Bacterial community structure of a pesticide-contaminated site and assessment of changes induced in community structure during bioremediation

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Abstract

The introduction of culture-independent molecular screening techniques, especially based on 16S rRNA gene sequences, has allowed microbiologists to examine a facet of microbial diversity not necessarily reflected by the results of culturing studies. The bacterial community structure was studied for a pesticide-contaminated site that was subsequently remediated using an efficient degradative strain *Arthrobacter protophormiae* RKJ100. The efficiency of the bioremediation process was assessed by monitoring the depletion of the pollutant, and the effect of addition of an exogenous strain on the existing soil community structure was determined using molecular techniques. The 16S rRNA gene pool amplified from the soil metagenome was cloned and restriction fragment length polymorphism studies revealed 46 different phylotypes on the basis of similar banding patterns. Sequencing of representative clones of each phylotype showed that the community structure of the pesticide-contaminated soil was mainly constituted by *Proteobacteria* and *Actinomycetes*. Terminal restriction fragment length polymorphism analysis showed only nonsignificant changes in community structure during the process of bioremediation. Immobilized cells of strain RKJ100 enhanced pollutant degradation but seemed to have no detectable effects on the existing bacterial community structure.

Introduction

Rapid analysis of diversity in complex microbial communities has remained an elusive but important goal in microbial ecology. The most widely applied methods currently consist of reducing microbial diversity to diversity of molecular taxonomic markers which can be generated by PCR on DNA isolated from the total community with primers against conserved gene regions. The use of the 16S rRNA gene as a target for microbial diversity is well established and has the advantage of being a phylogenetically and taxonomically relevant marker. Information from PCR-amplified 16S rRNA gene diversity can be used more quantitatively as well, in order to derive an idea of species richness (number of 16S rRNA gene fragments from a sample) and relative abundance (structure or evenness). The richness and evenness of biological communities reflect selective pressures that shape diversity within communities (Dunbar *et al.*, 2000). Measuring these parameters is most useful when assessing treatment impacts on community diversity, e.g. physical disturbances, pollution, nutrient addition, climate change, etc (Dunbar *et al.*, 2000), or the ability of a community to recover from disturbance and utilize resources efficiently (Chapin *et al.*, 1997).

Amplified ribosomal DNA restriction analysis (ARDRA) is a DNA fingerprinting technique based on restriction enzyme digestions and agarose gel electrophoresis of PCR-amplified 16S rRNA genes using primers for conserved regions (Massol-Deya *et al.*, 1995; Ross *et al.*, 2000a). ARDRA patterns have been used to estimate microbial diversity of various environments including soils and food (Ross *et al.*, 2000b; Cambon-Bonavita *et al.*, 2001; Lagace *et al.*, 2004). PCR amplification, cloning and DNA sequencing of 16S rRNA genes from community DNA have also been used to identify the relative abundances of microbial populations within given communities (McCaig *et al.*, 1999; Ross *et al.*, 2000b). Analysis of individual 16S rRNA gene clones in multiple libraries is an expensive method for comparison of a complex community that undergoes spatial and temporal variations; therefore, other methods such as

thermal or denaturing gradient gel electrophoresis (T/DGGE) or terminal fragment length analysis (T-RFLP), ribosomal intergenic spacer analysis (RISA), oligonucleotide fingerprinting of ribosomal RNA genes (OFRG), have been developed, which can more easily trace changes occurring in communities on temporal or spatial scales or as a response to environmental perturbation (Liu *et al.*, 1997; Ludeman *et al.*, 2000; Ikeda *et al.*, 2005; Jampachaisri *et al.*, 2005; Qu *et al.*, 2005). For example, T-RFLP of the 16S rRNA gene was used to fingerprint the microbial communities in denitrifying enrichment cultures for isolation of 4-chlorobenzoate and 4-bromobenzoate degrading microorganisms, and also to assess spatial and temporal changes in the bacterial community structure within an agricultural soil planted with transgenic and nontransgenic potatoes, and various other environments (Liu *et al.*, 1997; Dunbar *et al.*, 2000; Derakshani *et al.*, 2001; Kozdroj & van Elsas, 2001; Takai *et al.*, 2001; Song *et al.*, 2002). T-RFLP patterns can be considered as a representation of the operational taxonomic units (OTUs) present in a microbial community (Lukowl *et al.*, 2000); however, the technique does not provide a direct identification of each T-RF in the pattern. For this purpose, a combination of 16S rRNA gene cloning, screening of clones by RFLP and sequencing, and T-RFLP has been successful (Derakshani *et al.*, 2001; Takai *et al.*, 2001).

Quite often it is necessary to determine the soil microbial diversity of a particular location to reveal the effects of any deliberate anthropogenic activity carried out there. Bioremediation of contaminated sites has been accepted as a safer and more economic alternative to physical means of remediation (Maymo-Gatell & Schraa, 1997; Dua *et al.*, 2002), but it is not very clear what effects exogenously added microbes may have on the existing community composition. In this study we have determined the effects of exogenously added *p*-nitrophenol (PNP) degrading bacteria on the community composition in Indian soils. *p*-Nitrophenol is a metabolite arising from the conversion of the organophosphorous pesticides parathion and methyl parathion. Both pesticides are still prevalent in several countries, including India, despite being banned by the United States Environmental Protection Agency (USEPA). Via hydrolysis in soil they give rise to PNP, which is toxic to humans (Spain *et al.*, 2000) and has been listed as 'priority pollutant' by the USEPA (Keith & Telliard, 1979) (http://www.epa.gov/region1/npdes/permits/generic/prioritypollutants.pdf). Our attempts were to remediate a pesticide-contaminated soil site using the efficient PNP-degrading organism *Arthrobacter protophormiae* RKJ100. The bacterial community structure was profiled using T-RFLP so that any changes during implementation of the bioremediation could be assessed. Also, the community composition was determined by preparing a 16S rRNA gene library followed by sequencing of the individual clones.

