase	99.38	3e-108	6
	oprF	PP_2089	-	3563	OmpF family protein	100	0.0	2

Table 1. Continued

Group	Gene	Ref. strain ORF ID	Operon[a]	BIRD-1 ORF ID (PPUBIRD1)[b]	Product[c]	Protein Identity (%)	E-value	Reference[d]
Stress adaptation	*lolD*	PP_2155	PP_2154-PP_2156	3499	Lipoprotein releasing system ATP-binding protein	100	2e-152	6
	speB	PP_2196	PP_2195-PP_2196	3455	Agmatinase	99.69	0.0	14
Detoxification	*fepA*	PP_2242	PP_2242-PP_2243	3412	Outer membrane receptor FepA	98.52	0.0	14
	cti	PP_2376	PP_2376-PP_2378	3309	Fatty acid cis/trans isomerase	98.56	0.0	6
Transporters	*azlC*	PP_2385	PP_2383-PP_2385	3299	AzlC family protein)	99.57	2e-131	6
	-	PP_2543	-	3134	Amino acid ABC transporter permease	99.36	0.0	13
	-	PP_2561	-	0199*	Heme peroxidase	50.00	1e-12	6, 7
	-	PP_2669	PP_2667-PP_2669	3007	Hypothetical protein	98.48	0.0	6
	mexC	PP_2817	PP_2817-PP_2819	2337*	Multidrug efflux RND membrane fusion protein	50.15	6e-76	6
	proP	PP_2914	-	2820	Proline/glycine betaine transporter	99.60	0.0	14
	-	PP_3132	-	2601	Polysaccharide biosynthesis protein	99.34	0.0	6
	-	PP_3183	PP_3183-PP_3184	2306*	Electron transport protein SCO1/SenC	37.18	8e-29	6
	-	PP_3210	PP_3208-PP_3201	2543	ABC transporter permease	98.88	1e-133	6
	-	PP_3223	PP_3220-PP_3226	0936*	ABC transporter substrate-binding protein	25.10	1e-18	6
	ggt-1	PP_3535	PP_3534_PP_3535	2258	Gamma-glutamyltransferase	96.81	0.0	6
	-	PP_3583	PP_3582-PP_3583	2209	Acriflavin resistance protein	99.71	0.0	6
	-	PP_3668	-	2060	Catalase/peroxidase HPI	99.07	0.0	2
	gloA	PP_3766	-	1996	Lactoylglutathione Lyase	98.29	4e-117	13
	-	PP_3802	PP_3797-PP_3804	1979	Cation ABC transporter ATP-binding protein	98.39	4e-160	6
	-	PP_4002	PP_3999-PP_4004	1812	Recombination factor protein RarA	100	0.0	14
	-	PP_4034	PP_4034-PP_4035	1786	Allantoate amidohydrolase	98.13	0.0	14
	treZ	PP_4051	PP_4050-PP_4054	1774	Malto-oligosyltrehalose trehalohydrolase	97.59	0.0	16
	nuoL	PP_4129	PP_4119-PP_4131	1721	NADH dehydrogenase subunit L	99.50	0.0	14
	-	PP_4305	-	1552	Sulfate ABC transporter substrate-binding protein	99.10	0.0	13
	-	PP_4483	PP_4483-PP_4485	1418	Basic amino acid ABC transporter ATP-binding protein	99.61	1e-162	6
	-	PP_4615	-	4309	Phosphate-starvation -inducible E	99.36	3e-90	13

