RESEARCH ARTICLE

Survival and catabolic performance of introduced *Pseudomonas* strains during phytoremediation and bioaugmentation field experiment

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Abstract

A long-term field experiment was carried out to estimate the efficiency of bioaugmentation in combination with phytoremediation for oil shale chemical industry solid waste dump area remediation. Soil samples for microbiological and chemical analysis were collected during 3 years after bacterial biomass application. Microbial communities in soil samples were analysed using both culture-based and molecular methods. The survival of the introduced bacterial strains was confirmed by cultivation-based Box-PCR genomic fingerprints and denaturing gradient gel electrophoresis fingerprinting of the 16S rRNA and *lmPH* genes. The introduced bacterial strains as well as corresponding catabolic genes were recovered several years after biomass application, predominantly from the rhizosphere of birches. Soil samples from bioaugmented plots showed an elevated potential for degradation of phenolic compounds even 40 months after treatment. Based on our results we can conclude that the introduced *Pseudomonas* strains both survived, and their metabolic traits have persisted at the contaminated site over a long period of time.

Introduction

Soil bacteria are capable of performing a wide range of activities, some of which result in the mineralization of pollutants. Bioaugmentation, the addition of microorganisms to polluted soils to enhance the transformation rate of contaminants, has been proposed to take advantage of these bacterial abilities. According to Dejonghe *et al.* (2001), bioaugmentation should aim for the rearrangement of the group of organisms dominantly involved in the overall energy flux, so that specific catabolic traits necessary for the cleanup of pollutants are part of that active group. Bioaugmentation is performed either directly by introduced microorganisms (van Veen *et al.*, 1997) or via the transfer of catabolic genes (plasmid-mediated bioaugmentation) to indigenous bacteria (Top *et al.*, 2002). Regardless of the approach chosen, the survival and catabolic activity of introduced microorganisms are the key factors for successful bioaugmentation technologies.

Studies frequently observe that the improvement of the bioremediation activity is temporary, and the number of exogenous microorganisms decreases shortly after biomass addition to a site. There are several explanations for the death of introduced microorganisms, including both biotic and abiotic stresses. Abiotic stresses may include fluctuations or extremes in temperature, water content, pH and nutrient availability, along with low bioavailability or a potentially toxic level of pollutants in contaminated soil. Further, the added microorganisms almost always face competition for limited nutrients, together with indigenous organisms, along with antagonistic interactions including antibiotic production by competing organisms, and predation by protozoa and bacteriophages (Gentry *et al.*, 2004). In some cases, introduced bacteria may fail because of the inability to spread through the soil and reach the pollutant (Kuiper *et al.*, 2004).

Despite these difficulties, successful bioaugmentation has been applied to both nonvegetated and planted soils (Ruberto *et al.*, 2003; Singer *et al.*, 2003).

The use of traditional, culture-dependent approaches has provided most of our current understanding of the fate and survival of introduced strains in the environment. Currently,

FEMS MICROBIOLOGY ECOLOGY

the PCR amplification of the bacterial 16S rRNA gene is one of the most commonly used approaches for the detection of microorganisms in environmental samples. Molecular methods including terminal-restriction fragment length polymorphism (T-RFLP), denaturing gradient gel electrophoresis (DGGE), real-time PCR, FISH and RFLP with clone sequencing have been used to survey the presence and activity of introduced microorganisms (Boon *et al.*, 2000; Bathe & Hausner, 2006; Miyasaka *et al.*, 2006; Rahm *et al.*, 2006; Manzano *et al.*, 2007). In addition to 16S rRNA gene, reporter genes (*gfp*, *lux*) have also been used to quantitatively detect introduced bacteria (Boon *et al.*, 2000; Ripp *et al.*, 2000; Elvang *et al.*, 2001). In the case of bioaugmentation, reporter genes allow more sensitive monitoring of the presence and activity of introduced microorganisms and permit one to distinguish the inoculated strain from identical or similar indigenous bacteria. However, most of the studies concerning the survival and the impact of bioaugmented bacteria on the indigenous microbial community have been short term and performed in microcosm or in sequencing batch reactors (Gomes *et al.*, 2005; Baxter & Cummings, 2006; Jiang *et al.*, 2007).

We have previously shown the effect of bioaugmentation and phytoremediation on microbial community structure and activity in oil shale chemical industry solid waste vegetated with grass species (Truu *et al.*, 2003). Bioaugmentation was found to enhance plant growth and increase the intensity of biodegradation of oil products by up to 50%. Biodegradation activity increased due to the proliferation of specific microbial groups, changes in the taxonomic diversity of the bacterial community and catabolic genes (Truu *et al.*, 2007). The present study focuses on the survival and catabolic performance of the introduced bacterial strains during a 3-year bioremediation field experiment at an oil shale chemical industry solid waste depository area.