Materials and methods

Sampling sites

An agricultural site sprayed with pesticides (parathion/methyl parathion) and therefore contaminated with *p*-nitrophenol (initial *p*-nitrophenol level 9 μg g^{-1}) was identified and soil from this site was used for carrying out small-scale field studies. For the field studies *p*-nitrophenol contaminated soil was collected, transported to the site of study and added to four plots as described earlier (Labana *et al.*, 2005) (~300 kg of soil was added to each plot): Plot A, soil without carrier material or bacteria; Plot B, soil mixed with carrier material without bacteria; Plot C, soil mixed with inoculum without the carrier; Plot D: soil mixed with inoculum immobilized on carrier material (~300 g corn cob premixed with 2×10^6 bacteria g^{-1} soil or in total 6×10^{11} cells). Cells of strain RKJ100 suspended in 2 L of minimal medium were mixed with 300 g of corn cob powder and subsequently with 5 kg of soil. This inoculated soil was then thoroughly mixed with the rest of the soil. Corn cob powder was selected as carrier because of its porosity and highly adsorbing nature, which meant that it could provide a protective niche to the microorganisms.

Soil samples were collected from a depth of ~10 cm from three different random positions using a hollow pipe of ~2.5 cm diameter, pooled, mixed thoroughly and used for further analysis. Three such samples were collected from each plot. Sampling was done at the following time points: 0, 1, 2, 3, 5, 7, 10, 20 and 30 days.

Bacterium used for bioremediation

Arthrobacter protophormiae RKJ100 has the ability to utilize *p*-nitrophenol (Chauhan *et al.*, 2000), *o*-nitrobenzoate (Chauhan & Jain, 2000), *p*-hydroxybenzoate (Paul *et al.*, 2004) and 4-nitrocatechol (Chauhan *et al.*, 2000) as the sole source of carbon and energy. This bacterium also shows the ability to tolerate cobalt (Chauhan *et al.*, 2000).

Total DNA extraction from soil and PCR

Total bacterial community DNA was extracted using a FastPrep DNA isolation kit according to the manufacturer's instructions (BIO101, Q-Biogene Inc., CA). The DNA was further purified from gel using the Qiaquick gel extraction kit (Qiagen GmbH, Hilden, Germany). The concentration of DNA was measured spectrophotometrically. To amplify 16S rRNA gene fragments from the soil DNA, PCR was performed using 20–80 ng template DNA with universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′), which target universally conserved regions and permit the amplification of fragment of *c.* 1500 bp (Cheneby *et al.*, 2000). The

oligonucleotide primers were obtained from Biobasics, Markham, Ontario, Canada. PCR amplification was carried out in a personal thermocycler (Eppendorf, Hamburg, Germany). Reaction tubes contained 25 ng DNA, 1 U *Taq* DNA Polymerase (New England Biolabs, Beverly, MA), 1 × buffer (10 mM Tris-HCl [pH 9.0], 1.5 mM $MgCl_2$, 500 mM KCl, 10 mM deoxynucleoside triphosphate, and 20 pmol of each primer μL^{-1}. Initial DNA denaturation and enzyme activation steps were performed at 95 °C for 30 s, annealing at 50 °C for 1 min and extension at 72 °C for 2 min, and a final extension for 10 min at 72 °C. The presence and yield of PCR product was monitored by 1% agarose gel electrophoresis at 200 V for 1 h in 1 × Tris-acetate-EDTA buffer and stained with ethidium bromide.

Cloning, RFLP and sequencing

A 16S rRNA gene clone library was constructed for the Plot A (zero day) sample to study the existing bacterial community structure of the pesticide-contaminated site. For this purpose, 16S rRNA gene amplicons from five independent PCRs were pooled, ligated into the pGEM-T plasmid vector (Promega, Madison, WI), and transformed into *Escherichia coli* JM109 electrocompetent cells according to the manufacturer's instructions. The transformed cells were plated on Luria–Bertani agar plates containing 100 $\mu g\,mL^{-1}$ Ampicillin, 40 $\mu g\,mL^{-1}$ isopropyl-β-D-thiogalactopyranoside, and 40 $\mu g\,mL^{-1}$ 5-bromo-4-chloro-3-indolyl-β-D-galactoside. White clones were cultivated to analyze their plasmid content and about 200 clones containing inserts of the correct size (1.5 kb) were stored in 10% glycerol at − 80 °C. Two restriction enzymes (*Rsa*I and *Hae*III) were selected for digestion of the clones out of six randomly selected tetrameric restriction enzymes, e.g. *Sau*3AI, *Msp*I, *Hinf*III, *Rsa*I, *Hae*III and *Hha*I. Digestions were performed for 2 h at 37 °C in 25 μL reaction volumes containing 10 μL of the PCR product solution, 2.5 μL of the incubation buffer (1 ×) and 0.3 μL of the restriction enzyme (New England Biolabs). The reaction products were run on a 2% agarose gel and the restriction pattern of the clones was compared. Various phylotypes were identified on the basis of similarity in the restriction digestion pattern of 183 individual clones. Rarefaction analysis (Tipper, 1999) was used to confirm that the number of clone types had been exhausted after screening 183 individual clones. We are aware that any given RFLP pattern may represent sequences from multiple phylogenetic groups and may therefore not represent a true phylotype in the traditional sense. Therefore, we use the term phylotype to indicate groups for richness calculations. Phylotype richness (S) was calculated as the total number of distinct RFLP patterns in a soil. The Shannon–Weiner diversity index (Margalef, 1958) was calculated as follows: $H = \Sigma(\rho_i)(\log_2\rho - i)$, where ρ represents the proportion of a distinct RFLP pattern relative to the sum of all distinct patterns. Evenness was calculated from the Shannon–Weiner diversity index: $E = H/H_{max}$ where $H_{max} = \log_2(S)$. Partial 16S rRNA gene sequences (at least 450 bp) of one representative member of each phylotype were obtained using 16S rRNA gene primers 27F and 357F with the ABI PRISM Big Dye Terminator Cycle Sequencing kit and an ABI 310 automated DNA sequencer (Applied Biosystems, Foster City, CA).