Table 1. Continued

Group	Gene	Ref. strain ORF ID	Operon[a]	BIRD-1 ORF ID (PPUBIRD1)[b]	Product[c]	Protein Identity (%)	E-value	Reference[d]
Stress adaptation	-	PP_4646	-	4342	Oxidoreductase FAD/NAD(P)-binding domain-containing protein	99.61	4e-173	14
Detoxification	*cbrA*	PP_4695	PP_4695-PP_4696	4396	Multi-sensor signal transduction histidine kinase	99.90	0.0	14
	cbrB	PP_4696		4397	Fis family transcriptional regulator	99.58	0.0	14
Transporters	*pcnB*	PP_4697	PP_4697-PP_4698	4398	Poly(A) polymerase	98.91	0.0	14
	omlA	PP_4731	-	4434	SmpA/OmlA domain-containing protein	99.36	6e-87	14
	vacB	PP_4880	PP_4879-PP_4880	4669	Ribonuclease R	99.88	0.0	14
	putP	PP_4946	-	4729	Sodium/proline symporter	99.80	0.0	13, 17
	putA	PP_4947	-	4730	Pproline dehydrogenase/pyrroline-5-carboxylate dehydrogenase	99.62	0.0	17
	pstP	PP_5145	PP_5145-PP_5146	4936	Protein PtsP	99.87	0.0	14
	-	PP_5278	-	5072	Aldehyde dehydrogenase	100	0.0	2
	-	PP_5297	PP_5297-PP_5299	5090	Amino acid transporter	99.81	0.0	6
	spoT	PP_5302	PP_5301-PP_5304	5095	(p)ppGpp synthetase I SpoT/RelA	99.72	0.0	14
	-	PP_5311	-	5104	Signal transduction protein	99.79	0.0	19
	-	PP_5322	-	5114	Metal ion transporter, putative	99.55	0.0	14
	pstA	PP_5327	PP_5325-PP_5328	5119	Phosphate ABC transporter permease	99.82	0.0	14
	pstS	PP_5329	-	5121	Phosphate ABC transporter substrate-binding protein	98.79	0.0	3, 14, 16
	atpA	PP_5415	PP_5412-PP_5418	0061	ATP synthase F0F1 subunit alpha	99.61	0.0	2
DNA replication	*recJ*	PP_1477	PP_1476-PP_1477	4082	Single-stranded-DNA-specific exonuclease RecJ	98.95	0.0	6
recombination	-	PP_2565	-	3114	UvrD/REP helicase	99.16	0.0	6
	xthA	PP_2890	-	2845	Exonuclease III	98.89	0.0	2
repair	-	PP_3966	PP_3964-PP_3966	0990*	ISPpu14, transposase Orf1	33.33	0.011	6
	hsdM	PP_4741	PP_4741-PP_4742	4450	Type I restriction-modification system, M subunit	99.80	0.0	13
	purA	PP_4889	PP_4889- PP_4890	4676	Adenylosuccinate synthetase	100	0.0	2
	parC	PP_4912	PP_4912-PP_4915	4699	DNA topoisomerase IV subunit A	99.73	0.0	13
	-	PputW619_2699	PputW619_2699-PputW619_2670	2563	TraX family protein	72.49	4e-96	19

Table 1. Continued

Group	Gene	Ref. strain ORF ID	Operon[a]	BIRD-1 ORF ID (PPUBIRD1)[b]	Product[c]	Protein Identity (%)	E-value	Reference[d]
Secretion	*yidC*	PP_0006	PP_0006 – PP_0008	0071	Inner membrane protein translocase component YidC	100	0.0	13
	hlpA	PP_1449	PP_1449 – PP_1450	4110	Filamentous hemagglutinin	97.45	0.0	10
	hlpB	PP_1450	PP_1449 – PP_1450	4109	TPS family activation/ secretion protein	97.88	2e-131	10, 14
	-	PP_4514	-	1386	Patatin	99.20	0.0	1
	secB	PP_5053	PP_5053 – PP_5055	4841	Preprotein translocase subunit SecB	99.38	1e-106	13
	exbB	PP_5306	PP_5306 – PP_5308	5099	Ferric siderophore transport system protein ExbB	95.68	6.0E-154	9
	exbD	PP_5307		5100	Biopolymer transport protein ExbD	100	1.0E-88	9
	tonB	PP_5308		5101	TonB family protein	100	7.0E-53	9
Metabolism	*gabT*	PP_0214	-	0237	4-aminobutyrate aminotransferase	100	0.0	2, 4, 15
	soxG	PP_0326	PP_0322-PP_0327	0357	Sarcosine oxidase subunit gamma	99.52	3e-139	6
	aceE	PP_0339	-	0372	Pyruvate dehydrogenase subunit E1	99.77	0.0	13
	glcB	PP_0356	-	0392	Malate synthase G	99.59	0.0	2
	pykA	PP_1362	-	4200	Pyruvate kinase	100	0.0	2
	bglX	PP_1403	-	4156	Beta-glucosidase	99.18	0.0	6
	lecA	PP_1661	PP_1659-PP_1661	3956	LecA - dehydrogenase subunit	98.80	0.0	3, 16
	serC	PP_1768	PP_1678-PP_1679	3845	Phosphoserine aminotransferase	99.45	0.0	2
	-	PP_2009	-	3642	1-aminocyclopropane-1-carboxylate (ACC) deaminase	93.60	3e-149	2
	-	PP_2624	PP_2616-PP_2624	3066	Hypothetical protein	98.20	2e-106	6
	phaE	PP_3279	-	2493	Phenylacetate-CoA ligase	99.09	0.0	6
	phaB	PP_3283	PP_3280-PP_3284	2489	Enoyl-CoA hydratase	98.48	2e-163	6
	-	PP_3352	-	0106*	Arylsulfatase	24.90	1e-21	6
	hpd	PP_3433	PP_3433-PP_3434	2355	4-hydroxyphenylpyruvate dioxygenase	99.44	0.0	2

