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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIVISION DE NUEVAS CREACIONES
E. S. D.

Re: Expediente No. **07-069.903**

Solicitud de patente a nombre de **WESTINGHOUSE SAVANNAH
RIVER COMPANY, L.L.C.**

Yo, DILIA RODRIGUEZ D'ALEMAN, identificada como aparece al pie de mi firma, domiciliada en la carrera 11 No. 86-53, piso 6, obrando como apoderada de la sociedad **WESTINGHOUSE SAVANNAH RIVER COMPANY, L.L.C.**, domiciliada en Carolina del Sur, Estados Unidos de América, solicitante de la patente de la referencia, en respuesta al oficio No. 6127 notificado por fijación en lista el 08 de abril de 2011, me permito acompañar el siguiente documento solicitado por ese despacho:

1. SINGLETON D ET AL: "Microbial diversity in an acidic refinery sludge" ABSTRACTS OF THE GENERLA MEETING OF THE AMERICAN SOCIETY FOR MICROBIOLOGY, vol. 101, 2001, page 637.
2. PLAZA GRAZYNA ET AL: "Relationship between soil microbial diversity and bioremediation process at an oil refinery" ACTA MICROBIOLOGICA PLONICA, vol. 52, no. 2, 2003, pages 173-182.
3. DATABASE WPI Derwent Publications Ltd. London, GB: AN 2004-446832 XP002342187 BLOKHIN VA ET AL: "Strain of bacterium Pseudomonas alcaligenses MEV used for the removal of petroleum and its products from soil, ground and surface water" - & RU 2 228 953 C (BIOL-R) BIOL PREPARATIONS TOXICOLOGY & HYGIENE, 20 May 2004.
4. KORDA EST AL: "Petroleum hydrocarbon bioremediation: Sampling and analytical techniques, in situ treatments and commercial microorganisms currently used", APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, vol. 48, no. 6, December 1997 (1997-12), pages 677-686.
5. DAGHER FADI ET AL: "Comparative study of five polycyclic aromatic hydrocarbon degrading bacterial strains isolated from contaminated soils" CANADIAN JOURNAL OF MICROBIOLOGY, vol. 43, no. 4, 1997, pages 368-377.
6. SALLEH ABU BAKAR ET AL: "Bioremediation in petroleum hydrocarbon pollution" INDIAN JOURNAL OF BIOTECHNOLOGY, vol. 2, no. 3, July 2003 (2003-07), pages 411-425.

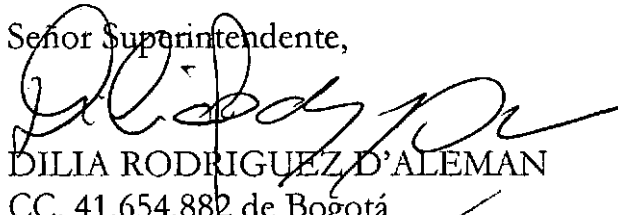
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7. ANDREONI V ET AL: "Bacterial communities and enzyme activities of PAHs polluted soils" CHEMOSPHERE, PERGAMON PRESS, OXFORD, GB, vol. 57, no. 5, November 2004 (2004-11), pages 401-412.

8. HAMANA KOEI ET AL: "Polyamine distribution profiles in newly validated genera and species with the Flavobacterium-Flexibacter-Cytophaga-Sphingobacterium complex", MICROBIOS, col. 106, no. Supplement 2, 2001, pages 105-116.

Espero con esto haber dado cabal cumplimiento a lo solicitado por ese despacho y de conformidad con lo indicado en los artículos 3º, inciso 3, 35 inciso 2 y 59 inciso 2 del CCA, al analizar este memorial, atentamente solicito se traten todas las cuestiones planteadas con motivo del mismo.

Señor Superintendente,



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s-Triazine compounds are extensively used throughout agriculture and industry. Among the *s*-triazine compounds that have gained wide use are atrazine and melamine. Atrazine is a herbicide used to control broad leaf weeds with coin and other crops, while melamine does not have phytotoxic properties and is commonly used in preparation of melamine urea resins. Both of these compounds are known to be biodegraded by different pseudomonads. *Pseudomonas* sp. ADP and *Pseudomonas* NRRL 12227 degrade atrazine and melamine, respectively. The genes encoding the first step in atrazine and melamine degradation, *atrA* and *trmA*, respectively, are 90% identical at the nucleotide level, yet the enzymes are very specific for the reactions catalyzed. It was hypothesized that other homologs exist in nature with highly similar sequences and activities, but with enough variation to help deduce structure-function correlations in these enzymes. In this context, bacterial enrichments were performed to isolate novel bacteria with the melamine degradation phenotype. Soil from a melamine manufacturing plant was used as an initial inoculum. Screening was performed using a plate-clearing assay in which 15 mg/ml of melamine was added to agar plates. As the bacteria degraded the melamine, clearing zones were produced around melamine-degrading bacteria. The consortia obtained from enrichments were shown by PCR to contain a homolog to the *atrA* genes. Primers specific for *trmA*, encoding a melamine deaminating enzyme which is 41% identical to *atrA* at the amino acid level, did not yield a PCR product. Sequencing the 16S rRNA genes of the consortia revealed the greatest population to belong to the *Acidovorax* genus.

Q-264. Microbial Diversity in an Acidic Refinery Sludge

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Sludge samples were obtained from a 100 year old Czechowice (C) refinery near Czechowice-Dziedzice, Poland. The aged sludge is acidic (pH 2) and composed of asphaltic highly contaminated with polycyclic aromatic hydrocarbons (PAHs). Additionally, other wastes such as spent catalysts, diatomaceous earth, silica gel, and coal fly ash was disposed of at this site. Approximately 120,000 tons of this waste was deposited in outlined lagoons 1 meters deep covering a total of 3.8 hectares. A total of 32 bacteria, 10 fungi, and several yeast spp. were isolated from the sludge on acidic minimum salts medium exposed to naphthalene vapor. The isolates were characterized by classical taxonomic criteria, BIOLOG[®], and analysis of the SSU rRNA genes. A number of the bacterial isolates were *Proteobacteria* related to *Ralstonia*, *Pseudomonas*, *Xanthomonas*, and *Acidithiobacillus* spp., and the most commonly encountered group possessed high nucleotide sequence similarity (90-100%) to *Ralstonia* sp. KN1 (eight isolates). In addition, isolates belonging to the *Actinobacteria* (high G+C gram positive), and *Firmicutes* (low G+C gram positive) were found. Several of the *Ralstonia* isolates and a *Bacillus* isolate grew with catechol, naphthalene, or fluorene as the sole carbon sources, indicating a high bioremediation potential. The fungal isolates that grew on naphthalene vapor appear to represent taxa that have not been previously reported to

degrade PAHs. This international project was part of a joint effort between the US Department of Energy and the Polish Institute for Ecology of Industrial Areas to develop efficient bioremediation strategies for acidic petroleum sludge impacted soils.

Q-265. Characterization of the Microbial Community in an Anaerobic Fluidized Bed Bioreactor Treating Pink Water Using t-RFLP

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Anaerobic fluidized-bed bioreactors (AFB) provide a potentially cost-effective alternative treatment for pink water, a wastewater contaminated with residual explosive chemicals. Two major components of pink water are trinitrotoluene (TNT) and cyclotrimethylene-trinitramine (RDX). The efficacy of AFB is dependent on the microbial community in the bioreactors, therefore it is important to characterize the bacteria present in the system in order to optimize pink water treatment. In an AFB little is known about which microorganisms are responsible for explosives degradation. In order to identify unique microorganisms in a TNT-fed reactor, terminal restriction fragment polymorphism (t-RFLP) analyses were performed. Two lab-scale AFBs were used: one reactor (+TNT) was fed a synthetic feed containing 11.57 mM of ethanol and 440 mM of TNT and the other (control) was fed ethanol only. Over the course of operation, the impacts of different conditions on the microbial community were evaluated. The changes consisted of: first, the TNT loading was doubled; second, the TNT feed was switched to RDX; and third, the RDX feed was switched back to TNT while the ethanol loading was decreased gradually, 50% every 6-7 weeks. The final ethanol loading was 6.25% of the initial concentration. DNA was extracted from reactor samples and amplified using PCR with a fluorescent-labeled 27f bacterial domain-specific primer and a 1492 universal primer. Three different restriction enzymes were used for fragment analysis. The t-RFLP-TAP program on the RDP web site was used to identify bacteria associated with individual terminal fragments. TNT was completely transformed to triaminotoluene even with the 6.25% ethanol loading, indicating that the TNT degrading bacteria were still present. Several unique fragments were identified in the +TNT reactor. Based on the t-RFLP data, one set of fragments (HhaI = 93, MspI = 132, and KpnI = 242), representing the predominant peak in the +TNT reactor, was identified as a *Cleobacter*. This suggests that a *Cleobacter* population may be important for TNT degradation in this reactor.

Q-266. Phenanthrene, Anthracene, and Biphenyl Degradation by *Mycobacterium* sp. PYR-1

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Cultures of *Mycobacterium* sp. PYR-1 were dosed with phenanthrene, anthracene, or biphenyl. After 6, 24, or 72 hr of incubation, they had degraded approximately 90%, 92%, and 95% of the added

phenanthrene, anthracene, and biphenyl, respectively. The metabolites were extracted and identified by uv-visible absorption, HPLC retention times, mass spectrometry, ¹H and ¹³C nuclear magnetic resonance (NMR) spectrometry, and by comparison to authentic compounds and literature data. When *Mycobacterium* sp. PYR-1 was grown in the presence of phenanthrene, the neutral pH ethyl acetate extract showed three metabolites, identified as *cis*- and *trans*-9,10-dihydroxy-9,10-dihydrophenanthrene and *cis*-1,3-dihydroxy-1,3-dihydrophenanthrene. Phenanthrene ring-fission products, isolated from acid extracts, were identified as 2,2-diphenic acid, 1-hydroxynaphthalic acid, and phthalic acid. Neutral pH extracts from anthracene-inoculated cells produced four metabolites, identified as *cis*-1,2-dihydroxy-1,2-dihydroanthracene, 6,7-benzocoumarin, 1-methoxy-2-hydroxynaphthalene, and 9,10-anthraquinone. A novel anthracene ring-fission product was isolated from acidified culture medium and was identified as 3-(2-carboxyvinyl)naphthalene-2-carboxylic acid. 6,7-Benzocoumarin was also found in that extract. Biphenyl produced one neutral pH metabolite, 4-hydroxybiphenyl, and two ring-fission products, benzoic acid and 4-hydroxybenzoic acid. NMR and mass spectral data confirmed the identification of the metabolites. The data suggest that parts of the pathways involved in the metabolism of phenanthrene, anthracene, and biphenyl by *Mycobacterium* sp. PYR-1 are similar to those of gram-negative bacteria, while other parts of the pathways are unique.

Q-267. Methoxyquinone Production and the Mechanism of 2,4,6-Trinitrotoluene (TNT) Degradation by *Gloeophyllum* Species

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Nitro-aromatic and nitro-substituted aromatic amines as well as nitro-esters have many applications in the chemical and pharmaceutical industries. In particular, the manufacturing and disposal of explosives worldwide has resulted in thousands of sites that are contaminated with these compounds. Brown-rot fungi are often potent degraders of organic compounds, however the biochemical mechanisms underlying these processes are largely unknown. Our recent discovery of dimethoxyquinones in liquid cultures of *Gloeophyllum trabeum* prompted our further investigations into their possible roles as redox cycling molecules to promote Fenton-like degradation. Various *Gloeophyllum* spp. were compared for their abilities to produce methoxyquinones, especially 2,5-dimethoxyhydroquinone (2,5-DHQ). Six of eight strains examined produced this compound in concentrations ranging from 1 ppm to 30 ppm. Salicylic acid was employed to trap Fenton-generated hydroxyl radicals in several cultures and was readily degraded by all of the strains examined. Small amounts of 2,3- and 2,5-dihydroxy benzoic acid, products expected from the reaction of salicylic acid and hydroxyl radicals, were observed using GC-MS analysis. *G. trabeum* also transformed TNT rapidly. However, mass balance studies which utilized ¹⁴C-UL-TNT indicated that 98% of added TNT remained in the culture supernatant and was not converted to ¹⁴CO₂. Analyses of culture extracts using electrospray MS/MS revealed several aromatic nitro-amines and nitro-aldehydes and their oligomeric coupling products.

Relationship between Soil Microbial Diversity and Bioremediation Process at an Oil Refinery

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Abstract

Microbial diversity in hydrocarbon-contaminated soil was characterized during a bioremediation project at an oil refinery. The project consisted of isolation and cultivation of microbes on laboratory media and the subsequent characterization of pure isolates. In a lagoon at the Czechowice Oil Refinery, Poland, a biopile with actively and passively aerated sections was constructed and has been operated since 1997. The bioremediation process has been continuously monitored by physical, chemical, and microbiological methods. One hundred and forty nine bacterial and fungal strains were isolated from site soils by standard procedures. Analysis of cultivable microorganisms revealed a diverse microbial population within the cultured isolates. Among isolated strains, *Pseudomonas* and *Chryseomonas* genera predominated in the bacterial population while *Candida*, *Fusarium*, and *Trichophyton* dominated the fungal population. This paper describes the application of traditional microbiological methods (plating and microscopic methods) to evaluate cultivable microbial diversity in bioremediated soil.

Key words: soil microbial diversity, bioremediation, oil refinery

Introduction

Bioremediation is the biological process of transformation or mineralization of organic compounds (both xenobiotic and naturally occurring) introduced into the environment to less toxic or innocuous forms (Hazen, 1997; Brigmon *et al.*, 2002). Bioremediation is an attractive, environmentally friendly, and relatively cost-effective alternative to conventional physicochemical soil treatment techniques (Bouwer *et al.*, 1994; Bartha, 1986). The success of bioremediation is largely determined by the metabolic potential of microorganisms to detoxify/utilize organic contaminants or mineralize them to CO₂, H₂O and biomass. The process is dependent on biodegradability and bioavailability of contaminants to microorganisms, as well as environmental parameters including nutrient availability. In addition, for bioremediation to be successful for a given site and contaminants the microbial

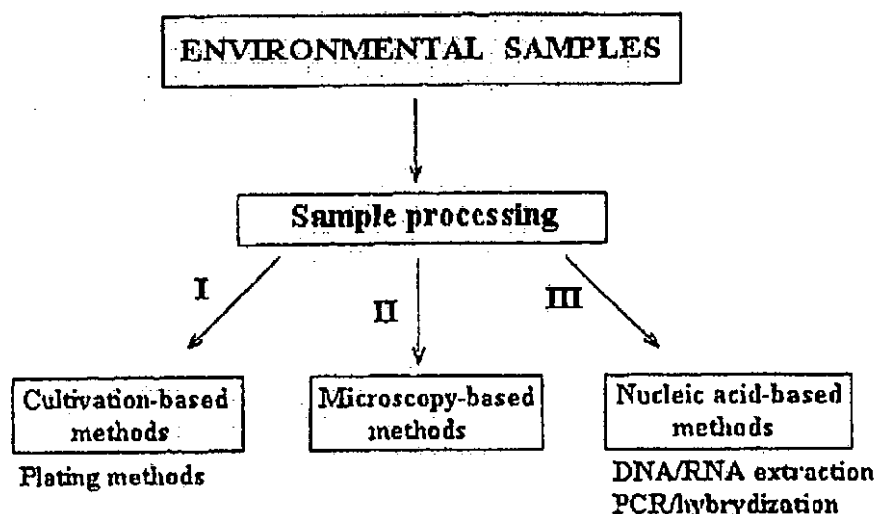


Fig. 1. Schematic of the approaches for detection of microorganisms in environmental samples (modified from Elsas *et al.* 1998).

community with specific biodegradation capacity must be present in the optimal concentrations and activity levels (Brigmon, 2001). Therefore, microbial characterization is critical to environmental restoration approaches utilizing bioremediation.

Fig. 1 illustrates current approaches used for detection and characterization of microorganisms in environmental samples. Of these, several (Fig. 1, I and II, in part) are considered to be traditional, as they rely on cultivation of isolated microorganisms and microscopy. On the other hand, the last decade has seen the implementation of a range of methods including molecular techniques based on the analysis of nucleic acids in environmental samples. The combination of culture-dependent and -independent approaches in the analysis of an environment may result in a more detailed description of the experimental system (Elsas *et al.*, 1998; MacNaughton *et al.*, 1999; Juck *et al.*, 2000).

Considering different existing bioremediation strategies and characteristics of the lagoon materials at the Czechowice-Dziedzice refinery (COR), the *ex situ* bioremediation technology in a biopile was selected to clean-up soil heavily contaminated with petroleum waste. The biopile with actively and passively aerated sections, referred as biopile 1 or engineered, was constructed in 1997 in the smallest lagoon at the COR site (Fig. 2) (IETU, 1999; Altman *et al.*, 1997). The purpose of the project was to evaluate novel technologies and research approaches to restoration of soils heavily contaminated with petroleum waste by comparing bioremediation processes under active *vs.* passive aeration and the removal rates of both readily available and recalcitrant petroleum hydrocarbons. This full-scale demonstration simultaneously remediated the soil contaminants to acceptable risk levels and created a permanent green zone, which has great public acceptance and greatly reduces the overall risk of the refinery to the city.

The purpose of the work described here was to evaluate the diversity of cultivable bacterial and fungal populations in the hydrocarbon-bioremediated soil.

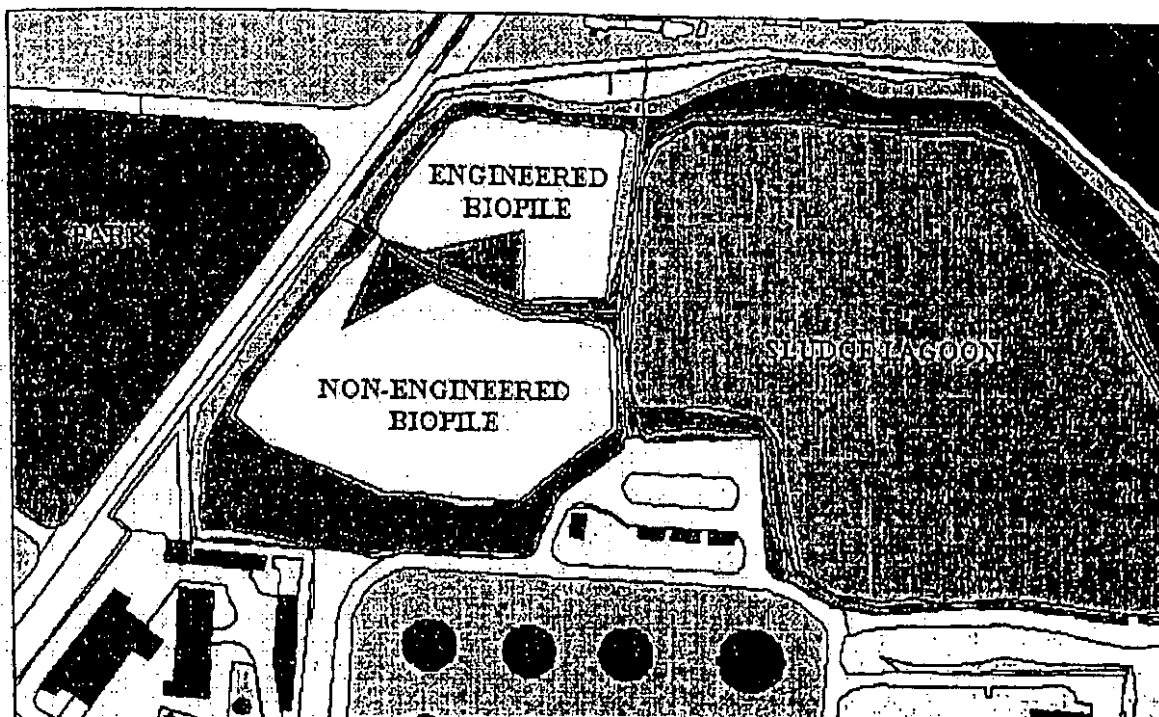


Fig. 2. Localization of biopiles at the COR site.

Experimental

Materials and Methods

Sample collection. The soil sampling strategy was presented in a previous report (IETU, 1999). The total number of sampling locations at the engineered biopile was twenty three. Soil was sampled from shallow (ca. 30–40 cm of depth) and deep (ca. 80–100 cm of depth) layers of the biopile.

Physico-chemical analyses. Each soil sample was examined for the following physico-chemical parameters: TPH – total petroleum hydrocarbons (non-polar aliphatic hydrocarbons); TPOC – total petroleum organic carbon (polar and non-polar aliphatic petroleum compounds); pH in H_2O ; pH in 1M KCl; electric conductivity; $N-NH_4$; $N-NO_2$; PO_4^{3-} ; and humidity. The parameter polycyclic aromatic hydrocarbons (PAHs) was the sum of the concentrations of the following PAHs: naphthalene, acenaphthalene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, benzo(g,h,i)perylene, dibenzo(a,h)anthracene, and indeno(1,2,3-cd)-pyrene. The parameters were measured according to ISO standards.

Microbial dehydrogenase activity – TTC method. Oxidation of petroleum by microbes is linked to electron transport and oxidative phosphorylation. Colorimetric reduction of triphenyltetrazolium chloride (TTC), a metabolic uncoupler, to a triphenyl formazan (TPF) crystal was used as a measure of oxygenic respiration. Soil samples were incubated with 3% TTC solution at 37°C for 24 h. The samples were then extracted with methanol, filtered (Qualitative Filter Paper Medium, DIAM 12,5 cm, PPH "POCh" S.A.), and the optical density was measured at 546 nm (red color) using a UV-VIS spectrophotometer (Varian) (Alef and Nannipieri, 1995). Values are presented as TPF mg/g dry weight of soil.

Isolation and identification of petroleum hydrocarbon-degrading microorganisms. Generally, the methods described in "Manual of Methods for General Bacteriology" (Gerhardt, 1981) were used for isolation of these microorganisms. These were namely: dilution method in phosphate buffer; most probable number (MPN) method combined with the petroleum and naphthalene enrichment

method with incubation in Biolog® boxes; enrichment and sequential enrichment methods in mineral medium (MM) supplemented with sterilized petroleum, naphthalene or other hydrocarbon derivatives in Erlenmeyer flasks.

The dilution and MPN methods were those of EPA U.S. (1978). Schlegel (2001) has described the enrichment and sequential enrichment methods. The composition of mineral medium (MM) was that of the "Manual of Methods for General Bacteriology" (Gerhardt, 1981), but the composition of microelements was that of Schlegel (2001). The MM medium was supplemented with sterilized hydrocarbon(s) (0.1% w/w), and soil (1–2% w/w) from the site. After four week of incubation in a rotary shaker at room temperature, microorganisms capable of degrading hydrocarbons were isolated using the 1:10 SMA (Standard Methods Agar) medium supplemented with vitamins and incubated in oil and/or naphthalene vapors.

Microbial strains were identified by plating on selective microbiological media and presumptively identified microscopically (Zeiss Axioscop, Germany). Identification and characterization of bacterial strains were performed according to standard procedures (Gerhardt, 1981). The following determinations were accomplished in order to determine the isolates taxonomic positions: 1. Morphological features observations (the Gram staining), 2. Culture features in the solid and liquid rich medium, 3. Motility and H₂S production, 4. Sporulation, 5. Biophysical factors in growth, *i.e.* pH, temperature, oxygen, and salinity. Physiological/biochemical tests based on the API identification systems (ID 32GN BioMérieux S.A.) also were performed. The identification was compared with "Bergey's Manual of Determination Bacteriology" (Holt *et al.*, 1994).

Subsequently, filamentous fungi strains were identified by analyzing their macro- and micromorphological characteristics and using selected taxonomic monographs (Sigler and Carminichael, 1976; Domsch *et al.*, 1980; Currah, 1985; Arx, 1986; Cano and Guarro, 1990; Guarro *et al.*, 1991). Macromorphological examination included the colony features, whereas micromorphological examination included microscopic features of the strains. Fungal strains were mostly examined using the methods recommended by de Hoog and Guarro (1995). Fungi were also isolated on hair bait (Vanbreuseghem, 1952) in the atmosphere of crude petroleum vapors. The strains were first examined on direct slides. Then, they were incubated on identification media, *e.g.* on MEA, CYA, PYE, Takashio agar (TK), rice (RA) and water (WA) agars. Yeasts were identified with the API 20 C AUX bioMérieux system and extended methods by Van der Walt (1970).

Results and Discussion

Changes in mean concentrations of petroleum hydrocarbons and microbial activities as measured by TTC in the soil samples during the 45-month bioremediation process are presented in Figure 3. Soil hydrocarbon concentrations decreased on average by 81% of the initial contaminant concentration over this time period. The first biodegradation stage (until the 7th month) was characterized by the considerable decrease of aliphatic hydrocarbons and high microbial activity in the biopile, as indicated by high dehydrogenase activity increase. The highest decrease of TPH (58%) and TPOC (49%) concentrations were observed in this stage of bioremediation process. The next considerable decrease of contaminant concentrations was observed between 39th and 44th months of bioremediation (Figure 3). The dehydrogenase activity observed at this stage was the highest during the whole bioremediation period. By the end of the 20th month of bioremediation the concentrations of TPH and all PAHs were below the Polish risk guidelines (PIOŚ, 1994). This study provides clear demonstration of the effective use of bioremediation for pollutant removal and environmental restoration.

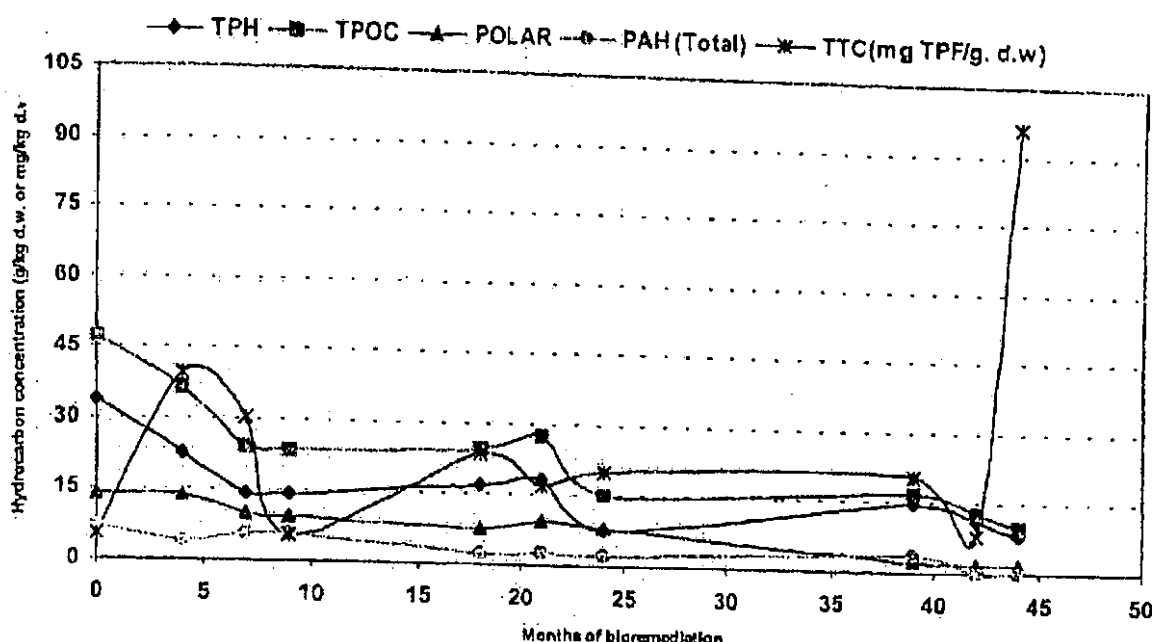


Fig. 3. Changes in the mean concentrations of petroleum-originating hydrocarbons and microbial activity measured in the soil during the bioremediation period.