To obtain a phylogenetic relationship between the various phylotypes, one representative member of each phylotype was selected and reamplified, digested with *Hae*III and a unweighted pair group method using arithmetic mean based dendrogram was constructed using Image Master ID Elite (version 4.0) (Amersham Lifesciences, Bucks, UK). For amplification, crude lysates of the selected clones were generated by suspending them in 100 μL water and 1 μL of the lysate was used as template in a PCR reaction as described above.

T-RFLP procedure

During the soil bioremediation process, soil samples were collected at time zero (before inoculation), and after 3, 7, 10, 20 and 30 days and analyzed for community composition changes by T-RFLP. For this, PCR was carried out using fluorescently labeled forward and reverse primers. The forward primer 8f (5′-AgA gTT TgA TCC Tgg CTC Ag-3′) and reverse primer 926r (5′-TCA ATT CCT TTR AgT TT-3′) were labeled with IRDye700™ (infrared dye 700) and IRDye800™ (Infrared Dye 800), respectively. LI-COR (Lincoln, NE) has developed IRDye700™ and IRDye800™ with carbodiimide (CDI) active groups that covalently bind to thymine (T) and guanine (G) on single- and double-stranded nucleic acid. These dye-labeled primers were procured from MWG-Biotech (Ebersberg, Germany). The PCR reactions were carried out in triplicate for each sample and resulting products were pooled, ethanol precipitated and resuspended in 20 μL of sterile water prior to restriction digestion. Digestions were carried out separately with the endonucleases *Hae*III and *Msp*I for 3 h using 10 μL of DNA and 5 U of enzyme to produce a mixture of variable length end-labeled 16S rRNA gene fragments. The choice of the enzyme was made as described before. The digested PCR product was precipitated and carefully dissolved in 6 μL of Tris-EDTA buffer and finally mixed with 4.5 μL of formamide loading buffer that consisted of 97.5% formamide, 2.5% 0.5 M EDTA and 0.3% acidic fuchsine red. This mixture was heated at 93.5 °C for 3 min and immediately chilled on ice and 1 μL was loaded onto a polyacrylamide gel (25 cm) for electrophoresis in a LiCOR4200L sequencing apparatus. Electrophoresis was carried out at 1500 V in 25 cm long 8% polyacrylamide gels (urea 7 M, long ranger solution 8%, 1 × TBE) in 1 × TBE (tris base 890 mM, boric acid 890 mM, EDTA 20 mM, pH 8.3 at 25 °C). Molecular

Table 1. List of phylotypes obtained through RFLP studies on 16S rRNA gene of a pesticide-contaminated site

	Phylotype		Nearest neighbor	
Clone cluster	Representative clone no.	Accession no.	Accession no.	% Identity
Acidobacterium	ISCB-2	AY596161	AF013560	92
	ISCB-5	AY596117	AF388329	95
	ISCB-19	AY596159	AF432679	92
	ISCB-30	AY596123	AY395118	98
	ISCB-32	AY596122	AY632472	97
	ISCB-36	AY596120	AF009990	95
	ISCB-37	AY596145	BSPZ95721	97
Alphaproteobacterium	ISCB-7	AY596156	AF423296	95
	ISCB-13	AY596118	AY221076	97
	ISCB-14	AY596158	AF423211	96
	ISCB-88	AY596131	AY309194	97
	ISCB-18	AY596129	AY242633	98
	ISCB-3	AY596127	AJ786022	94
	ISCB-51	AY596132	AY268257	94
	ISCB-59	AY596153	AF395032	96
	ISCB-49	AY596137	AY214737	97
Gammaproteobacterium	ISCB-60	AY596154	AY217472	96
	ISCB-17	AY596119	AY221611	88
Betaproteobacterium	ISCB-52	AY596139	AY177375	96
	ISCB-53	AY596133	AY367029	94
	ISCB-100	AY596147	AF423222	96
Deltaproteobacterium	ISCB-16	AY596128	AJ252630	88
	ISCB-47	AY596136	AY632530	96
Actinobacterium	ISCB-4	AY596163	AY186808	89
	ISCB-25	AY596126	AJ244329	96
	ISCB-1	AY596140	AF408952	97
	ISCB-10	AY596141	AJ243870	93
	ISCB-21	AY596143	AY150868	97
	ISCB-72	AY596155	AJ532728	96
Planctomycetes	ISCB-9	AY596157	AF465657	95
	ISCB-35	AY596121	AF392773	96
	ISCB-46	AY596135	AY214912	94
	ISCB-50	AY596138	AY465315	96
Bacteroidetes	ISCB-55	AY596150	AJ252591	95
	ISCB-29	AY596124	AY491561	97
	ISCB-11	AY596164	AF289157	97
	ISCB-23	AY596130	AF523333	86
Chloroflexi	ISCB-56	AY596151	AY289483	90
	ISCB-95	AY596146	AY037557	95
	ISCB-115	AY596134	AY221060	94
Verrucomicrobia	ISCB-58	AY596152	AB179534	97
Gammatimonadetes	ISCB-107	AY596148	AY218639	95
	ISCB-118	AY596162	AF545658	95
Cyanobacteria	ISCB-20	AY596142	AB074509	99
Unclassified unculturable	ISCB-110	AY596149	AF443583	94
	ISCB-77	AY596144	AY507695	92