Table 1. Continued

Group	Gene	Ref. strain ORF ID	Operon[a]	BIRD-1 ORF ID (PPUBIRD1)[b]	Product[c]	Protein Identity (%)	E-value	Reference[d]
Metabolism	*ilvE*	PP_3511	-	2284	Branched-chain amino acid aminotransferase	99.41	0.0	2
	glcE	PP_3746	PP_3745-PP_3749	2016	Glycolate oxidase FAD binding subunit	96.86	0.0	6
	-	PP_3786	PP_3781-PP_3788	0861*	Aspartate/tyrosine/aromatic aminotransferase	26.23	1e-34	6
	-	PP_3854	-	4480*	Endolysin - Muramidase	43.48	1.3	13
	-	PP_3923	-	1884	Phosphoglycerate mutase	98.58	7e-145	6
	icd	PP_4011	-	1803	Isocitrate dehydrogenase	99.28	0.0	2
	gnd	PP_4043	PP_4041-PP_4043	1777	6-phosphogluconate dehydrogenase	99.39	0.0	13
	aceA	PP_4116	-	1734	Isocitrate lyase	99.55	0.0	13
	sucC	PP_4186	PP_4185-PP_4186	1666	Succinyl-CoA synthetase subunit beta	100	0.0	2, 14
	-	PP_4588	-	1310	Nitroreductase	99.49	1e-131	6
	thiD	PP_4782	PP_4782-PP_4784	4565	Phosphomethylpyrimidine kinase	99.25	5e-151	6
	fda	PP_4960	-	4744	Fructose-1,6-bisphosphate aldolase	100	0.0	2
	hutU	PP_5033	-	4821	Urocanate hydratase	99.64	0.0	1, 2
	pgm	PP_5056	-	4844	Phosphoglyceromutase	100	0.0	13
	gltB	PP_5076	-	4865	Glutamate synthase subunit alpha	99.80	0.0	6
	ubiF	PP_5197	PP_5197-PP_5202	4992	2-octaprenyl-3-methyl-6-methoxy-1,4-benzoquinol hydroxylase	99.75	0.0	6
Protein synthesis folding degradation	*surA*	PP_0403	PP_0401-PP_0403	0441	Survival protein SurA	99.09	0.0	2
	tyrS	PP_0436	-	0473	Tyrosyl-tRNA synthetase	99.75	0.0	2
	clpB	PP_0625	-	0677	ATP-dependent Clp protease	99.77	0.0	2
	-	PP_0893	PP_0887-PP_0893	0944	Pfpi family intracellular protease	99.48	2e-118	2
	prc	PP_1719	PP_1718-PP_1719	3938	Carboxyl-terminal protease	99.72	0.0	2
	gltX	PP_1977	-	3692	Glutamyl-tRNA synthetase	100	0.0	13
	typA	PP_5044	-	4833	GTP-binding protein TypA	99.83	0.0	2, 14
Other	*fstA*	PP_1341	PP_1328 – PP_1342	4222	Cell division protein FtsA	99.77	0.0	2
	-	PP_1633	-	3983	Hypothetical protein	100	9e-77	3, 16
	-	PP_2076	PP_2075 – PP_2076	2172*	Hypothetical protein	24.52	1e-04	6
	-	PP_2298	-	3387	Hypothetical protein	97.32	3e-67	13
	-	PP_2550	-	3127	Hypothetical protein	100	7e-144	2
	-	PP_3855	-	4549*	Hypothetical protein	56.25	2.9	13
	-	PP_4306	PP_4306 – PP_4307	1546	Hypothetical protein	98.74	0.0	13
	-	PP_4634	-	4330	Hypothecical protein	96.82	0.0	13
	-	PP_5390	PP_5389 – PP_5392	3657*	Hypothetical protein	40	0.96	13