Materials and methods

Site description

The depository area consists of semi-coke mounds that have formed from solid waste of oil shale thermal treatment. These mounds have the shape of excentric cones, dark-gray or black in color and with a specific smell (Pae *et al.*, 2005). The mounds cover an area about 200 ha and contain up to 100 million tons of solid waste. According to Truu *et al.* (2003), solid waste is characterized by a high initial pH value, a high concentration of sulphides, Ca^{2+} and Mg^{2+} ions and a high amount of organic carbon (Table 1; Truu *et al.*, 2003). The organic carbon in this material is not similar to the organic carbon in regular soils because half of it is in the form of asphaltenic compounds, which are very recalcitrant to biodegradation. The composition of the mineral part of the solid waste consists mainly of calcite, dolomite and ettringite (Mötlep *et al.*, 2007). Solid waste has a granular texture. Distinctive layers or horizons characteristic of the regular soils, including the humic layer, are missing in the solid waste profile. There is no natural vegetation or undergrowth at the experimental area. Hence, the depository area is of serious environmental concern, as open deposition of the solid waste causes distribution of pollutants via air (dust). Liquid pollution from the dump area deteriorates surface water as well as the underlying aquifers (Truu *et al.*, 2002).

Table 1. Chemical properties of the solid waste at experimental area (Truu *et al.*, 2003)

Variable	Measured value
pH	8.0–11
Total nitrogen (%)	0.08
$P\text{-}PO_4^{3-}$ (mg kg^{-1})	12.3
K^+ (mg $kg^{-1)}$	799
Ca^{2+} (mg kg^{-1})	18 673
Mg^{2+} (mg kg^{-1})	826
Total organic carbon (%)	15.0–18.0
Oil products (mg kg^{-1})	340
Volatile phenols (mg kg^{-1})	0.30–0.34

Part of the older (10–15 years) depository area was planted with birches (*Betula pendula*) in 1998 (distance between birches is *c.* 1 m). During 2004–2006, 10-m^2 plots were established for bioaugmentation experiments at the flat part of the depository area, with two replicate plots per treatment. New plots were established each year in July based on the principle that the plots would have no influence on each other. The designations of the test plots are given in Table 2.

Bacterial strains and their characterization

The previously characterized *Pseudomonas* strains isolated from the nearby polluted area were selected for the bioaugmentation field experiment. According to species identification by BIOLOG GN microplates, rep-PCR genomic fingerprints and sequences of 16S rRNA and *carA* (subunit of carbamoyl-phosphate synthetase) genes, the strains are *Pseudomonas mendocina* (PC1), *Pseudomonas fluorescens* biotype F (PC17 and PC20), *P. fluorescens* biotype B (PC18) and *P. fluorescens* biotype C (PC24) (Heinaru *et al.*, 2000; Merimaa *et al.*, 2006). The presence of catabolic genes, the ability to grow on different aromatic compounds, plasmid profiles and *tra* genes of bacterial strains have been described previously (Table 3; Heinaru *et al.*, 2000).

Bioaugmentation experiment

Two different mixtures of *Pseudomonas* strains were used in the bioaugmentation experiment: a mixture of four strains

(PC1, PC18, PC20 and PC24 in 2004, 2005 and 2006) and a mixture of five strains (PC1, PC17, PC18, PC20 and PC24 in 2005). Before the introduction, strains were cultivated in Luria–Bertani (LB) medium. Cells from the stationary growth phase were mixed with 0.9% NaCl solution and inoculation was performed by spreading the mixture (20 L; strains in equal ratios; final concentration of each strain approximately 10^8 CFU mL^{-1}) onto the surface of experimental plots. The total amount of bacteria introduced into the plots was *c.* 10^{12} CFU m^{-2}. Inoculation was performed in July 2004, 2005 and 2006 only of the plots established in the same year.

In 2005, an amendment with additional mineral fertilizers was applied to the plots simultaneously with inoculation to study the effect of fertilizers on the survival of inoculated bacteria.

Sampling

Soil sampling was performed at the beginning of October during 2004–2007 from the plots established in the same year and from the plots established in previous years. In 2007, new plots were not established and sampling was performed only from old plots. Because of the very limited amount of birch roots, most of the soil is not influenced by roots and was defined as 'nonrhizosphere sample'. Soil samples collected directly from the surface of roots were defined as 'rhizosphere samples'.