Accession numbers of the partial sequences of representative clones of each sequence have been listed along with accession numbers of the closest BLAST hit. According to the BLAST results the phylotypes have been grouped into clone clusters.

weight markers for T-RFLP analysis were purchased from Microzone Limited, West Sussex, UK. Banding patterns on T-RFLP images were analyzed by the program RFLPScan (v.2.0, Scanalytics Inc., Rockville, MD). Migration distances corrected for smiling and relative intensities of bands (I_r) were recorded in the program and exported to EXCEL (Microsoft, Redmond, WA). Changes in overall diversity were calculated as the dissimilatory index, $1-\Sigma(I_{r,i})^2$ for $I_i=1$, n, with n being the total number of defined bands in the T-RFLP. The relative band intensity

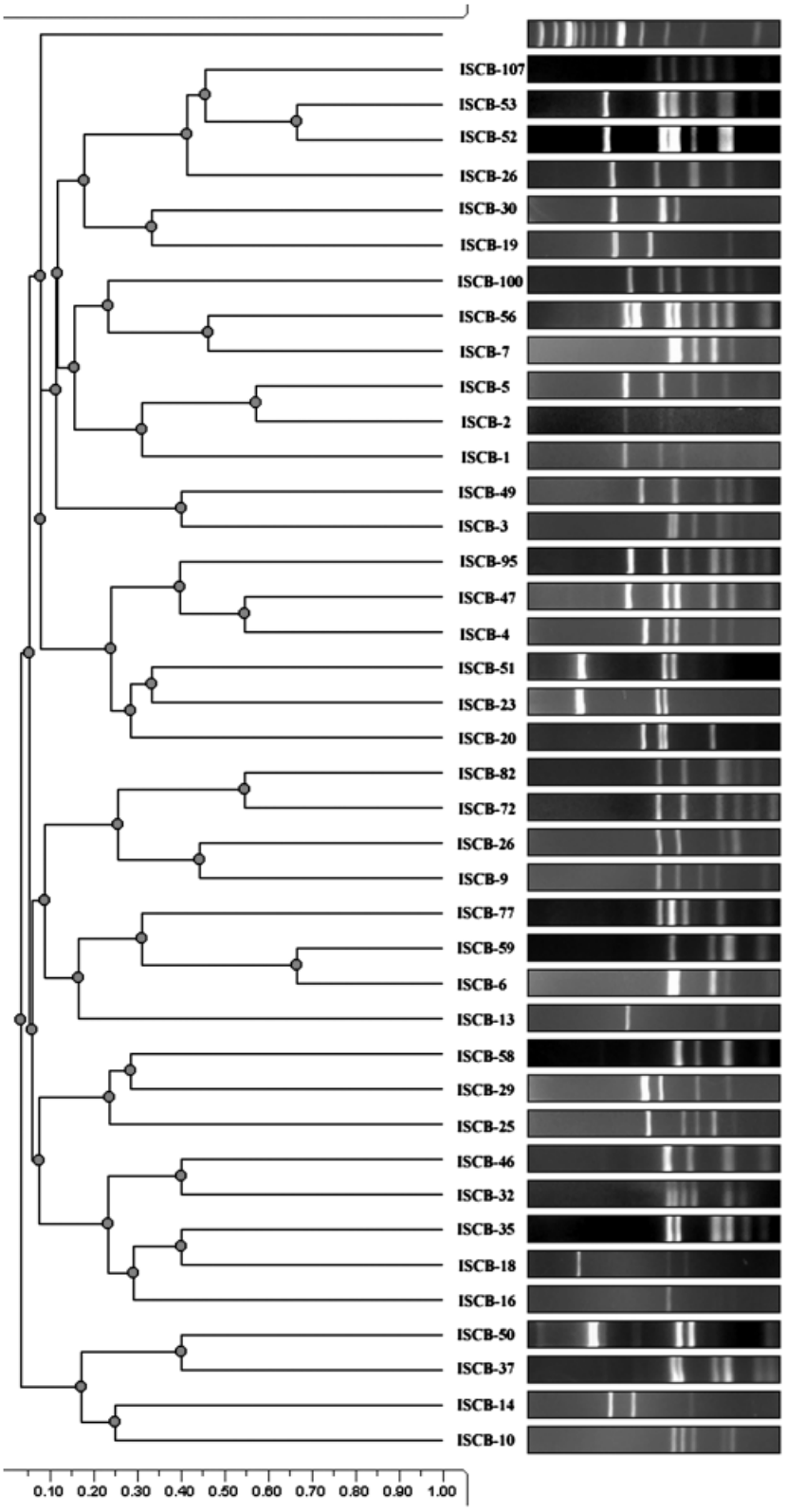

Fig. 1. Agarose-gel generated amplified ribosomal DNA restriction analysis dendrogram illustrating the relationship between some of the phylotypes obtained by comparing banding patterns of digested amplified 16S rRNA genes with *Hae*III endonuclease. The dendrogram was constructed using Image Master ID Elite (version 4.0) and grouped with unweighted pair group method using arithmetic mean.

