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Evolution of a degradative bacterial consortium during the enrichment of naphtha solvent

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L. CAVALCA, A. CONFALONIERI, S. LARCHER AND V. ANDREONI. 2000. A microbial mixed culture able to degrade naphtha solvent, a model of hydrocarbon aromatic mixture, was isolated from a hydrocarbon-polluted soil. Composition of the population was monitored by phenotypic and molecular methods applied on soil DNA, on whole enrichment culture DNA, and on 85 isolated strains. Strains were characterized for their 16S rDNA restriction profiles and for their random amplified polymorphic DNA profiles. Catabolic capabilities were monitored by phenotypic traits and by PCR assays for the presence of the catabolic genes methyl mono-oxygenase (*xyl*A,M), catechol 2,3 dioxygenase (*xyl*E) and toluene dioxygenase (*tod*C1) of TOL and TOD pathways. Different haplotypes belonging to *Pseudomonas putida*, *Ps. aureofaciens* and *Ps. aeruginosa* were found to degrade aromatic compounds and naphtha solvent. The intrinsic catabolic activity of the microbial population of the polluted site was detected by PCR amplification of the *xyl*E gene directly from soil DNA.

INTRODUCTION

Biological treatments for the removal of organic compounds from contaminated soil, water and reactors are based on the action of degrading microbial communities. When they are added to speed up degradation in contaminated environments, the assessment of time and efficiency of the biological process is linked to the evolution of bacterial consortia in terms of composition and catabolic activity. A combination of phenotypic and nucleic acid analyses in microbial ecology can address questions on type and abundance of specific degrading bacteria, describing evolutionary patterns in natural populations (Olsen *et al.* 1994). Moreover, the detection of catabolic genes directly in a contaminated environment can be predictive for the assessment of a bioremediation strategy (Walia *et al.* 1990; Joshi and Walia 1996). Restriction fragment length polymorphism (RFLP) of amplified 16S rDNA, largely used to characterize bacterial isolates (Heyndrickx *et al.* 1996), has also been applied to describe natural communities obtained from different habitats and from enrichment procedure (Liu *et al.* 1997; Massol-Deyà *et al.* 1997). In a recent paper Ward *et al.* (1997) pointed out that it is possible to monitor those cultures by applying molecular methods besides isolation and cultivation of strains, although comprehension of functions of an individual organism in a community is a requisite to improve the efficiency of the degradation in a system.

This work describes the evolution of a degrading consortium obtained from a polluted soil during the enrichment procedure characterizing both isolates and whole mixed culture by phenotypic and molecular methods.

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MATERIALS AND METHODS

Enrichment procedure

A 12-kg soil sample, used to select the bacterial culture analysed in the study, was collected at a depth of 18 m as an undisturbed core during the installation of vacuum wells of a bioventing plant for the bioremediation of the soil contaminated by BTEX (benzene, toluene, ethylbenzene and xylenes) and naphtha solvent from the grounds of a paint factory. A total of 50 g of soil was mixed with 450 ml of sterile NaCl solution (9 g l^{-1}) and shaken for 2 h at 30 °C. The soil suspension (10 ml) was seeded in a flask containing 200 ml of M9 mineral medium (Kunz and Chapman 1981) supplemented with 250 mg l^{-1} of naphtha solvent and incubated at 30 °C for 7 d on an alternative shaker. The culture was adapted to degrade the aromatic mixture by re-inoculating 1 ml of the cell suspension in 20 ml of M9 mineral medium supplemented with 250 mg l^{-1} of

naphtha solvent and then incubated at 30 °C. The adapted cells were subjected to 10 subsequent passages.

Strains and growth conditions

The culture, studied at the beginning of the enrichment procedure and after 10 passages, was serially diluted and seeded on plate count agar (Difco, Detroit, MI, USA). After 5 d incubation at 30 °C, colonies that appeared on the plates were isolated. Growth characteristics of pure isolates were studied in 100 ml flasks containing 20 ml of M9 mineral medium with 5 mg of various aromatic substrates as sole carbon and energy source and incubated at 30 °C. Growth and substrate utilization were registered as positive when the cultures became turbid in two subsequent transplants. Strains were maintained in 20% glycerol stock at – 20 °C after growth on aromatic compound. The isolates were identified according to Gram staining, catalase and oxidase tests, and API 20NE system (BioMérieux, Marcy-l'Etoile, France).

Catechol 2,3-dioxygenase (C23O) -positive colonies were identified by spraying plates with a 1-mol l^{-1} catechol solution. A positive reaction was indicated by the production of a yellow colouration due to the formation of 2-hydroxymuconic semialdehyde (Gibson 1971).