One hundred and forty-nine bacterial and fungal strains were isolated from the soil during the 1st bioremediation stage. (Table I). Isolated strains are aerobic/microaerophilic rods and cocci gram-negative bacteria from Group 4 (gram-negative aerobic microaerophilic rods and cocci) according to "Bergey's Manual of Determination Bacteriology". Most can grow under an air atmosphere (21% oxygen) and use oxygen as a terminal electron acceptor. For most bacteria optimum pH for growth lies between 6.5 and 7.5, and with temperature ranging from 10–45°C (mesophilic), with optimal growth at 30–37°C. Isolating anaerobic bacteria was not attempted here.

Bacteria from the genera *Pseudomonas*, *Chryseomonas*, *Sphingomonas*, and *Stenotrophomonas* were identified in examined samples. Most of them belonged to genus: *Pseudomonas* and *Chryseomonas*. In the genus *Pseudomonas*, five species: *Ps. cepacia* (2 isolates), *Ps. fluorescens* (20 isolates), *Ps. aureofaciens* (8 isolates), *Ps. mendocina* (2 isolates), and *Pseudomonas* sp. (8 isolates) were identified. *Pseudomonas* species have strictly respiratory type of metabolism, with oxygen as terminal electron acceptor but in some cases nitrate can be used as an alternate electron acceptor allowing anaerobic metabolism to occur. They are able to assimilate various substrates (glucose, mannose, mannitol, and citrate) as carbon sources. Some differences occur only in the case of N-acetylglucosamine and arabinose assimilation. None of the *Pseudomonas* isolates has capabilities to ferment glucose and to produce urease. All strains were oxidase-positive. *Pseudomonas cepacia* produced β -galactosidase but not urease. The genus *Chryseomonas* was represented by 2 species: *Ch. chlororaphis* (2 isolates) and *Ch. luteola* (8 species). Some of them were motile with H_2S production, but some of them were not. The isolated strains grew at temperature range between 4–45°C. Oxidase and urease tests were negative.

Table I
The list of species and strains isolated from the petroleum
contaminated soil of the refinery

Species	Number of strains
BACTERIAL STRAINS	
<i>Chryseomonas chlororaphis</i>	2
<i>Chryseomonas luteola</i>	8
<i>Pseudomonas aureofaciens</i>	8
<i>Pseudomonas cepacia</i>	2
<i>Pseudomonas fluorescens</i>	20
<i>Pseudomonas mendocina</i>	2
<i>Pseudomonas sp.</i>	8
<i>Sphingomonas paucimobilis</i>	2
<i>Stenotrophomonas maltophilia</i>	2
FUNGAL STRAINS	
<i>Aphanoascus keratinophilus</i>	6
<i>Aphanoascus reticulisporus</i>	3
<i>Candida saitoana</i>	14
<i>Candida guilliermondii</i>	3
<i>Candida sp.</i>	2
<i>Exophiala sp.</i>	6
<i>Fusarium solani</i>	11
<i>Geomyces pannorum</i>	3
<i>Geotrichum candidum</i>	3
<i>Microsporum gypseum</i>	5
<i>Microsporum sp.</i>	2
<i>Paecilomyces lilacinus</i>	3
<i>Penicillium javanicum</i>	4
<i>Phialophora sp.</i>	2
<i>Phoma sp.</i>	3
<i>Pseudallescheria boydii</i>	4
<i>Saccharomyces cerevisiae</i>	1
<i>Scopulariopsis brevicaulis</i>	1
<i>Trichophyton ajelloi</i>	15
<i>Trichophyton terrestris</i>	4
Total: 29 species	Total: 149 strains

Bacterial and fungal isolates also were tested for petroleum hydrocarbon removal rates (Ulfing et al., 1998). Some isolated bacterial strains found here, i.e., *Pseudomonas fluorescens*, *Sphingomonas paucimobilis* and *Stenotrophomonas*

Table II
Removal rates, mass losses, and biomass production for three bacterial strains used in the bioaugmentation study

Strains no.	Removal rates (g/l/24h)		Mass Losses (%)		Biomass production (g/l/24h)
	TPH	TPOC	TPH	TPOC	
1	0,0041	0,0000	13,1	0,00	0,0057
9	0,0000	0,0028	0,0	11,4	0,0055
14	0,0110	0,0081	35,1	18,7	0,0050
1+9	0,0093	0,0051	29,6	11,7	0,0020
9+14	0,0107	0,0088	34,1	20,3	0,0056
1+14	0,0135	0,0075	42,8	17,4	0,0038
1+9+14	0,0173	0,0186	55,0	42,8	0,0149

No 1 - *Sphingomonas paucimobilis*; No. 9 - *Stenotrophomonas maltophilia*;
No 14 - *Pseudomonas fluorescens*
TPH - total petroleum hydrocarbons; TPOC - total petroleum organic carbon

maltophilia have potential for hydrocarbon biodegradation. Then, they were used successfully in a bioaugmentation study of petroleum (Ulfig *et al.*, 1998). Hydrocarbon mass losses and removal rates for these bacterial strains are presented in Table II. Activity of the bacterial strains was evaluated in the cultures separately or together under different combinations. After 14 days of incubation the mass losses for *Pseudomonas fluorescens* were of 35% and 19% for TPH and TPOC, respectively. The highest mass losses of TPH (55%) and TPOC (43%) were in the mixed cultures, i.e., when *Sphingomonas paucimobilis*, *Stenotrophomonas maltophilia* and *Pseudomonas fluorescens* grew together.

Microscopic fungi were the second largest group of microorganisms isolated (Table I). *Candida saitoana*, *Fusarium solani* and *Trichophyton ajelloi* predominated in the fungal population. Fungal isolates from the genera *Aphanoascus*, *Microsporum*, *Scopulariopsis* and *Trichophyton* were keratinolytic. The role of these fungi in the assessment of soil contamination and biodegradation progress in a refinery biopile was demonstrated by Ulfig *et al.* (2002). The fungi are also able to remove hydrocarbons from the medium during degradation of proteins (Przystaś *et al.*, 2001). Many fungal isolates, e.g., *Candida saitoana* and *Penicillium javanicum*, were acidophilic or acid-tolerant. Characterization of these fungi was a subject of a separate study (Ulfig *et al.*, 2000).

Multidiscipline approaches to microbial ecology of bioremediation projects can be extremely useful. A study by Plaza *et al.* (2001) describes the molecular phylogeny of isolated cultured organisms from this biopile project. This study was a cultivation dependent approach followed by molecular analysis. A study at the Savannah River Site employed culture, molecular, phospholipid fatty acid, and immunological methods for examination of subsurface microbiology at site utilizing chemical oxidation (Kastner *et al.* 2000). The favorable results demonstrated that a better understanding of the subsurface microbiota would improve the potential coupling of chemical oxidation with bioremediation processes. Such hybrid technologies might prove

effective in remediation since oxidation processes can enhance the solubility of organic compounds and thus increase bioavailability. The results of the field study described here demonstrated the successful use of a biopile for bioremediation of petroleum-contaminated soil. The findings of this study support those conclusions by proving the isolation of an extensive list of hydrocarbon degrading microbial species from the remediated soils of that site.

We acknowledge that the approaches used in this study have several major limitations. Cultivation based methods strongly select for microbes that can grow on artificial media and laboratory growth conditions will undoubtedly differ substantially from those experienced in the environment. For the majority of organisms present in an environmental sample, nutritional requirements will not be met, while carbon stressed, dormant, or inactive populations will be quickly overgrown by opportunistic species. Recent advances in molecular biology have provided highly sensitive, alternative methods for the study of microbial communities (*e.g.*, Plaza *et al.*, 2001). Extensive organismal, genetic, and biochemical diversity has been described in essentially all environments, even those previously considered uninhabitable. Roughly 99% of microbial life is categorized uncultivable (Torsvik *et al.*, 1996; McCaig *et al.*, 2001; Watanabe and Baker, 2000), though we are still dependent on cultivation for the study of organisms. Successful cultivation of fortuitous, viable but nonculturable (VBNC), and ecologically significant taxa will be greatly improved by insight gained from molecular investigations. It should be noted, however, that none of these methods are comprehensive or unbiased. Therefore, a combination of molecular and traditional approaches will undoubtedly provide for more detailed descriptions of microbial communities.

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AB - RU2228953 NOVELTY - Strain of bacterium *Pseudomonas alcaligenes* MEV is isolated from wheat rhizosphere and deposited in All-Russian collection of industrial microorganisms, Moscow, at number B-8278. The culture *Pseudomonas alcaligenes* MEV produces biological surface-active substances (biosurfactants).

- DETAILED DESCRIPTION - Strain of bacterium *Pseudomonas alcaligenes* MEV is isolated from wheat rhizosphere and deposited in All-Russian collection of industrial microorganisms, Moscow, at number B-8278. The strain utilizes petroleum, mazut, diesel fuel, polycyclic aromatic hydrocarbons comprising from 2 to 4 benzene rings: naphthalene, phenanthrene, anthracene, fluoranthene, phenol. The strain is resistant to heavy metal ions: Pb, Zn, Mo, Fe, Cr. The culture *Pseudomonas alcaligenes* MEV produces biological surface-active substances (biosurfactants).

- USE - The strain can be used for preparing preparation for treatment of soils, ground and surface waters from petroleum, products of its processing and also in the combined pollution with petroleum hydrocarbons and metals.

- (Dwg.0/0)

C - C12N1/20 C12R1/38

IW - STRAIN BACTERIUM PSEUDOMONAS ALCALIGENES MEV REMOVE PETROL PRODUCT SOIL GROUND SURFACE WATER

IKW - STRAIN BACTERIUM PSEUDOMONAS ALCALIGENES MEV REMOVE PETROL PRODUCT SOIL GROUND SURFACE WATER

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TI - Strain of bacterium *Pseudomonas alcaligenes* MEV used for the removal of petroleum and its products from soil, ground and surface water

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MINI REVIEW

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Petroleum hydrocarbon bioremediation: sampling and analytical techniques, in situ treatments and commercial microorganisms currently used

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Abstract The sampling and analytical methods, along with available microorganisms, used for in situ hydrocarbon bioremediation are reviewed. Each treatment method is briefly described and its advantages and limitations pertaining to potential applications are evaluated. Bioremediation provides cost-effective, contaminant- and substrate-specific treatments equally successful in reducing the concentrations of single compounds or mixtures of biodegradable materials. In situ treatments rarely yield undesirable byproducts, but precautions and preliminary baseline tests are always recommended. Sampling methods should adhere to good laboratory and field practices and usually do not require highly trained personnel. Analytical methods vary in sensitivity, cost, duration of sample analysis and personnel training required. Voucher specimens of bacterial strains used in bioremediation exist in various repositories (e.g. ATCC, DSM, etc.) or are commercially available, and are usually covered by patent rights. Each one of these strains may yield spectacular results in vitro for specific target compounds. However, the overall success of such strains in treating a wide range of contaminants in situ remains limited. The reintroduction of indigenous microorganisms isolated from the contaminated site after culturing seems to be a highly effective bioremediation method, especially when microorganism growth is supplemented by oxygen and fertilizers.

ity to reduce the concentration and/or toxicity of various chemical substances such as petroleum products, aliphatic and aromatic hydrocarbons (including polyaromatic hydrocarbons and polychlorinated biphenyls), industrial solvents (phenols, benzene, acetone etc.), battery liquids, pesticides, and metals (arsenic, chromium, selenium, etc.).

Biodegradation is a natural process carried out by soil and aquatic microorganisms – mostly bacteria and fungi. Certain bacterial strains have a demonstrated ability to break down or transform the chemical substances present in petroleum products. Oil-spill bioremediation methods aim at providing favorable conditions of oxygen, temperature and nutrients to maximize biological hydrocarbon breakdown. Such methods have been applied successfully in restoring polluted seashores, airports, military operations, power plants, etc.

This paper is a short overview of the commonest petroleum hydrocarbon bioremediation methods currently used. Emphasis has been placed on sampling and analytical methodologies (Table 1), microorganisms (Table 2), and treatment enhancement by supplying nutrients, oxygen, etc. (Table 3).

Introduction

Bioremediation is a rapidly developing field of environmental restoration, utilizing natural microbial activ-

Sampling precautions

Hydrocarbon samples are subject to chemical, biological, and physical changes as soon as they are collected. Sample handling, preservation, and storage techniques should minimize changes in sample composition by retarding chemical and/or biological activity. Contamination of sediment samples during collection and handling should be avoided. A detailed procedure for sampling equipment operation and sample handling and storage should be clearly stated in the sampling plan. This may be accomplished by using standard operating procedures [U.S. Environmental Protection Agency (EPA) 1995]. For example, samples designated for hydrocarbon analysis should not come into contact with plastic surfaces.

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Sample contamination from vessel emissions and sampling apparatus must be avoided. Core samples are recommended for the determination of contamination at different depths. Water samples should be collected with either a non-contaminating pump or a discrete water sampler. When sampling with a pump, using a peristaltic pump or one with a magnetically coupled impeller can minimize contamination. Water samplers should be made of stainless steel or acrylic plastic, while seals should be Teflon-coated whenever possible. Prior to sample collection, water sampling devices should be rinsed by a solvent that does not react with the sampler material. For extended storage, freezing in solid CO₂ or a freezer is recommended. For shorter periods of storage, refrigeration at 4 °C is sufficient.

Hydrocarbon analysis

Sediment samples are dried overnight at 60 °C. Residual oil is extracted in chloroform for 8 h using a Soxhlet apparatus. Total oil content is estimated by weighing the dry residue after solvent evaporation. Subsequently, the extract is sonicated in 60 ml hexane (5 min, 40 kHz). The hexane-insoluble fraction contains asphaltenes, which are obtained by filtration on solvent-washed, preweighed Whatman GF/A glass-microfibre filters. The asphaltenes retained on the filters are, in turn, dried and weighed. The hexane-soluble fraction (maltenes) is separated by solid/liquid chromatography on a 15-cm length × 1-cm diameter silica gel column (100–200 mesh), activated overnight at 110 °C. The saturated petroleum hydrocarbons, aromatics and resins are successively eluted with 60 ml hexane, 60 ml 60:40 (v/v) mixture of hexane/dichloromethane and 60 ml methanol. After evaporation of the solvents, each fraction is weighed and the relative percentage of the four classes (saturates, aromatics, resins, and asphaltenes) can be calculated. The saturated and aromatic fractions are analyzed by computerized capillary gas chromatography using a gas chromatograph with a direct-injection port, equipped with a flame ionization detector and a capillary column (50 m × 0.32 mm, film thickness 0.25 µm); He at 0.8 bar is the carrier gas. The column temperature is programmed from 100 °C to 320 °C at 3 °C/min for the saturated fraction, and from 100 °C to 300 °C at 3 °C/min for the aromatics (Oudot 1994).

A variant of the aforementioned analytical procedure uses a column packed with silica gel, where the hexane-soluble fraction is partitioned into saturated, aromatic and polar fractions (Mille et al. 1991). Each fraction is weighed using a microbalance. Saturated hydrocarbons are analyzed by capillary gas chromatography. The apparatus used is equipped with a capillary column (30 m; 0.32 mm inner diameter), an on-column injector and a flame ionization detector. Temperature increase is programmed from 70 °C to 300 °C at 5 °C/min, with helium as the carrier gas at 0.5 bar. Aromatic hydrocarbons are analyzed by UV synchronous luminescence spec-

troscopy on a spectrophotometer. Polar compounds are analyzed by UV fluorescence and Fourier-transform infrared spectroscopy (FTIR) on a spectrophotometer, whereas asphaltenes are analyzed by FTIR.

The fraction containing the saturated and aromatic hydrocarbons is separated by capillary gas chromatography using standards. *n*-Alkanes are identified by comparing their retention times with those of standard *n*-alkanes ranging from *n*-C₁₁ to *n*-C₃₁. Concentrations of individual hydrocarbons are determined by calculating the surface area underneath the sample peak of the respective constituent, identified from its *R_F*. The content in each *n*-alkane (*n* < 31) is determined as the ratio of the area under the corresponding sample peak to the area under the peak of internal standards.

The aromatic fraction is characterized by gas chromatography/mass spectrometry (GC/MS) (Venosa et al. 1991). Some investigators (Huesemann 1995) utilize a comprehensive petroleum characterization procedure involving group-type-separation analyses, boiling-point distributions, and hydrocarbon typing by field desorption mass spectroscopy to determine initial and final concentrations of specified hydrocarbon classes.

Currently available information on analytical techniques employed for quantitative determination of petroleum hydrocarbons (Table 1) distinguishes methods for (a) the measurement and identification of contaminants, and (b) the measurement of microbial activity. The former are very important because they provide baseline information including the quantity and type of contaminants, distribution patterns, treatability, etc. The latter monitor biological activity and provide an assessment of biodegradation. When information from both methods is combined, the effectiveness of bioremediation can be evaluated.

Petroleum hydrocarbon contamination usually exists as a complex mixture of hydrocarbons. Despite the huge potential of microorganisms to degrade organic compounds under favorable conditions, no single species of microorganism can degrade all the components of a given oil (Office of Technology Assessment, 1991), and no oil-degrading "superbug" has been engineered. Currently, several organisms are known, each capable of degrading usually one or, at best, a few petroleum components at a time. Therefore, effective bioremediation of petroleum contamination requires a mixture of populations consisting of different genera each capable of metabolizing the respective compounds. Table 2 summarizes information on some commercially available bacterial and fungal strains used for petroleum hydrocarbon bioremediation. The bioremediation capacity of bacteria has been investigated more extensively because they are (1) easier to culture, (2) more amenable to molecular biology techniques, (3) capable of metabolizing chlorinated organics, (4) capable of mineralizing these chemicals and using them as carbon energy sources (Bouwer and Zehnder 1993). Although capable of metabolizing some aromatic contaminants, fungi require a primary growth substrate, such as glucose or cellulose to

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Table 1 Analytical Techniques

Name	Short description	Parameter measured	Applicable to	Advantages	Constraints	Reference
Soil respirometry	The rate of crude oil biodegradation in a soil matrix is assessed by measuring the rate of carbon dioxide production and oxygen consumption in a soil respirometer	Microbial activity	Soils with low organic matter content	Appears to provide accurate estimates of contaminant biodegradation kinetics	The addition of petroleum hydrocarbons to high-organic-carbon soils may result in enhanced biodegradation of soil organic matter that cannot be accounted for in a control experiment	Huesemann and Moore 1994
Electrolytic respirometry	An electrolytic respirometry apparatus is comprised of a temperature-controlled water bath containing measuring units, a recorder for digital indication and direct plotting of the oxygen uptake velocity curves, and a cooling unit for the conditioning and continuous recirculation of water bath volume. Each measuring unit is comprised of a reaction vessel with a carbon dioxide absorber mounted inside, an oxygen generator connected by tubing, and pressure indicators connected electronically to the unit. Several measuring units can be monitored simultaneously and data stored in a computer. The measuring units are interconnected by tubing, forming an air-sealed system. Depletion of oxygen by microbial activity within each measuring unit creates a drop in pressure. Oxygen production is triggered by the reduced pressure and sufficient oxygen is produced to counterbalance oxygen loss. The electrical current used to generate the oxygen is measured by the digital recorder, and the data are converted directly into mg/l oxygen uptake. The CO ₂ produced by microbial activity is absorbed by soda lime. The nitrogen/oxygen ratio in the gas phase above the sample is maintained constant throughout the experiment, and there is no depletion of oxygen	Microbial activity	Soils with low organic matter content	Atmospheric pressure fluctuations do not adversely affect the results	Same as above	Venosa et al. 1991
Gas chromatography	This technique separates a complex mixture of organic materials into its components. The sample extract is injected into a heated chamber, in which the mixture is concentrated at the head of a separating column. The mixture is then carried through the column by an inert gas (mobile phase). As the column is heated, the analytes pass through absorbent materials (stationary phase). From the chromatogram and the digital information contained in the quantification report, analytes contained in the sample can be accurately identified and quantified	Contaminant identification and determination	Complex extracts of oil, containing hundreds, even thousands, of components	Satisfactory separation of complex mixtures, high sensitivity, accurate identification, quantification	Experimental conditions (especially the velocity of the carrier gas) should be continuously monitored and kept stable	U.S. EPA 1995; Hutchins and Wilson 1994

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Table 1 (Contd.)

Name	Short description	Parameter measured	Applicable to	Advantages	Constraints	Reference
Gas chromatography/ flame ionization detection	In the MS chamber, compounds separated by GC are bombarded by electrons and broken down into characteristic fragments called ions. Compounds are identified on the basis of the electrical current required to ionize the fragments. A sensor accumulating data on electrical current required to ionize that mass detects the mass of charged fragments (i.e., their molecular mass). The relative intensity of this current over all the different masses recorded for a particular compound generates its mass spectrum. The pattern of fragmentation ions in a mass spectrum has a characteristic appearance used to distinguish one compound from another. In addition, the intensity of the current recorded for one characteristic ion over time gives rise to its mass chromatogram, which is used to quantify the concentration of the analyte as it elutes from the gas chromatograph. This characteristic ion is called the quantification ion. The mass chromatograms for all ions detected in a sample can be superimposed onto a reconstructed ion chromatogram (RIC), also called a total ion chromatogram. The RIC is a graphic display of the total ionization current resulting from all mass fragments of all compounds detected throughout the analysis.	Contaminant identification and determination	Identification of compounds eluted from a GC column	GC/MS enables positive identification. The RIC can be compared with the chromatograms produced by other detectors and provides an indication of the relative composition of components in the sample mixture analyzed by GC/MS.	Expensive equipment, complicated experimental procedure	U.S. EPA 1995
Gas chromatography/ flame ionization detection	The flame ionization detector (FID) responds to ions produced by the burning of compounds (separated by gas chromatography) in a H ₂ /air flame. FID response depends on the number of ions produced by a compound. Since this varies considerably between compound classes, FID response factors vary correspondingly.	Contaminant identification and determination	Identification of almost all organic compounds, after their elution from a GC column	FID response is linear in a wide range of concentrations and not influenced by temperature changes	Carbon compounds detected must be oxidizable. A few organic compounds (e.g., formic acid, acetaldehyde) exhibit poor sensitivity	Hatziananou 1989; Price et al. 1994; Karazek and Clement 1988; Sveum et al. 1994
Luminescence techniques	Bioluminescence is a natural phenomenon associated with several microorganisms. Reporter gene constructs, comprised of luciferase structural genes and contaminant-specific promoter sequences, are inserted into a bacterial host. Their expression is induced by the contaminant hydrocarbon of interest. Light emitted by such systems can be measured and used to monitor bacterial metabolic activity using the specific contaminant	Microbial activity	Determination of the aromatic content of a complex mixture of hydrocarbons. The aromatic content is important because of its toxicity against weathering. Provides preliminary assessment of contaminant bioavailability for biodegradation	High sensitivity and reasonable selectivity. Direct examination of oil mixtures in water is possible under favorable circumstances. Does not require the disruption of cellular activities or structures; provides real-time data on cellular catabolic activities in vivo	Severe and not quantifiable quenching of light emission in soil slurries compared to artificial, soil-free media. Some bacteria do not exhibit bioluminescence. Limited application in multi-species oil-degrading microbial communities and under field conditions of poorly controlled environmental parameters	Grisson et al. 1985; Sanseverino et al. 1994; Hastings et al. 1985; Heizer et al. 1992

Fluorescence analysis	During fluorescence analysis of a compound, excitation and emission spectra are recorded. These two spectra can be corrected through appropriate modifications, and used for the identification of the compound	Contaminant identification and determination	Oil fingerprinting	This technique has become a part of a basic and routine analytical procedure, because of the considerable success in identification following oil spills in coastal waters. This procedure involves the use of TLC, IR and GC measurements	A conventional fluorescence spectrum merely represents one section across a three-dimensional matrix determined by the dependence of intensity on the two wavelength parameters of emission and excitation. The subjectivity imposed by the choice of a single excitation wavelength can lead to the exclusion of valuable conventional fluorescence spectra. Although valuable as a rapid means of discrimination between some suspect sources this method is severely limited in its ability to differentiate between hydrocarbon mixtures of similar composition	Grigson et al. 1985; Hatzizoiannou 1989
Use of internal petroleum biomarkers	This analytical approach is used to quantify the extent of oil weathering from biological or physical processes. Provided that all concentration data are expressed on an oil-weight basis, the increase in the internal biomarker concentration relative to the concentration in the source oil is proportional to the amount of oil lost through weathering and biodegradation. The method is gravimetric and relies on GC/FID and GC/MS to determine total oil or specific analyte concentrations	Contaminant identification and determination	Both field and laboratory studies of petroleum and refined products. Provides quantitative information about the degradation of petroleum in highly variable environments	Field data indicate that the use of an internal chemical indicator reduces spatial variability of oil data when compared to other mass-balance approaches. This allows effective degradation monitoring by reducing the number of samples required	The application of an internal chemical indicator to monitor oil weathering or degradation is based on the following assumptions: (1) the source of oil contamination has been identified and is primarily a single source; (2) the chemical indicator is not formed or degraded during biodegradation, and (3) the extraction efficiency of the chemical marker is the same as the rest of the oil	Douglas et al. 1994; Prince et al. 1994
TPH/infrared spectroscopy - TPH/gas chromatography	Total petroleum hydrocarbons/IR spectroscopy (EPA 418.1 modified for soils; U.S. EPA 1986, 1989) is the method where an oiled sample is solvent-extracted. The total mass of dissolved hydrocarbon is subsequently quantified by comparing the infrared absorption of the extraction liquid against that of a defined hydrocarbon mixture. Gas chromatographic methods for petroleum hydrocarbon analysis include the American Society for Testing and Materials (ASTM) method 3328-78 (ASTM 1989) and EPA modified method 8015 (US EPA 1986, 1989). Both methods use GC/FID to measure petroleum hydrocarbons	Contaminant identification and determination	Measurements of total petroleum hydrocarbons (TPH) for the assessment/screening of diesel-fuel-contaminated soils, as well as for determining site closure	Low technical complexity, quick quantification	The mixture in which the diesel fuel bioremediation is being performed can affect both analytical methods. Matrix effects are site-specific. Neither analytical method can overcome these limitations. Part of the difficulty is the use of a non-compound-specific measurement to assess progress. One way to overcome this difficulty may be through the use of target or specific marker compounds	Troy and Jergert 1994

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Table 3 In situ treatments

Treatment	Description	Applicable to	Advantages	Constraints	Reference
Bioaugmentation	Bioaugmentation involves the addition of oxygen, water and mineral nutrients (usually combinations of nitrogen, phosphorus, and trace metals)	Groundwater, soils	Acceleration by as much as 100-fold of the rate of production of organisms already present at the site.	Nutrient application requires current ecological monitoring studies. Although dilution, tidal mixing etc. should minimize the potential for adverse ecological effects, certain components of the fertilizer could be toxic to some species. In addition, algal blooms could occur as a result of the sudden availability of nitrogen and phosphorus.	Orzech et al. 1991; U.S. EPA 1989
Bioventing	This remediation technique combines conventional advective soil venting with biodegradation. The soil-venting phase of the process takes advantage of the highly volatile nature of the components present in the hydrocarbon mixture. In the biodegradation phase, the less volatile high-molecular-mass hydrocarbon compounds are broken down and the more volatile organics removed during conventional venting.	Soils	Adds full range of petroleum hydrocarbons. Is among the most effective methods of supplying indigenous microorganisms with enough oxygen to support degradation of hydrocarbon contaminants.	A shallow water table prevents diffusion of gases and makes application of bioventing less feasible. Furthermore, the solid matrix must allow for sufficient air movement.	Van Eyk 1994; Reisinger et al. 1994
Bioaugmentation	This method involves the direct application of microorganisms originating from (a) the remediation site (b) an off-site vendor (c) genetic engineering. The microorganisms have been cultured and adapted, while their degrading ability can be enhanced for specific contaminants and site conditions.	Groundwater, soils	One of the most effective bioremediation techniques.	The possibility of adverse ecological effects from the introduction of off-site or genetically engineered microorganisms should be examined and minimized.	Orzech et al. 1991; Leavitt and Brown 1994
Surfactants	Synthetic or biogenic substances are used to increase the aqueous solubility of solid hydrocarbons, and emulsify liquid hydrocarbons.	Solid and liquid aliphatic and aromatic hydrocarbons (including hydrophobic organic compounds)	Enhancement of contaminant accessibility to microorganisms, nutrients, and possibly oxygen. Improvement of the bioremediation efficiency.	Surfactant use is still controversial. Synthetic surfactants may adversely affect the permeability of the microbial cell membrane, thus reducing or eliminating the biodegradative potential of indigenous microorganisms. Surfactant injection must be carefully controlled to be ecologically acceptable and to offer more economical and physical advantages than disadvantages.	Ducieux et al. 1994; Churchill et al. 1995

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Table 3 (Contd.)