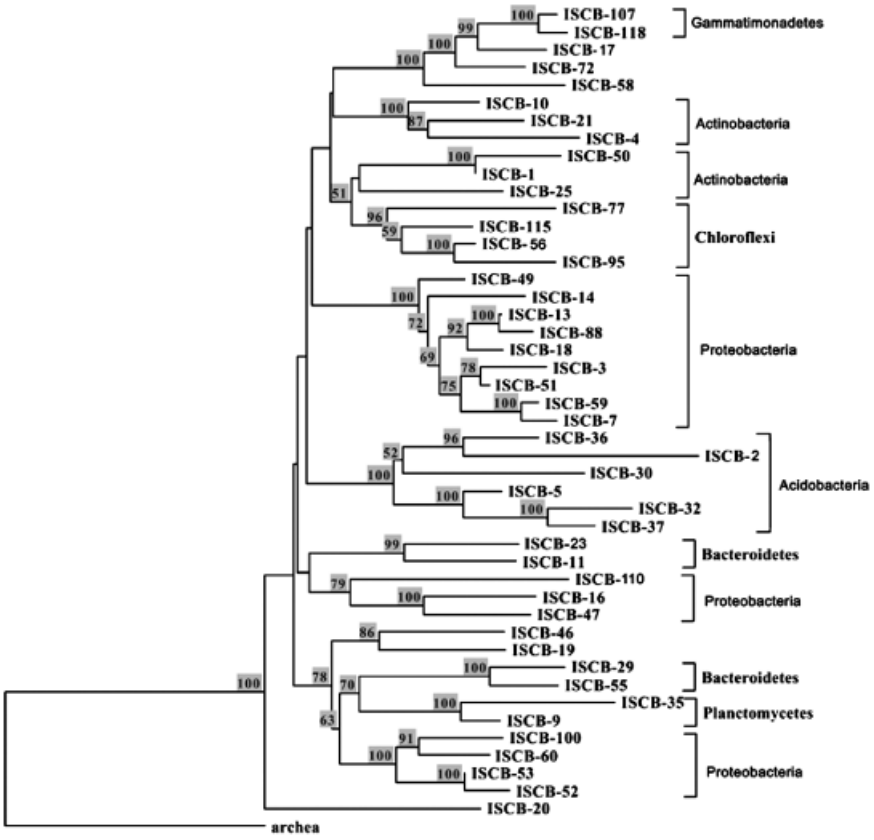

Fig. 2. Phylogenetic tree constructed for partial 16S rRNA gene of representative clones of each phylotype as suggested by restriction fragment length polymorphism data. A neighbor-joining analysis with Jukes–Cantor correction and bootstrap support was performed on the gene sequences. Bootstrap values are given at nodes when they exceed 50% and refer to the clusters to the right of each number. 16S rRNA gene of *Archea* has been taken as an outgroup.

data for the different time and sampling points were further analyzed by principal component analysis using the program R with functions PRINCOMP or DUDI PCA.

Analysis of the sequence data

Partial 16S rRNA gene sequences were initially analyzed using the BLAST search facility (www.ncbi.nlm.nih.gov/blast/blast.cgi) and RDPII analysis software (www.ce.msu.edu/RDP/html/analyses.html). The sequences were submitted to the NCBI (National Centre for Biotechnology and Biotechnology Information) and GenBank accession numbers were obtained for all of them. The closest match and GenBank accession numbers of each sequence are listed in Table 1. Clustal X (Thompson *et al.*, 1997) was used to align these sequences. Sequence dissimilarities were converted to evolutionary distances according to the method of Jukes & Cantor (1969). Dendrograms were constructed with a neighbor-joining algorithm using TREECON version 1.3b (Van de Peer & De Wachter, 1994).

Reagents and standards

p-Nitrophenol was obtained from Sigma Chemical Co. (St Louis, MO). All other chemicals were of highest purity grade available locally.

Results and discussion

Bacterial community structure of the pesticide-contaminated site

About 200 clones were screened, out of which 183 showed the presence of 16S rRNA gene inserts. Following digestion with two enzymes, *Hae*III and *Rsa*I, the clones were grouped into 46 distinct phylotypes on the basis of banding pattern similarities. The dendrogram in Fig. 1 shows the relatedness of some of the various phylotypes obtained following restriction digestion with *Hae*III. The rarefaction analysis curve indicated that the entire diversity has been sufficiently covered. For this community a Shannon's diversity index (H) was calculated of 2.23 and a species evenness (E) of 81.5. The evenness and dominance values approximate to the maximum possible values, as most of the sequence types were recovered only once.

Representative members of each phylotype were partially sequenced. The affiliation of clone sequences to those of described taxa indicate the presence of mainly aerobic species, belonging to *Proteobacteria*, although not always deducible with a high degree of confidence. Some 16S rRNA gene sequences point to facultative anaerobes (rhodopseudomonads) as well as obligate anaerobes, e.g. *Pelobacter*. Some members of *Proteobacteria* and *Actinobacteria* are