The % identity column corresponds to the degree of identity obtained after pairing the protein sequence of the reference strain to the nucleotide sequence of *Pseudomonas putida* BIRD-1 using TBlastn. [a]Predicted operons, [b]orthologues and [c]products according to Pseudomonas Genome Database (http://www.pseudomonas.com/) (Winsor *et al.*, 2009). *Non predicted as an orthologue. [d]References: 1) Barret *et al.*, 2011; 2) Cheng *et al.*, 2009; 3) Duque *et al.*, 2013; 4) Espinosa-Urgel and Ramos, 2001; 5) Martínez-Gil *et al.*, 2010; 6) Matilla *et al.*, 2007; 7) Matilla *et al.*, 2010; 8) Matilla *et al.*, 2011; 9) Molina *et al.*, 2005; 10) Molina *et al.*, 2006; 11) Nielsen *et al.*, 2011; 12) Nilsson *et al.*, 2011; 13) Ramos-González *et al.*, 2005; 14) Reva *et al.*, 2006; 15) Revelles *et al.*, 2004; 16) Roca *et al.*, 2013; 17) Vílchez *et al.*, 2000; 18) Winsor *et al.*, 2009; 19) Wu *et al.*, 2011; 20) Yousef-Coronado *et al.*, 2008.

Table 2. Identity (%) of rhizosphere competence determinants identified in sequenced strains of *Pseudomonas putida*.

Group	Gene	Ref. strain ORF ID	KT2440	BIRD-1	F1	UW4	W619	DOT-T1E	S16	GB-1	PC9	Idaho
Adhesion	*lapD*	PP_0165	100	98	93	70	86	93	89	91	90	93
	lapC	PP_0166	100	99	97	78	87	96	94	95	94	97
Attachment	*lapB*	PP_0167	100	99	92	78	88	92	92	91	92	92
	lapA	PP_0168	100	99	82	39	36	70	68	72	48	86
Biofilm formation	*lapH*	PP_0805	100	99	87	24	80	91	23	84	23	87
	lapF	PP_0806	100	87	86	44	65	87	38	80	29	88
	algD	PP_1288	100	99	100	85	97	100	96	98	97	100
	-	PP_1789	100	22	22	22	25	22	75	22	22	22
	-	PP_1790	100	43	21	26	24	50	92	22	50	29
	-	PP_1791	100	20	26	28	27	26	93	20	27	27
	-	PP_1792	100	30	36	28	64	61	78	29	25	62
	-	PP_1793	100	26	27	29	60	59	75	27	28	60
	-	PP_1794	100	0	0	35	49	74	74	38	22	74
	-	PP_1795	100	22	23	25	84	97	96	34	23	97
	bcsQ	PP_2634	100	98	90	24	38	90	38	79	28	88
	bcsA	PP_2635	100	98	95	29	27	95	27	90	27	95
	bcsB	PP_2636	100	98	93	34	28	91	45	87	45	91
	bscC	PP_2637	100	99	96	24	35	96	31	88	31	92
	peaG	PP_3139	100	96	91	34	79	91	87	89	87	92
	peaH	PP_3140	100	98	78	31	58	78	67	73	66	78
	peaI	PP_3141	100	95	92	50	68	93	80	86	80	40
	galU	PP_3821	100	99	100	92	96	100	97	91	97	100
	hemN	PP_4264	100	99	100	83	94	100	98	97	99	100
	-	PP_4934	100	100	97	89	96	97	96	96	96	96
	ndvB	PputW619_2133	41	79	42	61	100	31	36	22	36	0
Flagella	*fliA*	PP_4341	100	100	100	90	96	100	99	99	99	100
	flhB	PP_4352	100	99	78	62	77	77	80	81	78	92
	fliO	PP_4356	100	100	79	70	90	78	96	74	95	78
	fliN	PP_4357	100	99	90	88	85	90	88	87	87	91
	fliL	PP_4359	100	100	82	64	76	81	79	81	79	91
	fliI	PP_4366	100	99	99	87	96	99	99	99	99	100
	fliF	PP_4369	100	99	88	77	84	88	87	87	86	90
	fleQ	PP_4373	100	100	100	93	96	100	97	99	98	100
	fliD	PP_4376	100	46	38	37	40	35	54	40	36	50