Treatment	Description	Applicable to	Advantages	Constraints	Reference
Hydrogen peroxide	Hydrogen peroxide can be used as an efficient alternative oxygen source to enhance in situ biodegradation	Biodegradation of aromatic compounds and volatile fatty acids. Subsurface applications	Provides oxygen at a rate up to two orders of magnitude faster than diffusers (systems to saturate injected water with air). The growth of aerobic bacteria in general and hydrocarbon-degrading bacteria in particular, as well as the breakdown of gasoline are significantly enhanced by the addition of H_2O_2 .	Although H_2O_2 has been used successfully in a number of cases, problems, including too rapid decomposition, gas blockage, and ineffectiveness, have been encountered elsewhere	Lu 1994a; Brown and Norris
Nitrate	Nitrate is used as an alternative electron acceptor, instead of aeration of the injected water or amendment with H_2O_2	Subsurface applications	Nitrate is relatively inexpensive, highly soluble in water, does not adsorb in soil matrices, and does not decompose. Thus it can be readily distributed within an aquifer	Nitrate concentrations in groundwater typically are limited by regulatory standards to 46 mg/l. Another disadvantage is that nitrate is effective for fewer classes of compounds than oxygen. Nitrate-utilizing bacteria do not degrade aliphatic compounds. Benzene is not readily degraded under denitrifying conditions	Huchins and Wilson 1994; Brown and Norris 1994b; Butterman et al. 1994
Fertilizer application (water-soluble or oleophilic)	Fertilizer application stimulates microbial metabolism by supplying the indigenous oil-degrading microorganisms with nutrients (N, P, K, etc.)	Soil, groundwater, sediments	Acceleration of the natural biodegradation process, especially in sites with low nutrient content	A toxicology assessment is required to evaluate the possibility of harmful biological effects of excessive nutrient application	Prince et al. 1994; Sveum et al. 1994

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co-oxidize these compounds. However, because fungi can not further metabolize the products of co-oxidation, mixed cultures with bacteria are required for complete mineralization of the organic contaminant (Bouwer and Zehnder 1993).

The origin of the organisms to be used in bioremediation is debatable. Indigenous microorganisms isolated from the contaminated site are certainly adapted to the climatic, physicochemical and nutrient conditions prevalent therein. Bioremediation by these microorganisms is expected to proceed at increased rates after nutrient addition and/or seeding with enriched microorganism cultures. However, these microbial communities may not include the full range of species or enzymes required for effective oil biodegradation. Commercially available preparations of oil-biodegrading microorganisms usually include many species and have an increased potential to degrade various oil components effectively. Suppliers claim that these mixtures can be custom-made for the specific oil or environmental conditions and can also be easily produced for emergency situations.

The commonest methods of in situ hydrocarbon bioremediation are tabulated in Table 3. The addition of N_2 , P or oxygen in various forms is thought to be sufficient to enhance the growth of microbes capable of utilizing carbon in the form of petroleum hydrocarbons (Alexander 1991). However, bioremediation is not a panacea against organic contamination. The spectacular results of laboratory experiments cannot always be transferred directly to the field. Many compounds that are easily metabolized in vitro are often not broken down efficiently in contaminated soils and aquifers. This is probably due to reduced contaminant bioavailability caused by adsorption on soil particles or solution in nonaqueous-phase liquids (Alexander 1991). Another bioremediation-restricting factor is the patchy distribution of bacterial populations in the soil. This natural heterogeneity of environmental conditions on the microhabitat scale results in localization of the hydrocarbon-degrading bacteria.

In contrast to the wide public acceptance of bioremediation using indigenous microorganisms, the public and the scientific community are somewhat reluctant to accept the method of seeding with introduced microorganisms. The main reasons for this (Atlas 1977) are (1) the lack of controlled experiments demonstrating superior performance of introduced microorganisms compared to indigenous ones, (2) the time lag between microorganism application and hydrocarbon breakdown, (3) the lack of information on microorganism pathogenicity to humans, genetic stability and toxicity of metabolic byproducts, (4) the large quantities of microorganisms required for frequent application to the contaminated site, (5) the logistics of culture preparation and mixing just prior to application on site, and (6) the fate of these microorganisms once they have completed their role in bioremediation.

Future research should be directed towards the improvement of existing and the devising of innovative

methods of bioremediation. Attention should be paid to the factors governing bioavailability of organic contaminants and methods to increase availability and microorganism activity, monitoring of bioremediation, the ecology and fate of introduced microorganisms, the transfer of laboratory findings to the field, the elucidation of fertilizer action, the discovery of new, more efficient fertilizers and ways of application, the coupling of major biochemical cycles, such as nitrogen and phosphorus, with bioremediation cases, and the effects of other environmental factors (e.g. temperature and salinity fluctuations, wave action, oxygen availability, nitrogen-fixing bacteria, etc.); most importantly, all of the above should be investigated in the field under realistic conditions.

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Comparative study of five polycyclic aromatic hydrocarbon degrading bacterial strains isolated from contaminated soils

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Abstract: Five polycyclic aromatic hydrocarbon (PAH) degrading bacterial strains, *Pseudomonas putida* 34, *Pseudomonas fluorescens* 62, *Pseudomonas aeruginosa* 57, *Sphingomonas* sp. strain 107, and the unidentified strain PL1, were isolated from two contaminated soils and characterized for specific features regarding PAH degradation. Degradation efficiency was determined by the rapidity to form clearing zones around colonies when sprayed with different PAH solutions and the growth in liquid medium with different PAHs as sole source of carbon and energy. The presence of plasmids, the production of biosurfactants, the effect of salicylate on PAH degradation, the transformation of indole to indigo indicating the presence of an aromatic ring dioxygenase activity, and the hybridization with the SphAb probe representing a sequence highly homologous to the naphthalene dioxygenase ferredoxin gene *nahAb* were examined. The most efficient strain in terms of substrate specificity and rapidity to degrade different PAHs was *Sphingomonas* sp. strain 107, followed by strain PL1 and *P. aeruginosa* 57. The less efficient strains were *P. putida* 34 and *P. fluorescens* 62. Each strain transformed indole to indigo, except strain PL1. Biosurfactants were produced by *P. aeruginosa* 57 and *P. putida* 34, and a bioemulsifier was produced by *Sphingomonas* sp. strain 107. The presence of salicylate in solid medium has accelerated the formation of clearing zones and the transformation of indole by *Sphingomonas* sp. strain 107 and *P. aeruginosa* 57 colonies. Plasmids were found in *Sphingomonas* sp. strain 107 and strain PL1. The SphAb probe hybridized with DNA extracted from each strain. However, hybridization signals were detected only in the plasmidic fraction of *Sphingomonas* sp. strain 107 and strain PL1. Using a polymerase chain reaction (PCR) approach, we determined that several genes encoding enzymes involved in the upper catabolic pathway of naphthalene were present in each strain. Sequencing of PCR DNA fragments revealed that, for all the five strains, these genes are highly homologous with respective genes found in the *pah*, *dox*, and *nah* operons, and are arranged in a polycistronic operon. Results suggest that these genes are ordered in the five selected strains like the *pah*, *nah*, and *dox* operons.

Key words: polycyclic aromatic hydrocarbons, biodegradation, polymerase chain reaction, naphthalene catabolic genes.

Résumé : Cinq souches bactériennes capables de dégrader les hydrocarbures aromatiques polycycliques (HAP), *Pseudomonas putida* 34, *Pseudomonas fluorescens* 62, *Pseudomonas aeruginosa* 57, *Sphingomonas* sp. souche 107 et la souche PL1 non identifiée, ont été isolées à partir de deux sols contaminés. Nous avons étudié leurs caractéristiques face à la dégradation des HAP. L'efficacité de dégradation a été déterminée en se basant sur la rapidité de formation de zones d'éclaircissement autour de colonies lors de la vaporisation avec différentes solutions de HAP, ainsi que par la croissance bactérienne en milieu liquide avec différents HAP comme seule source de carbone et énergie. Nous avons aussi examiné la présence de plasmides, la production de biosurfactants, l'effet du salicylate sur la dégradation des HAP, la transformation de l'indole en indigo indiquant la présence de l'activité d'une dioxygénase du noyau aromatique, et l'hybridation avec la sonde SphAb représentant une séquence hautement homologue au gène *nahAb* codant pour la ferrédoxine de la naphthalène dioxygénase. La souche la plus efficace pour la spécificité de substrats et la rapidité à dégrader différents HAP était *Sphingomonas* sp. souche 107, suivie de la souche PL1 et de *P. aeruginosa* 57. Les souches les moins efficaces étaient *P. putida* 34 et *P. fluorescens* 62. Chaque souche a transformé l'indole en indigo, excepté la souche PL1. *Pseudomonas putida* 34 et *P. aeruginosa* 57 ont produit des biosurfactants, tandis qu'une activité bioémulsifiante a été observée chez *Sphingomonas* sp. souche 107. La présence de salicylate en milieu solide a accéléré la formation de zones d'éclaircissement et la transformation de l'indole par les colonies de *Sphingomonas* sp. souche 107 et *P. aeruginosa* 57. Des plasmides ont été trouvés chez *Sphingomonas* sp. souche 107 et la souche PL1. La sonde SphAb a hybridé avec l'ADN extrait de chacune des souches. Cependant, des signaux d'hybridation ont été détectés uniquement dans la fraction plasmidique de *Sphingomonas* sp. souche 107 et de la souche PL1. Par la réaction

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en chaîne de la polymérase (PCR), nous avons déterminé que plusieurs gènes codant pour la voie catabolique supérieure du naphthalène étaient présents dans chaque souche. La séquence des produits de PCR a révélé que, pour toutes les souches, ces gènes sont hautement homologues aux gènes respectifs retrouvés dans les opérons *dax*, *pah* et *nah*, et qu'ils sont arrangés en un opéron polycistronique. Nos résultats suggèrent également que ces gènes soient ordonnés dans les cinq souches de façon similaire aux opérons *pah*, *dax* et *nah*.

Mots clés : hydrocarbures aromatiques polycycliques, biodégradation, réaction en chaîne de la polymérase, gènes responsables du catabolisme du naphthalène.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are widespread pollutants resulting from human activities. They are released in large quantities from fossil fuel combustion and various industrial processes. Several PAHs are known or suspected to be toxic, mutagenic, and carcinogenic. Exposure to PAHs can be hazardous to human health, and therefore, their elimination is considered a priority in many countries (White 1986).

Many bacterial strains have been isolated for their ability to degrade and use PAHs as source of carbon and energy. The catabolic pathway of naphthalene has been elucidated for *Pseudomonas putida* G7, while for other PAHs this has been partly done (see reviews in Yen and Serdar 1988; Cerniglia 1992; Pothuluri and Cerniglia 1994). Naphthalene catabolism in *P. putida* G7 involves hydroxylation of one aromatic ring to produce 1,2-dihydroxy-1,2-dihydronaphthalene by the naphthalene dioxygenase. This multimeric enzyme is composed of a reductase (encoded by *nahAa*), a ferredoxin (*nahAb*), and two subunits of iron-sulfur protein (*nahAc* and *nahAd*). After the dehydrogenation of the 1,2-dihydroxy-1,2-dihydronaphthalene to 1,2-dihydroxynaphthalene (by a dehydroxygenase encoded by *nahB*), the hydroxylated ring is cleaved by the 1,2-dihydroxynaphthalene dioxygenase (*nahC*). Further reactions involving an isomerase (*nahD*), a hydratase-aldolase (*nahE*), and a dehydrogenase (*nahF*) lead to the formation of salicylate and pyruvate (Eaton and Chapman 1992; Eaton 1994). Salicylate is further transformed to catechol, which is then degraded by *ortho* or *meta* cleavage depending on the bacterial strains; this is referred to as the lower pathway (Williams and Sayers 1994). The catabolic pathway of naphthalene to salicylate, referred to as the upper pathway, occurs also with other PAHs. For example, it is involved in the transformation of phenanthrene and anthracene to 1-hydroxy-2-naphthoic acid and 2-hydroxy-3-naphthoic acid, respectively, in *Pseudomonas fluorescens* SR (Menn et al. 1993).

Genes encoding the lower and upper pathways in *P. putida* G7 are arranged in two respective polycistronic operons, *sal* and *nah*, located on the plasmid NAH7. Control of expression of the two operons is regulated by the *nahR* gene product. The activity of NahR depends on the intracellular concentration of salicylate (Schell 1985). PAH-degradation genes have been isolated from other pseudomonads. These genes are arranged in polycistronic operon such as (i) the *nah* operon on the pDTG1 plasmid in *P. putida* NCIB 9816-4 (Harayama and Rekik 1989; Simon et al. 1993; Eaton 1994); (ii) the *pah* operon located on the chromosome in *P. putida* OUS82 (Takizawa et al. 1994); (iii) the *ndo* operon located on plasmid in *P. putida* NCIB 9816 (Kurkela et al. 1988); and (iv) the *dax* operon located on a plasmid in *Pseudomonas* strain C18 (Denome et al. 1993). Nucleotide and the deduced

respective genes of the *nah*, *pah*, *ndo*, and *dax* operons. The gene order of the *nah* operon is *nahAa-nahAb-nahAc-nahAd-nahB-nahF-nahC-nahQ-nahE-nahD* (Eaton 1994). This arrangement occurs also in the *pah*, *dax*, and *ndo* operons. PAH-degradation genes are encountered in other *Pseudomonas* species. For example, strong hybridization signals were found between a *nah* probe and plasmids recovered from three *Pseudomonas* strains involved in the mineralization of naphthalene, phenanthrene, and anthracene (Sanseverino et al. 1993a). Similar results were observed with strains of *Pseudomonas stutzeri* involved in degradation of naphthalene (Rossello-Mora et al. 1994). The only published study of PAH catabolic genes from nonpseudomonads is on *Comamonas testosteroni* strains that have the ability to degrade naphthalene, phenanthrene, and anthracene. Although the PAH catabolic pathway is similar to pseudomonads, Goyal and Zylstra (1996) showed that no hybridization signal was detected with the *nah* probe in these bacterial strains and that the gene arrangement was different. These observations suggest that substantial differences occur at the gene level in *C. testosteroni* that may reflect a different approach of PAH degradation such as gene regulation and enzymatic specificity.

We were interested in finding highly efficient, PAH-degrading bacterial strains with a broad range of substrate specificity. We were also interested in determining whether important changes at the gene level can be found in these strains that could be related to the efficiency of PAH degradation. In this paper, we investigated five bacterial strains chosen from a pool of 65 PAH-degrading bacterial isolates derived from two contaminated soils. Comparison of these strains revealed differences in their substrate specificity and their efficiency to degrade PAHs.

Materials and methods

Isolation of PAH-degrading bacterial strains

Soils came from a sandpit heavily contaminated with oil refinery wastes and an area contaminated with aromatic hydrocarbons. Soil samples were agitated with four volumes of Bushnell-Haas minimal salt medium (BH) at 150 rpm for 10 days and 30°C in presence of 100 ppm of different PAHs such as phenanthrene, fluorene, pyrene, fluoranthene, and chrysene. Dilutions of soil slurry were inoculated on BH medium plates supplemented with 0.05% w/v yeast extract (BHY). Colonies were chosen for their capacity to form clearing zones and (or) produce a color change when sprayed with different PAH solutions (Sylvestre 1980). These solutions were prepared (2% w/v in acetone) with naphthalene, three-ring PAHs (dibenzothiophene, fluorene, phenanthrene, and anthracene), and four-ring PAHs (fluoranthene, pyrene, and benz[a]anthracene). PAHs were purchased from Aldrich Chemical Co. (Milwaukee, Wis.). Colonies were also examined for the expression of aromatic ring dioxygenase activity by the formation of blue indigo when they were transferred

checked at regular time intervals for the appearance of clearing zones and blue-indigo color.

Identification of bacterial strains

PAH-degrading bacterial strains were characterized based on colony morphology and pigmentation, Gram staining, API (Analytical Profile Index) 20E strips (bioMérieux Vittek, Inc., Hazelwood, Mo.), and several physiological and biological tests (Krieg and Holt 1984).

Effect of salicylate on PAH degradation

Bacteria were plated on BHY solid medium containing 100 µg/mL of sodium salicylate (Aldrich Chemical, Milwaukee, Wis.) and incubated at 30°C, except at 25°C for strain PL1, for 1–4 days. Colonies were sprayed with the same PAH solutions as enumerated before. They were checked at regular time intervals for the appearance of the clearing zones. Controls were BHY agar plates without salicylate inoculated with bacteria.

Growth in liquid culture with PAH as sole source of carbon

Bacterial strains were grown at 30°C (or 25°C, strain PL1) on BHY agar plates for 1–4 days. Three to five colonies were picked and added to 25 mL of BH medium containing 200 ppm of respective PAH and agitated in the dark at 200 rpm and 30°C (or 25°C, strain PL1). The PAHs used were the same as enumerated before. Cultures were regularly checked for bacterial growth (turbidity), appearance of color (reflecting appearance of intermediates), and disappearance of nonsolubilized PAH crystals. Negative controls were noninoculated media.

Biosurfactant production

Presence of surface tension lowering activity in bacterial cultures was determined with the drop-collapsing test (Jain et al. 1991) and measured with a Fisher tensiometer by the De Noy ring method. Emulsifying activity was estimated visually as described by Déziel et al. (1996). The positive control was the biosurfactant-producing strain *Pseudomonas aeruginosa* 19SJ (Déziel et al. 1996).

Measurement of PAHs degradation in liquid culture

The 25-mL cultures were acidified with 10 N HCl and extracted with three volumes of 10 mL diethyl ether. The final volume of the extraction was adjusted to 25 mL and dehydrated with anhydrous sodium sulfate. Residual PAHs were determined by gas chromatography (GC) (Hewlett Packard, model 5890A) with a flame ionization detector. Finally, the residual concentration of PAHs was calculated in comparison to an internal standard (9-methyl anthracene).

Plasmid extraction

Plasmids from *Sphingomonas* sp. strain 107, strain PL1, and *Escherichia coli* were extracted by alkaline lysis protocol and purified either by polyethylene glycol precipitation or a CsCl gradient as described by Sambrook et al. (1989).

Total DNA extraction

Bacterial cells were centrifuged at 8000 × g for 10 min, resuspended in 50 mM Tris-HCl (pH 8.0) – 20 mM EDTA – 150 mM NaCl with 5 mg lysozyme/mL, and incubated 10 min at 37°C. Sodium dodecyl sulfate (SDS), β-mercaptoethanol, and proteinase K were added to a final concentration of 2%, 1%, and 50–100 µg/mL respectively, and incubated 2–3 h at 45°C. The solution was then extracted twice with phenol (equilibrated with Tris-HCl pH 8.0), twice with phenol – chloroform – isoamyl alcohol (50:49:1), and once with chloroform – isoamyl alcohol (24:1). After RNase treatment (5 µg/mL for 15 min at 37°C), DNA was precipitated with

Polymerase chain reaction (PCR)

Consensus sequences used to generate oligonucleotides specific for PAH-degradation genes were deduced from the alignment between sequences available from *pah*, *nah*, and *dax* operons using the PILEUP program of GCO (Genetics Computer Group, University of Wisconsin). Universal eubacterial primers for 16S rRNA gene are the following: pA, 5'-AGAGTTTGATCCTGGCTCAG-3' (location 8–27 based on the *E. coli* 16S rRNA sequence); pH, 5'-AAGGAGGTGATCCAGCCGCA-3' (location 1541–1522) (Bruce et al. 1992). Oligonucleotides were synthesized using a Gene Assembler (Pharmacia, Baie-d'Urfé, Que.).

Amplification was accomplished in a 50-µL reaction volume with 10 mM Tris-HCl (pH 9.0), 1.5 mM MgCl₂, 50 mM KCl, 200 µM dNTP, 10 pmol of each primer, and 2.5 U of *Taq* DNA polymerase (Pharmacia). Amplifications were done in a DNA thermal cycler 480 (Perkin-Elmer) at 80°C for 3 min (in which 50–100 ng of template DNA was added), 94°C for 5 min, and 55°C for 5 min; then 30–35 cycles at 72°C for 2 min, 94°C for 40 s, and 55°C for 1 min; and finally, an extension time of 10 min at 72°C. Amplification products were examined by 1.0–1.8% agarose gel electrophoresis.

PCR for amplification of large DNA fragments (more than 2 kb) was accomplished in a 50-µL volume as recommended by the manufacturer (Expand PCR, Boehringer Mannheim, Laval, Que.) with 50 mM Tris-HCl (pH 9.2), 1.75 mM MgCl₂, 16 mM (NH₄)₂SO₄, 350 µM dNTP, 15 pmol of each primer, 100–200 ng of template DNA, and 2.5 U of thermostable DNA polymerases. Amplifications were done in a DNA thermal cycler 480 (Perkin-Elmer) at 94°C for 2 min; 10 cycles at 94°C for 30 s, 55°C for 30 s, and 68°C for 4 min; then 25 cycles at 94°C for 30 s, 55°C for 30 s, and 68°C for 4 min with 20-s extension time/cycle; and finally an extension time of 7 min at 68°C.

Cloning of PCR products

Amplified DNA were recovered from agarose gel electrophoresis with a Naf solution and a silica matrix column as described by the manufacturer (Geneclean II, BIO 101 inc. LaJolla, Calif.). They were ligated to the TA vector (pCRII) and transformed in the *E. coli* INV αF' host (Invitrogen, San Diego, Calif.) as recommended by the manufacturer, or ligated into the *Sma*I-digested pTZ18U (Bio-Rad) vector and transformed in *E. coli* XL1 blue as described by Sambrook et al. (1989).

DNA manipulations

Restriction endonuclease digestions and DNA separation on agarose gel electrophoresis were carried out as described by Sambrook et al. (1989). DNA was transferred from agarose gels on nylon charge membrane with ammonium acetate solution as described by Rigaud et al. (1987). The SphA probe was prepared by PCR amplification of a 291-bp DNA fragment with the ferredoxin primers (FerD – FerG, Fig. 3) using the plasmid of *Sphingomonas* sp. strain 107. This 291-bp fragment was labelled by random primer extension with digoxigenin-11-dUTP (DIG) according to the manufacturer specifications (Boehringer Mannheim, Laval, Que.). Hybridization and detection conditions were also done according to the manufacturer specifications (Boehringer Mannheim) with the hybridization temperature carried out at 63°C with 5 × SSPE (20 × SSPE stock solution is 200 mM phosphate buffer (pH 7.0) – 3 M NaCl – 20 mM EDTA) in the hybridization buffer. The membrane was washed twice for 30 min at 63°C in 0.5 × SSPE – 0.1% SDS. Sequencing were performed on double-stranded DNA by the dideoxynucleotide method (Sanger et al. 1977).

Results

Identification of PAH-degrading bacterial strains selected
Sixty-five bacterial strains were isolated for their ability to

Fig. 1. 16S rRNA gene sequence analysis of *Sphingomonas* sp. strain 107. The 16S rRNA gene sequences of strain 107 and six *Sphingomonas* were aligned. The most divergent region of these 16S rRNA gene sequences is illustrated. Only the nucleotide changes are shown compared with *S. yanoikuyae* 16S rRNA gene sequence. Dots represent identity. Bars represent a gap. YANOI, *S. yanoikuyae* (GenBank accession No. D13728); 107, *Sphingomonas* sp. strain 107; *S. sp.*, *Sphingomonas* sp. (GenBank accession No. D16148); TERRA, *S. terrae* (GenBank accession No. D13727); PARAP, *S. parapaucimobilis* (GenBank accession No. D13724); ADHAE, *S. adhaesiva* (GenBank accession No. D16146); PAUCI, *S. paucimobilis* (GenBank accession No. D16144).

	930		980
YANOI	GACATCCCTATCGCGGATCGTGAGACACTTTCCTTCAGTTCGGCTGGATAGGTGACAGG		
107			
<i>S. sp.</i>	t.tcca.....tgga.a.....		
TERRA	tg.g.....t.acca.....tgg.....t.....		
PARAP	gtcagga..at.tcca.....tgga.ct....ct.cg....ac.g.aac.....		
ADHAE	gtcagga..at.tcca.....tgga.ct....nnnn.cg....ac.g.aac.....		
PAUCI	gg..gga..ac.tcca.....tgga..t....ttcgg....acctacac.....		

several PAH solutions. Identification of these strains revealed that most of them were pseudomonads, mainly *P. aeruginosa*, *P. fluorescens*, and *P. putida*. Some *Sphingomonas* sp. strains were identified. With one strain, named PL1, the identification was uncertain (see below). Relative to each species, we have chosen for the comparative study the most efficient bacterial strain based on two characteristics. The first one was the spectrum of substrate specificity tested by the formation of clearing zones around colonies, indicating PAH degradation. The second characteristic was the relative speed of appearance of these clearing zones. These strains are 34, 57, 62, 107, and PL1.

Strain 34 is Gram negative and produces pyoverdine pigment but no pyocyanin. It grows at 5°C but not at 42°C, and uses acetamide as source of carbon but not sorbitol, trehalose, or geraniol. Together with API, it was identified as a *P. putida* species. Strain 57 is Gram negative and produces pyocyanin and pyoverdine pigments. It does not grow at 5°C but grows at 42°C. It uses acetamide and geraniol as source of carbon but not trehalose or sorbitol. With API, it was identified as a *P. aeruginosa* species. Strain 62 is Gram negative, produces pyoverdine but not pyocyanin, grows at 5°C but not at 42°C, and uses trehalose and sorbitol as sources of carbon but not geraniol or acetamide. It was identified as a *P. fluorescens* species. Strain 107 is a Gram-negative rod bacterium, forming yellow-pigmented colonies that become sticky and adhesive after 3–4 days of incubation at 29°C on tryptic soy agar plates. Excellent similarity with *Pseudomonas paucimobilis*, now *Sphingomonas paucimobilis*, was obtained with the API strips. Its 16S rRNA gene was amplified by PCR and cloned, and part of the gene was sequenced. The resulting sequence was highly homologous to the 16S rRNA gene sequence of *Sphingomonas* species. The most divergent region in the 16S rRNA gene sequence between *Sphingomonas* species (Fig. 1) is identical between strain 107 and *Sphingomonas yanoikuyae*, suggesting that strain 107 is a *S. yanoikuyae* species.

Strain PL1 is a nonpigmented, Gram-negative, motile, coccobacillus (0.4–0.5 to 0.7–1.1 µm) bacterium. Electron micrographs showed one flagellum per cell (data not shown). Identification tests with API strips showed that strain PL1 is oxidase positive. It was negative for β-galactosidase, arginine dihydrolase, ornithine decarboxylase, urease, tryptophan deaminase, gelatinase, and amylase; it was negative for production of H₂S and indole and for nitrate reduction. It

hydrates. Further testing with the Biolog identification system (Microlog GN; Harward, Calif.) also showed that strain PL1 did not use any carbohydrates. It uses methyl pyruvate, acetate, cis-aconitate, citrate, formate, α-hydroxybutyrate, β-hydroxybutyrate, α-ketobutyrate, α-ketoglutarate, D- and L-lactate, malonate, propionate, succinate, bromo succinate, succinamate, alaninamide, and D and L-alanine. It does not use any other amino acid (results were borderline for some of them). Tests with the API strips showed that strain PL1 belonged to the *Alcaligenes* genus. The Biolog system identified strain PL1 as *Oligella ureolytica/urethralis* (formerly *Moraxella urethralis* [Neisseriaceae family, *Moraxella* genus]; Krieg and Holt 1984; Rossau et al. 1987), with a similarity index of 0.738 that is below the index (0.75) to be considered as acceptable for species identification. Indeed, *O. ureolytica/urethralis* has no flagella and does not use malonate and citrate. Furthermore, it was found primarily in human samples (Hansen et al. 1975). Further tests are needed such as G+C content, fatty acid composition, and rRNA gene sequence analysis to identify strain PL1.