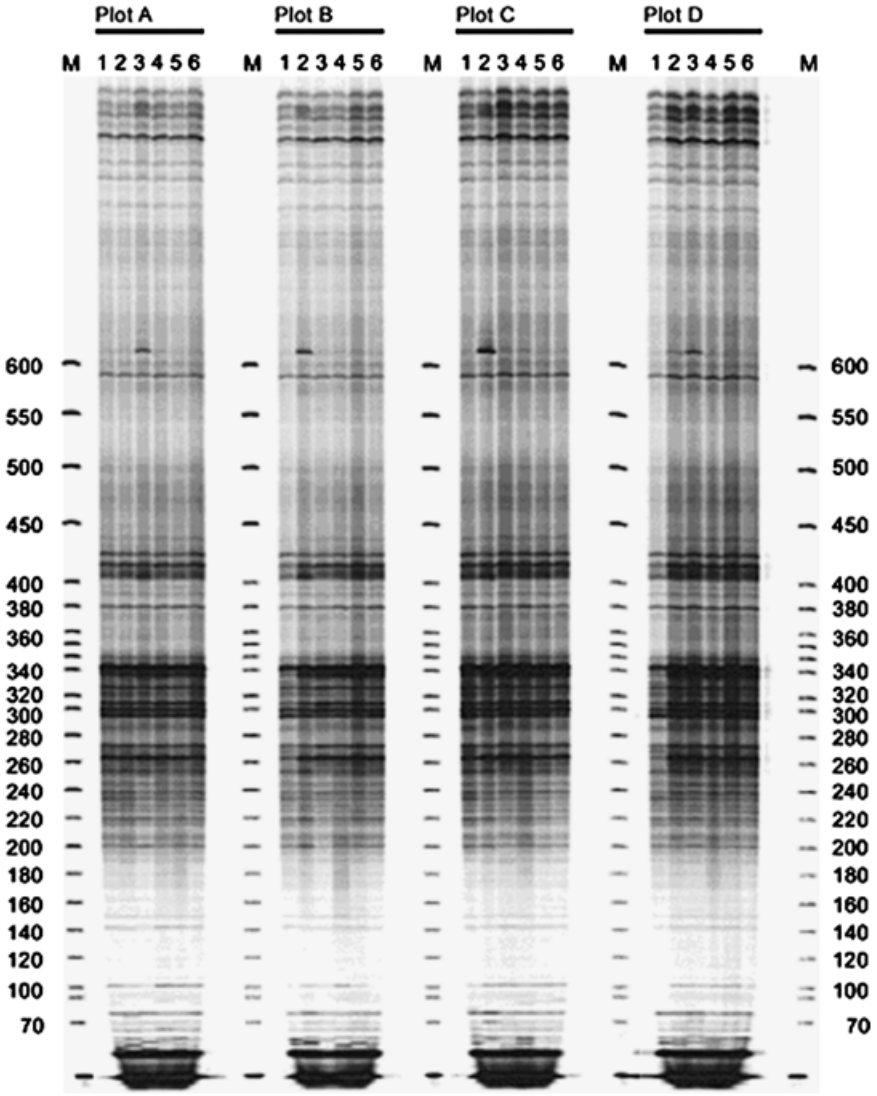

Fig. 3. Terminal restriction fragment length polymorphism pattern, with respect to the 8f primer, of the microbial community structure of the contaminated soil during bioremediation with *Arthrobacter protophormiae*. Lanes 1–6 represent different time points, i.e. 0, 3, 7, 10, 20 and 30 days, respectively. Lane M represents molecular weight marker in bases.

known to be important degraders of toxic compounds and might therefore be expected to form significant populations in such a pesticide-contaminated site (Kotouckova *et al.*, 2004; Leys *et al.*, 2005; Nagata *et al.*, 2005) . The other dominant taxa present in this contaminated site were *Acidobacterium* and *Planctomycetes*. The degree of similarity of clone sequences and the 16S rRNA gene sequence of its closest relative in the database was in the range 85–99% (Table 1). To summarize the total bacterial diversity at the site we grouped 16S rRNA gene sequences forming an exclusive cluster with its nearest neighbours (clone clusters) as shown by BLAST search, and clone clusters comprised one or more phylotypes. This method of analyzing diversity underestimates the actual number of species (Stackebrandt & Goebel, 1994) but serves as a helpful working summary. The sequences contain the variable regions (positions around 100–200) that discriminate well between closely related species. At the intraphylum level, individual species show about 8% difference because in different taxa the variable regions are located at different regions of the 16S rRNA gene (Stackebrandt & Rainey, 1995). For some pairs of organisms belonging to different phyla, similarities based upon partial sequences are slightly higher than those based upon almost complete sequences. With some exceptions, similar values determined for a partial sequence of 16S rRNA gene differed significantly among taxa. Therefore, in the phylogenetic tree (Fig. 2) most of the related sequences could be grouped as distinct branches, although some sequences have shown anomalous clustering.

On the basis of complete 16S rRNA gene sequences a bin similarity value of 97.5% indicates the presence of two distinct species (Stackebrandt & Goebel, 1994). As differences in similarity values between partial and almost complete sequences are less significant for highly related