Table 2. Continued

Group	Gene	Ref. strain ORF ID	KT2440	BIRD-1	F1	UW4	W619	DOT-T1E	S16	GB-1	PC9	Idaho
Flagella	*fliC*	PP_4378	100	65	62	61	57	65	54	66	68	44
	flgL	PP_4380	100	67	46	46	51	45	90	64	45	77
	flgD	PP_4389	100	99	54	54	54	54	82	84	55	90
	flgB	PP_4391	100	93	93	80	95	93	97	99	97	100
Chemotaxis	-	PP_4331	100	99	89	66	81	87	75	75	76	38
	-	PP_4988	100	98	91	58	80	91	87	88	87	92
Regulators	-	PP_0700	100	99	86	27	63	89	81	80	80	86
	colS	PP_0902	100	99	88	67	83	88	86	86	87	88
Sensor proteins	*rpoN*	PP_0952	100	99	98	89	96	98	97	97	97	98
	-	PP_1066	100	100	100	87	97	100	98	98	98	100
	gacS	PP_1650	100	99	90	69	82	91	86	88	86	91
	-	PP_2070	100	90	97	36	34	98	38	36	38	35
	-	PP_2127	100	97	97	61	72	96	89	91	89	100
	-	PP_3640	100	92	92	29	25	92	87	92	88	91
	-	PP_4295	100	99	100	86	96	100	99	100	99	100
	pfrI	PP_4424	100	34	34	33	34	34	35	40	35	34
	-	PP_4508	100	99	96	79	92	29	93	94	29	98
	-	PP_4959	100	99	98	70	88	98	92	94	92	99
	-	PP_4966	100	100	89	69	83	89	86	87	86	89
	phoR	PP_5321	100	98	97	81	92	97	95	96	95	97
Stress adaptation	-	PP_0063	100	99	100	82	98	100	98	99	97	100
	-	PP_0109	100	98	77	57	72	78	78	76	78	78
	cyoE-1	PP_0110	100	98	77	67	74	77	76	76	76	78
Detoxification	-	PP_0196	100	99	95	82	92	95	94	94	93	95
	-	PP_0296	100	99	91	75	88	91	89	90	89	90
Transporters	-	PP_0368	100	100	100	94	98	100	99	99	99	100
	-	PP_0373	100	100	7675	100	77	76	77	77	77	76
	-	PP_0383	100	99	97	88	92	97	94	94	94	100
	-	PP_0486	100	98	93	32	81	92	87	31	87	94
	-	PP_0670	100	95	84	24	75	84	76	80	77	85
	-	PP_0721	100	92	86	75	86	86	93	86	93	86
	cyoE-2	PP_0816	100	100	92	72	90	91	91	91	91	92
	-	PP_0906	100	99	91	79	86	91	89	90	89	91
	phoP	PP_1186	100	98	81	72	79	81	81	80	81	82