Nonrhizosphere samples were taken from the plots with a soil corer (Ø = 2 cm) from the 0–15-cm layer and a composite sample was made for each plot. For a composite sample, 20 subsamples were randomly collected from each plot. Composite samples were sieved (Ø < 2 mm) and used for further analysis (Fig. 1).

Birch roots with attached soil particles were removed from plots by hand and placed into sterile plastic bag. Soil particles were removed from the surface of 1 g of roots (wet weight) by vortexing for 5 min at maximum speed in 10 mL of 0.9% NaCl solution. Before centrifugation, the solution was used for culture-dependent analyses. Pellets obtained from centrifugation (10 000 ***g*** for 10 min) were used for DNA extraction (Fig. 1).

Table 2. Designation of the studied test plots

Plot establishment and inoculation	No. of plots established	No. of strains used in inoculation	Designations of plots
2004	2	4	C+, 1(4)*
2005	5	4, 5	C+, 2(4), 3(5), 2f(4), 3f(5)
2006	3	4	C+, 4(4), 5(4)

*Number of introduced strains in parentheses.
f, amendment with additional fertilizers; C+, planted control; 1–5, sampling sites.

Culture-dependent analyses for the detection of introduced strains

Soil and rhizosphere samples were used for serial dilutions and plating selective media. Representative colonies were obtained on R2A agar or minimal media containing M9 salts and trace elements supplemented with 2.5 mM phenol, 5 mM salicylate or naphthalene in vapour phase. For purification, colonies were picked up from these agar media and restreaked onto R2A agar until a pure culture was obtained

Table 3. Characteristics of bacterial strains used in bioaugmentation experiment (Heinaru *et al.*, 2000)

Strain	Degradation of substrates	Aromatic ring cleavage enzymes	Key enzyme of phenol degradation	*p*-Cresol degradation pathway	Accession number for 16S rRNA gene sequence in GenBank
Pseudomonas mendocina PC1	Phe^+ *p*-Cre^+ $Benz^+$ Pob^+ *m*-Tol^+	C23O C12O PC34O	mPH	C23O	AF232713
Pseudomonas fluorescens F PC17	Phe^+ *p*-Cre^+ $Benz^+$ Pob^+ Sal^+	C12O PC34O	mPH	PC34O	AY538263
Pseudomonas fluorescens B PC18	Phe^+ *p*-Cre^+ $Benz^+$ Pob^+ Sal^+	C23O C12O PC34O	mPH	PC34O	AF228366
Pseudomonas fluorescens F PC20	Phe^+ *p*-Cre^+ $Benz^+$ Pob^+ Sal^+ Nah^+	C23O C12O PC34O	sPH	C23O	AY538264
Pseudomonas fluorescens C PC24	Phe^+ *p*-Cre^+ $Benz^+$ Pob^+ *m*-Tol^+	C12O PC34O	sPH	PC34O	AF228367

Phe^+, *p*-Cre^+, $Benz^+$, Pob^+, *m*-Tol^+, Sal^+, Nah^+: phenol, *p*-cresol, benzoate, *p*-hydroxybenzoate, *m*-toluate, salicylate and naphthalene. C12O, catechol 1,2-dioxygenase; PC34O, protocatechuate 3,4-dioxygenase; MPH, multicomponent phenol hydroxylase; SPH, single-component phenol hydroxylase (encoded by the *pheA*).

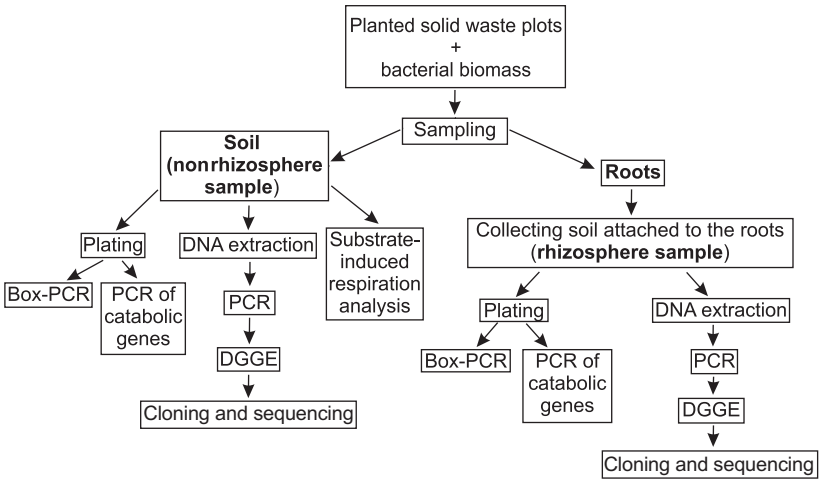

Fig. 1 Schematic representation of sampling and subsequent analyses of soil samples.