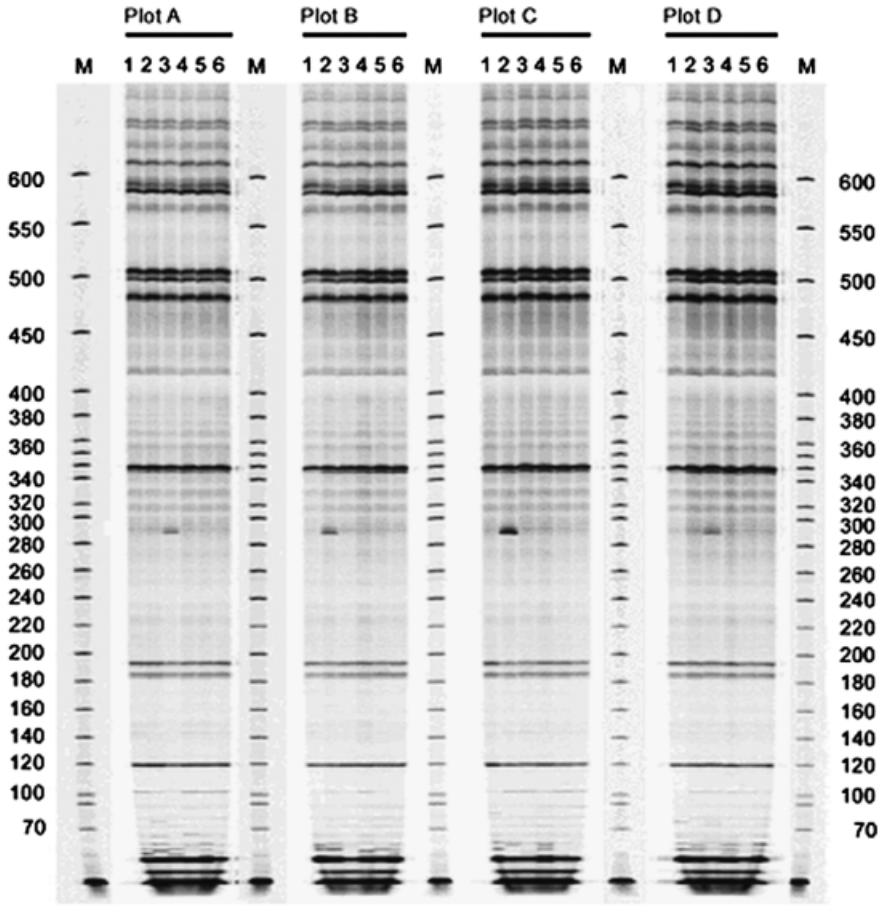

Fig. 4. Terminal restriction fragment length polymorphism pattern, with respect to the 926f primer, of the microbial community structure of the contaminated soil during bioremediation with *Arthrobacter protophormiae*. Lanes 1–6 represent different time points, i.e. 0, 3, 7, 10, 20 and 30 days, respectively. Lane M represents molecular weight marker in bases.

organisms than for unrelated organisms, it is assumed that the level of ∼98% similarity to its nearest neighbor signals the presence of potentially new species thriving in the pesticide-contaminated site (Brambilla *et al.*, 2001). Clone numbers 101 and 77 show less than 95% similarity to their nearest neighbour and may indicate the presence of some typical species found solely in that particular site.

Depletion of PNP and stability of *Arthrobacter protophormiae*

To assess the efficiency and suitability of the bioremediation technique as well as the organism used for this purpose, PNP depletion in the experimental plot as compared to the control plots was determined and survival of the introduced organism was monitored. It is important to monitor the fate of the inoculant population to determine the survival of introduced bacteria as well as to determine its influence on the ecosystem and soil functionality. In plot D, to which *A. protophormiae* immobilized on corn-cob was added, the number of viable *A. protophormiae* cells remained essentially stable throughout a period of 30 days (Labana *et al.*, 2005). In plot C, to which freely suspended cells of *A. protophormiae* were added, the population started declining after 5 days, and after 30 days the decrease was more than two log units compared to the initial bacterial count of 2×10^6 CFU g^{-1} soil (Labana *et al.*, 2005).

Depletion of *p*-nitrophenol in the soil under field conditions was clearly stimulated by the addition of the bacteria (Maymo-Gatell & Schraa, 1997). In the plot to which immobilized *A. protophormiae* was added (Plot D), *p*-nitrophenol was completely depleted within 5 days, whereas free cells (Plot C) could deplete only 75% of PNP in this time; in control plots the PNP persisted even after 30 days.

Bacterial population dynamics within the plots during the process of bioremediation

Fifty-nine bands could be discerned on T-RFLP gels with the 8f700 marker and 23 on gels with the 926r800 marker, both of which were subsequently used in statistical analysis. No obvious visual changes were observed in the T-RFLP profiles of the contaminated soil during the process with respect to the forward primer (8f) (Fig. 3) except for a major T-RF appearing at the samples collected at either day 3 or day 7 from all the plots. The corresponding T-RFs (∼290 bases)

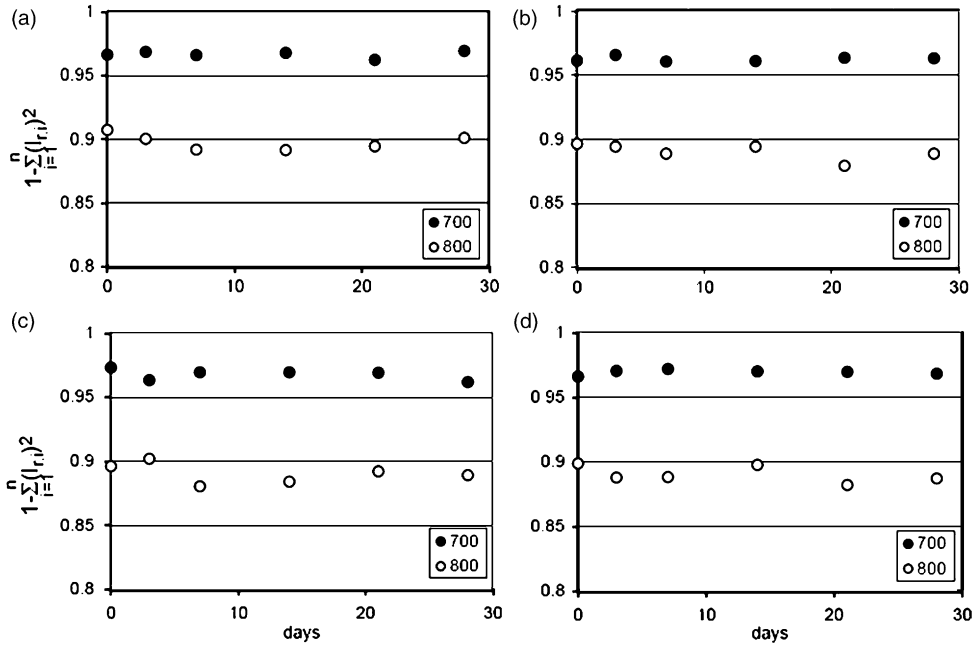

Fig. 5. Dissimilatory diversity indices calculated on the basis of the relative band intensities in the 8f700 and 926r800 terminal restriction fragment length polymorphisms. (a) Uninoculated control, (b) control plot, amended with corn cob powder, (c) plot inoculated with cells in liquid culture, (D) plot inoculated with cells immobilized in corn cob powder.