Table 2. Continued

Group	Gene	Ref. strain ORF ID	KT2440	BIRD-1	F1	UW4	W619	DOT-T1E	S16	GB-1	PC9	Idaho
Stress adaptation	*oprD*	PP_1206	100	82	96	44	82	95	93	80	93	96
	-	PP_1271	100	98	86	61	83	87	85	85	85	87
	aapJ	PP_1297	100	99	96	85	94	96	95	95	96	25
Detoxification	*fpr*	PP_1638	100	100	100	97	99	100	99	98	99	100
	relA	PP_1656	100	99	100	90	99	100	99	100	99	34
Transporters	-	PP_1874	100	99	97	71	90	98	94	93	94	100
	oprF	PP_2089	100	100	97	83	81	97	83	96	83	97
	lolD	PP_2155	100	100	100	84	98	100	99	99	99	100
	speB	PP_2196	100	99	99	84	44	100	97	98	97	100
	fepA	PP_2242	100	98	98	24	88	98	95	98	94	99
	cti	PP_2376	100	98	99	74	93	99	96	98	95	99
	azlC	PP_2385	100	99	91	71	86	90	88	89	88	90
	-	PP_2543	100	99	98	55	95	98	97	97	97	98
	-	PP_2561	100	50	88	78	52	88	49	83	81	88
	-	PP_2669	100	98	93	90	29	94	89	91	89	94
	mexC	PP_2817	100	50	48	44	48	48	48	75	91	98
	proP	PP_2914	100	99	94	37	90	93	91	92	91	94
	-	PP_3132	100	99	81	0	67	81	75	79	77	88
	-	PP_3183	100	37	98	65	36	96	90	94	90	99
	-	PP_3210	100	98	34	28	83	86	33	30	30	87
	-	PP_3223	100	25	25	26	26	25	26	37	26	97
	ggt-1	PP_3535	100	96	95	63	84	95	88	90	88	34
	-	PP_3583	100	99	89	74	86	89	87	89	87	90
	-	PP_3668	100	99	94	76	89	94	88	92	88	94
	gloA	PP_3766	100	98	99	80	93	99	95	98	96	99
	-	PP_3802	100	98	99	79	91	99	94	94	96	99
	-	PP_4002	100	100	100	91	96	100	99	99	99	100
	-	PP_4034	100	98	96	82	89	96	91	94	93	77
	treZ	PP_4051	100	97	94	63	84	95	89	91	88	95
	nuoL	PP_4129	100	99	94	86	93	94	93	94	93	93
	-	PP_4305	100	99	94	87	92	94	93	93	93	95
	-	PP_4483	100	99	96	90	95	96	94	94	94	96
	-	PP_4615	100	99	92	65	65	92	88	91	88	92
	-	PP_4646	100	99	99	79	95	99	98	99	98	100

Table 2. Continued

Group	Gene	Ref. strain ORF ID	KT2440	BIRD-1	F1	UW4	W619	DOT-T1E	S16	GB-1	PC9	Idaho
Stress adaptation	*cbrA*	PP_4695	100	99	92	85	91	92	91	91	91	95
	cbrB	PP_4696	100	99	93	84	90	93	91	91	91	93
	pcnB	PP_4697	100	98	93	80	90	93	90	92	91	93
Detoxification	*omlA*	PP_4731	100	99	87	62	83	83	82	86	81	83
	vacB	PP_4880	100	99	93	76	90	93	90	91	90	95
Transporters	*putP*	PP_4946	100	99	92	80	88	92	90	88	89	92
	putA	PP_4947	100	99	96	87	94	96	95	96	95	96
	pstP	PP_5145	100	99	98	91	96	98	97	97	97	38
	-	PP_5278	100	100	96	84	94	96	95	96	95	96
	-	PP_5297	100	99	85	33	79	84	81	83	81	86
	spoT	PP_5302	100	99	98	92	97	98	97	97	97	98
	-	PP_5311	100	99	100	80	90	100	96	97	96	100
	-	PP_5322	100	99	91	79	87	91	88	90	88	91
	pstA	PP_5327	100	99	86	72	82	86	83	84	84	86
	pstS	PP_5329	100	98	89	82	88	89	89	89	89	90
	atpA	PP_5415	100	99	97	93	96	98	96	97	96	98
DNA replication recombination repair	*recJ*	PP_1477	100	98	92	82	90	92	91	91	91	92
	-	PP_2565	100	99	93	65	85	94	91	92	91	94
	xthA	PP_2890	100	98	99	90	96	99	96	96	96	99
	-	PP_3966	100	33	29	29	99	27	89	34	31	58
	hsdM	PP_4741	100	99	89	44	27	30	23	28	28	25
	purA	PP_4889	100	100	100	88	99	100	99	100	99	100
	parC	PP_4912	100	99	96	85	93	97	94	95	94	97
	-	PputW619_2699	28	72	29	37	100	48	69	27	69	33
Secretion	*yidC*	PP_0006	100	100	94	77	92	94	90	93	90	94
	hlpA	PP_1449	100	97	96	23	72	96	81	84	81	96
	hlpB	PP_1450	100	97	90	30	65	91	75	33	76	93
	-	PP_4514	100	99	97	33	93	97	94	97	94	98
	secB	PP_5053	100	99	100	87	97	100	97	98	97	100
	exbB	PP_5306	100	95	77	78	73	79	75	77	75	79
	exbD	PP_5307	100	100	100	83	99	100	99	99	99	100
	tonB	PP_5308	100	100	73	62	87	73	72	72	72	73
Metabolism	*gabT*	PP_0214	100	100	100	85	97	100	99	99	100	100
	soxG	PP_0326	100	99	100	80	95	100	96	98	97	100