(the purity of all isolated strains was examined microscopically). The isolates were characterized by morphology, Gram test, oxidase test, growth on King's B medium and on different aromatic substrates. In addition, the presence of different catabolic genes was tested for. The primers and PCR conditions used for the amplification of fragments of the genes coding for the large subunit of multicomponent phenol hydroxylase (*lmPH*) and catechol-2,3-dioxygenase (C23O) have been described earlier (Merimaa *et al.*, 2006). The bacterial isolates were also tested by spraying with 0.5% catechol (w/v) for rapid distinguishing of strains possessing *meta*-cleavage activity under induced conditions (growth on phenol), and under uninduced conditions (growth on R2A) for the discrimination of PC20 from other strains. The isolates with colony characteristics similar to the bioaugmentation strains were selected for further Box-PCR analysis using BOXA1R primer (Table 4). The colony rep-PCR was performed as described in Heinaru *et al.* (2000). Similarity values based on densitometric curves of the gel tracks were calculated using the Pearson correlation coefficient.

DNA extraction and PCR conditions

Microbial DNA was extracted from 0.3 g of the samples (wet weight) with UltraClean Soil DNA kit (Mo Bio Laboratories Inc.) according to the manufacturer's instructions. Extracted DNA was quantified with a spectrophotometer (Nanodrop 1000) and stored at $-20\,^{\circ}C$.

Based on the 16S rRNA gene sequences of previously described *Pseudomonas* species, the taxon-specific primer pair PseF/PseR (Table 4) was designed for the amplification of the variable V3 region in 16S rRNA gene. The first PCR amplification was carried out with the primer pair PseF/PseR using 1 μL (1–5 ng) of extracted DNA as the template. Tenfold diluted PCR products were then used as a template (0.5 μL) for the next amplification, using the primer pair PRBA338f/PRUN518r (Table 4). *lmPH* gene fragments were amplified under the same conditions, first using 1 μL extracted total DNA for primers Phe00/Phe212, followed by amplification with primers PheGC/Phe212. Primers PRBA338f and PheGC contained a GC clamp (40 bp) at the 5′ end to enable DGGE analysis. The PCR mixture included $1\times$ PCR buffer [with $(NH_4)_2SO_4$], 200 μm concentrations of each dNTP, 2.5 mm $MgCl_2$, 20 pmol of both primers, 60 ng μL^{-1} bovine serum albumin and 0.5 U of Taq DNA polymerase (Fermentas). The reaction conditions of the first PCR with primers PseF/PseR and Phe00/Phe212 were as follows: 95 °C for 5 min, 35 cycles of 95 °C for 1 min, 57 °C for 30 s (65 °C for 30 s for *lmPH*), 72 °C for 1 min and a final extension of 72 °C for 5 min. The reaction conditions of the second PCR with primers PRBA338f/PRUN518r and PheGC/Phe212 were as follows: 95 °C for 2 min, 30 cycles of 95 °C for 30 s, 53 °C for 30 s (65 °C for 30 s for *lmPH*), 72 °C for 30 s and a final extension of 72 °C for 5 min. The PCR products were quantified in 2% (w/v) agarose gel by comparison with the standard (100-bp DNA size marker, Fermentas) using EASY WIN32 software (Herolab GmbH).

DGGE analysis

Approximately 500 ng of the amplified 16S rRNA and *lmPH* gene fragments were applied for DGGE analysis using the method elaborated by Muyzer *et al.* (1993). The DCode DGGE system (Bio-Rad Laboratories) was used for electrophoresis as recommended by the manufacturer. A 10% (v/v) polyacrylamide gel (acrylamide : bisacrylamide = 37.5 : 1 in $1\times$ TAE buffer) with linear DNA denaturing gradient was used. The denaturing gradient was formed with deionized formamide and urea [100% denaturant agent is 7 M urea and 40% (v/v) deionized formamide]. Gels were electrophoresed in a $1\times$ TAE buffer for 11 h at a constant

Table 4. Characteristics of PCR primers and GC clamp used in the study. The GC clamp is attached to the end of the 5′ end of the PRBA338f and PheGC primers

Primer	Primer sequence (5′ → 3′)	Size (bp)	References
PseF	GAGAAAGCAGGGGACCTTCG	408	This study
PseR	CCGGGGMTTTCACATCCAAC		This study
PRBA338f*	ACTCCTACGGGAGGCAGCAG	180	Ovreas *et al.* (1997)
PRUN518r	ATTACCGCGGCTGCTGG		Muyzer *et al.* (1993)
BOXA1R	CTACGGCAAGGCGACGCTGACG		Louws *et al.* (1994)
Phe00	CRATYGACGARCTGCGYCA	170	Heinaru *et al.* (2005)
Phe212	GTTGGTCAGCACGTACTCGAAGGAGAA	209	Watanabe *et al.* (1998)
PheGC	CRATYGACGARCTGCGYCA		Heinaru *et al.* (2005)
GC clamp	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG		

temperature of 60 °C and a constant voltage of 100 V. Gels were stained in MilliQ water containing 0.5 μg L^{-1} ethidium bromide and destained twice in MilliQ water before UV transillumination.