Characteristics of the five PAH-degrading bacterial strains selected

We compared these five strains on the following characteristics: (i) presence of plasmids on which catabolic genes might be located; (ii) presence of an aromatic ring dioxygenase activity such as the naphthalene dioxygenase by the appearance of blue indigo color in presence of indole (the relative speed of appearance of the color was also measured), (iii) the effect of salicylate added in the BHY solid medium on the time required for the appearance of clearing zones and the transformation of indole; (iv) production of biosurfactants that might increase the availability of PAHs; and (v) the time required to observe bacterial growth in BH liquid medium with phenanthrene as a sole source of carbon and energy. Table 1 summarizes the characteristics of the five selected bacterial strains.

The less efficient bacterial strains *P. fluorescens* 62 and *P. putida* 34 had a narrower spectrum of substrate specificity than the other strains. Formation of clearing zones when sprayed with different PAHs and transformation of indole to indigo took more than a day. Addition of salicylate in the solid medium neither decreased the time required for the formation of clearing zones and the conversion of indole, nor

Table 1. Characteristics of the five selected PAH-degrading bacterial strains.

	<i>P. putida</i> 34	<i>P. fluorescens</i> 62	<i>P. aeruginosa</i> 57	Strain PL1	<i>Sphingomonas</i> sp. strain 107
Plasmid	No	No	No	Yes	Yes
Biosurfactant	Yes	No	Yes	No	Yes ^a
Indole ^b	+	+	+++	-	+++
Specificity ^d					
Naphthalene	+	+	++	++	++
Dibenzothiophene	-	+/-	++/-	++/-	++ +/-
Fluorene	+/y	+/y	++/y	++/y	+++/y
Phenanthrene	+	+	++	++	+++
Anthracene	+	+	+	+	++
Fluoranthene	-	-	-	+/-	+/-
Pyrene	-	-	-	-/br	-/br
Benz[a]anthracene	-	-	-	+	+
Salicylate ^c	No	No	Yes	No	Yes

^aBioemulsifying activity.^bAppearance of blue indigo color of colonies transferred on 1 mM indole plate.^cRelative speed of appearance of blue indigo or relative speed of appearance of clearing zones and color: +, more than 1 day; ++, less than 1 day; +++, less than 1 h; -, absence of blue indigo or clearing zones after 14 days of incubation; o, orange; y, yellow; br, brown.^dAppearance of clearing zones and color of colonies sprayed with different PAHs.^eEffect of salicylate on the speed of forming clearing zones and producing indigo color.

In BH medium containing phenanthrene, bacterial growth was apparent after more than a week. The culture had an orange coloration characteristic of degradation intermediates and nonsolubilized phenanthrene crystals had disappeared. Biosurfactants were produced by *P. putida* 34 and no plasmid was observed in both strains.

Pseudomonas aeruginosa 57 and strain PL1 colonies formed clearing zones faster than the preceding strains. It took less than a day to see clearing zones with naphthalene, dibenzothiophene, phenanthrene, and fluorene, and more than a day with anthracene. No clearing zones were formed with any four-ring PAHs tested with *P. aeruginosa* 57. Strain PL1 colonies formed clearing zones with biphenyl, acenaphthylene, and dibenzofuran, and with the four-ring PAHs fluoranthene and benz[a]anthracene. A brown coloration with pyrene was apparent, indicating formation of degradation intermediates. However, in liquid medium with pyrene, fluoranthene, or benz[a]anthracene as sole source of carbon, no growth of strain PL1 was observed after 3 weeks of incubation. Both strains grew in phenanthrene-BH liquid medium after 5-6 days, concomitant with the presence of orange coloration and the disappearance of nonsolubilized phenanthrene crystals. Analysis by gas chromatography showed that 100% of phenanthrene and 50% of fluorene were degraded after 8 days in strain PL1 liquid cultures containing these respective PAHs as substrate.

Pseudomonas aeruginosa 57 transformed indole to indigo in less than a day. We observed that clearing zones and formation of indigo appeared more rapidly when this strain was plated on BHY solid medium containing salicylate. However, strain 57 colonies did not form clearing zones with new PAHs in the presence of salicylate. Finally, strain 57 produced biosurfactants and no plasmid was observed. Strain PL1 did not transform indole to indigo. Salicylate had no effect on the formation of clearing zones. Extrachromosomal DNAs were extracted from strain PL1. However, this DNA never pro-

duced the usual two plasmidic bands but rather a broad band of 5-10 mm on CsCl gradient. Figure 2C (lane 11) shows that this DNA preparation was composed of 5- to 10-kbp DNA fragments and a DNA smear of 2-4 kbp long. The nature of such DNA remains to be clarified.

For *Sphingomonas* sp. strain 107, the time required for the formation of clearing zones and for the transformation of indole was in general faster than the other strains. Formation of clearing zones was observed in less than a day with naphthalene and anthracene, and in less than an hour with dibenzothiophene, fluorene, and phenanthrene. Transformation of indole was observed in less than an hour, usually 10 min after transferring colonies on indole plates. Clearing zones were seen with fluoranthene and benz[a]anthracene. A brown color was observed on colonies sprayed with pyrene. *Sphingomonas* sp. strain 107 colonies also formed clearing zones with biphenyl, acenaphthene, acenaphthylene, and dibenzofuran. No clearing zone was observed when sprayed with the five-ring PAH benzo[a]pyrene. Bacterial growth in phenanthrene-BH liquid medium was apparent after 4 days with the usual orange coloration and disappearance of phenanthrene crystals. In contrast to strain PL1, *Sphingomonas* sp. strain 107 was able to grow in BH liquid medium with fluoranthene and pyrene as substrate. Addition of salicylate in BHY solid medium accelerated the formation of clearing zones and transformation of indole. A plasmid was found in this strain (Fig. 2A). Digestion with different restriction enzymes revealed that the plasmid is approximately 55 kbp long.

Sphingomonas sp. strain 107 cultures were grown in BH liquid medium with 200 ppm of pyrene. After 7 days of incubation, 20 ppm of pyrene was degraded. As pyrene is poorly soluble in water, Tween 80 (200 µg/mL) was added to increase the availability of pyrene for microbial degradation. The same was done with 200 ppm of fluoranthene. After 7 days of incubation, 40 ppm of pyrene and 25 ppm of fluoranthene

were degraded. With the three-ring PAHs fluorene and phenanthrene, no trace of these PAHs were present after 10 days of incubation in BH with Tween 80. Finally, addition of salicylate (100 $\mu\text{g/mL}$) in the BH with Tween 80 showed an increase in pyrene degradation to 95 ppm after 7 days of incubation. Analysis of pyrene degradation intermediates by GC – mass spectrometry showed several peaks. However, none of these intermediates have been identified yet.

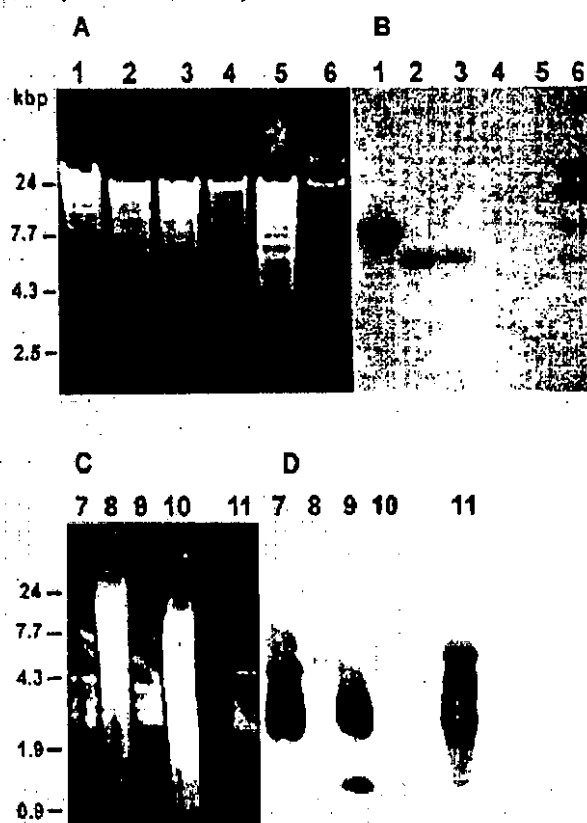
Hybridization with the *nahAb*-related probe

PCR on the *Sphingomonas* plasmid with the FerD and FerG primers specific to the naphthalene dioxygenase ferredoxin gene such as *nahAb*, *pahAb*, and *daxA* produced a DNA fragment of approximately 300 bp. Cloning and sequencing of this DNA fragment revealed a homology of 92% with the *nahAb* nucleotide sequence. This fragment was used as a probe, named SphAb, to detect the presence of ferredoxin gene in the other four strains. Total and plasmid DNAs were extracted from each strain, digested with *Hind*III, and hybridized with the SphAb probe. Hybridization signals were observed with DNA extracted from each strain (Fig. 2). The SphAb probe hybridized exclusively to the plasmid DNA of strain PL1 and *Sphingomonas* sp. strain 107. However, hybridization signals were observed with total DNA of these two strains when a longer exposure of the hybridized membrane was done (data not shown). These signals corresponded to the plasmid DNA that coextracted with the chromosomal DNA. Several hybridization DNA fragments were observed with the *Sphingomonas* plasmid DNA that correspond to the digested and different forms of the undigested circular plasmid. A weaker hybridization signal was observed with *P. putida* DNA that might be the result of divergence between the probe and the target sequence. Interestingly, the hybridization signals on the plasmid fraction of strain PL1 did not correspond to one of the major DNA fragments but rather to a DNA fragment not visible by ethidium bromide staining. It also produced a strong hybridization signal on the DNA smear.

PCR amplification of PAH-degradation genes

Seven oligonucleotides were synthesized based on consensus sequences found between respective genes in the *pah*, *dax*, and *nah* operons. These oligonucleotides were used as primers to do PCR on total and plasmidic DNA extracted from each strain. If PAH-degradation genes are present in these DNA and if they are arranged in polyclonic operon and ordered like the *pah*, *dax*, and *nah* operons, the length of the PCR products can be predicted based on available nucleotide sequences of these three operons (Fig. 3). Oligonucleotides (Fig. 3) are FerG and FerD, specific for the naphthalene dioxygenase ferredoxin gene; Red and Isp, specific for the gene encoding the reductase and the iron-sulfur small subunit, respectively, of naphthalene dioxygenase; DioG and DioD, specific for the 1,2-dihydroxynaphthalene dioxygenase gene; and Iso, specific for the *daxI* gene and homologous to *nahD* (encoding the 2-hydroxychromene-2-carboxylate isomerase). To confirm that PCR amplified PAH-degradation genes, several PCR products with the predicted length were cloned, and one or both extremities of these products were sequenced. In all cases, nucleic acid sequence analysis confirmed the presence of the expected PAH-degradation gene. Results of PCR experiments are summarized in Table 2.

Fig. 2. Hybridization of the total and plasmid DNA with the SphAb probe. Total and plasmid DNA were run on a 0.7% agarose electrophoresis gel and stained with ethidium bromide (A, C). DNAs were transferred on a nylon membrane and hybridized with the DIG-labelled SphAb probe (B, D). *Hind*III-digested total DNA of *P. aeruginosa* 57 (lane 1), *P. fluorescens* (lane 2), *P. putida* 34 (lane 3), *Sphingomonas* sp. strain 107 (lane 4), and strain PL1 (lane 5). Plasmid DNA from *Sphingomonas* sp. strain 107 (lane 6). Plasmid DNA from strain PL1 digested with *Hind*III (lane 7), *Eco*RI (lane 9), or undigested (lane 11). Total DNA from strain PL1 digested with *Hind*III (lane 8) or *Eco*RI (lane 10).



PCR with the FerG–FerD and DioG–DioD primers showed that the naphthalene dioxygenase ferredoxin and 1,2-dihydroxynaphthalene dioxygenase genes are present in all strains. Amplification using the FerG–Isp primers produced in all strains a 2.2-kbp DNA fragment as predicted by the *nah*, *dax*, and *pah* operons. This suggests that the gene order of that fragment would be (using nomenclature for the *nah* operon) *nahAb-nahAc-nahAd*. Amplification using the FerG–Iso primers gave an unexpected result. A 4.2-kbp DNA fragment was obtained in all strains instead of the predicted 8.4-kbp DNA fragment. The latter fragment was observed once with *P. putida* 34 in addition to the 4.2-kbp DNA fragment. Sequence of the extremities of the 4.2-kbp fragment revealed that the Iso primers annealed in all strains to the same location in the *nahF* gene (aldehyde dehydrogenase). During the amplification of a large DNA fragment, this annealing site was preferentially amplified. Therefore, failure to obtain the 8.4-kbp DNA fragment might be due to PCR

Fig. 3. Strategy to determine the gene arrangement of PAH-degradation genes by PCR. Gene arrangement of the PAH-degradation genes is based on the *nah*, *dax*, and *pah* operons as described by Eaton (1994). Letters in the boxes correspond to the nomenclature of the *nah* operon from the NAH7 plasmid. Localization and orientation of primers are represented by arrows. Lines under the operon are the predicted DNA fragments and their length (numbers beside in bp) that should be generated by PCR with respective combination of primers. Primer sequences are shown with their location on the *nah* operon (Red primer; Simon et al. 1993) or the *dax* operon (all the other primers, Denome et al. 1993). ¹DioG and ¹DioD are consensus sequences between *nahC* and *nahH* (catechol 2,3-dioxygenase; Ghosh et al. 1987) sequences. ²The Iso sequence was derived from *daxJ* only.

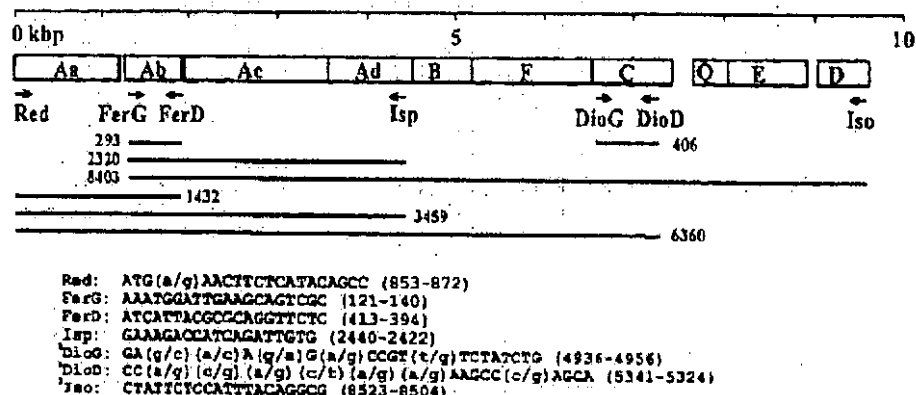


Table 2. DNA fragments obtained after PCR with primers specific to PAH-degradation genes on total and plasmidic DNA isolated from the selected bacterial strains.

Primers	Predicted DNA ^a fragment length (bp)	<i>P. putida</i> 34	<i>P. fluorescens</i> 62	<i>P. aeruginosa</i> 57	Strain PL1 ^b	<i>Sphingomonas</i> sp. strain 107 ^b
FerG-FerD	293	+	+	+	+	+
DioG-DioD	406	+	+	+	+	+
FerG-Isp	2320	+	+	+	+	+
FerG-Iso	8403	+	-	-	-	-
		4188 ^d	4188	4188	4188	4188
Red-FerD	1432	-	-	+	+	+
Red-Isp	3459	-	-	+	+	+
Red-DioG	6360	-	-	+	+	+

^aBased on the *nah* and *dax* operons (Simon et al. 1993; Denome et al. 1993).

^bPCR products generated only with the plasmidic DNA preparation.

^c+, positive amplification of the predicted DNA fragment; -, no amplification.

^dUnexpected 4188-bp DNA fragment generated with FerG-Iso primers (see text).

conditions used to amplify large DNA fragment. The length of a PCR product, predicted by the *nah* operon and flanked by the FerG sequence and the *nahF* sequence recognized by the Iso primer, is 4188 nucleotides. This suggests that the gene order in this 4.2-kbp fragment is *nahAb-nahAc-nahAd-nahB-nahF*. Combinations of the Red-FerD, Red-Isp, and Red-DioD primers generated the predicted 1.4-, 3.6-, and 6.4-kbp DNA fragments, respectively, with *Sphingomonas* sp. strain 107, strain PL1, and *P. aeruginosa* 57. No amplification was observed with these primers on *P. putida* 34 and *P. fluorescens* 62. This suggests that in the former three strains the gene order would be *nahAa-nahAb-nahAc-nahAd-nahB-nahF-nahG*. In *P. putida* and *P. fluorescens* the reductase gene might be missing in the operon or the reductase sequence might be different. All these results suggest that several PAH-degradation genes are present in all the studied strains and are arranged in a polycistronic operon. Our results also predict that the gene order in the five strains should be similar to the *pah*, *dax*, and *nah* operons.

Sequence homology

As mentioned before, several PCR products were cloned and their extremities were sequenced. Compared with the corresponding genes in the *pah*, *nah*, and *dax* operons, nucleotide sequences were 90–95% identical, and the deduced amino acid sequences were 93–100% identical (Fig. 4). Most of the differences were punctual and scattered through the sequences.

Discussion

Efficiency of PAH degradation of five bacterial strains isolated from contaminated soil were compared with each other. The most efficient strain in terms of (i) substrate specificity, (ii) rapidity of forming clearing zones around colonies when sprayed by PAH solutions, and (iii) bacterial growth in liquid medium with different PAHs as sole source of carbon and energy was *Sphingomonas* sp. strain 107, followed by the strain PL1 and *P. aeruginosa* 57. The least efficient strains were the *P. putida* 34 and *P. fluorescens* 62.

Addition of salicylate in solid medium has accelerated the formation of clearing zones by *Sphingomonas* sp. strain 107 and *P. aeruginosa* 57. It has also accelerated the transformation of indole to indigo. No such effects were observed with the three other strains. However, salicylate did not broaden the range of PAH specificity for *Sphingomonas* sp. strain 107 and *P. aeruginosa* 57. This is the first report that shows the effect of salicylate on PAHs other than naphthalene. Salicylate is known to be the inducer of the expression of genes encoding the upper and lower pathway of the naphthalene catabolism in the NAH7 plasmid (Schell 1985). Our results suggest that gene regulation via salicylate might occur in *Sphingomonas* sp. strain 107 and *P. aeruginosa* 57. Ogunseitan and Olson (1993) have shown that addition of 50 µg salicylate/g of soil contaminated with naphthalene increased the naphthalene mineralization. Sanseverino et al. (1993b) have shown that the addition of salicylate in four contaminated soils during a slurry treatment increased the *nahA* mRNA levels in only one soil. However, they suggest that this transcript was already induced in the other soils because of high concentration of naphthalene.

The addition of synthetic or biologically produced surfactants has been shown to enhance PAH biodegradation (Tiehm 1994; Hunt et al. 1994). *Sphingomonas* sp. strain 107 secretes a bioemulsifier, whereas *P. aeruginosa* 57 and *P. putida* 34 produce extracellular surface-active agents. The ability to produce biosurfactants is often linked to the utilization of hydrophobic substrates (Hommel 1990). The capacity of forming clearing zones around the PAH-degrading bacterial colonies sprayed with PAHs may be caused by the production of biosurfactants (Burd and Ward 1996). In our study, clearing zones were generated whether the bacterial strains were producing surfactants or not. Moreover, we observed a correlation between the appearance of clearing zones with a specific PAH and the bacterial growth on liquid medium with this PAH as sole source of carbon and energy. The only exception was strain PL1 with pyrene, benz[a]anthracene, and fluoranthene. Finally, there is apparently no correlation between production of biosurfactants and the efficiency of PAH degradation by a bacterial strain as shown with *P. putida* 34.

A fivefold increase of pyrene degradation by *Sphingomonas* sp. strain 107 cultures occurred with the addition of a synthetic surfactant (Tween 80) and salicylate in the BH liquid medium. One explanation of this increase is that the addition of a surfactant would have solubilized more pyrene in solution making it available more rapidly for microbial degradation, and the addition of salicylate would have stimulated the expression of PAH-degradation genes. However, salicylate and Tween 80 could have been used as carbon sources, resulting in an higher cell concentration for the degradation of pyrene.

Hybridization with a *nahAb*-related probe and PCR with primers specific for PAH-degradation genes revealed that each strain contained several of these genes. Cloning and sequencing of PCR products showed high homology between PAH-degradation gene sequences found in the five selected strains and the respective gene sequences in the *nah*, *pah*, and *dox* operons. It is interesting to notice that although strain PL1 did not transform indole to indigo, genes encoding the naphthalene dioxygenase were present and parts of these genes that we sequenced are highly conserved. Indole might not be a substrate for the naphthalene dioxygenase in strain PL1 or

might not enter into the cell. Results obtained by PCR suggest that PAH-degradation genes are arranged in a polycistronic operon in each strain. These same results predict that these genes are ordered like the *pah*, *nah*, and *dox* operons.

PAH-degradation genes were found on a plasmid in *Sphingomonas* sp. strain 107 and on extrachromosomal DNAs in strain PL1. No plasmids were found in *P. aeruginosa* 57, *P. putida* 34, and *P. fluorescens* 62. This study is the second one to show a PAH catabolic function on a plasmid in the *Sphingomonas* genus. Stillwell et al. (1995) have shown that *Sphingomonas* sp. strain F199 has a 180-kbp plasmid, pNL1, implicated in the degradation of PAHs. Also, no previous study showed gene arrangement and sequences of PAH-degradation genes from *P. aeruginosa*. This is the second study on PAH-degradation genes in nonpseudomonads. Although identification tests were inconclusive in determining the species with certainty, strain PL1 did not show any characteristics of pseudomonads. The only study on PAH-degradation genes in nonpseudomonads was with three *Comamonas testosteroni* strains capable to degrade phenanthrene, naphthalene, and anthracene. Using the whole *nah* operon as a probe, Goyal and Zylstra (1996) did not observe any hybridization signal with total DNA from these three strains. They also cloned the chromosomal region responsible for naphthalene and phenanthrene degradation from one of these strains and showed that the gene order was different than the *nah* operon. Moreover, this region was present in one of the other strains but absent in the third one, suggesting that two different sets of genes for the degradation of phenanthrene exist among the three *C. testosteroni* strains.

In our study, we showed that pseudomonad PAH-degradation genes can be found in other bacterial genera, and are highly conserved in their arrangement and their sequences. However, as these genes are located on a plasmid, *Sphingomonas* sp. strain 107 and strain PL1 could have obtained them by conjugation with pseudomonads.

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Bioremediation of Petroleum Hydrocarbon Pollution

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Uncontrolled and catastrophic releases of petroleum pose ecological and environmental repercussions as a lot of hydrocarbon components are toxic and persistent in terrestrial and aquatic environments. Several physico-chemical methods of decontaminating the environment have been established and employed. Biological degradation, a safe, effective and an economic alternative method, is a process of decay initiated by biological agents, specifically in this case by microorganisms. Bioremediation refers to site restoration through the removal of organic contaminants by microorganisms. Biodegradation of hydrocarbons is largely carried out by diverse bacterial populations, which are ubiquitously distributed in the environment. The most commonly reported genera of hydrocarbon-degraders include *Pseudomonas*, *Acinetobacter*, *Nocardia*, *Vibrio* and *Achromobacter*. The factors, that influence the rates of microbial degradation of hydrocarbons, include temperature, pH, salinity, oxygen, nutrients, and physical and chemical composition of petroleum. Due to the complexity of crude oil, biodegradation involves the interaction of many different microbial species. It could be attributed to the effects of synergistic interactions among members of the consortium.

Keywords: bioremediation; petroleum; hydrocarbon; biodegradation; parameters

Introduction

Large amounts of hydrocarbon contaminants are released into the environment as a result of human activities. While releases like industrial emissions can be controlled and carefully regulated, catastrophic releases like major spillage from tankers, pipelines and storage tanks are largely accidental and unavoidable and occur frequently in present times. Such releases often pose severe, immediate, as well as long-term ecological and environmental repercussions, since a lot of hydrocarbon components are toxic and persistent in terrestrial and aquatic environments. Several physico-chemical methods of decontaminating the environment have been established and employed (Table 1). However, such methods are usually expensive and labour-intensive and often involve the risk of spreading the pollution because the waste would require disposal elsewhere. A better way would be to use biodegradation. Biodegradation of hydrocarbons is often relatively slow under normal conditions due to the complex interaction that involves the hydrocarbons, the environment and the composition of the microbial community. The emphasis of research now is to

exploit the hydrocarbon degradation abilities of the microbial population to raise the rates of biodegradation found naturally to significantly higher rates. Bioremediation, the degradation or stabilization of contaminants by microorganisms, is claimed as a safe, effective and economic alternative method of environmental clean up.

Crude oil

Crude oil is liquid petroleum in its unrefined state. The principle chemical elements (90% of the weight of crude oil) are carbon and hydrogen, which are combined in a series of compounds called hydrocarbon. Only 31 hydrocarbons account for 75% of the weight of the whole distillate fraction (55°-145°C) of an Oklahoma crude (Tiratsoo, 1973). Other components in crude oil are sulphur, nitrogen and oxygen compounds in very small proportions. Sulphur compounds include mercaptans, thiophenes and thioethers. Oxygen occur in crude in mainly asphaltenes and naphthanic acids while N compounds are homologues of pyridine or alkylated quinolines. Other elements found in crude oil in trace amounts include iron, silicon, aluminium and nichrome.

Biodegradation and Bioremediation

The biodegradation denotes complete microbial mineralization of complex materials into simple

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Table 1—Physico-chemical methods for the decontamination of hydrocarbon-contaminated soil

Method	Principles	Variations of technique	Comments
Thermal	Evaporation and/or destruction of hydrocarbons	Rotating tube furnace; fluidized bed furnace; incinerators	Excavation needed; top-soils only; off-gases must be treated; expensive
Extraction	Removal of hydrocarbons into solution	Percolating towers; scrubbing towers; fluidized beds	Excavation needed; top-soils only; extract must be disposed of; efficiency unknown; hydrocarbons may be bound to soils; very expensive; little data
Steam stripping	Removal of volatiles	Rotating drum; <i>in situ</i>	Volatiles only; potential for subsoils; steam must be treated; little data
Hot-air stripping	Removal of volatiles	Rotating drum; <i>in situ</i>	Volatiles only; potential for subsoils; little data
Chemical oxidation	Alteration of pollutant to ease removal	<i>In situ</i> ; reaction chambers	No information for hydrocarbons
Groundwater control	Pumping of aquifer to prevent flow	Pumping with/without physical containment; direct removal of compounds on water	Prevents migration of hydrocarbons; no removal of compounds in unsaturated zone; efficient, useful to prevent pollution spread during biotreatment; widely used
Immobilization	Binding hydrocarbons <i>in situ</i>	Chemical bonding; soil solidification	Expensive; not widely tested; does not remove pollutants
Flooding	Raising hydrocarbons to surface on top of water table	—	Risk of spreading pollution; inefficient
Adsorption	Groundwater pumped through activated carbon	—	Expensive; waste requires disposal; efficiency unknown
Detergent extraction	Excavated soil or <i>in situ</i> soil is flushed with surfactant	—	Efficiency unknown; expensive

Morgan & Watkinson, 1989.

inorganic constituents such as carbon dioxide, water and minerals as well as cell biomass. In aquatic and terrestrial environments, the biodegradation of crude oil and other petroleum complexes predominantly revolves around the actions of bacterial and fungal populations.

Bioremediation refers to site restoration through the removal of organic contaminants by microorganisms. It is a process that exploits the natural metabolic versatility of microorganisms to degrade environmental contaminants. At present, bioremediation revolves around either stimulating indigenous microbial populations by environmental modifications or introducing exogenous microbial populations that are known degraders to a contaminated site, a process also known as seeding. Bioremediation potentially offers a number of advantages such as destruction of contaminants, lower treatment costs, and greater safety and less environmental disturbance (Table 2).

Bioremediation is not the universal remedy for organic contamination. Growth and survival of microorganisms is affected by environmental factors like temperature, composition of the contaminant, soil type and nutrient and water availability. These factors

affect the application of bioremediation as a process of clean-up. Similarly, petroleum hydrocarbons greatly vary in their susceptibility to metabolic breakdown by bacteria. This can limit the scope and effectiveness of bioremediation.