Table 2. Continued

Group	Gene	Ref. strain ORF ID	KT2440	BIRD-1	F1	UW4	W619	DOT-T1E	S16	GB-1	PC9	Idaho
Metabolism	*aceE*	PP_0339	100	99	99	93	98	99	98	98	98	100
	glcB	PP_0356	100	99	96	81	93	96	96	95	96	97
	pykA	PP_1362	100	100	100	87	98	100	99	99	99	100
	bglX	PP_1403	100	99	99	76	95	100	97	98	98	28
	lecA	PP_1661	100	98	97	22	94	97	27	26	27	98
	serC	PP_1768	100	99	99	81	97	99	96	97	97	100
	-	PP_2009	100	93	79	53	67	78	74	75	74	80
	-	PP_2624	100	98	99	42	39	98	34	89	84	99
	phaE	PP_3279	100	99	98	24	94	98	96	98	96	100
	phaB	PP_3283	100	98	93	33	81	93	90	89	91	95
	-	PP_3352	100	24	99	42	88	46	25	23	25	99
	hpd	PP_3433	100	99	99	90	98	99	97	99	97	99
	ilvE	PP_3511	100	99	100	84	98	99	99	99	99	37
	glcE	PP_3746	100	96	94	69	82	94	86	89	87	95
	-	PP_3786	100	26	26	27	26	26	26	26	26	26
	-	PP_3854	100	43	45	26	28	59	91	39	72	74
	-	PP_3923	100	98	92	62	81	93	88	89	88	94
	icd	PP_4011	100	99	100	90	98	99	99	98	99	100
	gnd	PP_4043	100	99	96	69	92	96	95	94	94	96
	aceA	PP_4116	100	99	100	95	98	100	99	98	99	100
	sucC	PP_4186	100	100	97	93	97	97	97	95	97	97
	-	PP_4588	100	99	99	85	25	99	96	99	95	99
	thiD	PP_4782	100	99	91	83	88	90	89	89	89	44
	fda	PP_4960	100	100	100	98	99	100	100	99	100	100
	hutU	PP_5033	100	99	95	88	94	95	94	95	95	95
	pgm	PP_5056	100	100	94	81	89	94	91	92	91	95
	gltB	PP_5076	100	99	100	89	97	100	98	99	98	100
	ubiF	PP_5197	100	99	99	86	92	99	98	97	98	100
Protein synthesis folding degradation	*surA*	PP_0403	100	99	96	80	92	96	92	95	92	96
	tyrS	PP_0436	100	99	89	81	86	89	87	87	87	89
	clpB	PP_0625	100	99	92	86	91	100	91	91	91	43
	-	PP_0893	100	99	93	78	88	93	89	92	91	93
	prc	PP_1719	100	99	97	85	95	97	95	95	95	96
	gltX	PP_1977	100	100	100	91	98	99	99	99	99	100

Table 2. Continued

Group	Gene	Ref. strain ORF ID	KT2440	BIRD-1	F1	UW4	W619	DOT-T1E	S16	GB-1	PC9	Idaho
	typA	PP_5044	100	99	96	91	95	96	92	95	92	40
Other	*fstA*	PP_1341	100	99	98	92	97	98	97	98	97	98
	-	PP_1633	100	100	99	63	88	100	95	93	95	100
	-	PP_2076	100	24	89	35	31	86	38	81	45	25
	-	PP_2298	100	97	98	45	94	98	94	95	93	100
	-	PP_2550	100	100	92	71	86	92	86	90	86	92
	-	PP_3855	100	56	28	43	68	70	42	35	36	28
	-	PP_4306	100	98	99	66	94	100	96	95	95	99
	-	PP_4634	100	96	97	65	86	98	90	90	91	99
	-	PP_5390	100	40	74	0	74	41	44	74	44	40

The % identity columns corresponds to the degree of identity obtained after pairing the amino acid sequence of the reference strain (*P.putida* KT2440) to the amino acid sequence of other sequenced *Pseudomonas putida* using BlastP.

Conclusions

Results in this study allows us to present the subsequent conclusions:

1. *Pseudomonas putida* BIRD-1 is able to solubilise organic and inorganic phosphate through the production of phosphatases and inorganic acids, respectively, is able to solubilise iron through the production of siderophores and synthesises IAA. Moreover, is able to adhere to seeds and colonize roots efficiently.