A linear denaturing gradient of 38–49% was used for 16S rRNA gene fragments and a linear denaturing gradient of 50–70% for *lmPH* gene fragments. Representative bands (16S rRNA and *lmPH* gene fragments that demonstrated identical migration properties with fragments in reference lanes) were excised from the polyacrylamide gel and eluted in a buffer containing 0.5 M NH_4Ac, 10 M MgAc, 1 mM EDTA and 0.1% sodium dodecyl sulphate, resuspended in MilliQ and reamplified as described above.

Cloning and sequencing

To verify whether the detected rRNA and *lmPH* genes sequences from soil samples show a similarity to sequences from the introduced strains, reamplified 16S rRNA and *lmPH* gene fragments were ligated into pTZ57R using the InsT/Aclone™ PCR Product Cloning Kit (Fermentas), and the constructs were transformed into *Escherichia coli* ΔH5α competent cells (Inoue *et al.*, 1990). The *E. coli* cells containing the vector plasmid with inserted PCR product were selected on LB medium at 37 °C, containing ampicillin 15 μg mL^{-1}, isopropyl β-D-thiogalactopyranoside 48 μg mL^{-1} and 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside 80 μg mL^{-1}. Nucleotide sequencing of the cloned inserts was carried out on an ABI Prism TM 377 DNA sequencer (Perkin-Elmer) using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and the protocols provided by the manufacturer.

Substrate-induced soil respiration analysis

Nonrhizosphere samples with different bioaugmentation ages (sampling in October 2007, inoculation in 2004, 2005 and 2006) were used for a substrate-induced soil respiration test to assess the changes in the catabolic activity of the microbial community using an OxiTop respirometric system (WTW). The test was performed in two replicates in 250-mL bottles containing 10 g of sample (wet weight) suspended in 90 mL of Bushnell–Haas minimal medium at 22 °C for 7 days, with an initial phenol concentration of 2.5 mM. Oxygen consumption was measured on the basis of the pressure drop in the bottles following CO_2 trapping by absorbent (soda lime, Fluka). Basal respiration without substrate was used as a control for all samples under the same conditions. After subtracting the blank values, biodegradation was expressed as a percentage of the theoretical oxygen demand (ThOD) of the phenol [the ratio of biological oxygen demand (BOD) to ThOD]. The time course of phenol removal was subject to kinetic data analysis, using a sigmoid function:

$$y = \frac{C}{1 + e^{-k(t-S)}}$$

where y corresponds to BOD/ThOD (%), t is the time (h), k is pseudokinetic constant (h^{-1}), C (the limiting value of y) and S are adjustable parameters. The calculations were performed using SIGMAPLOT 10 software.

To study the phenol removal efficiency of mixture of strains at different concentrations in soil, an experiment with varying concentrations of mixed population (final concentration 3.1×10^2–3.1×10^5 CFU mL^{-1}) and control soil (10 g) was performed under the same conditions.

Results

Detection of introduced strains using culture-dependent analyses

The presence of the strains used in bioaugmentation was tested as described in Table 5: in 2005 (4 months after treatment), 2006 (4 months after treatment in 2006 and 16 months after treatment in 2005) and 2007 (16 months after treatment in 2006). The isolates obtained from samples were characterized by morphology, phenotype (including catabolic properties) and genotype (the presence of key catabolic genes, REP-fingerprints). The presence of different aromatic ring cleavage dioxygenases and phenol hydroxylases was

useful in distinguishing the colonies of bioaugmented strains (Table 3).

A comparison of the REP-fingerprints of the introduced strains and the strains isolated from bioaugmented soil samples is shown in Fig. 2. Most important is that introduced *Pseudomonas* strains were detected only in rhizosphere samples. Only one strain, PC20, was found in all tested bioaugmented plots (Table 5), whereas the strain PC17 was not detected in any analysed plots. Untreated samples were used as the control in all 3 years and the introduced strains were not found in these cases. Thus, our analysis confirmed the survival of four of five introduced strains in the rhizosphere samples, and the PC20 strain in particular.