Microorganisms that Metabolize Crude Oil and Other Petroleum Products

The common occurrence of metabolically active bacterial and fungal populations in areas that are contaminated with hydrocarbons strongly suggests that these microorganisms are able to utilize hydrocarbons as their carbon and/or energy sources. Some members of these populations degrade alkanes, some aromatics, while others decompose both paraffinic and aromatic hydrocarbons, transforming them into products such as carbon dioxide, water and biomass or other less-harmful end-products (Table 3). Due to the complexity of crude oil, biodegradation involves the interaction of many different microbial species.

In marine environments, biodegradation of hydrocarbons is largely carried out by diverse bacterial populations, which are ubiquitously distributed in the oceans. The most commonly

Table 2—*In situ* treatments in bioremediation

Treatment	Description	Applicable to	Advantages
Biostimulation	The addition of oxygen, water and mineral nutrients (usually combinations of nitrogen, phosphorus and trace metal(s))	Groundwater, soils	Acceleration by as much as 100-fold of the reproduction of indigenous organisms
Bioventing	Combines conventional soil venting with biodegradation. The less volatile compounds are biodegraded and the volatile components are vented off in conventional venting.	Soils	Addresses full range of petroleum hydrocarbons. Effective method of supplying indigenous microorganisms with oxygen to support degradation
Bioaugmentation	Direct application of microorganisms originating from (a) the remediation site (b) an off-site vendor (c) genetic engineering.	Groundwater, soils	One of the most effective bioremediation techniques. The microorganisms have been cultured and adapted and their degrading capacity can be enhanced for specific contaminants and site conditions.
Surfactants	Synthetic or biogenic substances are used to increase the aqueous solubility of solid hydrocarbons and emulsify liquid hydrocarbons.	Solid and liquid aliphatic and aromatic hydrocarbons	Enhancement of contaminant accessibility to microorganisms, nutrients and possibly oxygen.
Fertilizer application	To stimulate microbial metabolism by supplying indigenous oil-degraders with nutrients (N, P, K etc)	Soil, groundwater, sediments	Acceleration of natural biodegradation process, especially in nutrient-deficient areas.

Korda *et al.*, 1997.

reported genera of hydrocarbon-degraders include *Pseudomonas*, *Acinetobacter*, *Nocardia*, *Vibrio* and *Achromobacter* (Floodgate, 1984).

Metabolic Pathways and Products of Hydrocarbon Degradation

Biodegradative enzymes are often encoded on plasmids. However, most studies on plasmid-encoded pathways of hydrocarbon degradation have been limited to members of the *Pseudomonas* species (van Bailen *et al.*, 1994; Kostal *et al.*, 1998; McBeth, 1989). Chromosome encoded degradation is also reported (Table 4). The alkane-degradative systems in *Acinetobacter* sp. appear to be located on the chromosome (Singer & Finnerty, 1984a; Watkinson & Morgan, 1990). Similar findings were recorded for a psychrotrophic *Rhodococcus* sp. strain Q15. Plasmidless strains demonstrated slower rates of alkane mineralization, suggesting that the Q15 plasmid may carry genes that have a positive impact on hydrocarbon catabolism in some other ways (Whyte *et al.*, 1998).

Microbial Degradation of Alkanes

Alkanes are generally easily biodegraded, particularly those of relatively shorter chains due to

their lower hydrophobicity. Straight chain alkanes (C₁₀-C₂₄) are the most rapidly degraded (Atlas, 1981). *A. calcoaceticus* strain and *Nocardioforms* demonstrated good growth in n-alkanes with up to 30 and 40 carbon atoms respectively (Radwan *et al.*, 1999). With an increase in the carbon-chain length, bioavailability of alkanes is reduced due to the decreased solubility in aqueous media. Shorter chains of alkanes usually evaporate easily during the weathering processes especially in warmer temperatures.

In *Pseudomonas* sp. and *Acinetobacter* sp., the initial degradation of alkane begins with the oxidation of the terminal methyl group and results in the formation of an alcohol, which is then dehydrogenated via the aldehyde to the corresponding carboxylic acid, which can then be metabolized in the β -oxidation pathway of fatty acids (May & Katapodis, 1990; Lal & Khanna, 1996). In *A. calcoaceticus* S19, octadecane was converted to octadecanol and octadecanoic acid; the corresponding aldehyde was, however, not detected (Bajpai *et al.*, 1998). Another pathway (Fig. 1) was proposed for *Acinetobacter* sp. HO1-N, involving an n-alkyl hydroperoxide, which is reduced to alcohol (Singer & Finnerty, 1984b). Some

Table 3—Microorganisms that degrade hydrocarbons

Compound	Microorganism	References
Alkanes	<i>Acinetobacter</i> sp.	Bajpai <i>et al.</i> , 1998 Foght <i>et al.</i> , 1990 Marin <i>et al.</i> , 1996 Razak <i>et al.</i> , 1999 Foght <i>et al.</i> , 1990
	<i>Actinomyces</i> , <i>Arthrobacter</i> group	
	<i>Bacillus</i> sp.	Kim <i>et al.</i> , 2000 Sorkhoh <i>et al.</i> , 1995 Surzhko <i>et al.</i> , 1995
	<i>Candida</i> sp.	Surzhko <i>et al.</i> , 1995
	<i>Micrococcus</i> sp.	Rambeloarisona <i>et al.</i> , 1984
	<i>Planococcus</i>	Engelhardt <i>et al.</i> , 2001
	<i>Pseudomonas</i> sp.	Barathiy & Vasudevan, 2001 Li & Poole, 1999 Sekelsky & Shreve, 1999 Sorkhoh <i>et al.</i> , 1995
	<i>Rhodococcus</i> sp.	Bruchheim <i>et al.</i> , 1997 Sorkhoh <i>et al.</i> , 1995
	<i>Streptomyces</i>	Barabás <i>et al.</i> , 2001
	<i>Pseudomonas</i> sp.	Hubert <i>et al.</i> , 1999 Juteau <i>et al.</i> , 1999 Lee & Gibson, 1996
Mono- aromatics	<i>Ralstonia</i> sp.	Lee & Lee, 2001
	<i>Rhodococcus</i> sp.	Juteau <i>et al.</i> , 1999 Kim <i>et al.</i> , 2002
	<i>Sphingomonas</i> sp.	Shien <i>et al.</i> , 1998
Poly- aromatics	<i>Alteromonas</i> sp.	Zaidi & Imam, 1999
	<i>Arthrobacter</i> sp.	Grifoli <i>et al.</i> , 1992
	<i>Bacillus</i>	Aitken <i>et al.</i> , 1998 Annweiler <i>et al.</i> , 2000
	<i>Mycobacterium</i> sp.	Boldrin <i>et al.</i> , 1993
	<i>Penicillium</i>	Tongpin & Pickard, 1999
	<i>Janthidium</i>	Boonchan <i>et al.</i> , 2000
	<i>Phanaerochaete</i> <i>chrysosporium</i>	Barclay <i>et al.</i> , 1995
	<i>Pseudomonas</i> sp.	Widada <i>et al.</i> , 2002 Dagher <i>et al.</i> , 1997

Rhodococcus sp. oxidize n-alkanes by both the terminal, like *Pseudomonas* sp. and *Acinetobacter* sp., as well as the sub-terminal pathways where the alkane is oxidized via a monooxygenase to the corresponding secondary alcohol, then to a ketone and finally to a fatty acid (Whyte *et al.*, 1998).

Microbial Degradation of Monoaromatic Hydrocarbons

Benzene degradation begins with oxidation of the molecule by way of a three-enzyme system, which introduces two hydroxy groups into the molecule

forming a cis-dihydriol, which is dehydrogenated to yield catechol. The aromatic ring of catechol can be cleaved oxidatively in two different ways, the ortho-cleavage or the meta-cleavage to produce muconic acid or semialdehyde respectively (Cerniglia, 1984; Muller, 1992).

Toluene, a pollutant that biodegrades rapidly relative to other BTEX compounds in the aerobic environment, has been studied almost entirely in *Pseudomonas* sp. (Applegate *et al.*, 1998; Hubert *et al.*, 1999; Lee & Gibson, 1996). Strains of other genera such as *Acinetobacter*, *Azoarcus*, *Mycobacterium*, *Nevskia*, *Pseudonocardia* and *Rhodococcus*, that could degrade toluene, have also been isolated (Juteau *et al.*, 1999).

Depending on the organism, toluene can either be initially oxidized at the methyl group to benzoic acid or on the aromatic ring to form a dihydrodiol with a cis-configuration. *Pseudomonas putida* ml-2 and *P. aeruginosa* initiates the oxidation of toluene at the methyl group (Fig. 2), while *P. mendocina* at the C4-position on the aromatic ring (Cerniglia, 1984; Gibson, 1987). The degradation of xylenes by *Pseudomonas* sp. proceeds by oxidation at the methyl group, akin to that of toluene, to the corresponding methylbenzyl-alcohols, tolualdehydes, toluic acid and methyl catechol (Davey & Gibson, 1974).

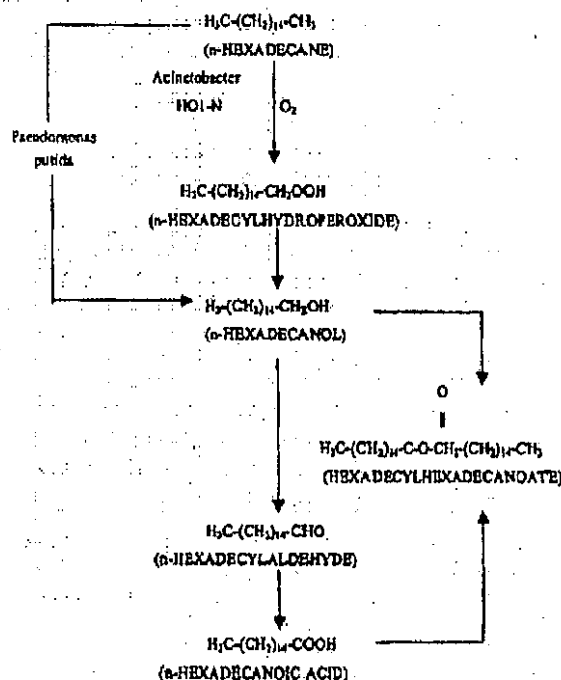


Fig. 1—Pathways of hexadecane metabolism in *Pseudomonas putida* and *Acinetobacter* HOI-N. Singer & Finnerty, 1984b.

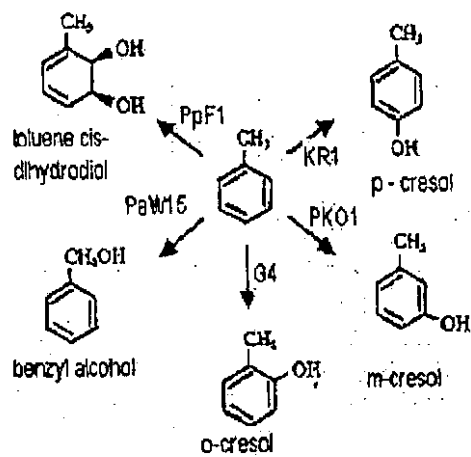


Fig. 2—Initial reactions in the five pathways for aerobic degradation of toluene in strains: *P. putida* F1; *P. putida* PaW15; *B. cepacia* G4; *R. pickettii* PKO1; and *P. mendocina* KR1. *P. putida* F utilizes a dioxygenase-initiated pathway for toluene degradation. G4, PKO1, and KR1 initiate toluene degradation with toluene 2-, 3-, and 4-monooxygenases, respectively. PaW15 carries the TOL plasmid and oxidizes the methyl group of toluene (Parales *et al.*, 2000).

Microbial Degradation of Polyaromatic Hydrocarbons

Biodegradability of polyaromatic hydrocarbons (PAHs) (Table 5) is generally inversely related to the number of fused benzene rings (Cerniglia & Heitcamp, 1989). Half-lives in soil and sediment of the three-ring phenanthrene molecule may range from 16 to 126 days while for the five-ring benzo[a]pyrene they may range from 299 to more than 1400 days (Shuttleworth & Cerniglia, 1995). The persistence of higher molecular weight PAHs is due largely to their low water solubility and resonance energy of their structures (Cerniglia, 1992).

Naphthalene, the simplest form of PAHs, is the most readily degraded PAH. Bacteria initially oxidize naphthalene by incorporating both atoms of molecular oxygen into the aromatic molecule to form cis-1,2-dihydroxy-1,2-dihydronaphthalene, which is then converted to 1,2-dihydroxynaphthalene. Salicylaldehyde and pyruvate are produced through ring cleavage of 1,2-dihydroxynaphthalene. The aldehyde is then oxidized to salicylate that is subsequently converted to catechol (Cerniglia, 1984), which is further oxidized via the ortho or meta pathway as per described earlier for benzene.

In naphthalene-degradation by the thermophilic *Bacillus thermoleovorans*, mesophilic microorganisms might have a different pathway for polyaromatic degradation compared to mesophiles. Annweiler *et al* (2000) reported intermediates such as 2,3-dihydroxynaphthalene, 2-carboxynnamic acid, and phthalic and benzoic acid were identified in addition to the typical metabolites of naphthalene degradation known from mesophiles in the pathway of *B. thermoleovorans*.

The utilization of fluoranthene, a HMW PAH, as a sole source of carbon and energy is possible in a seven-member bacterial consortium (Mueller *et al.*, 1989). Cometabolism was also seen – the community was able to transform other HMW PAHs when grown on fluoranthene. Cooxidation is potentially an important process for the bioremediation of PAHs in soil (Keck & colleagues, 1989). Pyrene metabolized by a variety of bacteria (Tables 3 & 5) through different pathways (Fig. 3) for the different bacteria (Cerniglia, 1992; Kelley *et al.*, 1993; Weissenfels *et al.*, 1991).

Catabolism of a PAH molecule starts via the oxidation of the PAH to a dihydrodiol by a multicomponent enzyme system. The dihydroxylated

Table 4—Some genes and plasmids encoding hydrocarbon degradation

Microorganism	Hydrocarbon compound	Gene	Location	Reference
<i>Pseudomonas oleovorans</i>	C ₅ -C ₁₂ alkanes	alkB	Plasmid	van Bellen <i>et al.</i> , 1994
<i>Rhodococcus</i> sp. strain Q15	C ₁₀ -C ₂₁ alkane, branched alkanes, cyclohexane	thcA	Chromosome	Whyte <i>et al.</i> , 1998
<i>Acinetobacter</i> sp. HO1-N	Alkanes		Chromosome	Singer & Finnerty, 1984a
<i>Pseudomonas</i> sp.	Alkanes	alkA, alkB	OCT plasmid	Singer & Finnerty, 1984a
	Naphthalene	nahA-M	NAH7, pND140, pND160 plasmids	Singer & Finnerty, 1984a
	Aromatics		TOL plasmid	Singer & Finnerty, 1984a

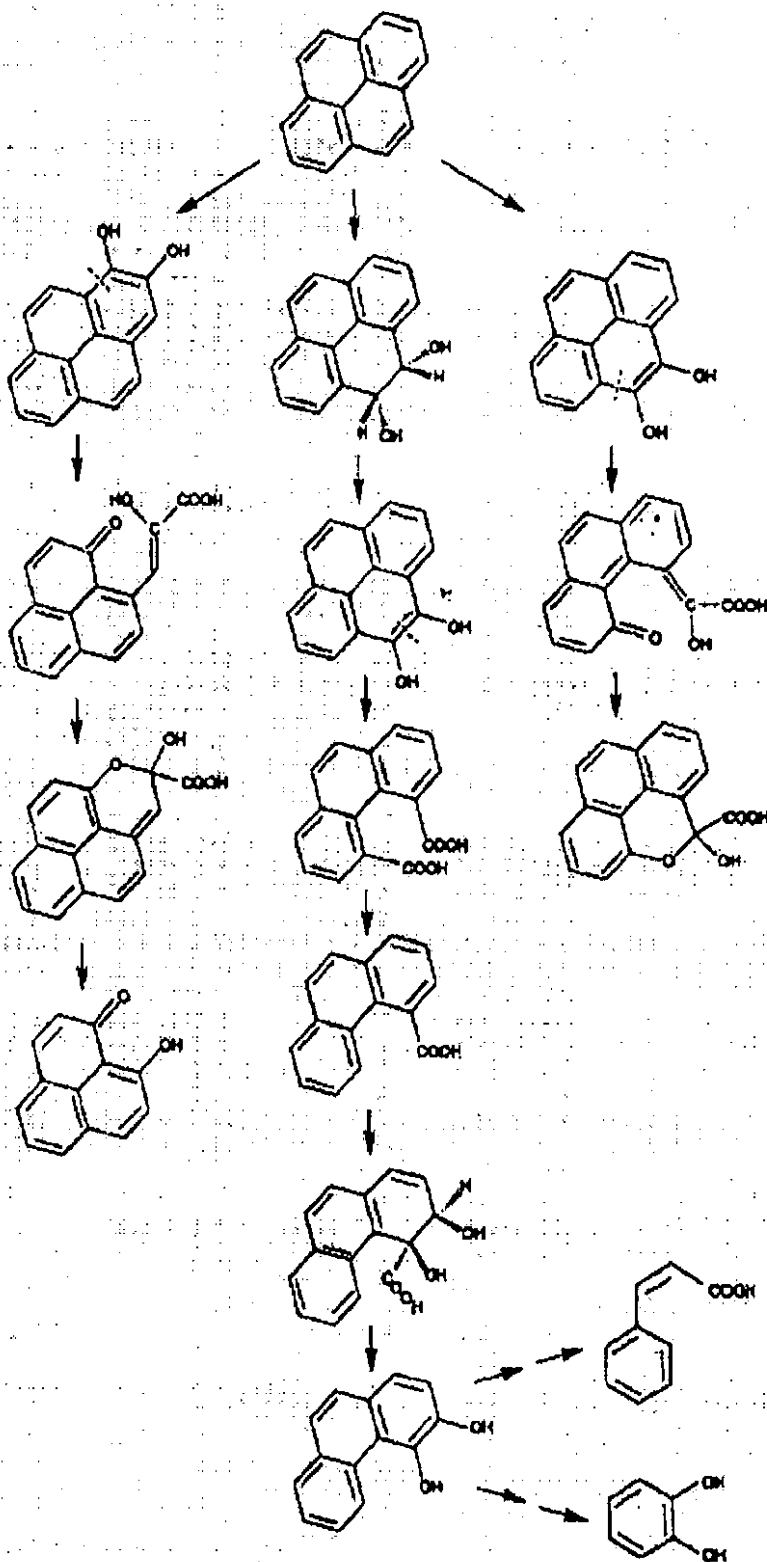


Fig. 3—Several different pathways of microbial degradation of pyrene

Table 5—Polyaromatic hydrocarbons and the microorganisms that degrade them

Compound	Microorganisms	Reference
Naphthalene	<i>Mycobacter calcoaceticus</i>	Kelley <i>et al</i> , 1991
	<i>Pseudomonas paucimobilis</i>	Kuhm <i>et al</i> , 1991
	<i>P. putida</i>	Ahn <i>et al</i> , 1998
	<i>Rhodococcus</i> sp.	Grund <i>et al</i> , 1992
	<i>Bacillus thermoleovorans</i>	Annweiler <i>et al</i> , 2000
Anthracene	<i>P. paucimobilis</i>	Mueller <i>et al</i> , 1990
	<i>Rhodococcus</i> sp.	Grund <i>et al</i> , 1992
	<i>Mycobacterium</i> sp.	Tongpin & Pickard, 1999
	<i>Cycloclasticus pugetii</i>	Dyksterhouse <i>et al</i> , 1995
	<i>Stropharia rugosoannulata</i>	Steffen <i>et al</i> , 2002
Phenanthrene	<i>Alcaligenes faecalis</i>	Kiyohara <i>et al</i> , 1990
	<i>Aerobacter ptychromogenes</i>	Keuth & Rehm, 1991
	<i>Pseudomonas</i> sp.	Widada <i>et al</i> , 2002
	<i>B. cereus</i>	Aitken <i>et al</i> , 1998
	<i>Rhodococcus</i> sp.	Surovtseva <i>et al</i> , 1997
Fluoranthene	<i>Mycobacterium</i> sp.	Kelley <i>et al</i> , 1991
	<i>P. paucimobilis</i>	Mueller <i>et al</i> , 1990
	<i>Rhodococcus</i> sp.	Walter <i>et al</i> , 1991
Pyrene	<i>Rhodococcus</i> sp.	Walter <i>et al</i> , 1991
	<i>Sphingomonas</i> sp.	Dagher <i>et al</i> , 1997
	<i>Mycobacterium</i> sp.	Churchill <i>et al</i> , 1999
Chrysene	<i>Rhodococcus</i> sp.	Walter <i>et al</i> , 1991
	<i>Pseudomonas</i> sp.	Aitken <i>et al</i> , 1998
	<i>Sphingomonas</i> sp.	Aitken <i>et al</i> , 1998

intermediates may then be processed through either an ortho cleavage or a meta cleavage type of pathway, leading to intermediates such as protocatechuates and catechols, which are then further converted to tricarboxylic acid cycle intermediates (van der Meer *et al*, 1992).

Factors influencing Rates of Microbial Degradation of Hydrocarbons

The principle abiotic factors that influence the rates and extent of microbial transformations are temperature, pH, moisture level (for soil), and salinity of the environment, and chemical composition, concentration and physical state of the contaminant hydrocarbon.

Temperature

The temperature can act on both the metabolic activity of the microbial populations as well as on the physical and chemical nature of petroleum. The low temperatures are typically associated with little or no biodegradation of many organic substrates (Alexander, 1999). The biodegradation of petroleum

hydrocarbons has been reported in a variety of cold terrestrial and marine ecosystems, including Arctic (Braddock *et al*, 1997), alpine (Margesin, 2000) and Antarctic soils (Baraniecki *et al*, 2002), Arctic seawater (Siron *et al*, 1995) and Antarctic seawater and sediments (Delille & Delille, 2000). Microorganisms are reported to degrade successfully diesel oil at 10°C (Margesin & Schinner, 1998). A strain of psychrotrophic *Rhodococcus* sp. is reported to mineralize short-chain alkanes at 0°C (Whyte *et al*, 1998, 1999). However, using psychrotrophic and/or psychrophilic populations with increasing incubation temperatures (5-20°C) would remarkably increase the rates of biodegradation. Conversely, the lower temperature threshold for significant oil biodegradation is around 0°C (Siron *et al*, 1995).

Petroleum biodegradation usually declines due to the suppression of the microbial growth rates and metabolic activities under low temperatures and toxic components in crude oil. At low temperatures, the viscosity of oil increases, reducing the degree of oil spreading in soil and water. Low temperatures also retard the volatilization of short chain alkanes (<C₁₀)

and hence increasing their solubility and amount in water and soil, respectively. Consequently, their microbial toxicity is increased (Leahy & Colwell, 1990). Many long chain alkanes solidify below 10°C, with many forming crystals at 0°C (Margesin & Schinner, 2001), thus reducing their bioavailability to the microbial forms.

With the increase in enzymatic activity of mesophilic and thermophilic microorganisms associated with increasing temperatures, it is expected that biodegradation rates to be enhanced to a certain extent, typically in the range of 30° to 40°C. Above 40°C, the membrane toxicity of hydrocarbons is increased, thus hindering biodegradation (Bossert & Bartha, 1984). Of 368 isolates, belonging predominantly to *Bacillus stearothermophilus*, purified from desert samples taken from oil-contaminated areas in Kuwait, two strains effectively degraded crude oil at 60°C (Sorkhohi *et al.*, 1993). Annweiler *et al.* (2000) described a thermophilic *B. thermoleovorans* growing on naphthalene at 60°C. Being able to overcome membrane toxicity, thermophilic microorganisms benefit from the amplified bioavailability of hydrocarbons as their solubility increases with water temperature.

pH

Biodegradation rates are highest at a pH near neutrality (Alexander, 1999; Leahy & Colwell, 1990; Zaidi & Imam, 1999). In acidic soil environments, biodegradation of crude oil is usually dominated by fungal populations, which are generally more tolerant to low pH environments (Jones *et al.*, 1970). Stapleton *et al.* (1998) reported an indigenous microbial population consisting of a fungus, yeast and several bacteria to be actively degrading mono- and PAHs in the run-offs of a coal pile storage area at pH 2. It was postulated that initial fungal attacks on the hydrocarbons might have produced intermediates for further degradation for the bacteria. At the other extreme, alkaliphilic bacteria, that were effectively degrading phenol from wastewater optimally (pH, 7.5-10.6), have been isolated from highly alkaline lake and industrial effluents (Kanekar *et al.*, 1999; Sarnaik & Kanekar, 1995).

Salinity

In general, many soil, freshwater and estuarine isolates can survive in salinities comparable to seawater salinity. However, few freshwater or terrestrial species (ZoBell, 1973) are able to

reproduce in seawater (salinity, 3.5%). Due to salt requirements, marine isolates grow poorly in salinities lower than 1.5 to 2%. In a seawater bacterial community, maximum crude oil biodegradation was seen at 0.4 M NaCl (natural seawater) while significant biodegradation was seen between 0.1 to 2.0 M NaCl. The biodegradation of hydrocarbons in the marine environment is mainly attributed by *Pseudomonas* sp., Enterobacteria and a few Gram-negative aerobes (Bertrand *et al.*, 1993). The reduced capacity for biodegradation in hypersaline niches would be attributed more to a general reduction in microbial activity associated with extreme environments rather than the effects on the hydrocarbon forms (Ward & Brock, 1978). Whitehouse (1984) reported an inverse relationship between salinity and solubility of PAHs resulting from the salting-out effects occurring in the solution and solid phases.

Oxygen

Molecular oxygen is fundamental in hydrocarbon degradation. In the aquatic ecosystems, biodegradation is limited by oxygen deficiency particularly when an oil spill is limited to a thin slick on the water surface. However, in aquatic sediments, water movement is severely restricted by the small pore sizes and heterotrophic activity generally renders all but the thin surface layer anaerobic. Any oil sinking below 1-2 cm layer of topsoil is expected to persist for long time. Sinking oil are usually tar balls that are generally recalcitrant to biodegradation. In soil, availability of oxygen is determined by the rates of microbial respiration, the type of oil, and the presence of available substrates that can lead to oxygen depletion (Bossert & Bartha, 1984).

Stoichiometrically, 3.1 mg/l oxygen is required to biodegrade 1 mg/l hydrocarbons. As groundwater is saturated with dissolved oxygen at 6-12 mg/l (depending on temperature), fully saturated groundwater can be expected to degrade 2-4 mg/l of hydrocarbons. These calculations however do not take into consideration microbial cell growth. In this case, the mass of oxygen required to degrade 1 mg of benzene decreases to 1.03 mg (Curtis & Lamme, 1998).

Anaerobic biodegradation occurs in limited oxygen and can amount to a significant decrease in contamination through the actions of anaerobic populations such as sulphate-reducing bacteria, metal-reducing bacteria, methanogens and denitrifiers. A

novel sulphate-reducing bacterium was isolated from fuel-contaminated subsurface soil that could mineralize 80% of toluene carbon to CO₂ under strictly anaerobic condition using sulphate as the electron acceptor (Beller *et al.*, 1996). So & Young (1999) isolated and characterized an alkane-degrading sulphate-reducing bacterium from an estuarine sediment chronically contaminated with petroleum. Strain AK-01 was reported to be able to catabolize alkanes (C₁₃-C₁₈). Anaerobic benzene degradation coupled to nitrate reduction to nitrogen gas was observed in enrichment cultures developed from soil and groundwater microcosms (Burland & Edwards, 1999). While their ecological significance has been regarded as minor and negligible (Atlas, 1981). Anaerobic populations play a significant role in the transformation of petroleum hydrocarbons in anaerobic environments (Curtis & Lamney, 1998; Rooney-Varga *et al.*, 1999; Vargas *et al.*, 2000).