Degradation studies by enrichment culture and isolates

In order to study the degradation of naphtha solvent, the mixed culture was grown as shaken liquid cultures in 100 ml vials, each containing 15 ml of M9 mineral medium supplemented with 250 mg l^{-1} of naphtha solvent. Each vial was inoculated with 1 ml of culture suspension to an optical density at 600 nm ($O.D._{600}$) of 0·15, after growth on the same medium. The vials were then sealed with Teflon-coated grey butyl rubber stoppers and aluminium crimps and incubated at 30 °C on an alternative shaker. Uninoculated medium controls were included. Three replicates were used. After different incubation times, appropriate sample and control vials were used to determine the extent of the degradation and the production of metabolites. Samples were mixed with an equal volume of methanol, withdrawn from vials using a sterile syringe and stored at – 20 °C until HPLC qualitative analysis.

The degradation of *m*-xylene by *Pseudomonas putida* CM 337, and of benzene and toluene by *Pseudomonas aureofaciens* CM 332, chosen as representative strains harbouring two different catabolic pathways, was studied with resting cells induced with the appropriate substrate. After growing for 18 h, cells were harvested by centrifugation at 5 °C for 10 min at 15 000 *g*, washed three times and resuspended in 10 mol l^{-1} phosphate buffer, pH 7·0, to obtain an $O.D._{600}$ of 1·77. The reaction was carried out in 100 ml vials containing 16 ml of the reaction mixture, which consisted of a 1-ml cell suspension in 10 mol l^{-1} phosphate buffer, appropriate micromoles of substrate, and 15 ml of 10-mol l^{-1} phosphate buffer (final $O.D._{600}$ of 0·24). Vials were sealed with Teflon-faced butyl stoppers, incubated at 30 °C and treated as described. Two replicates were used for each trial, and uninoculated medium controls were included.

Analytical methods

A Beckman model DU 640 spectrophotometer (Beckman Instruments, Fullerton, CA, USA) was used for determination of cell growth and protein concentrations. Protein contents were determined by the method of Bradford (1976). HPLC analyses were carried out according to Origgi *et al.* (1997).

Chemicals

Benzene, toluene, and *o*-, *m*- and *p*-isomers of xylene, propylbenzene and 1,2,4-trimethylbenzene (purity > 99%) and 1,3,5-trimethylbenzene (purity > 98%) were purchased from Merck (Darmstadt, Germany); 1-ethyl-2-methylbenzene, 1-ethyl-3-ethylbenzene, ethylbenzene (purity > 99%) and 1-ethyl-4-methylbenzene (purity > 90%) from Aldrich Chemie (Steinheim, Germany); and 1,2,3-trimethylbenzene (purity > 95%) from Flucka Chemie AG (Switzerland). Naphtha solvent (Chimedi Marketing Services, Sesto San Giovanni, Italy) was made up of: 1-ethenyl-2-methylbenzene (30%), isomers of trimethylbenzene (15%), 1-ethyl-4-methylbenzene (20%), propylbenzene (15%), styrene (10%), ethylbenzene (5%) and other aromatic compounds with various alkyl groups in the range of C_9–C_{10} (5%).

Molecular methods

DNA extraction. Total DNAs from the whole enrichment culture (grown on M9 liquid medium and naphtha solvent) and from pure isolates (grown on plate count broth [Difco]) were extracted by Chelex 100®-SDS boiling lysis (De Lamballerie *et al.* 1992). Total DNA from soil sample was extracted by the freeze-thaw and SDS lysis method (Tsai and Olson 1992) with minimal modifications.

Primers and polymerase chain reaction (PCR) conditions. *Xyl*A,M genes were amplified in a 25-μl reaction volume made up of: 2·5 μl of 10 × buffer, 2·5 mmol l^{-1} of $MgCl_2$, 200 μmol l^{-1} of each nucleotide (dNTPs, Pharmacia Applied Biotech, Uppsala, Sweden), 7·5% dimethylsulphoxide (Sigma, St Louis, MO, USA), 0·25 μmol l^{-1} forward

primer (5′-GGG TTT GGC TTG GGG CGG CAA CA-3′) and 0·25 μmol l^{-1} reverse primer (5′-CCC GCG CAA CAC CAA GTC GAA CGA-3′). After a hot start denaturing step at 94 °C for 3 min, 1 U of *Taq* polymerase was added. The following thermal profile was then used: 94 °C for 1 min, annealing at 57 °C for 1 min, 72 °C for 2 min for 35 cycles, an extension step at 72 °C for 10 min. Amplification of *xyl*E was performed in a 50-μl mixture containing: 5 μl of 10 × buffer, 1·25 mmol l^{-1} $MgCl_2$, 200 μmol l^{-1} dNTPs (Pharmacia Applied Biotech), 1·2 μmol l^{-1} forward primer (5′-GTN YTN GGN TTY TAY YTN GCN GAR-3′) and 1·2 μmol l^{-1} reverse primer (5′-NCK RTT NCC NSW NGG RTC RAA-3′), 1 U of *Taq* polymerase, and 2 μl of template DNA. The thermal profile was as follows: 94 °C for 1 min, *Taq* was then added to the PCR mixture and followed by 30 cycles at 92 °C 40 s, 53 °C 50 s and 72 °C 1 min, and by one cycle at 72 °C for 7 min. Amplification of the internal fragment of the *tod*C1 gene was carried out according to the protocol reported by Whyte *et al.* (1996).