Table 2. Number of significantly different ($P < 0.05$)* pairwise compared relative band intensities

Treatment pair	700 profile	800 profile
A–B	9	3
A–C	14	3
A–D	16	7
B–C	14	4
B–D	20	6
C–D	8	3
	$n = 56$	$n = 23$

*T-test pairwise determination.

also appeared in the T-RFLP profile with respect to the reverse primer (926r) (Fig. 4). These T-RFs (with respect to 926r primer) that were generated with *Hae*III digestion did not match with the T-RFs generated from *in silico* amplification and digestions (*Hae*III, *Msp*I and *Rsa*I) of RDP II alignments (version 8.0) and hence cannot be identified. Specific T-RFs originating from the inoculated *A. protophormiae* strain RKJ100 could not be discerned on the profiles (229 bp with the forward labeled primer $8f_{700}$ and 25 bp from the reverse labeled primer $926r_{800}$). The dissimilatory diversity index generated on the basis of the T-RFLPs both for the forward and reverse primer did not change significantly over the course of the incubation, nor between different treatments (Fig. 5). In the relative band intensities of various bands in pairwise *t*-test comparisons of similar bands between treatments (relative band intensity averaged for all time sampling points) some minor changes were observed but they were not consistent for a particular band size or treatment (Table 2). Finally, principal component analysis of the relative band intensities for all the generated bands in the $8f_{700}$ and $926r_{800}$ profiles showed a slight clustering of treatments A and B vs. C and D in the 700-generated profile (Fig. 6a), whereas the 800-generated profile showed a tendency for the first sampling point of all treatments to be different from the rest (samples A1, B1, C1 and D1 in Fig. 6b). Taken together, we conclude that none of the treatments (strain inoculation and/or addition of corncob powder) significantly influenced the parameters species richness and evenness of the community composition in the soils.

The study demonstrated for the first time that bioaugmentation of contaminated soil with *A. protophormiae* stimulated complete degradation of *p*-nitrophenol under field conditions. Analysis of the bacterial community composition by T-RFLP for the amplified 16S rRNA gene showed no significant changes in total microbial diversity parameters during the bioremediation process, except for a few individual visual bands on T-RFLP (Figs 3 and 4). Other subtle changes were revealed by comparing relative band intensities, which seemed to suggest a differentiation between inoculated and noninoculated soils (Fig. 6, Table 2). These results suggest that changes in community were not as significant as compared to results obtained during degradation of phenol in a fed-batch culture by a nonadapted complex microbial community (Guieysse *et al.*, 2001), and phenol- and phenol-plus-trichloroethene-fed sequencing batch reactors by a phenol degrading community (Ayala-Del-Rio *et al.*, 2004). In both these studies community analysis by T-RFLP of the 16S rRNA genes showed changes

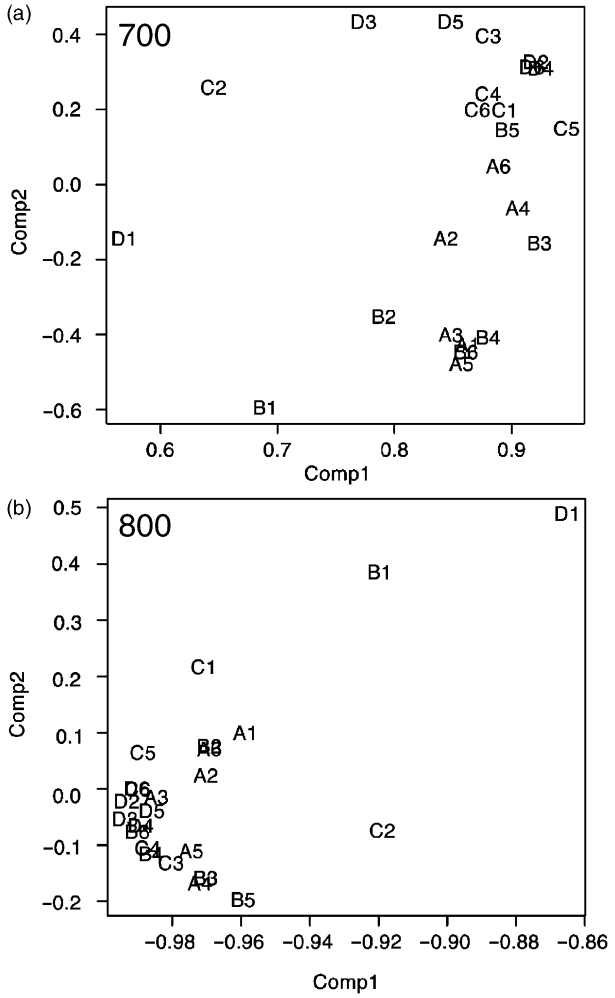

Fig. 6. Nonscaled principal component analysis plots of the two first components explaining 83% (a) and 97% (b) of the total variance in the relative band intensities among all bands in the terminal restriction fragment length polymorphisms. Data input from the bands generated with (a) the IRD-700 labeled end ($n = 59$) and (b) the IRD-800 labeled end ($n = 23$). Data point labeling correspond to the four different treatments and sampling times (see legend to Figs 3 and 4).

in community composition during the initial phases of the experiment and became stable thereafter. However, both these *in vitro* studies lasted for over 3 months, whereas in this study complete bioremediation of *p*-nitrophenol-contaminated soil using *A. protophormiae* took only few weeks under natural conditions. Bioremediation using *A. protophormiae* RKJ100 may thus offer a solution for contaminated sites where *p*-nitrophenol degradation does not occur naturally or is too slow to be practical. This study also showed the practicality of using T-RFLP for investigating complex community structure on a time scale that requires rapid analysis and in a sample where the number of bacterial species may be quite large. This study can also serve as a model for understanding the degradation of other, more complex or structurally related organic compounds in soil.

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