Nutrients

In any environment, the ratio of C:N:P must be maintained at about 120:10:1 to sustain any microbial activity (Thomas *et al.*, 1992). Typically, the supply of nutrients other than C exceeds the requirements of the resident microbial populations given the little readily available C. However, the situation changes considerably if a compound that is potentially readily utilizable is introduced into the environment in significant amounts. During oil spills, the sudden or continuous loading of high amounts of C upsets this ratio by producing high C:N or C:P ratios or both, thus detrimental to microbial growth. Furthermore, waters in the oceans, lakes, rivers, soils and aquifers containing oil or petroleum typically have too low concentrations of these inorganic nutrients at the interface between the water insoluble pollutants and the aqueous phase to support the microbial activity that could accelerate degradation (Alexander, 1999).

Due to low levels of N and P in seawater, crude oil degradation following marine oil spills to be slow unless N or P or both are added to stimulate the indigenous microbial populations (Rosenberg *et al.*, 1992; Zaidi & Imam, 1999). Both organic (fertilizers) and inorganic (salts) sources of N and P have been investigated for their roles in stimulating biodegradation in both aquatic and terrestrial environments. Marine biodegradation performed in mesocosm systems or enclosures demonstrated that inorganic salts are effective, it is especially difficult to

overcome the dilution effect associated with adding water-soluble salts to an open water body. The concentration of salts of N and P introduced into surface waters at or very near the oil-water interface rapidly declines because of the water volume and movements, thus making the addition of these salts to stimulate biodegradation less efficient and economical in bioremediation efforts.

The addition of N and P through organic sources was extensively studied through the use of fertilizers following the Exxon Valdez spill. From three types of fertilizer formulations, the oleophilic fertilizer produced the most far-reaching effects in stimulating biodegradation within 2 to 3 weeks on the cobble shorelines at Prince William Sound (Atlas, 1995; Pritchard & Costa, 1991). Oleophilic fertilizers remain associated with the oil and stimulate hydrocarbon-degrading microorganisms. In contrast, the same oleophilic formulation (Inipol EAP22), when applied to a lagoon system on the coast of Kings Bay, Norway, did not result in any difference in the degradation rates of an Arabian light crude oil compared to the untreated area. Hence, Inipol EAP22 was not effective for treatment of oil slicks on open water in Arctic because the nutrients within the formulation dispersed fairly rapidly from the slick (Swannell *et al.*, 1996).

Chemical Composition of Petroleum

Due to variable chemical structures and molecular weights, petroleum hydrocarbons differ in their susceptibility to microbial attack as follows: n-alkanes > branched alkanes > low-molecular-weight aromatics > cyclic alkanes (Perry, 1984; Leahy & Colwell, 1990). Biodegradation rates are highest for saturates, followed by light aromatics with high-molecular-weight aromatics and polar compounds being highly recalcitrant to biodegradation (Leahy & Colwell, 1990; Obuekwe *et al.*, 2001). Fedorak & Westlake (1981) reported a more rapid degradation of aromatic hydrocarbons compared to n-alkanes. Cooney *et al.* (1985) observed that more naphthalene than hexadecane was degraded in water and sediment mixtures.

Physical State of the Hydrocarbon

The oil undergoes a series of actions such as evaporation, dissolution, photooxidation, and emulsification with water to form emulsions or mousse and perhaps eventually the formation of tar

balls. The formation of oil mousse increases the surface area of the oil and thus making it more available for microbial attack (Al-hadhrami *et al.*, 1995; Leahy & Colwell, 1990). Tar balls, however, are exceptionally recalcitrant to degradation due to their solid state and low surface area to volume ratios.

PHAs are highly persistent in the environment due to their high hydrophobicity and hence low availability to biodegrading microorganisms. Biodegradation of low molecular PAHs occurs readily when PAHs are in aqueous phase (Volkerling *et al.*, 1992). Therefore, the low solubility and dissolution rates of the larger complex PAHs may limit their availability for biodegradation. Phenanthrene and anthracene have water solubilities of 1.29 and 0.07 mg/l respectively (Poeton *et al.*, 1999). Phenanthrene in solution form is more biodegradable than in crystalline form (Bouchez *et al.*, 1995).

Concentration of the Petroleum or Hydrocarbons

While the rates of microbial uptake and biodegradation of water-soluble compounds are usually proportional to the concentration of the compound, the same cannot be said of compounds with low aqueous solubility and those which can exert membrane toxicity at high concentrations. The biodegradation rates of high molecular weight PAHs such as naphthalene and phenanthrene are related to their aqueous solubility rather than their concentrations in a given solution.

On the other hand, high concentrations of highly soluble or volatile organic compounds may be detrimental to microbial forms due to their toxicity. Dibble & Bartha (1972) found that biodegradation activities in oil sludge occurred between oil concentrations of 1.25 and 5% and were best at 5%. Oil loadings (>5%) lead to a decline in microbial numbers due to increase in toxicity. Del' Arco & Franca (2001) supported this using sandy sediment deliberately contaminated with petroleum. Tarabily & Khalid (2002) reported complete cessation of activity when the concentration of water-soluble fractions exceeded 50%. In addition to toxicity, high concentrations of petroleum may also inhibit microbial growth by upsetting the C:N:P ratios. Oxygen limitation may also hinder microbial growth when a thick layer of oil forms on the surface of a water body, preventing oxygen transfer into the aqueous phase.

Adaptation

Prior exposure to hydrocarbon contamination confers adaptation to microbial population. Often this lead to enhanced mineralization. Grosser *et al.* (1991) recorded 55% pyrene mineralization in soil when the inoculum was grown on pyrene compared to 1% mineralization by indigenous population. Al-hadhrami *et al.* (1995) also reported higher metabolic activity when the inoculum was grown with crude oil as a substrate compared to nutrient broth.

Petroleum Biodegradation by Microbial Consortia

Many biodegradations require more than a single species. Individual microorganisms can metabolize only a limited range of hydrocarbon substrates, so assemblages of mixed populations with overall broad enzymatic capacities are required to bring the rate and extent of petroleum biodegradation further. Microbial strains belonging to various genera have been detected in petroleum-contaminated soil or water (Dagher *et al.*, 1997, Radwan *et al.*, 1999, Sorkhoh *et al.*, 1995, Surovtseva *et al.*, 1997). This strongly suggests that each strain or genera have their roles in the hydrocarbon transformation processes.

Further evidence for the cooperation of mixed cultures in biodegradation is apparent when Sorkhoh *et al.* (1995) observed a sequential change of the composition of oil-degrading bacteria over a period of time in oil-contaminated sand samples. Similar observations were reported in sequential enrichments in medium containing residual crude oil (Ventakeswaran & Harayama, 1995). After exhaustive growth of one strain on crude oil, the residual oil supported the growth of a second and third strain of bacteria (Horowitz *et al.*, 1975). When grown together in a mixed culture or sequentially, there was over 75% oil transformation.

To maximize petroleum biodegradation, many studies employed mixed bacterial or bacterial-fungal cultures (Mishra *et al.*, 2001, Shelton *et al.*, 1999, Van Hamme *et al.*, 2000, Vifias *et al.*, 2002). Rambeloarisoa *et al.* (1984) demonstrated a consortium of 8 strains made up of members of 6 genera to be able to effectively degrading crude oil. Only 5 of these strains were able to grow in pure cultures using a variety of hydrocarbons. However, when the other 3 strains were removed from the consortium, the effectiveness of the mixed culture was remarkably reduced. Márquez-Rocha *et al.* (2001) demonstrated diesel-biodegradation effectiveness of

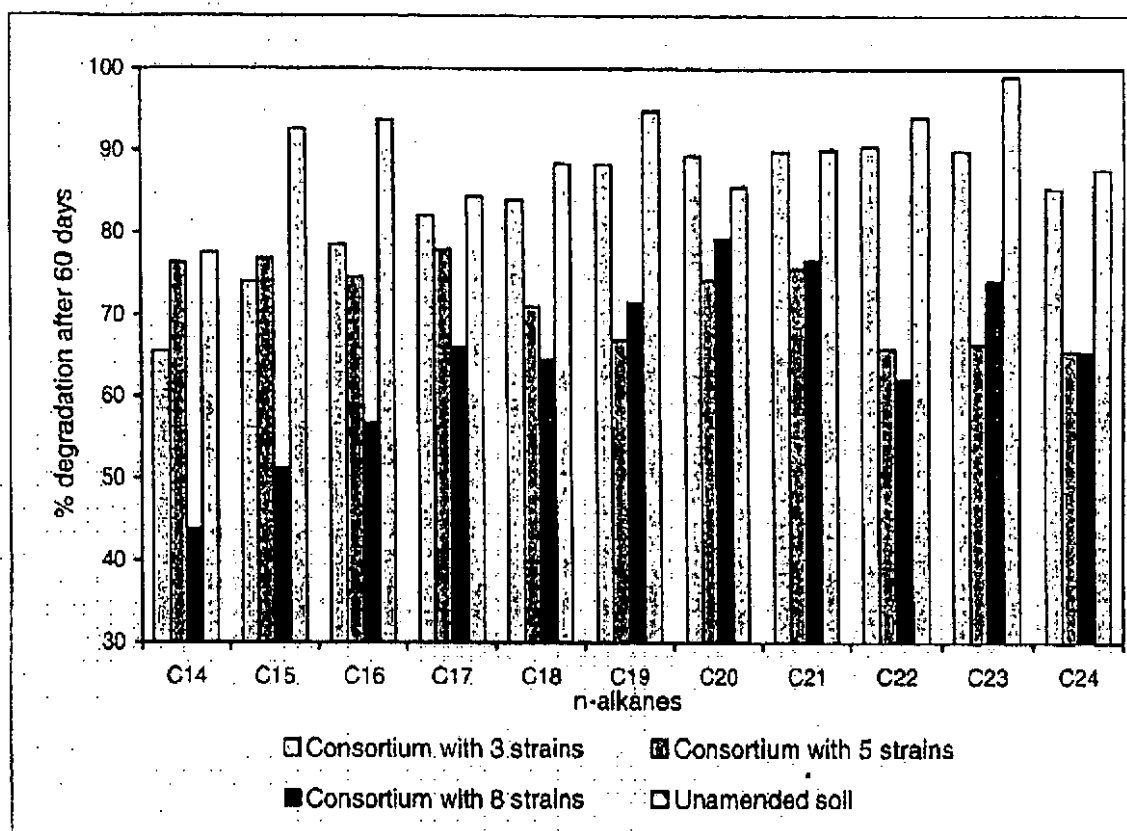


Fig. 4—The degradation patterns of n-alkanes in soil by microbial consortia of petroleum-degrading bacteria. Un-amended soil serves as the background degradation by indigenous microbial population and physical weathering.

85, 91 and 96% using a bacterial consortium, a white rot fungus, *Pleurotus ostreatus*, and a combination of the bacterial consortium and *P. ostreatus*, respectively. The biodegradation rate of diesel was also highest in the bacterial and fungus mixture. Thus, each member in a microbial community has significant role and may need to depend on the presence of other species or strains to be able to survive.

The degradative capacity of any microbial consortium is not necessarily the result of mere adding together of the capacities of the individual strains forming the association. Surovtseva *et al* (1997), when removing three strains from an association, saw a decrease in efficiency of the consortium's degradation of aromatic fractions.

Komukai-Nakamura *et al* (1996) reported the sequential degradation of Arabian light crude oil by two different genera. *Acinetobacter* sp. T4 biodegraded alkanes and other hydrocarbons producing the accumulation of metabolites. Following that, *Pseudomonas putida* PB4 began to grow on the

metabolites and finally degraded aromatic compounds in the crude oil. Further, Nadarajah *et al* (2002) suggested that single pure bacterial cultures would require the use of sterile fermenters and culture media, which have high capital and operating costs, hence using a mixed bacterial culture offers an economically feasible bioremediation process.

In present study of 3 microbial consortia consisting of 3, 5 and 8 bacterial strains obtained from hydrocarbon-polluted soils, it was seen that the consortium consisting the highest number of bacterial strains was most effective at removing weathered diesel from soil (Fig. 4). The diesel degradation was measured by measuring the residual n-alkanes in the soil. A reduction (up to 22%) was seen in tetradecane in the un-amended soil (not inoculated with microbial mixture). The addition of the microbial mixture resulted in enhanced degradation of the middle- and long-chain alkanes in the soil. With the addition of the consortium of 8 strains (predominantly *Bacillus* and *Pseudomonas* Spp.), 43-79% of n-alkanes remained after 60 days of incubation.

The advantages of employing mixed cultures as opposed to pure cultures in bioremediation have been attributed to the effects of synergistic interactions among members of the association. It is possible that one species removes the toxic metabolites (that otherwise may hinder microbial activities) of the species preceding it. It is also possible that the second species is able to degrade compounds that the first is able to degrade only partially (Alexander, 1999). Further research should be directed towards understanding the roles of individual members in influencing the effectiveness of a microbial association.

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Bacterial communities and enzyme activities of PAHs polluted soils

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Abstract

Three soils (i.e. a Belgian soil, B-BT, a German soil, G, and an Italian agricultural soil, I-BT) with different properties and hydrocarbon-pollution history with regard to their potential to degrade phenanthrene were investigated. A chemical and microbiological evaluation of soils was done using measurements of routine chemical properties, bacterial counts and several enzyme activities. The three soils showed different levels of polycyclic aromatic hydrocarbons (PAHs), being their contamination strictly associated to their pollution history. High values of enzyme activities and culturable heterotrophic bacteria were detected in the soil with no or negligible presence of organic pollutants. Genetic diversity of soil samples and enrichment cultures was measured as bands on denaturing gradient gel electrophoresis (DGGE) of amplified 16S rDNA sequences from the soil and enrichment community DNAs. When analysed by Shannon index (H'), the highest genetic biodiversity ($H' = 2.87$) was found in the Belgian soil B-BT with a medium-term exposition to PAHs and the poorest biodiversity ($H' = 0.85$) in the German soil with a long-term exposition to alkanes and PAHs and where absence, or lower levels of enzyme activities were measured. For the Italian agricultural soil I-BT, containing negligible amounts of organic pollutants but the highest Cu content, a Shannon index = 2.13 was found.

The enrichment of four mixed cultures capable of degrading solid phenanthrene in batch liquid systems was also studied. Phenanthrene degradation rates in batch systems were culture-dependent, and simple (one-slope) and complex (two-slope) kinetic behaviours were observed. The presence of common bands of microbial species in the cultures and in the native soil DNA indicated that those strains could be potential *in situ* phenanthrene degraders. Consistent with this assumption are the decrease of PAH and phenanthrene contents of Belgian soil B-BT and the isolation of phenanthrene-degrading bacteria.

From the fastest phenanthrene-degrading culture C_{B-BT}, representative strains were identified as *Achromobacter xylosoxidans* (100%), *Methylobacterium* sp. (99%), *Rhizobium galegae* (99%), *Rhodococcus aetherivorans* (100%), *Stenotrophomonas acidaminiphila* (100%), *Alcaligenes* sp. (99%) and *Aquamicrobium defluvium* (100%). DGGE-profiles of culture C_{B-BT} showed bands attributable to *Rhodococcus*, *Achromobacter*, *Methylobacterium rhizobium*, *Alcaligenes* and *Aquamicrobium*.

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The isolation of *Rhodococcus aetherovorans* and *Methylobacterium* sp. can be consistent with the hypothesis that different phenanthrene-degrading strategies, cell surface properties, or the presence of xenobiotic-specific membrane carriers could play a role in the uptake/degradation of solid phenanthrene.

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Keywords: Soil chemical/enzymatic characteristics; DGGE; Bacterial diversity; Phenanthrene consumption; Batch liquid systems

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are widespread in nature (i.e. soil, water and sediments) because of several polluting anthropogenic activities (Samanta et al., 2002). They have been recognised as a potential health risk due to their intrinsic chemical stability, high recalcitrance to different types of degradation and high toxicity to living organisms (Alexander, 1999).

PAHs present in soil may exhibit a toxic activity towards different plants, microorganisms and invertebrates. Microorganisms, being in intimate contact with the soil environment, are considered to be the best indicators of soil pollution. In general, they are very sensitive to low concentrations of contaminants and rapidly respond to soil perturbation. An alteration of their activity and diversity may result, and in turn it will reflect in a reduced soil quality (Schloter et al., 2003). Soil enzyme activities are the driving force behind all the biochemical transformations occurring in soil. Their evaluation may provide useful information on soil microbial activity and be helpful to establish effects of soil specific environmental conditions (Dick et al., 1996).

Numerous research efforts are being dedicated to the search of proper remediation technologies to remove as much as possible contaminants from the environment or to transform them into less toxic compounds. Bioremediation appears to be an appealing technology to approach the recovery of PAH-polluted sites (Harayama, 1997). Several microorganisms are capable to mineralise a large variety of PAHs and/or to break down them to their less-toxic metabolites (Cerniglia, 1992). The very low water-solubility of PAHs and the slow mass-transfer rates from solid phase may limit their availability to microorganisms, thus hindering natural attenuation microbial processes. However, some bacteria degrade sorbed PAHs at different rates, indicating organism-specific bioavailability (Grosser et al., 2000).

Bioremediation of PAH contaminated sites rely either on the presence of autochthonous degrading bacteria which capabilities might be stimulated in situ (Margesin and Schinner, 1997), or on the inoculation of selected microorganisms with desired catabolic traits in bioaugmentation techniques (Straube et al., 1999). When microorganisms are added to speed up degradation in contaminated environments, the duration assessment and biological process efficiency depend on the

evolution of bacterial communities in terms of composition and catabolic activity. Denaturing gradient gel electrophoresis (DGGE) analysis of 16S rRNA genes represents a powerful tool to study the bacterial community structures in complex environments as well as in enrichment cultures (Muyzer and Smalla, 1998). However, the combination of both culture-independent and culture-dependent techniques might provide useful and complementary information on the structure of microbial communities.

Soils with different pollution history were preliminary characterized in terms of their chemical properties, enzymatic activity and culturable heterotrophic bacteria. Site characterization is a pre-requisite when dealing with any remediation approach of a polluted site (Smith and Mason, 1999). Indeed, chemical and biochemical properties may assist in the analysis of the ability for the soil to be recovered (Margesin et al., 2000). Moreover, the enrichment and selection of bacterial phenanthrene-degrading cultures, capable of degrading solid phenanthrene in batch liquid systems were performed. The kinetics of phenanthrene disappearance by enriched cultures, the comparison of their degradation rates and their species composition were also investigated, as assessed by DGGE analysis of PCR-amplified 16S rDNA gene fragments. The enrichment of such cultures is a necessary step to obtain microorganisms with the desired catabolic traits, usable in the bioaugmentation of polluted soils.

2. Materials and methods

2.1. Chemicals

Phenanthrene was at >96% purity (Sigma Aldrich, Germany). Solvents at 99.9% purity and all the other chemicals, reagent grade were supplied by Analaar, BDH Ltd. (Germany), unless otherwise stated.

2.2. Soil description and sampling

Three soils having a different pollution history were studied. Namely:

- (1) A German soil, G, polluted by a long-term exposition (>50 years) to alkanes and PAHs, leading to the formation of a typical light non-aqueous phase

- liquid (LNAPL) contamination (Saccomandi and Gianfreda, 2001). The soil is from Turingia (Germany) and its pollution is dated back to II World War. The site is still heavily contaminated because no remediation actions were implemented on it.
- (2) An Italian agricultural soil, I-HT, from the North of Italy, with no or negligible presence of pollutants.
- (3) A Belgian soil, B-BT, from a fluvial canal of Bruxelles (Belgium), characterised by a medium-term (<3 years) exposition to PAHs. The soil was subjected to an accidental pollution event that caused a spread distribution of PAHs on its surface. The soil was sampled after 3 years from the pollution event.

Italian and Belgian soil samples were taken random by ram-drilling at a depth of 5–15 cm. German soil was drawn from within the LNAPL phase, immediately above the water table (at a depth ranging from 5.5 to 7.6 m below soil surface). Soil samples were packed on-site into sealed polythene bags and transported to the laboratory, stored dark and cooled (4 °C). Samples were homogenised, sieved to <0.2 mm and stored at 4 °C until used.

Investigations were performed also on Italian (I-AT) and Belgian (B-AT) soils after bioremediation pilot experiments. Soils were treated aerobically in a bioreactor for 5 months; the experimental procedure adopted and the obtained results are under a patent. Unfortunately, no further information was provided by the site's owner. German soil was not treated because previous laboratory investigations demonstrated that any effort to bioremediate it was unsuccessful (Saccomandi and Gianfreda, 2001).

2.3. Determination of chemical and microbiological properties

The soils were characterized with respect to both physical and chemical as well as microbiological properties. In particular, a set of enzyme activities (e.g. dehydrogenase, fluorescein diacetate hydrolase, arylsulphatase, phosphatase and urease) and culturable heterotrophic bacterial cell number were determined. Molecular biodiversity of total bacterial populations was also analysed, according to methods described below (Section 2.6).

Chemical and physical analyses were performed on air-dried and sieved (<2 mm) samples according to standard techniques (Methods of Soil Chemical Analysis, 1996). Soil organic C was determined by the method of dichromate oxidation, pH was measured by glass electrode in 1:2.5 H₂O suspensions, total N was measured by the standard Kjeldahl method. Particle size distribution was assessed by the pipette-method. Overall content of PAHs and alkanes of German soil was determined according to Saccomandi and Gianfreda (2001).

Heavy metals were determined by atomic adsorption spectroscopy (AAS) after acid digestion with HF/HNO₃.

Enzyme activities were determined on fresh moist soils sieved <2 mm. The arylsulphatase (ARYL) and phosphatase (PHO) activities were determined according to Tabatabai and Bremner (1970) and Sannino and Gianfreda (2001), respectively. Specific substrates (*p*-nitrophenyl derivatives) and buffers were used for each enzyme. Urease (UR) activity was measured as described by Kundeler and Gerber (1988). Dehydrogenase (DH) assays were performed using soluble tetrazolium salt (TTC) as an artificial acceptor (Trevors, 1984). The activity of fluorescein diacetate hydrolase (FDAH) was assessed as described by Adam and Duncan (2001). A unit (U) of ARYL, DH and PHO enzyme activity was defined as the micromoles of substrate transformed at 30 °C h⁻¹ by 1 g of dried soil. The FDAH and UR activities were expressed as micrograms of substrate hydrolysed at 30 °C h⁻¹ by 1 g of dried soil. Control tests with autoclaved soils were carried out to evaluate the spontaneous or abiotic transformation of substrates.

To enumerate culturable heterotrophic bacteria, 10 g of each soil sample were suspended in 45 ml sterilised Na₂P₂O₇ (0.2 g l⁻¹ in bidistilled water) in 300 ml glass bottles for 1 h on a shaker, in order to separate bacteria from soil particles. One millilitre of supernatant obtained after 10 min sedimentation was then 10-fold serially diluted in NaCl 9 g l⁻¹ solution. Appropriate dilutions were plated onto 10% strength Tryptic Soy Agar medium for a total heterotrophic bacterial count; 100 µl ml⁻¹ cycloheximide were added to the medium to inhibit the growth of eukaryotes. The plates were incubated at 28 °C for 8 days and then counted.

Unless otherwise specified, all results reported are averages of triplicate determinations.

2.4. Enrichment and isolation of phenanthrene-degrading cultures

Freshly prepared phenanthrene stock solution in acetone (20 mg ml⁻¹) was added to 500 ml glass bottles. The acetone was allowed to evaporate before adding 100 ml of autoclaved M9 mineral salt medium (Kunz and Chapman, 1981) to have a final concentration of 200 mg l⁻¹ phenanthrene. Then 10 g of soil samples were added to a series of bottles. The bottles were teflon-stoppered and incubated in the dark at 25 °C with agitation on a reciprocal shaker at 96 rpm for 3 weeks. Periodically (3 weeks) 10 ml aliquots of grown cultures were transferred into fresh medium under the same conditions.

Different bacteria were isolated from the enrichment cultures. The isolates were grown on M9 liquid medium containing 100 mg l⁻¹ phenanthrene. Pure cultures were identified by 16S rDNA gene nucleotide sequence analysis according to the method below described.

2.5. Measurements of phenanthrene utilisation rates

The mixed cultures were grown at 25 °C with shaking in 500 ml bottles containing 100 ml M9 mineral medium supplemented with 200 mg l⁻¹ phenanthrene. Four bottles for each culture were prepared. At each sampling time the concentration of phenanthrene was determined on duplicate sacrificial bottles and the other two bottles were utilised to perform protein content analysis (Bradford, 1976) and to extract total DNA (see below). Two bottle-controls (without bacteria) were run in parallel to account for the abiotic loss of phenanthrene.

The extraction and quantification of phenanthrene was determined as follows. Culture broths were extracted three times with 50 ml CH₂Cl₂; the organic layers were collected, dried with Na₂SO₄, filtered and the solvent was removed under reduced pressure. The residue was solved in 2 ml of ethyl acetate and 4 ml of a solution of dodecanol in ethyl acetate (5 mg ml⁻¹) were added as internal standard for gas chromatographic analyses. The aqueous phase was acidified by conc. HCl (pH 2) and extracted three times with 50 ml ethyl acetate; the organic layers were collected and processed as before described.

Gas-chromatographic analyses were carried out using a DANI 1000 Gas-chromatograph, equipped with a FID detector (hydrogen 0.9 bar, air 1.0 bar and nitrogen 1.0 bar) and a fused silica capillary column WCOT-CP-SIL 8 CB Chrompack (25 m × 0.32 mm ID), carrier helium (0.8 bar), and injection temperature 300 °C, detection 300 °C, initial oven temperature 140 °C (3 min), temperature increase 10 °C min⁻¹, final isotherm 250 °C, injection volume 2 µl. The dodecanol *R_t* was 6.9 min and the phenanthrene *R_t* was 11.3 min. Detector signal output was monitored by computer and all chromatograms and data were generated and processed by Dani Data Station version 1.7 software.

2.6. Molecular methods

DNA was extracted from soil samples, enrichment cultures and isolated strains. Soil DNA and enrichment culture DNA were extracted by a bead-beating method (MOBIO, USA) and by BIO101 method (Resnova, Italy), respectively, according to the manufacturer instructions. According to Cavalea et al. (2002), proteinase K (1 mg ml⁻¹) was used to extract DNA from strains.

PCR amplification of the 16S rDNA was performed on the extracted DNA, by using eubacterial universal primers P27f and P1495r referred to *E. coli* nucleotide sequence of 16S rDNA gene (Cavalea et al., 2002). Nested PCR reaction for V3 amplification was carried out according to Muyzer and Smalla (1998). V3 PCR products from soil, enrichment culture and bacterial isolates DNAs were characterized by a DGGE run on a vertical acrylamide gel in a DCODE Universal Mutation Detec-

tion System (Biorad). DGGE was performed with 8% (wt/vol) polyacrylamide gels in TAE buffer (20 mM Tris acetate pH 7.5, 10 mM sodium acetate, 0.5 mM Na₂-EDTA) with a linear chemical gradient ranging from 35% to 65%. Denaturant solutions were prepared by mixing the appropriate volumes of two 0–100% denaturant stock solutions (7 M urea, and 40% vol/vol formamide (Amersham Biosciences, Sweden). Gels were run at a constant voltage of 70 V for 16 h at 55 °C. Gels were stained in a 0.5 mg l⁻¹ ethidium bromide solution and documented with GelDoc System (Biorad). Bands of interest were excised from DGGE using an UV transilluminator. The excised bands were suspended into 200 µl of PCR water, reamplified and sequenced. The nucleotide sequences of 16S rDNA of the resulting amplicons and of isolates were determined according to the Perkin Elmer ABI Prism protocol (Applied Biosystems, USA). Primers used in the PCR reaction for sequencing products were the same of those in normal 16S rDNA PCR reactions. The forward and reverse samples were run on an Applied Biosystems 310A sequence analyser. The sequences were compared with similar sequences of reference organisms deposited in public domain databases.

DGGE analyses were performed to compare the bacterial community structures of soils and enrichment cultures. Although the technique could be associated with a variety of PCR biases (Wintzingerode et al., 1997; Fromin et al., 2002), it provides comprehensive information on the global patterns of microbial diversity (Torsvik and Overås, 2002). However, to minimize biases, DGGE analyses were performed on samples treated using identical methods in which DNA extraction and amplification biases are supposed to occur homogeneously.