Randomly amplified polymorphic DNA (RAPD) analysis. RAPD of all isolated bacteria was performed by adding 1 μl of template DNA to a 24-μl PCR mixture consisting of 2·5 μl of 10 × buffer, 1·25 mmol l^{-1} $MgCl_2$, 200 μmol l^{-1} dNTPs (Pharmacia Applied Biotech), 1 U of *Taq*-polymerase and 1·2 μmol l^{-1} of three individual 10-mer primers (Hansen and Winding 1997) used one at a time: OPA 2 (5′-TGC CGA GTC G-3′), OPA 9 (5′-GGG TAA CGC C-3′), OPA 10 (5′-GTG CCG AGC TG-3′). The thermal profile was made up of 45 cycles: 5 s denaturing at 94 °C, 1 min annealing at 32 °C and 2 min extension at 72 °C.

16S rDNA PCR-RFLP analysis. 16S rDNAs were amplified as previously described (Andreoni *et al.* 1998). All the PCR reagents were supplied by Promega (Madison, WI, USA) unless otherwise stated. Amplifications were all carried out on a Cetus 2400 apparatus (PE, Applied Biosystems, Foster City, CA, USA). Five microlitres of the amplified products was analysed on 1·5% agarose gels, visualized by a standard procedure (Sambrook *et al.* 1989) and photographed by a Polaroid camera. Eight microlitres of the amplified products was digested with 5 U of four restriction endonucleases, *Hha*I, *Hinf*I, *Rsa*I and *Taq*I (Pharmacia Applied Biotech). Each sample was incubated separately in the presence of the corresponding enzyme buffer overnight at 37 °C. Ten microlitres of restriction products was separated on 3% agarose gel and processed as already stated. Restriction profiles were compared with published sequences analysed by free program WebCutter© (www.medkem.gu.se/cgi-bin/cutter/cutter). Accession numbers of 16S rDNA sequences used in the study are: Z76656(*Ps. aureofaciens*), Z76651(*Ps. aeruginosa*), X93997(*Ps. putida*), X95923(*Xanthomonas maltophilia*), and M22509(*Alcaligenes xylosoxidans denitrificans*).

Tod*C1 and* bed*C1 restriction analysis.* Owing to a high homology at primer annealing sites, we could have indeed amplified the *tod*C1 gene or the *bed*C1 gene. The compared *Hpa*II maps of amplicones showed the presence of point mutations in four to five *Hpa*II restriction sites, which made it possible to ascertain the origin of the amplified product. Restriction analysis of the amplified products was carried out to evaluate whether strains could carry *tod*C1 or *bed*C1 genes. The *Hpa*II restriction maps of the *tod*C1 gene from *Ps. putida* F1 (GenBank accession number, J04996) and from *bed*C1gene of *Ps. putida* ML2(GenBank accession number, L04642) were obtained by using WebCutter© software.

Southern hybridization. Catabolic gene PCR products were tested for nucleotide homology to known gene sequences through Southern analysis. A probe for *xyl*E was produced by PCR from the *Ps. aeruginosa* strain C56 previously isolated in our laboratory, *tod*C1 from *Escherichia coli* pDTG601 (Zylstra and Gibson 1989), and *xyl*A,M from *E. coli* pGSH2836 (Harayama et al. 1989). PCR fragments of *xyl*A,M, *xyl*E and *tod*C1 genes of isolated strains were transferred to Nylon membranes (Boehringer Mannheim, Mannheim, Germany) by osmotic pressure. The membranes were then washed in 5 × sodium saline citrate and air dried. DNA was cross-linked to the membrane by u.v. irradiation for 3 min. Hybridization was carried out under conditions of high stringency (0·1 × sodium saline citrate, 0·1% SDS, 68 °C), and detection was realized using the Dig Luminescent Detection Kit and CDP Star as chemiluminescent substrate (Boehringer Mannheim). The reaction conditions were as described above, except that the dNTPs were as follows: dATP, dCTP and dGTP, 200 mmol l^{-1} each; 26 mmol l^{-1} dTTP; 13 mmol l^{-1} Dig 11-dUTP (Boehringer Mannheim). For each probing, 2 μl of the amplified product was heat-denatured and incorporated in 10 ml of hybridization solution.