Detection of rRNA gene sequences corresponding to inoculated strains

Based on the migration of 16S rRNA gene fragments in polyacrylamide gel we found that although the total 16S rRNA gene sequence is highly conserved within genus *Pseudomonas*, the V3 region was variable enough to distinguish the inoculated strains from each other in reference lanes (Fig. 3). All bands in soil sample lanes migrating onto the same position as the bands that corresponded to the introduced strains in the reference lanes were excised from the gel, cloned and sequenced. In all cases, these sequences showed 100% identity to the respective reference strain.

Based on the DGGE fingerprints of samples from control plots established in different years, no differences were found (data not shown). For that reason, samples from control plots established in 2004 were selected. The gel image shows that the strains PC1, PC17, PC20 and PC24, but not PC18, were detected in the soil samples collected in the year of inoculation (Fig. 3). Only three strains were detected from soil samples taken in 2007 but inoculated in previous years: PC24 (samples 4, 1*), PC18 (samples 4, 1, 1*) and PC20 (samples 4, 1, 4*). In the soil samples of the control areas, no bands corresponding to the introduced strains were obtained.

In 2005 we also applied additional mineral fertilizers to the soil to improve the catabolic performance of the indigenous bacterial populations and the survival of the introduced bacteria as well as plant growth, but no signifi-

Table 5. Detection of introduced *Pseudomonas* strains in the rhizosphere samples using cultivation-based methods

Year of inoculation	Year of sampling	Bioaugmentation age (months)	Strains detected by cultivation
2005	2005	4	PC1, PC18, PC20, PC24
2005	2006	16	PC1, PC18, PC20, PC24
2006	2006	4	PC1, PC18, PC20, PC24
2006	2007	16	PC20

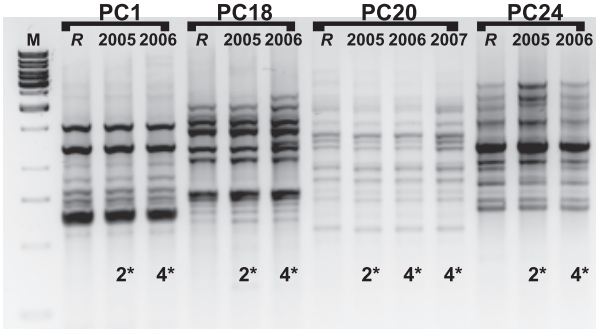

Fig. 2 Negative gel image of Box-PCR fingerprints of the selected bacterial colonies and reference strains separated on 1.5% (w/v) agarose gel. M, DNA size marker (1 kb); R, reference strain; 2, 4, sampling sites (2, inoculation in 2005; 4, inoculation in 2006); *rhizosphere sample. Years designate sampling time.

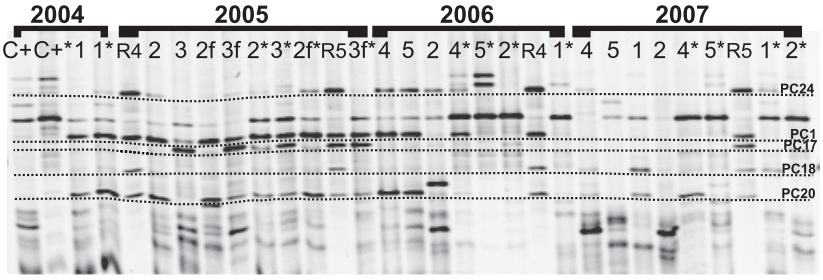

Fig. 3 Negative gel image of DGGE fingerprint of separated *Pseudomonas* species-specific 16S rRNA gene fragments (10% polyacrylamide gel, linear denaturing gradient 38–49%). C+, planted control; *rhizosphere sample; f, amendment with additional fertilizers; 1–5, sampling sites [1 (4), inoculated in 2004; 2 (4) and 3 (5), inoculated in 2005; 4 (4) and 5 (4), inoculated in 2006; number of introduced strains in parentheses]; reference lanes R4 (contains bacterial strains PC24, PC1, PC18 and PC20) and R5 (contains bacterial strains PC24, PC1, PC17, PC18 and PC20). Years designate sampling time; dotted line indicates excised, cloned and sequenced 16S rRNA gene fragments.