Shannon index (*H'*) (Magurran, 1988) was used to evaluate the biodiversity of both soils and enrichment cultures, and Sorensen index (*S*) (Magurran, 1988) to evaluate the similarity within soils (native vs. treated soil) and within the deriving cultures.

The Shannon index of soils was calculated on the basis of the number and intensity of bands present on DGGE samples, run on the same gel, as follows: $H' = -\sum P_i \log P_i$, where P_i is the importance probability of the bands in a gel lane. P_i was calculated as follows: $P_i = n_i/N$, where n_i is the band intensity for each individual band and N is the sum of intensities of bands in a lane. Statistical comparison of different DGGE profiles was done with the GelDoc software package. This latter assumes that the population size is proportional to the thickness of bands. Gel analysis included conversion of the scanned gel image and normalization in order to correct shift within or between gels, so that bands or peaks of the same molecular size have the same physical position relative to a standard. Once all banding profiles were in a standardized analysis format, each band could be described by its position on the gel and by its relative intensity.

3. Results and discussion

3.1. Physico-chemical and microbiological properties of soils

The chemical and physical properties of a soil as well as the evaluation of its pollution degree may help to estimate the impact of pollutants on the quality of soil under investigation, if they are complemented with the measurement of biological properties (Margesin et al., 2000).

Tables 1 and 2 summarise the physical and chemical properties of investigated soils and the amounts of both organic and inorganic pollutants.

The moderate-high amounts of carbonate and the pH values (measured in H_2O), ranging from 2.68 to 5.36 and from 6.73 to 8.19, respectively, indicate a sub- to moderate-alkaline character of soils (Table 1). At the measured pH range soil microbial growth and its activity are usually favoured. As discussed by Smith and Doran (1996), soil pH can provide valuable information on the availability and toxicity of several elements, including Fe, Al, Mn, Cu, Cd and others to plants and microorganisms.

German and Italian soils showed comparable amounts of clay, silt and sand fractions (Table 1) whereas Belgian soil had a very low amount of both clay (~7%) and silt (~8%) and a predominant presence of sand (>80% as total of coarse and fine fractions). According to USDA classification (Soil Survey Staff, 1993), German and Italian soils can be classified sandy clay loam soils while Belgian is a typically loamy sand soil.

In Belgian and mainly in German soil before treatment (B-BT and G) total organic C values, and consequently organic matter contents, were very high, being influenced by organic pollutant contamination. Thus, their values did not represent natural, endogenous soil organic matter levels, possibly present in the soil in the absence of any contamination. Considering the low amounts of N measured in both soils, the C/N ratios (11.5 and 26.3 for B-BT and G soils, respectively) were higher than those normally found in unpolluted soils. When hydrocarbon-polluted soils are considered, much higher C/N ratios, ranging from a minimum value of 9:1 to a maximum of 200:1, are, however, needed to obtain a consistent microbial growth and resulting hydrocarbon degradation (Bewley, 1996).

The physical and chemical properties of Belgian and Italian soils were also measured after the bioremediation treatment (Table 1). As expected, no significant variations of clay, silt and sand fractions were noted. The 2-fold higher amounts of both N and available K measured in B-AT are likely the result of nutrient supply during the biological treatment.

According to the current European Union regulation (Commission of the European Communities, 1986)

Table 1
Physical-chemical properties of study soils

Soil	pH (H_2O)	CaCO ₃ (%)	Moisture (%)	Clay (%)	Silt (%)	Coarse sand (%)	Fine sand (%)	O.C. (g kg ⁻¹)	O.M. (g kg ⁻¹)	Total N (g kg ⁻¹)	C/N	P-Okem (mg kg ⁻¹)	Available K (mg kg ⁻¹)
<i>Before treatment</i>													
German (G)	6.73a [*] (±0.23)	3.07a (±0.15)	13.0a (±0.90)	24.7a (±1.5)	15.0a (±0.97)	22.0a (±0.99)	38.3a (±1.0)	11.1a (±1.50)	19.1a (±1.20)	0.422a (±0.09)	26.3a (±3.45)	Trace	nd
Italian (I-BT)	7.67b (±0.56)	3.93a (±0.21)	14.5a (±1.04)	22.5a (±2.0)	24.5b (±2.4)	19.5b (±1.30)	33.4b (±1.10)	7.70b (±1.20)	13.3b (±1.90)	2.20b (±0.50)	3.5b (±0.45)	33.7a (±4.50)	337b (±17.8)
Belgian (B-BT)	8.19b (±1.10)	2.93a (±0.09)	11.0a (±0.85)	6.94b (±0.54)	7.75c (±0.63)	40.6c (±3.20)	45.3c (±3.60)	8.2b (±1.60)	14.1b (±2.20)	0.71c (±0.09)	11.5c (±1.20)	15.0b (±1.50)	224c (±13.4)
<i>After treatment</i>													
Italian (I-AT)	7.77b (±0.61)	5.36b (±0.35)	18.5b (±1.20)	21.6b (±2.0)	23.9b (±2.70)	18.9a (±1.40)	35.6b (±3.10)	7.9b (±1.50)	13.6b (±2.10)	2.85d (±0.60)	2.8b (±0.38)	30.5a (±3.90)	574d (±20.7)
Belgian (B-AT)	8.17b (±0.60)	2.68a (±0.10)	11.0a (±0.91)	6.90b (±0.54)	8.88c (±0.74)	38.6c (±1.93)	45.9c (±2.30)	8.20b (±0.90)	14.1b (±1.50)	1.40c (±0.80)	5.8d (±0.40)	20.2c (±0.80)	495c (±18.5)

* For each variable different letters alongside columns refer to significant differences ($P < 0.05$).

^a Values in parentheses represent standard deviation.

Table 2
Amounts of inorganic and organic pollutants of study soils

Soil	Inorganic (mg kg ⁻¹)					Organic (mg kg ⁻¹)		
	Cu	Zn	Cr	Ni	Fe	Alkanes	PAH	Phenanthrene
<i>Before treatment</i>								
German (G)	145a [*] (±8.7) ^a	88.0a (±9.4)	14.0a (±2.7)	39.0a (±8.2)	6.1a (±3.6)	290 (±10.1)	94a (±6.4)	14a (±2.6)
Italian (I-BT)	301b (±21.5)	121b (±9.6)	72.4b (±6.5)	75.5b (±8.5)	40.3b (±5.4)	nd ^b	nd ^b	nd
Belgian (B-BT)	50.2c (±5.3)	124b (±7.5)	83.9c (±5.3)	55.4c (±6.5)	39.0b (±5.6)	nd	30.8b (±3.2)	4.7b (±0.7)
<i>After treatment</i>								
Italian (I-AT)	290b (±19.3)	265d (±12.1)	70.8b (±5.8)	85.7b (±9.1)	25.9c (±3.2)	nd	nd	nd
Belgian (B-AT)	52.9c (±8.5)	329c (±17.5)	67.4b (±6.4)	65.6d (±7.6)	33.4d (±2.4)	nd	8.9c (±0.87)	0.7c (±0.6)

^a For each variable different letters alongside columns refer to significant differences ($P < 0.05$).

^b Values in parentheses represent standard deviation.

referring to agricultural soils, investigated soils showed levels of heavy metals all below the maximum permitted concentrations, except for copper in Italian soil that was about twice the safe limit (150 mg kg⁻¹ soil).

A different situation holds when organic pollutants are considered. German soil resulted heavily polluted by high concentrations of alkanes and PAHs. BTEX and phenols were also detected (data not shown), thus confirming the presence of a LNAPL widespread pollution (Saccomandi and Gianfreda, 2001). In contrast, these pollutants were not detected in Italian soils. Belgian soil presented a detectable amount of PAHs

(30.8 mg kg⁻¹), being phenanthrene relatively the most abundant (Table 2).

The activities of five enzymes and the heterotrophic bacteria of the investigated soils are reported in Table 3. Arylsulphatase and phosphatase release sulfate and phosphate, the main plant and microbial available S and P forms from various organic sulfate and phosphate esters (Nannipieri et al., 2002). Urease catalyses the hydrolysis of urea to carbon dioxide and ammonium and it is widely distributed in microorganisms, plants and animals (Nannipieri et al., 2002). Dehydrogenase activity typically occurs in all intact, viable

Table 3
Enzymic activities and microbial counts of study soils

Soil	ARYL ($\mu\text{mol g}^{-1} \text{h}^{-1}$)	PHO ($\mu\text{mol g}^{-1} \text{h}^{-1}$)	UR ($\mu\text{g g}^{-1} \text{h}^{-1}$)	DH ($\mu\text{g g}^{-1} \text{h}^{-1}$)	FDAH ($\mu\text{g g}^{-1} \text{h}^{-1}$)	Total heterotrophs CFU (g ⁻¹)
<i>Before treatment</i>						
German (G)	nd	4.10a (±0.045)	nd	18.9a (±2.1)	nd	3.9×10^5 a (±4.0 × 10 ⁴)
Italian (I-BT)	0.388a [*] (±0.07) ^a	2.20b (±0.31)	18.4a (±1.7)	0.748b (±0.03)	186a (±6.45)	4.9×10^5 b (±4.0 × 10 ⁴)
Belgian (B-BT)	0.014b (±0.003)	0.35c (±0.21)	nd	nd	8.52b (±0.91)	2.3×10^5 c (±2.0 × 10 ⁴)
<i>After treatment</i>						
Italian (I-AT)	0.555c (±0.09)	3.84d (±0.40)	18.8a (±1.6)	1.27c (±0.08)	197c (±6.51)	3.9×10^5 d (±5.0 × 10 ⁴)
Belgian (B-AT)	0.265d (±0.02)	2.90b (±0.1)	nd	0.049d (±0.01)	162d (±5.56)	5.8×10^5 e (±6.0 × 10 ⁴)

nd = not detected; ARYL = arylsulphatase, PHO = phosphatase, UR = urease, DH = dehydrogenase, FDAH = fluorescein diacetate hydrolase.

^a For each variable different letters alongside columns refer to significant differences ($P < 0.05$).

^b Values in parentheses represent standard deviation.

microbial cells. Thus, its measurement is usually related to the presence of viable microorganisms and their oxidative capability (Trevors, 1984). Fluorescein diacetate hydrolase (FDAH) has been often used as a sensor and functional indicator of soil health (Adam and Duncan, 2001). Being the fluorogenic substrate uptaken by active cells and then transformed by a large arrays of hydrolytic enzymes, the enzyme has been considered a measure of the soil microorganism activity (Killham and Staddon, 2002).

Enzyme activities and total heterotrophs, mainly for Belgian and German soils, are in agreement with the results obtained with soils contaminated by similar pollutants (Kiss et al., 1998; Margesin et al., 2000). The German soil was the most contaminated compared to Belgian and Italian soils, having the lowest number of heterotrophs (Table 3).

After the biological treatment an increase in CFU of only one order of magnitude was measured in both Belgian and Italian soils (Table 3). As reported by Margesin et al. (2000) total number of heterotrophs of PAHs polluted soils did not greatly increase after biological remediation actions, whereas the relative amounts of specific pollutant-degrading bacteria increased to a detectable extent.

Enzyme activities also confirmed that the Italian soil showed the highest microbiological activity. All the measured enzymes were present at moderate to high range levels, usually found in agricultural soils (Nannipieri et al., 2002). The relatively low dehydrogenase activity measured in this soil (which seems to contradict the high values of both FDAH activity and total microorganisms) could be explained by the possible interference exerted by the high Cu content (Table 2) on the analytic assay used. Indeed, Cu may react abiotically with the triphenylformazan, the end product of DH catalysis, thus resulting in a underestimation of the soil dehydrogenase activity (Chander and Brookes, 1991).

Although the influence of other factors deriving from natural and anthropogenic events cannot be ruled out (Gianfreda and Bollag, 1996), the complete absence and/or the very low enzymatic activities of both German and Belgian soils could be also partly due to the presence of PAHs in soils. As extensively reviewed by Kiss et al. (1998), even moderate levels of hydrocarbon contamination may cause a significant decline of several soil enzyme activities, showing each enzyme a different sensitivity to the presence of pollutants. Although the interpretation of enzyme activities of soil is complex because both extracellular and intracellular enzyme activities contribute to the overall soil enzyme activity, some hypotheses might be advanced. In soil, non-polar organic compounds, such as hydrocarbons, may likely exert different effects on microbiological properties. Hydrocarbons may be toxic to soil microorganisms which may reflect in a consistent reduced enzymatic activity;

and/or they may cover both organic-mineral and cell surfaces, thus hindering the interaction between enzyme active sites and soluble substrates with adverse effect on enzyme activity expression (Kiss et al., 1998). Moreover, a synergistic negative effect on soil enzyme activities exerted by the simultaneous presence of heavy metals cannot be ruled out.

After bioremediation, enzyme activities of Italian and Belgian soils increased to a moderate and a more detectable extent, respectively.

3.2. Biodiversity of soils

In our analysis, the number of DGGE bands was taken as an indication of species in each sample. The relative surface intensity of each DGGE band and the sum of all the surfaces for all bands in a sample were used to estimate species abundance (Fromin et al., 2002; Sekiguchi et al., 2002). DGGE profiles of soils are shown in Fig. 1. Many DGGE bands were observed in the profiles, thus indicating the presence of different bacterial populations and different relative abundance species in soils. As indicated by the values of Shannon indices, contamination of soils appeared to affect their genetic diversity: German soil and native Belgian soil B-BT showed the poorest ($H'_G = 0.85$) and the highest ($H'_{B-BT} = 2.87$) biodiversity, respectively. For the Italian agricultural soil I-BT, containing negligible amounts of organic pollutants but the highest Cu content, a Shannon index = 2.13 was found.

After treatment, a loss of bacterial species diversity occurred in Belgian soil with a H'_{B-BT} equal to 1.13. Furthermore, the bacterial community of the native soil B-BT showed a marked different pattern when compared with its treated B-AT counterpart. Indeed, the S index of similarity was equal to 0.18. Only few bands ("a" and "b" in Fig. 1) were in common between the two soils, indicating the survival of some predominant species.

On the contrary, for Italian soils only negligible differences in DNA patterns ($S = 0.56$) were evidenced between the native I-BT and its treated I-AT counterpart ($H'_{I-AT} = 2.14$), indicating that the bioremediation did not substantially change the community structure of the native one.

3.3. Enrichment of phenanthrene-degrading mixed cultures and determination of degradation kinetics

The diversity encountered in the bacterial communities of the study soils prompted us to perform enrichments on phenanthrene from all soil samples in order to obtain cultures with different potential strategies to degrade phenanthrene.

Attempts to enrich phenanthrene degrading bacteria from the German soil were unsuccessful (Saccamundi

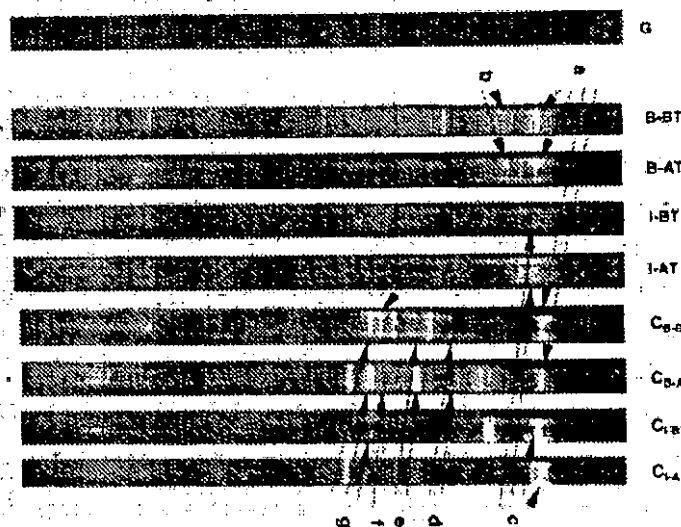


Fig. 1. DGGE analysis of PCR-amplified 16S rDNA gene-V3 fragments from soil samples and from enrichment cultures after six transplants on fresh phenanthrene. Bands were designated as described in the text. G, German soil; B-BT, Belgian soil before treatment; B-AT, Belgian soil after treatment; I-BT, Italian soil before treatment; I-AT, Italian soil after treatment; C_{B-BT} , C_{B-AT} , C_{I-BT} , C_{I-AT} , enrichment cultures from the corresponding soil samples.

and Gianfreda, 2001). The presence of highly bound residues in the old-contaminated German soil could have represented a constraint in phenanthrene bioavailability to bacteria thus impairing the possibility to isolate degrading microorganisms.

Four mixed bacterial cultures, named C_{B-BT} and C_{B-AT} and C_{I-BT} and C_{I-AT} were instead selected from the Belgian and Italian soils before and after the biological treatment, respectively.

All cultures enriched from Belgian and Italian soils grew on phenanthrene when added as sole C and energy source and turbidity of culture broths increased during incubation.

Fig. 2 shows the disappearance of 200 mg l^{-1} crystalline phenanthrene and the corresponding protein contents within 21-d incubation of the selected cultures. A time course analysis of phenanthrene may provide an estimate of first order uptake/degradation rate constant according to the following expression: $X_t = X_0 e^{-kt}$, where X_t is the concentration of phenanthrene in mg l^{-1} , k is the uptake/degradation constant and t is the time.

When phenanthrene degradation data of Fig. 2 were reported in a semilog plot, a one-slope behaviour was observed for C_{B-BT} and C_{I-AT} cultures, while a typical two-slope occurred for C_{B-AT} and C_{I-BT} , suggesting a more complex kinetics of phenanthrene degradation by these cultures (data not shown). This could imply that for culture C_{B-BT} and C_{I-AT} the whole phenanthrene degradation process is dominated by a single, straightforward, key step, whereas for cultures C_{B-AT} and

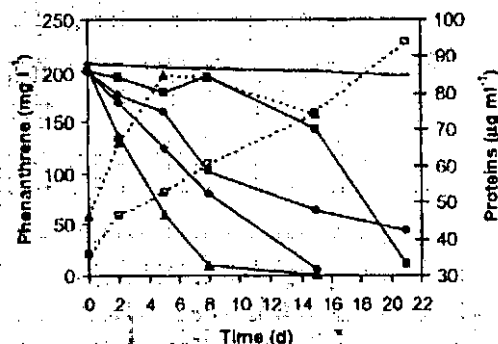


Fig. 2. Phenanthrene disappearance (solid lines, full symbols) by bacterial cultures C_{B-AT} (■), C_{I-AT} (●), C_{B-BT} (▲), C_{I-BT} (◆), free-cell control (—○), and bacterial growth (dotted lines, empty symbols) in C_{B-BT} and C_{B-AT} samples as determined by the protein content. Each value is the mean of two determinations.

C_{I-BT} a complex mechanism, involving a slower intermediate step, occurred.

Table 4 reports the degradation constants calculated by means of a non-linear regression routine applied to phenanthrene degradation data of Fig. 2. The first step-kinetics occurring for C_{B-AT} and C_{I-AT} , characterized by low degradation constants, could suggest a slower utilisation of phenanthrene within the first 8 days. In particular, the very low k_1 value (0.020 d^{-1}) cal-

Table 4
Values of phenanthrene (200 mg l^{-1}) disappearance constants calculated for the cultures enriched from the study soils

Culture	$k_1 (\text{d}^{-1})$	$k_2 (\text{d}^{-1})$	R^2
C_{B-BT}	0.369	—	0.95
C_{B-AT}	0.020	0.297	0.99
C_{I-BT}	0.113	0.510	0.99
C_{I-AT}	0.076	—	0.98

k_1 and k_2 calculated by a non-linear regression routine according the equation $X_t = X_0 \exp(-kt)$ where X_t is the concentration of phenanthrene in mg l^{-1} , k is the uptake or transformation constant and t is time.

culated for C_{B-AT} could indicate the presence of a slow phenanthrene mass transfer resulting in a hampered PAH utilisation. By contrast, the mixed culture C_{B-BT} almost completely utilized 200 mg l^{-1} phenanthrene (more than 90%) within 10 days. Longer times were required for complete degradation by C_{I-AT} and C_{B-AT} (Fig. 2 and Table 4). All cultures degraded phenanthrene without the appearance of any metabolites in culture broths.

The protein content patterns of culture broths confirmed the ability of strains to utilise phenanthrene as the sole C source. The profiles of protein contents vs. phenanthrene disappearance of cultures C_{I-AT} and C_{I-BT} were the same as C_{B-BT} (data not shown).

C_{B-AT} protein content seems to confirm that the consumption rate by this culture was limited by dissolution dynamics. Indeed, the growth rate of C_{B-AT} , evaluated as protein content ($2.33 \text{ } \mu\text{g ml}^{-1} \text{ d}^{-1}$) in the exponential (0–21 d) growth phase was lower than that measured for C_{B-BT} ($7.78 \text{ } \mu\text{g ml}^{-1} \text{ d}^{-1}$) in the exponential (0–5 d) growth phase. The different behaviour of C_{B-BT} compared to C_{B-AT} , enriched from the same soil after the biotreatment, could be referred to a different species composition of the cultures (Fig. 1). The former contained probably bacteria with different PAH-degrading strategies or with different cell surface properties. A bacterial adhesion to solid phenanthrene and subsequent solubilisation at the level of the cell wall could be hypothesised. Similar mechanisms have been suggested for degradation of solid hydrophobic chemicals (palmitic acid) by *Pseudomonas* strains (Thomas and Alexander, 1987).

Culture C_{I-BT} , obtained from the Italian agricultural soil I-BT, was for the first 8 days metabolically less active than culture C_{B-BT} obtained from the Belgian contaminated soil B-BT. A faster degradation occurred, however, in the last incubation period (k_2 value for C_{I-BT} higher than k_1 value for C_{B-BT} , Table 4). A different phenanthrene-degrading culture was selected from the Italian biotreated soil I-AT and its degradation rate was slower than that of I-BT (Fig. 2 and Table 4).

3.4. Biodiversity of enrichment cultures

The DGGE profiles of the mixed cultures analysed after six 21-d-incubation transplants on phenanthrene, when cultures were supposed to be stable and used also for degradation kinetic experiments, are shown in Fig. 1. DGGE profiles of enrichment cultures were less complex than soil profiles, due to the selective pressure represented by the presence of fresh phenanthrene.

All the cultures showed DGGE profiles that indicated a different bacterial species composition, as evidenced by the presence of peculiar bands in each culture (Fig. 1). Sørensen similarity values calculated from DGGE profiles revealed that there were significant differences in species composition of cultures from each native and treated soil ($S = 0.33$ for C_{B-BT} vs. C_{B-AT} and 0.25 for C_{I-BT} vs. C_{I-AT}). Some bands were in common among enrichment cultures, indicating the presence of similar bacterial species, such as band "g" in C_{B-BT} , C_{B-AT} and C_{I-BT} . Other bands were visible in the enrichment-culture DNA profiles and in the corresponding soil samples (band "a" in C_{B-BT} and C_{B-AT} , in B-BT and B-AT, and band "c" in C_{I-BT} and C_{I-AT} , in I-BT and I-AT). All these bands belong to species that could be relevant in situ phenanthrene degraders and that have been enriched during the transplant procedure. Four bands ("d", "e", "f" and "g") were in common among DNA profiles of C_{B-BT} and C_{B-AT} , thus confirming their presence in the native and treated Belgian soils (Fig. 1).

The differences encountered in the DGGE profiles could reflect the different degradative kinetics of the four cultures. The presence of different species could assure a probable existence of different mechanisms for efficient assimilation/uptake of soluble or solid phenanthrene.

Colonies with different morphologies were isolated from the fastest degrading culture C_{B-BT} after growth on 0.1% tryptic soy broth agar plates. Representative strains of C_{B-BT} , identified on the basis of 1200 nucleotides sequence homologies with entries in GenBank-EMBL databases, belong to: *Achromobacter xylosoxidans* (100%), *Methylobacterium* sp. (99%), *Alcaligenes* sp. (99%), *Rhizobium galegae* (99%), *R. aetherovorus* (100%), *Aquaspirillum defluatum* (100%) and *Stenotrophomonas acidaminiphila* (100%). When these strains were checked for the capability of growing on 100 mg l^{-1} crystalline phenanthrene as sole C source, the growing strains had different growth behaviour. While *R. aetherovorus* produced a diffuse turbidity of culture broths (data not shown), *Methylobacterium* sp. grew in contact with the phenanthrene crystals, as revealed by microscopic examination. This implies that the low solubility of phenanthrene was limiting the growth, and the few cells freely present in the culture broth were probably those sloughed off from the crystal surfaces.

The presence of strains within the culture C_{B-BT} during the time course of phenanthrene degradation was

followed by DGGE analysis. During the degradation process, no change was evidenced in the bacterial components of C_{B-BT} (Fig. 3) but some bands increased their relative intensity.

C_{B-BT} bands were correlated to the isolated strain bands (Fig. 3) on the basis of their electrophoretic mobility. Theoretically, bands at the same position in the electrophoresis pattern contain DNA fragments with identical sequences. Band "h" had the same electrophoretic mobility of *R. aetherovorans*, band "l" the same of *R. galegae* and *Aquamicrobium defluvium*, band "m" the same of *Methylobacterium* sp., band "n" the same of *Alcaligenes* sp. and of one of the two bands of *A. xyloxydans* and band "p" the same of the other band of *A. xyloxydans*. Bands corresponding to *R. aetherovorans* and *A. xyloxydans* increased their relative intensity during phenanthrene degradation suggesting that these strains represent active members of the culture and are likely involved directly or indirectly in the utilization of phenanthrene as C and energy sources.

The overlapping of amplified PCR products cannot confirm that sequences of these isolates are identical to the sequences of corresponding DGGE enrichment culture bands.

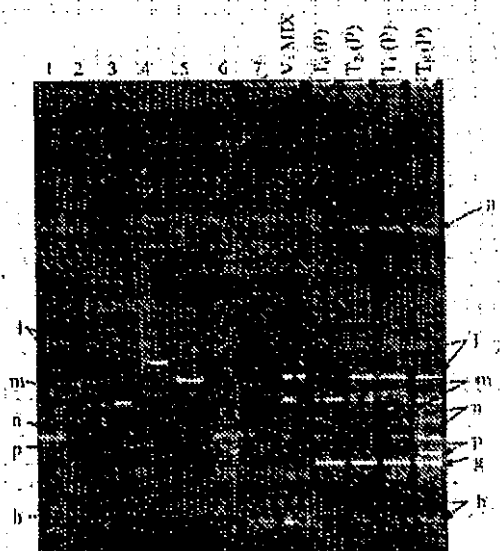


Fig. 3. DGGE analysis of V3 fragments obtained from uncharacterized bacterial culture C_{B-BT} and bacterial isolates from the culture. Lanes $T_0(P)$ to $T_{16}(P)$ show the profiles obtained from C_{B-BT} after 0, 2, 4 and 8 day growth in presence of phenanthrene; lane V3 MIX contains the separation pattern of a mixture of fragments of seven isolates, i.e., *Alcaligenes* sp. (lane 1); *Rhizobium galegae* (lane 2); *Methylobacterium* sp. (lane 3); *Simotrophomonas acidaminiphila* (lane 4); *Aquamicrobium defluvium* (lane 5); *Achromobacter xyloxydans* (lane 6) and *R. aetherovorans* (lane 7).

Band corresponding to *St. acidaminiphila* has never been retrieved in culture C_{B-BT} DGGE profiles. This could be due either to its low cell number in the culture or to the DNA applied extraction method. Conversely, species corresponding to bands "a" and "g" in the DGGE profiles of culture C_{B-BT} were not recovered among isolates, and their sequence types were identified as *Pseudomonas* and *Arthrobacter*, respectively. The amplification of these bands may be due to biases in selective PCR amplification (Heuer and Smalla, 1997). The bands corresponding to *P. putida* and *Ralstonia* sp. have approximately the same relative intensity during incubation time, suggesting that these species do not increase during phenanthrene degradation.

4. Conclusions

The results, here presented, all indicate that soils highly contaminated by hydrocarbons displayed different microbiological properties. In particular the higher/lower the pollutant content, the smaller/the greater are the activities of some enzymes related to nutrient cycling and the viable bacterial cell numbers. The different microbiological properties of the soils probably reflect the different bacterial diversity as assessed by DGGE profiles of the 16S rDNA genes.