Statistical methods

Sizes of fragments were estimated by using a linear regression equation between the molecular mass of the DNA ladder and the log of the distance covered by fragments within the same gel run. Similarity among strains was estimated from the proportion of shared restriction fragments of 16S rDNA or of RAPD amplified bands and examined using Jaccard's coefficient to compute the distance matrix (NTSYSpc 2·0, Exeter Software, New York, NY, USA).

The dendrogram was constructed from the distance matrix by the unweighted pair group method with an arithmetic mean (UPGMA) (Sneath and Sokal 1973) using the NTSYS-pc 2·0 analysis package (Rohlf 1987).

RESULTS

Catabolic genes in polluted soil

Before enriching the culture, the *xyl*E gene, which codes for C23O and is responsible for the *meta* ring cleavage of toluene, *m*-xylene and *p*-xylene, was detected by PCR amplification of DNA obtained from soil used as inoculum and predicted the presence of genes for the mineralization of aromatic hydrocarbons within the microflora of the contaminated site. Also DNA extracted from the whole bacterial community during subsequent enrichment steps was positive to the *xyl*E probe indicating that the consortium was still maintaining this ability.

Naphtha solvent degradation

Degradation of naphtha solvent by the mixed culture studied at the beginning and after 10 subsequent passages, qualitatively monitored by HPLC analyses, is reported in Fig. 1. At the beginning of the enrichment procedure, the fraction corresponding to 3-ethyltoluene, 4-ethyltoluene, propylbenzene and 1,2,4-trimethylbenzene was partially degraded in 30 d (Fig. 1b). Utilization of the naphtha components was accompanied by the formation of two metabolites, m_1 and m_2. The m_1 metabolite, on the basis of HPLC retention time of an authentic sample (6·4 min), could correspond to 3,4-dimethylbenzoic acid resulting from the degradation of 1,2,4-trimethylbenzene. Other naphtha fractions such as 1,2,3-trimethylbenzene, 1,3,5-trimethylbenzene and 1-ethenyl-4-methylbenzene were not used. Bacterial culture at the 10th passage completely degraded in 24 h the fraction corresponding to 3-ethyltoluene, 4-ethyltoluene, propylbenzene and 1,2,4-trimethylbenzene, which represented 40% of the total naphtha mixture (Fig. 1c). The degradation capability of the consortium did not change subsequently, suggesting that it was stable in its catabolic activities.

Characterization of microbial isolates

From early transplant on naphtha, 14 strains were isolated according to their different colony morphology to briefly describe the diversity of bacterial genera present in the consortium. The isolates were identified to belong to species normally retrieved in soils: *Ps. aureofaciens*, *Ps. aeruginosa*, *Ps. putida*, *Alc. xylosoxidans denitrificans*, *X. maltophilia* and one unidentified Gram-positive strain. The restriction analysis by using four endonucleases, *Hha*I, *Rsa*I, *Hinf*I and *Taq*I, confirmed the phenotypic characterization, and the strains were grouped in six 16S rDNA types. The map location of restriction sites in the 16S rDNA genes were consistent with other known 16S rDNA