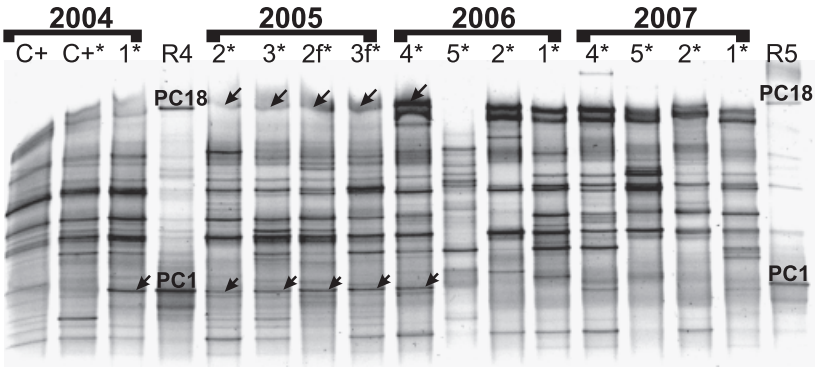

Fig. 4 Negative gel image of DGGE fingerprint of separated *lmPH* gene fragments in rhizosphere samples (10% polyacrylamide gel, linear denaturing gradient 50–70%). C+, planted control; *rhizosphere sample; f, amendment with additional fertilizers; 1–5, sampling sites [1 (4), inoculated in 2004; 2 (4) and 3 (5), inoculated in 2005; 4 (4) and 5 (4), inoculated in 2006; number of introduced strains in parentheses]; reference lanes R4 and R5, strains PC18 and PC1. The years designate sampling time; arrows indicate excised, cloned and sequenced *lmPH* gene fragments.

Table 6. Kinetic parameters of phenol removal obtained in 7-day respirometric test with unplanted control, planted control, planted soil samples of different bioaugmentation age in months and mixture of strains in pure culture

Sample	k (h^{-1})	S (h)	C (BOD/ThOD %)	R^2(%)
Control	0.07 (0.002)	101.7 (0.49)	83.8 (0.2)	98.3
Control+	0.18 (0.01)	86.2 (4.38)	90.8 (0.2)	98.3
40 months	0.26 (0.001)	47.1 (1.46)	93.9 (0.2)	99.2
28 months	0.24 (0.04)	64.6 (1.03)	93.3 (0.2)	99.1
16 months	0.26 (0.01)	72.3 (0.23)	94.9 (0.2)	98.8
Mixture of strains (1.5×10^3 CFU g^{-1} dw)	0.44 (0.02)	53.6 (0.19)	91.4 (0.2)	99.4

Mean and SD values (in parentheses) of the parameters are shown.

cant differences were obtained compared with the unfertilized plots (Fig. 3).

Detection of catabolic genes (*lmPH*) corresponding to the inoculated strains

The multicomponent phenol hydroxylase gene was used as one of the molecular markers because it has been reported previously that *mPH* genes are predominant in bacteria isolated from a phenol-polluted area (Peters *et al.*, 1997). Only rhizosphere samples were subjected to PCR-DGGE analysis for the detection of phenol-degrading genes. The gel image shows that *lmPH* gene fragments corresponding to inoculated strains PC1 and PC18 were mainly found in soil samples in the year of inoculation (Fig. 4). Subsequent cloning and sequencing showed that the *lmPH* gene fragments (indicated by arrows in Fig. 4) sharing identical electrophoretic mobility with the fragments derived from the introduced strains in reference lanes were 100% identical. In the soil samples from the control areas, bands corresponding to the *lmPH*s of the introduced strains were not obtained.

Aerobic biodegradation of phenol

Results from the phenol biodegradation test showed that even 40 months later, the potential catabolic performance of microbial community in soil samples with added bacterial biomass remained higher than that of the planted and unplanted control. The kinetic parameters presented in Table 6 show that although soil samples with different bioaugmentation age demonstrate similar pseudo-first-order constants of degradation of phenol, the rate of degradation is $>$ 30% higher than in the planted control samples and three times higher than in unplanted soil. The degree of biodegradation (value of BOD/ThOD) was also higher in bioaugmented samples than in control samples and in the unplanted control in particular. The smallest value of parameter S (shortest lag phase) was obtained with a soil sample of 40 months of bioaugmented age compared with samples with latter inoculations and controls. In respirometric tests with varying inoculum density, the pseudo-first-order constant values obtained were positively related to the density of the bacterial mixture in suspension, but we cannot transfer this relationship directly to data obtained with field samples as contact time between soil and introduced bacteria are too different in these two cases.

Discussion

As mentioned earlier in the introduction, there are two key factors for successful bioaugmentation: the survival and the catabolic activity of introduced microorganisms. It is obvious that conditions in soil are not favourable for these factors. For example, excessive Ca^{2+} ions in rhizosphere solution limit microbial activity and may prevent

germination of plant seeds and reduce plant growth rates (White & Broadley, 2003). This latter is important in the context of rhizoremediation, as rhizosphere plays an essential role in the metabolic activity and survival of the microbial community, particularly in such extreme conditions. However, it has been shown that toxic properties of soil decrease over time mostly due to a decrease in free calcium ion concentration (Raave *et al.*, 2007).