2. *Pseudomonas putida* BIRD-1 is able to survive in the rhizosphere due to the ability to metabolize a wide range of compounds released by plant roots as carbon or nitrogen sources, such as amino acids, organic acids, sugars, flavonoids, citrate, succinate and lignin-derived compounds; and to overcome rhizosphere-induced stresses such as oxidative stress induced by reactive oxygen species, and its genome encodes for 16 genes involved in oxidative stress response. Reactive oxygen species are a product of root cells respiration, then redundancy of genes to deal with these compounds points out the importance of these enzymes for rhizosphere fitness.

3. *Pseudomonas putida* BIRD-1 is able to survive in soil at low humidity rates through the production of trehalose, compared to the reference strain *P. putida* KT2440, a relevance trait for surviving in the environment, especially in Mediterranean climate and semiarid conditions.

4. *Pseudomonas putida* BIRD-1 is able to survive, proliferate and exert its plant growth-promoting effects in harsh environments, such as burned ecosystems and petroleum-hydrocarbon polluted soils.

5. The use of *Pseudomonas putida* BIRD-1, in consortium with other bacteria, in rhizoremediation treatments for restoring burned ecosystems improves ecosystem resilience and does no affect indigenous microbiota.

6. The use of *Pseudomonas putida* BIRD-1, in consortium with other bacteria, in rhizoremediation treatments for restoring petroleum-hydrocarbon polluted soils enhances the establishment of vegetation, which favours the increase of bacterial populations with ability for degrading these complex toxic compounds.

7. The complex genomic machinery involved in survival and proliferation in the rhizhosphere is found being well conserved in *Pseudomonas putida* BIRD-1, as well as among sequenced *Pseudomonas putida* strains.

Los resultados de este estudio nos permiten presentar las siguientes conclusiones:

1. *Pseudomonas putida* BIRD-1 es capaz de solubilizar fosfato orgánico e inorgánico a través de la producción de fosfatasas y ácidos inorgánicos, respectivamente, es capaz de solubilizar hierro a través de la producción de sideróforos y sintetiza IAA. Por otra parte, es capaz de adherirse a semillas y de colonizar raíces de manera eficiente.

2. *Pseudomonas putida* BIRD-1 es capaz de sobrevivir en la rizosfera debido a la capacidad para metabolizar una amplia gama de exudados radiculares como fuentes de carbono o nitrógeno, tales como aminoácidos, ácidos orgánicos, azúcares, flavonoides, citrato, succinato y compuestos derivados de la lignina, y para superar estreses inducidos por la rizosfera, tales como el estrés oxidativo provocado por especies reactivas del oxígeno, y su genoma codifica 16 genes implicados en la respuesta al estrés oxidativo. Las especies reactivas de oxígeno son un producto de la respiración de las células radiculares, por lo que la redundancia de los genes para hacer frente a estos compuestos destaca la importancia de estos enzimas para la supervivencia en la rizosfera.

3. *Pseudomonas putida* BIRD-1 es capaz de sobrevivir en el suelo con niveles bajos de humedad a través de la producción de trehalosa, en comparación con la cepa de referencia *P. putida* KT2440, un rasgo de

importancia para sobrevivir en el medio ambiente, especialmente en el clima mediterráneo y condiciones semiáridas.

4. *Pseudomonas putida* BIRD-1 es capaz de sobrevivir, proliferar y ejercer su actividad promotora del crecimiento vegetal en ambientes hostiles, como ecosistemas quemados y los suelos contaminados con hidrocarburos de petróleo.

5. El uso de *Pseudomonas putida* BIRD-1, en consorcio con otras bacterias, en tratamientos rhizoremediation para restaurar ecosistemas quemados mejora la resiliencia del ecosistema y no afecta a la microbiota indígena.

6. El uso de *Pseudomonas putida* BIRD-1, en consorcio con otras bacterias, en tratamientos rhizoremediation para la restauración de suelos contaminados con hidrocarburos derivados del petróleo mejora la capacidad de establecimiento en el suelo de la vegetación, lo que favorece el aumento de las poblaciones de bacterias con capacidad para degradar estos compuestos tóxicos complejos.

7. La compleja maquinaria genómica implicada en la supervivencia y la proliferación en la rizosfera, se encuentra bien conservada en *Pseudomonas putida* BIRD-1, así como entre cepas secuenciadas de *Pseudomonas putida*.

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