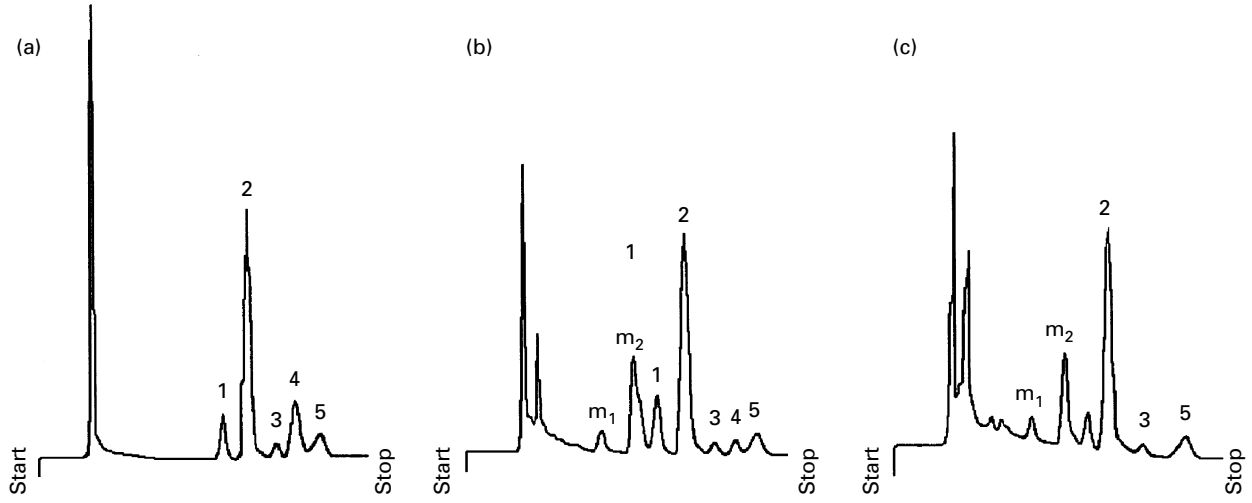

Fig. 1 HPLC chromatograms showing the degradation of naphtha solvent by the enrichment culture at zero time (a); after 30 d incubation by the early passage culture (b); and after 24 h incubation by the 10th passage culture (c). Peaks: unidentified compound (1), 1-ethenyl,2-methylbenzene (2), 1,2,3-trimethylbenzene + 2-ethyltoluene (3), 1,2,4-trimethylbenzene + 3-ethyltoluene + 4-ethyltoluene + propylbenzene (4), 1,3,5-trimethylbenzene (5), 3,4-dimethylbenzoic acid (m_1), unidentified metabolite (m_2)

Table 1 Growth characteristics of degrading strains isolated at an early stage of enrichment and PCR amplification of catabolic genes

Strain	Aromatic compounds													PCR amplifications		
	Naphtha	T	*o*-X	*m*-X	*p*-X	B	EB	n-PB	iso-PB	1,2,4 TMB	1,2,3 TMB	1,3,5 TMB	2-ET	3-ET	4-ET	*Xyl*A,M	*Xyl*E	*Tod*C1
Ps. aeruginosa CM323	+	+	−	−	−	−	+	+	+	+	−	−	−	−	+	−	−	+
Ps. putida CM337	+	+	−	+	+	−	−	−	−	+	+	−	−	−	−	+	+	−
Ps. aureofaciens CM312	+	+	−	−	−	+	+	+	−	−	+	−	−	+	−	−	−	−
Ps. aureofaciens CM332	+	+	−	−	−	+	+	+	+	+	−	+	−	−	+	−	−	+
Ps. aureofaciens CM334	+	+	−	−	−	−	+	+	+	−	+	−	−	−	−	−	−	−

T – toluene, X – xylene, B – benzene, EB – ethylbenzene, PB – propylbenzene, TMB – trimethylbenzene, ET – ethyltoluene
+ – growth after a 3-d incubation ($O.D._{600nm} > 0{\cdot}5$), ± – scarce growth ($O.D._{600nm} < 0{\cdot}2$), − – absence of growth.

sequences of the *Pseudomonas* genus previously studied (Laguerre *et al.* 1994) and by type strain sequence data reported in GenBank. Catabolic activities of the 14 isolates were evaluated by recording aerobic growth responses on naphtha solvent, naphtha constituents and on BTEX, which were prevalent contaminants of the soil. Strains belonging to *Alc. xylosoxidans denitrificans*, *X. maltophilia* and to the Gram-positive group did not show any degradation abilities.

*Xyl*A,M, *xyl*E and *tod*C1 genes were specifically amplified in degrading isolates (Table 1). *Ps. putida* CM337 gave amplifications with *xyl*A,M and *xyl*E probes but not with the *tod*C1 probe. *m*-Xylene degradation by resting cells of the *Ps. putida* CM337 grown on *m*-xylene occurred with a degradation rate of 67·20 $nmol\,min^{-1}$ (mg protein)$^{-1}$ through the formation of 3-methylbenzoic acid. The *Ps. aureofaciens* strain CM332 and the *Ps. aeruginosa* strain CM323 gave amplification of the *tod*C1 but not of *xyl*A,M and *xyl*E genes. Resting cells of the *Ps. aureofaciens* CM 332 grown on benzene oxidized benzene with a degradation rate of 62·31 $nmol\,min^{-1}$ (mg protein)$^{-1}$ and produced *cis*-3,5-cyclohexadiene-1,2-diol, catechol and phenol. Toluene was oxidized via 3-methylcatechol, with a degradation rate of 160·84 $nmol\,min^{-1}$ (mg protein)$^{-1}$.

Seventy-one strains were isolated at the 10th passage, and the degrading microbial community was characterized for species composition to evaluate whether the enhanced degradative capability was correlated to a shift within the microbial consortium from the early to the 10th transplant. 16S rDNA restriction analysis showed that the microbial population at this stage was made up of four species. 16S rDNA restriction profiles of the 71 isolates were compared to the 16S rDNA fingerprints of the classified strains previously isolated at the early stage of the enrichment procedure. According to the comparisons, this consortium was made up of: 33 strains of *Ps. aeruginosa*, 24 strains of *Ps. putida*, nine strains of *Alc. xylosoxidans denitrificans* and five strains of *Ps. aureofaciens. X. maltophilia* and Gram-positive strains were no longer isolated. The distribution of catabolic genes in the 71 isolates composing the selected culture was studied (Fig. 2). The *tod*C1 gene for toluene dioxygenase was retrieved in the 24 *Ps. putida*, in the five *Ps. aureofaciens*, in the nine *Alc. xylosoxidans denitrificans*, and in 12 of the 33 *Ps. aeruginosa* isolates. Five *Ps. Putida* strains (CM2, CM6, CM40, CM61, CM62), which had already shown *tod*C1 amplification were found to possess also *xyl*A,M and *xyl*E genes, hence harbouring two catabolic pathways.