In the current experiment, the presence of 16S rRNA and *lmPH* gene fragments corresponding to the introduced *Pseudomonas* strains were found in both nonrhizosphere and rhizosphere samples. The survival of the introduced bacteria was confirmed mainly in the rhizosphere samples. Bioaugmented samples demonstrated an elevated phenol biodegradation as long as 40 months after the treatment, implying a contribution of the introduced strains to soil remediation.

It is generally accepted that the most crucial stage in bioaugmentation is strain selection. It has been suggested that the best way to increase the survival of the inoculum is to look for candidate microorganisms from the same ecological niche as the polluted area (El Fantroussi & Agathos, 2005). Such microorganisms are more adapted to the biotic and abiotic conditions in polluted environments. It is also easier to incorporate them to the local microbial community. In the current study, the origin of the strains may be one of the reasons for their long-term survival, as the set of bacteria was isolated from a nearby area with similar conditions concerning pollutants. The best survival among introduced bacteria was demonstrated by strain PC20, whereas strain PC17 only survived for a very short period. This confirms previous reports that different strains, even when they are genetically very similar, have different survival capabilities when introduced into the soil (Goddard *et al.*, 2001).

A phytoremediation field experiment performed with grass species at the same depository area revealed the impact of vegetation on the biodegradation efficiency and on the taxonomic and metabolic diversity of the bacterial community in soil (Juhanson *et al.*, 2007; Truu *et al.*, 2007). According to the results, vegetation also contributed to the survival of the introduced bacterial strains. It is known that because of the exudates from the roots, the microbial community in the rhizosphere is characterized by higher metabolic activity and survival (Kuiper *et al.*, 2004). Also, the contact between microorganisms associated with the roots and the contaminants in soil is increased, resulting in an increased biodegradation rate of the pollutants and improvement of physical and chemical properties of soil (Kuiper *et al.*, 2001). The improvement of the physical and chemical conditions in the rhizosphere zone may be one of the reasons for the better survival of the strains compared with the nonrhizosphere.

In addition to the physicochemical parameters in the polluted environment, the physiological state of the introduced microorganism also influences its survival and effectiveness. Cunliffe *et al.* (2006) demonstrated that pretreatment of the inoculum had a dramatic impact on the survival and metabolic activity of the inoculum in polluted soil. In the current experiment, before introduction of the strains to the soil, strains were cultivated in complex media, which, according to Cunliffe *et al.* (2006), may improve biodegradation of contaminants and survival of the strains.

Plasmid-mediated bioaugmentation has been suggested as an alternative strategy where the survival of the introduced donor strain is no longer needed once catabolic genes are transferred and expressed in indigenous bacteria (Top *et al.*, 2002). More important than the survival of introduced bacteria is the survival of their catabolic traits. This alternative approach has also been suggested in the context of rhizoremediation, as the rhizosphere may be a habitat that allows a higher frequency of catabolic gene transfer as well as higher metabolic activity compared with bulk soils, both of which are necessary for a successful plasmid-mediated bioaugmentation (Top *et al.*, 2002). This study did not address the question whether and to what extent horizontal gene transfer contributed to the overall efficiency of biodegradation. Nevertheless, conditions for the transfer of genetic information are present. Survival of the introduced strains was found particularly in the rhizosphere and it is known that four *Pseudomonas* strains used in our bioaugmentation experiment (PC17, PC18, PC20 and PC24) contain plasmids that carry catabolic genes. PC20 has two large plasmid replicons, a conjugative naphthalene plasmid and a smaller plasmid bearing the *pheBA* operon encoding the enzymes for phenol degradation via the *ortho* pathway of catechol degradation. Therefore, transfer of catabolic genes is possible but not proven in this case. Successful plasmid-mediated bioaugmentation of organic contaminants and transfer of catabolic genes to the indigenous bacteria has been demonstrated in soils and in activated sludge reactors (Top *et al.*, 1998; Bathe *et al.*, 2005).

To summarize our results, introduced *Pseudomonas* strains both survived and their metabolic traits persisted at the contaminated site over a long period of time. In addition to the survival of the strains, the introduction of bacterial biomass as well as plants facilitated the development of a functional and stable microbial community with an elevated capacity for degradation of pollutants in soils. Nevertheless, it is important to emphasize that estimation of the gene transcripts (catabolic genes or amount of 16S rRNA gene) would be necessary to obtain stronger evidence regarding the survival and metabolic activity of the introduced bacterial strains.

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