The *Hpa*II restriction profile of *tod*C1 putative genes amplified from isolated strains was identical to that of the *tod*C1 amplified gene from *Ps. putida* F1. This allowed us to ascertain that they possessed a toluene dioxygenase, which conferred benzene and toluene-degrading pheno-

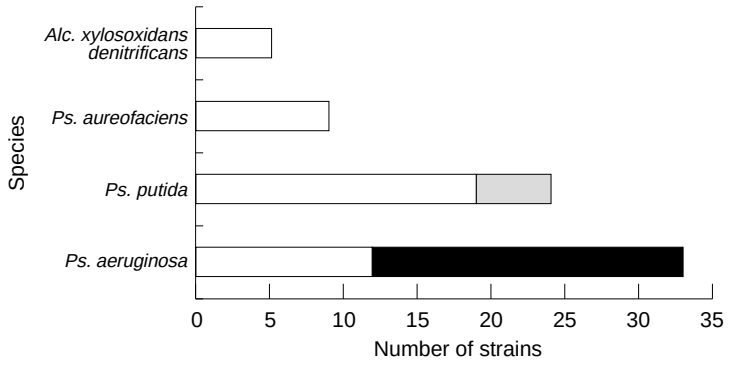

Fig. 2 Microbial species present in the mixed culture at the 10th passage and distribution of catabolic genes among the 71 isolates. *Tod*C1 (□), *Xyl*A,M, *Xyl*E and *Tod*C1 (▦), no genes amplified (■)

types, and not a benzene dioxygenase specific for benzene degradation.

A *meta* ring cleavage activity was confirmed by a positive spot test in all the strains carrying a TOL-like or a TOD-like pathway. Twenty-one *Ps. aeruginosa* strains negative to catabolic genes did not present any *meta* ring cleavage activity.

Genetic diversity of the degrading community by RAPD analysis

All 71 isolates were characterized by RAPD analysis to describe the genetic structure of the degrading community and evaluate whether the selective pressure of the naphtha solvent led to the selection of particular best fitting strains. Three different decamers (OPA2, OPA9 and OPA10) allowed the discrimination of strains within the *Ps. putida*, *Ps. aeruginosa*, *Ps. aureofaciens* and *Alc. xylosoxidans denitrificans* species, according to Hansen and Winding (1997). Sixteen polymorphic profiles were found out of a total of 24 *Ps. putida* isolates (Fig. 3a): nine strains presented the same haplotype, accounting for up to 38% of the *Ps. putida* population; the remaining 15 strains had 15 different haplotypes and formed 60% of the *Ps. putida* population. *Ps. aeruginosa* strains were grouped in 10 polymorphic profiles: 15 strains, 45% of total *Ps. aeruginosa* strains, had the same profile (Fig. 3b), and the 18 remaining strains were grouped in nine different profiles. Five *Ps. aureofaciens* isolates were grouped in four polymorphic types (Fig. 3c), since two strains (CM1, CM17) presented the same haplotype. Nine *Alc. xylosoxidans denitrificans* isolates were grouped in five different RAPD types (Fig. 3d). All these data underline different degrees of biodiversity within these species which could reflect a different response to environmental pollution.

Evolution of microbial consortia by total 16S rDNA-RFLP

The 16S rDNA gene population was amplified from total DNAs of the whole community at the beginning of the enrichment and after 10 subsequent passages on M9 fresh medium supplemented with naphtha, in order to describe the evolution of the microbial consortium without isolation procedures and successive cultivation biases. Comparison of the *Hinf*I-16S rDNA restriction profiles (Fig. 4) revealed shifts in its structure. The 70 and 500 bp bands characteristic of *Ps. putida* and the 120 and 210 bp bands characteristic of *Ps. aureofaciens* and of *Ps. aeruginosa* were always present, as confirmed by isolation of strains belonging to the species; *X. maltophilia* and Gram-positive bands at 170 and 290 bp, respectively, were not detected at the early or after 10 passages. Such data indicate that few cells of the strains were present, and their DNAs were not amplified. *X. maltophilia* and Gram-positive strains were no longer isolated at the 10th step. *Alc. xylosoxidans denitrificans* was isolated at the early passage but it was not visible in the restriction profile of the community, probably because it was present in a small number. At the 10th passage, two characteristic bands at 320 and 670 bp were seen, and nine *Alc. xylosoxidans denitrificans* strains were indeed isolated.

DISCUSSION

The presence of *xyl*E in soil DNA gave evidence of the presence of degrading bacteria in the contaminated site, prior to isolation of strains or degradation analysis. The effective presence of these micro-organisms was confirmed by selecting a degradative enrichment culture whose components were isolated and identified at species level by phenotypic traits and by PCR-RFLP analyses of 16S rDNA

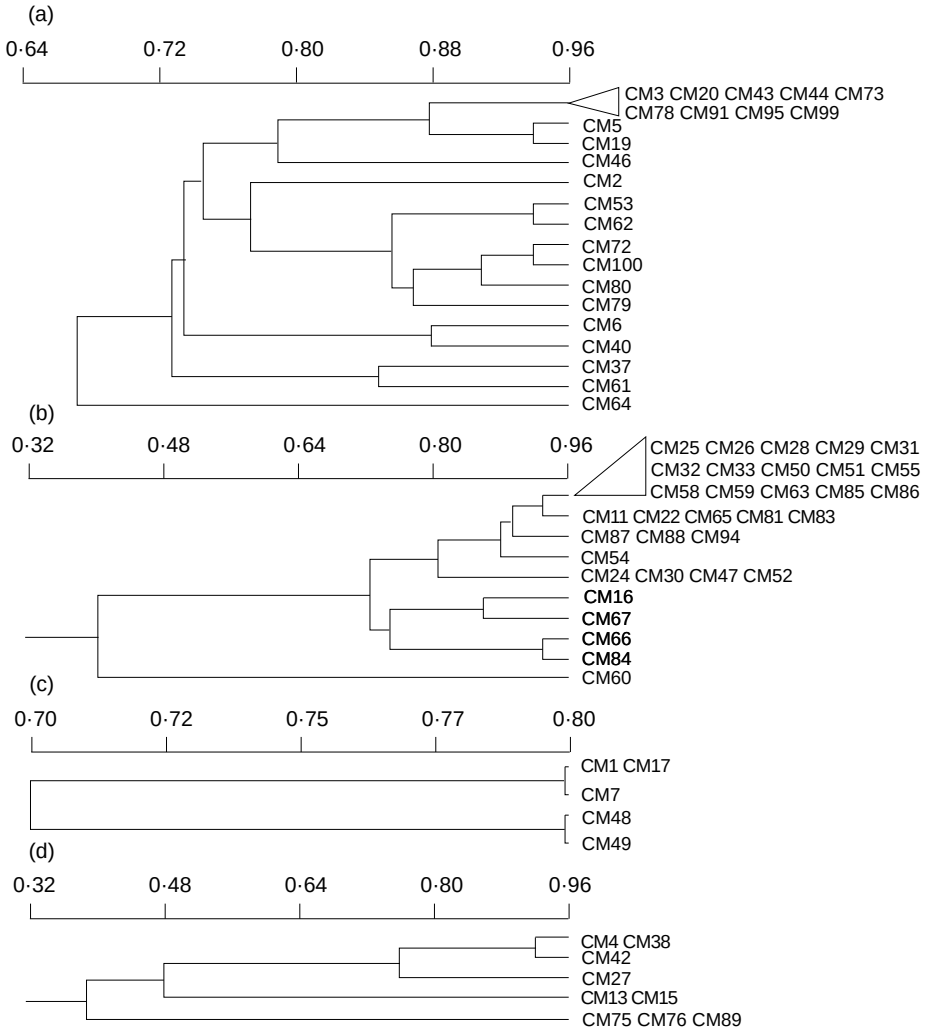

Fig. 3 Cluster analysis of isolates from the enriched culture (10th passage), on the basis of RAPD profiles obtained using OPA 2 and OPA 10 decamers. The Jaccard's coefficient was used to compute the distance matrix and the UPGMA method was used to build the similarity tree from the distance matrix. *Pseudomonas putida* haplotypes (a), *Ps. aeruginosa* haplotypes (b), *Ps. aureofaciens* haplotypes (c), *Alcaligenes xylosoxidans denitrificans* haplotypes (d)

genes. These restriction profiles allowed rapid detection of the species composition of the culture in subsequent passages and to follow the evolution of the bacterial community during the enrichment procedure without cultivation of strains according to Liu *et al.* (1997). Direct analysis of 16S-RFLPs of the whole bacterial community is a useful tool to check the representativeness of isolated strains, since populations enriched in liquid cultures may be unable to develop on solidified media (Ward *et al.* 1997). The enrichment procedure led to a simplification of the degrading consortium which was ultimately composed of *Ps. putida*, *Ps. aeruginosa*, *Ps. aureofaciens* and *Alc. xylosoxidans denitrificans* strains, the most abundant species present in contaminated environments (Hook *et al.* 1992). Early passage strains of *X. maltophilia* and Gram-positive group which were negative to catabolic probes were no longer retrieved from the consortium, whereas some *Ps. aeruginosa* strains negative to catabolic probes were still present at the

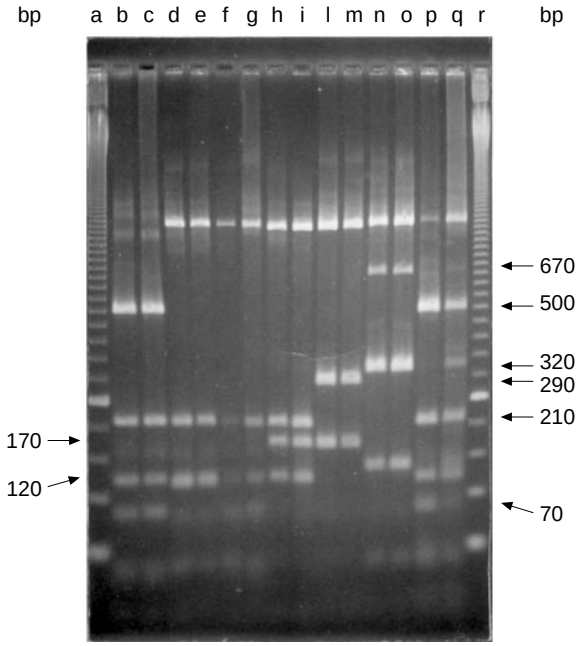

Fig. 4 *Hinf*I digestion profile of 16S rDNA of *Pseudomonas putida* (lanes b–c), *Ps. aureofaciens* (lanes d–e), *Ps. aeruginosa* (lanes f–g), *Xanthomonas maltophilia* (lanes h–i), Gram-positive strains (lanes l–m) and *Alcaligenes xylosoxidans denitrificans* (lanes n–o). Restriction profiles of total DNA extracted from the enrichment culture at an early passage (lane p) and after 10 passages (lane q). 50 bp DNA ladder (lanes a–r)

final passage. These non-degrading strains may utilize intermediates of hydrocarbon degradation (Golovleva *et al.* 1992), playing a key role in metabolic cross-feeding with reference to catabolism of organic compounds and also produce biosurfactants, which are recognized to enhance the degradation of aliphatic and aromatic hydrocarbons in microbial populations (Desai and Banat 1997).

*Xyl*A,M, *xyl*E and *tod*C1 genes were retrieved in strains able to grow on aromatic hydrocarbons. The presence of TOL-like pathways in strains able to grow on toluene, *m*-xylene and *p*-xylene was confirmed by amplification of *xyl*A,M and *xyl*E genes and by the formation of 3-methyl benzoic acid during *m*-xylene degradation by resting cells of *Ps. Putida* CM337 grown on *m*-xylene. *Tod*C1 gene was retrieved from strains able to grow on toluene and benzene. In *Ps. aureofaciens* CM332 the formation of 3-methylcatechol during toluene degradation and of *cis*-3,5-cyclohexadiene-1,2-diol, catechol and phenol during benzene degradation indicated that strain CM332 possessed not only a dioxygenase system but also a monoxygenase system in accord with Gibson *et al.* (1989) and Kitayama *et al.* (1996). The absence of a *xyl*E amplification signal in strains possessing a TOD-like pathway can be due to the lack of homology at primers annealing sites between *tod*E and *xyl*E genes. The enrichment procedure favoured the selection of strains carrying a TOD-like pathway, since it was present in 70·4% of the strains. The TOL pathway was retrieved only in *Ps. putida* strains, whereas the TOD pathway was present also in *Ps. aureofaciens*, *Ps. aeruginosa* and *Alc. xylosoxidans denitrificans* strains. All these micro-organisms are known to be versatile degraders (Gibson *et al.* 1989; Harayama *et al.* 1989; Golovleva *et al.* 1992; Moon *et al.* 1996; Dutta and Gunsalus 1997). *Ps. putida* strains isolated at early passage harboured only *xyl*A,M and *xyl*E genes, whereas those isolated at the 10th transplant possessed both TOL- and TOD-like genes. This characteristic extended their metabolic capabilities, conferring to these strains the possibility to utilize both benzene and toluene. The presence of catabolic routes is not linked just to a specific best-fitting RAPD haplotype or strain. Transposons and insertion sequences often flank degradative operons located on plasmids and chromosomes, possibly leading to diffusion of catabolic abilities between strains within a community when a selective pressure is imposed (Wydham *et al.* 1994).

The investigation demonstrated that during the enrichment procedure a microbial consortium able to degrade 40% of naphtha solvent in 24 h was obtained and that most isolates carried catabolic genes. Both TOL and TOD pathways were present, widening the degradation abilities of the consortium. The application of molecular methodology permitted the simultaneous monitoring of populations inhabiting the culture during the enrichment procedure by using 16S rDNA signatures for the species present. Such a consortium when used as inoculum in soil microcosm studies or *in situ* experiments, can easily be monitored by molecular analysis for its bioremediation and colonization potentials.

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