Phenanthrene-degrading mixed cultures were enriched from all soils except the old heavily contaminated German soil. When tested in liquid batch systems using solid phenanthrene as C and energy source, cultures showed different kinetic behaviours, probably because of a different species composition, as evidenced by DGGE 16S rDNA profiles. The presence of different species could indicate a probable existence of different mechanisms for efficient assimilation/uptake of soluble or solid phenanthrene, as observed for C_{B-BT} culture that contained more than one phenanthrene-degrading bacterium. The simultaneous presence in the culture of *Rhodococcus* and *Methylobacterium* strains might be explained with the capability to use phenanthrene under different conditions such as dissolved, solid associated, and perhaps surfactant-associated, according to different substrate-degrading strategies. C_{B-BT} culture also contained bacteria that do not use phenanthrene, suggesting that the phenanthrene-degraders themselves may be associated with bacteria using metabolites of phenanthrene. The presence of some DGGE bands with the same electrophoretic mobility and the presence of degrading strains belonging to the same species in all the enrichments are indicative of their degradative role in the cultures. The isolation of bacteria from B-BT soil, that are able to grow on phenanthrene, is consistent with the observed decrease of PAH and phenanthrene contents of soil after the biotreatment and suggests that aerobic phenanthrene biodegradation was occurred. The finding

that a number of bacteria identified in culture C_{HAT} degrade phenanthrene supports this assumption. The isolation of *R. aerivorans* and *Methylobacterium* sp. can be consistent with the hypothesis that different phenanthrene-degraders inhabiting soils and enrichment cultures may be adapted to different phenanthrene bioavailabilities. The use of these species in microcosm bioaugmentation trials could help in evaluating their in situ catabolic behaviour to degrade phenanthrene in highly polluted soils.

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Polyamine distribution profiles in newly validated genera and species within the *Flavobacterium*-*Flexibacter*-*Cytophaga*-*Sphingobacterium* complex

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Key words: polyamine, spermidine, homospermidine, *Flavobacterium*, *Flexibacter*, *Cytophaga*, *Sphingobacterium*

Abstract

Cellular polyamines of 58 strains belonging to the *Flavobacterium*-*Flexibacter*-*Cytophaga*-*Sphingobacterium* complex were analysed by HPLC. Homospermidine was found in all species of *Flavobacterium*, *Chryseobacterium*, *Empedobacter*, *Myroides*, *Cellulophaga*, *Saiegentibacter*, *Psychroserpens* and *Galidibacter* of the family Flavobacteriaceae. *Flavobacterium ferrugineum* located outside of this family also contained homospermidine. *Cytophaga fermentans* and *C. xylanolytica* belonging to the family Bacteroidaceae contained spermidine. *Cytophaga mariniflava* and *C. latercula* belonging to Flavobacteriaceae contained homospermidine. The *Cytophaga hutchinsonii*/*C. aurantiaca* group contained homospermidine which was the major polyamine in *Flexibacter maritimus*/*F. ovolyticus* of the family Flavobacteriaceae. The *Flexibacter sancti*/*F. filliformis*/*Cytophaga arvensicola* group, *F. elegans*, *F. ruber*, *F. canadensis*, *F. flexilis* and *F. tractuosus*, were located separately in different six clusters, and contained homospermidine. The *Flexibacter littoralis*/*F. polymorphus*/*F. aggregans* group contained spermidine, which was detected in *Flexibacter roseolus* belonging to a divergent cluster. *Sphingobacterium* and *Pedobacter* species of the family Sphingobacteriaceae contained homospermidine. Polyamine profiles serve, as a phenotypic chemotaxonomic marker, for the classification of this complex.

Introduction

The *Cytophaga*-*flavobacter*-*bacteroides* (CFB) phylum tentatively comprise five subgroups, *Bacteroides* (family Bacteroidaceae), *Flavobacter* (Flavobacteriaceae), *Cytophaga*, *Sphingobacter* (Sphingobacteriaceae), and *Saprospira* (Saprospiraceae). The latter four subgroups are included in the *Flavobacterium*-*Flexibacter*-*Cytophaga*-*Sphingobacterium* complex (Paster *et al.*, 1994; Weller *et al.*, 2000). In this complex, seven new genera *Chryseobacterium*, *Persicobacter*, *Empedobacter*, *Flammeovirga*, *Myroides*, *Pedobacter* and *Cellulophaga* have been created (Bowman, 2000; Johansen *et al.*, 1999; Nakagawa and Yamasato, 1996; Nakagawa *et al.*, 1997; Steyn *et al.*, 1998; Vancanneyt *et al.*, 1996; Vandamme *et al.*, 1994). New *Marinilabilia* species were formerly classified as *Cytophaga* species, belong to the *Bacteroides* subgroup (Nakagawa and Yamasato, 1996). *Cytophaga* species are widespread in the *Bacteroides*, *Cytophaga* and *Flavobacter* subgroups. *Flexibacter* species are extensively distributed among the *Flavobacter*, *Cytophaga* and *Saprospira* subgroups.

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Cellular polyamines of the members of these new eight genera and the original four genera *Flavobacterium*, *Flexibacter*, *Cytophaga* and *Sphingobacterium* have been analysed (Hamana and Matsuzaki, 1990, 1991; Hamana *et al.*, 1995). On the other hand, we have reported the polyamine patterns of another eighteen genera *Bergeyella*, *Capnocytophaga*, *Chitinophaga*, *Cyclobacterium*, *Flectobacillus*, *Flexithrix*, *Haliscomenobacter*, *Lewinella*, *Microscilla*, *Ornithobacterium*, *Polaribacter*, *Psychroflexus*, *Riemerella*, *Runella*, *Saprospira*, *Spirosoma*, *Sporocytophaga* and *Weeksella*, validated as members of this complex (Hamana and Nakagawa, 2001). Distribution of the diamines, putrescine, cadaverine and agmatine, and the triamines, spermidine and homospermidine, serve for chemotaxonomy as phenotypic markers within this complex (Hamana and Matsuzaki, 1990, 1991; Hamana *et al.*, 1995; Hamana and Nakagawa, 2001; Nakagawa and Yamasato, 1996; Nakagawa *et al.*, 1997).

The isolation of new species, reclassification among the eight new genera and the original four genera, have been continued (Bernardet *et al.*, 1996; Fujita *et al.*, 1996; Haack and Breznak, 1993; McCammon and Bowman, 2000; McCammon *et al.*, 1998; Mudarris *et al.*, 1994; Shivaji *et al.*, 1992; Vancanneyt *et al.*, 1996). Therefore, polyamine profiles of 58 strains (34 species) belonging to the twelve genera and the recently published three genera, *Salegentibacter* (McCammon and Bowman, 2000), *Psychroserpens* (Bowman *et al.*, 1997) and *Gelidibacter* (Bowman *et al.*, 1997) of this complex, were analysed anew for chemotaxonomic evaluation of their polyamine profiles.

Materials and methods

The organisms were cultured aerobically or anaerobically at their optimum growth temperatures (10–30°C), in nutrient broth (NB; Nissui Pharmaceutical Co., Tokyo, Japan), brain heart infusion broth (BHIB; Nissui Pharmaceutical Co.), trypticase soy broth (TSB; BBL, Becton Dickinson, Cockeysville, U.S.A.), synthetic 199 medium (polyamine-free; Nissui Pharmaceutical Co.), 199 medium dissolved in 70% sea water (199SW) and marine broth (MB) (Difco Laboratory, Detroit, U.S.A.). The media designated IFO, JCM, NCIMB and ATCC were found in the catalogues for culture strains. Culture media are shown in Table 1.

Stationary phase cells were harvested by centrifugation. The pellets of the cells were homogenized in equal volumes of cold 1.0 M perchloric acid (PCA). Polyamines extracted into 0.5 M PCA were analysed by high performance liquid chromatography (HPLC) on an

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L6000 high speed liquid chromatograph (Hitachi Co., Tokyo, Japan), using cation-exchange resin (Hamana *et al.*, 1995). Acid hydrolysis of cells with 6 M HCl, at 110°C, for 24 h, was performed to detect polyamines covalently linked in cells. The PCA extract was hydrolysed with 1 M NaOH, at 110°C for 20 h, to destroy guanidinoamines.

Results and discussion

Cellular concentrations of the polyamines detected in the newly analysed 58 strains (34 species) within the previously validated twelve genera and three new genera (total 15 genera) are shown in Table 1, in comparison with the other species previously reported. Spermine was incorporated from the media containing spermine, except for polyamine-free 199, 199SW and K101 media. Distribution of major polyamines obtained in our previous (Hamana and Nakagawa, 2001) and present studies are shown on a phylogenetic tree of the CFB phylum (Figure 1).

Bacteroides subgroup (family Bacteroidaceae)

Two *Cytophaga* species *C. fermentans* and *C. xylanolytica*, were phylogenetically located in the *Bacteroides* subgroup (Paster *et al.*, 1994). We have reported the occurrence of spermidine in *C. fermentans* as the major polyamine (Hamana *et al.*, 1995). In this work, *C. xylanolytica* contained spermidine as the major polyamine (Table 1). Other *Cytophaga* species belonging to other subgroups ubiquitously contained homospermidine as the major polyamine (Figure 1). Indeed, the occurrence of spermidine in *Marinilabilia* species has been used for the reclassification of *Cytophaga agarovorans* and *C. salmonicolor* into the new genus *Marinilabilia* of this subgroup (Nakagawa and Yamasato, 1996).

Genus *Flexibacter*

Flexibacter aurantiacus which was transferred into the genus *Flavobacterium* as *F. aurantiacus* (Bernardet *et al.*, 1996) and the *Flexibacter maritimus*/*F. ovolyticus* group belonging to the family Flavobacteriaceae, contained homospermidine. *Flexibacter aggregans* and *F. tractuosus* species were heterogeneous in their polyamine profiles (Table 1). The type strain of *F. tractuosus* contained homospermidine. The type strain of *F. aggregans* and the *F. litoralis*/*F. polymorphus* group contained spermidine and was located in the same cluster (Figure 1). *Flexibacter roseolus* contained cadaverine and spermidine as a unique polyamine profile, but this *Flexibacter* species was located in the most divergent cluster within this complex. Other *Flexibacter* species, *F. canadensis*, *F. elegans*, *F. filiformis*, *F. sancti*, *F. ruber* and *F. flexilis*, were distributed from the three families (subgroups) Bacteroidaceae, Flavobacteriaceae

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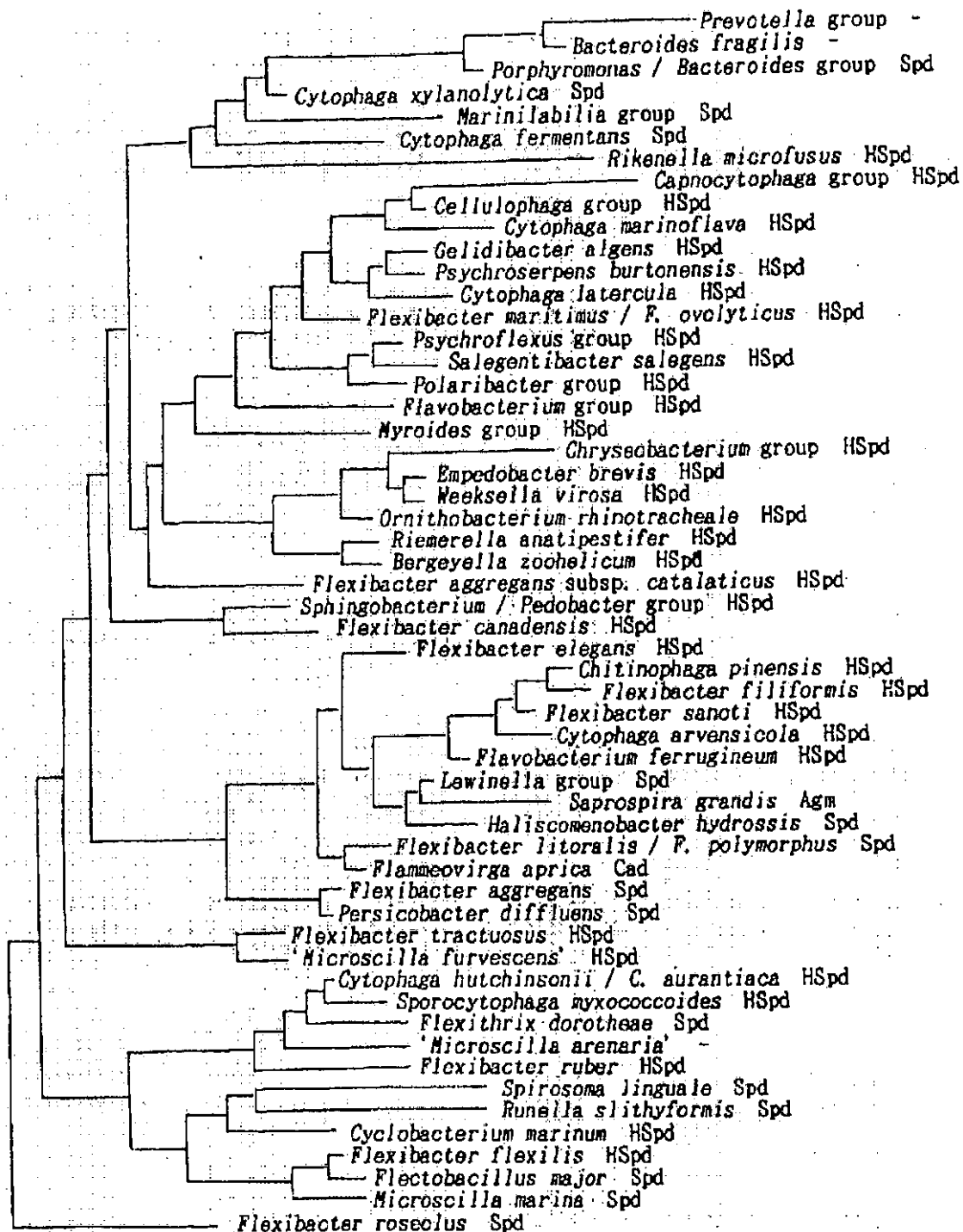


Figure 1 Polyamine types on the phylogenetic tree for CFB phylum. The tree is based upon 16S rRNA sequence comparisons by Sly et al. (1999), Bowman (2000), McCammon and Bowman (2000) and Weller et al. (2000) which were roughly combined. -, Cellular polyamines were not detected. For abbreviations of polyamines see Table 1.

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and Sphingobacteriaceae, and contained homospermidine. The phylogenetic position of *F. japonensis* containing homospermidine is not clear.

Genus *Flavobacterium* and *Flavobacter* subgroup (family Flavobacteriaceae)

The newly analysed *Flavobacterium* species, *F. allerginae* (Bernardet *et al.*, 1996) and *F. hibernum* (McCammon *et al.*, 1998) and the reclassified species from the genera *Cytophaga* and *Flexibacter*, contained homospermidine as the major polyamine (Table 1). *Cytophaga marinoflava*, *C. latercula*, *Flexibacter maritimus* and *F. ovolytica* belonging to this subgroup also contained homospermidine (Table 1 and Figure 1). Two *Myroides* species transferred from *Flavobacterium* and the new *Gelidibacter*, *Psychroserpens* and *Salegentibacter* species, analysed in the present report, and the new genus *Cellulophaga* comprising some species which were transferred from *Cytophaga* (Johansen *et al.*, 1999; Bowman, 2000), contained homospermidine (Table 1).

Homospermidine was the major polyamine in *Chryseobacterium* species including *C. indoltheticum* and *C. scophthalmum* (Mudarris *et al.*, 1994), *Empedobacter* species (Table 1) and another seven genera, *Capnocytophaga*, *Polaribacter*, *Psychroflexus*, *Weeksella*, *Ornithobacterium*, *Riemerella* and *Bergeyella* of this subgroup, as reported previously (Hamana and Nakagawa, 2001; Figure 1). *F. resinovorum* which was reported by Delaporte and Daste (1956) but phylogenetically has not been validated, contained spermidine, suggesting that this organism does not belong in this subgroup. Thus, homospermidine was the major polyamine in the flavobacteria belonging to the 17 genera (except for *Cytophaga* and *Flexibacter*) located in the *Flavobacter* subgroup, at the present time (Figure 1).

F. ferrugineum phylogenetically located in a different cluster divergent from this subgroup, also contained homospermidine (Figure 1). Within nonvalidated flavobacteria listed in ATCC and/or NCIMB catalogues, '*Flavobacterium buchnei*' and '*F. sulfureum*' contained homospermidine, whereas '*F. marinoviriosum*' and '*F. tirrenicum*' contained spermidine (Table 1).

***Sphingobacter* subgroup (family Sphingobacteriaceae)**

The genera *Sphingobacterium* and *Pedobacter* consists of a cluster, and comprise the Sphingobacteriaceae (Figure 1) according to Steyn *et al.* (1998). A species of *Sphingobacterium*, *S. antarcticus* (Shivaji *et al.*, 1992), analysed in the present work, as well as another five *Sphingobacterium* species and two *Pedobacter* species, contained homospermidine as the major polyamine (Table 1). *Flexibacter canadensis* which is phylogenetically close to this subgroup also contained homospermidine (Figure 1).

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Table 1 Cellular concentrations of polyamines in *Flavobacterium*-*Flexibacter*-*Cytophaga*-*Sphingobacterium* complex

Organism	Strain number		Polyamines ($\mu\text{mol/g}$ wet weight):					
			Medium	Put	Cad	Spd	HSpd	Spm Agm
<i>Cellulophaga lytica</i> (<i>Cytophaga lytica</i>)	ATCC 23178 ^T	(c)	199	-	-	-	1.20	-
	DSM 2039 ^T		MB	0.08	-	-	2.30	0.07 0.19
	DSM 2040 ^T		MB	0.03	-	-	2.40	0.04 0.15
	ATCC 23167		IFO 333	0.88	-	-	2.10	- 0.05
	ATCC 23174		199SW	0.05	-	-	2.15	- 0.16
			MB	-	-	-	0.70	0.05 -
	ATCC 23176		199SW	0.15	-	-	2.55	- 0.35
<i>uliginosa</i> (<i>Cytophaga uliginosa</i>)			MB	-	-	-	0.50	0.10 -
	IAM 14312 ^T	(c)	199SW	-	-	-	1.10	- -
<i>Chryseobacterium balustinum</i> (<i>Flavobacterium balustinum</i>) <i>gleum</i> (<i>F. gleum</i>) <i>indologenes</i> (<i>F. indologenes</i>) <i>indoltheticum</i> (<i>F. indoltheticum</i>) <i>meningosepticum</i> (<i>F. meningosepticum</i>) <i>scophthalmum</i> (<i>F. scophthalmum</i>)	JCM 1278 ^T	(a)	199	-	-	-	1.08	- 0.02
	JCM 2410 ^T	(a)	199	-	-	-	2.40	- -
	ATCC 29897 ^T	(b)	199	0.01	-	-	5.90	- 0.03
	NCIMB 2220 ^T		NB	-	-	-	1.29	- -
	IFO 12535 ^T	(a)	199	0.01	-	-	1.92	- 0.01
	NCIMB 13483 ^T		TSB	-	-	-	1.60	- 0.02
<i>Cytophaga arvensicola</i> <i>aurantiaca</i> <i>fermentans</i> <i>hutchinsonii</i> <i>latercula</i> <i>marinoflava</i> <i>xylanolytica</i>	IAM 12550 ^T	(c)	199	0.02	-	-	1.90	- -
	IAM 12553		IAM-B55	-	-	-	1.05	- -
	IAM 14300 ^T	(c)	199SW	0.01	-	-	1.20	- -
	IAM 14302 ^T	(c)	199SW	0.08	-	2.05	-	- -
	IAM 12607 ^T	(c)	199	0.01	-	-	5.32	- -
	NCIMB 10782		IFO 276	-	-	-	1.80	- -
	ATCC 23177 ^T		199SW	0.20	0.20	-	2.60	- -
	IFO 14170 ^T		199	0.01	-	-	1.02	- 0.01
	NCIMB 397 ^T		199SW	-	-	-	2.50	- -
	ATCC 51429 ^T		ATCC1937	-	-	1.07	-	- -
<i>Empedobacter brevis</i> (<i>Flavobacterium breve</i>)	JCM 6812 ^T	(a)	199	0.02	-	-	2.40	- -
<i>Flammeovirga aprica</i> (<i>Cytophaga aprica</i>)	ATCC 32126 ^T	(c)	199	-	2.00	-	-	- -
<i>Flavobacterium aquatile</i> <i>allerginae</i> (<i>Cytophaga allerginae</i>) <i>aurantiacus</i> (<i>Flexibacter aurantiacus</i>) <i>aurantiacus</i> subsp. <i>excathedrus</i> (<i>F. aurantiacus</i> subsp. <i>excathedrus</i>) <i>brachiophilum</i> (<i>brachiophila</i>) <i>'buchnei'</i> <i>columnare</i>	IAM 12316 ^T	(a)	199	-	-	-	1.24	- 0.01
	ATCC 35408 ^T		NB	-	-	-	1.35	- 0.04
	ATCC 23107 ^T	(c)	199	-	-	-	0.85	- -
	ATCC 23086		IFO 272	-	0.16	-	1.65	- -
	IFO 15030 ^T	(c)	CB	-	-	0.03	0.52	0.01 -
	NCIMB 1786		NB	-	-	-	0.90	- -
	IAM 14301 ^T	(c)	199	0.02	-	-	5.00	- -

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Table 1 Continued

Organism	Strain number	Polyamines ($\mu\text{mol/g}$ wet weight):					
		Medium	Put	Cad	Spd	HSpd	Spm Agm
(<i>Cytophaga columnaris</i>)							
(<i>Flexibacter columnaris</i>)							
<i>ferrugineum</i>	IFO 14992 ^T	(c) 199	0.01	-	-	0.92	-
<i>flavense</i>	IFO 14960 ^T	(c) 199	0.01	-	-	0.66	-
(<i>Cytophaga flavensis</i>)	IAM 12375 ^T	(c) 199	0.01	-	-	1.30	-
<i>hibernum</i>	ATCC 51468 ^T	ATCC1913	-	-	-	1.20	-
<i>hydatis</i>	IAM 12365 ^T	(c) 199	0.01	-	-	3.68	-
(<i>Cytophaga aquatilis</i>)							
<i>johnsonae</i>	IAM 14304 ^T	IAM-B56	-	-	-	0.80	-
(<i>Cytophaga johnsonae</i>)	JCM 7449	NB	-	-	-	0.92	-
	JCM 7450	NB	-	-	-	0.68	-
	JCM 7451	NB	-	-	-	0.70	-
	JCM 8514 ^T	NB	-	-	-	0.66	-
' <i>merlinovrosium</i> '	NCIMB 563	BHIB	-	-	0.70	-	0.03 0.02
<i>pectinovorum</i>	IAM 14307 ^T	(c) 199	0.40	-	-	2.10	-
(<i>Cytophaga pectivora</i>)							
<i>psychrophilum</i>	IAM 14308 ^T	(c) 199SW	0.05	-	-	2.10	-
(<i>Cytophaga psychrophila</i> NCIMB 1947)							
(<i>Flexibacter psychrophilus</i> ATCC 49418)							
<i>saccharophilum</i>	IAM 14309 ^T	(c) 199SW	-	-	-	0.80	-
(<i>Cytophaga saccharophila</i>)							
<i>resinovorum</i>	NCIMB 8787 ^T	NB	-	-	0.66	-	-
<i>succinicans</i>	IFO 14805 ^T	(c) 199	0.01	-	-	2.50	-
' <i>sulfureum</i> '	JCM 7462	(c) 199	0.01	-	-	1.68	-
' <i>tirrenicum</i> '	NCIMB 1766	BHIB	-	0.02	1.05	-	0.10 0.04
	ATCC 15997	(b) 199	0.02	-	0.74	-	- 0.08
<i>xanthum</i>	IAM 12026 ^T	(b) 199	0.12	-	0.16	1.70	- 0.75
(<i>Cytophaga xantha</i>)							
<i>Flexibacter aggregans</i>	NCIMB 1389	MB	-	-	1.10	-	- 0.15
	NCIMB 1391	199	-	-	-	1.42	- 0.17
		MB	-	-	-	1.40	- 0.19
	NCIMB 1432	199	-	-	-	1.10	- 0.38
		MB	-	-	-	1.33	- 0.10
	NCIMB 1443 ^T	MB	-	-	1.25	-	-
	NCIMB 1464	199	-	-	0.77	-	- 0.50
		MB	-	-	1.73	-	0.05 0.40
' <i>aggregans</i> subsp. <i>cataliticus</i> '	NCIMB 1418	199	-	-	-	1.00	-
(<i>Microscilla aggregans</i> subsp. <i>catalitica</i> ATCC 23190)	MB		-	-	-	1.45	-
<i>aurantiacus</i> subsp. <i>copepodarum</i>							
	NCIMB 1394	IFO 333	-	1.45	-	-	-
<i>canadensis</i>	ATCC 29591 ^T	(c) 199	0.01	-	-	1.03	-
<i>elegans</i>	IFO 15055 ^T	(c) 199	0.01	-	-	0.80	-
<i>filiformis</i>	ATCC 29495 ^T	(c) 199	-	-	-	0.89	-
<i>flexilis</i>	ATCC 23079 ^T	(c) K101	0.01	-	-	1.14	-
	ATCC 23089	IFO 272	-	-	-	1.45	-
	ATCC 23096	IFO 272	-	-	-	1.45	-
' <i>flexilis</i> subsp. <i>pelliculosus</i> '	ATCC 23098	IFO 272	-	-	-	1.50	-
<i>japonensis</i>	JCM 9735 ^T	199	-	-	-	1.90	-
<i>litoralis</i>	ATCC 23117 ^T	IFO 333	-	-	1.85	-	- 0.45
<i>maritimus</i>	NCIMB 2154 ^T	MB	-	-	0.03	0.80	- 0.27
(<i>Flavobacterium maritimus</i>)	IAM 14317 ^T	(c) 199	0.01	0.38	-	0.40	-
	NCIMB 2158	MB	-	-	0.02	0.70	- 0.51

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Table 1 Continued

Organism	Strain number	Medium	Polyamines ($\mu\text{mol/g}$ wet weight):					
			Put	Cad	Spd	HSpd	Spm	Agm
<i>(Cytophaga marina)</i> <i>ovolyticus</i> <i>polymorphus</i> <i>(polymorpha)</i> <i>roseolus</i> <i>ruber</i> <i>sancti</i> <i>tractuosus</i> <i>(Microscilla tractuosa)</i>	NCIMB 2153	(c) 199SW	-	0.40	-	0.50	-	-
	IAM 14318 ^T	MB	-	-	-	0.50	-	0.08
	ATCC 27820 ^T	ATCC 754	-	-	1.03	-	0.05	-
	ATCC 23088 ^T	IFO 341	-	1.45	2.75	-	-	-
	ATCC 23087	IFO 332	-	2.30	0.75	-	-	-
	NCIMB 1436 ^T	NCIMB 218	0.01	-	-	1.45	-	-
	ATCC 23103 ^T	(c) K101	-	-	-	0.35	-	-
	ATCC 23092 ^T	(c) 199	-	-	-	0.60	-	-
	ATCC 23090	IFO 272	-	-	-	1.12	-	-
	ATCC 23097	IFO 272	-	-	-	2.00	-	-
NCIMB 1393	199	-	-	-	-	1.80	-	0.10
	MB	-	-	-	-	1.80	-	0.04
	NCIMB 1416	199	-	0.02	-	1.72	-	0.02
	MB	-	-	-	-	1.46	-	0.07
	NCIMB 1429	MB	-	-	-	1.15	-	0.13
	ATCC 23168 ^T	IFO 333	-	-	-	2.15	-	0.65
	ATCC 23145	MB	-	-	0.82	-	0.02	-
	ATCC 23151	MB	-	-	-	0.75	0.01	-
	ATCC 23152	199SW	-	-	0.55	0.02	-	-
	MB	-	-	-	0.47	0.05	0.01	-
ATCC 23191	MB	-	-	-	0.70	-	-	-
	ATCC 700364 ^T	MB	-	-	-	0.74	-	0.02
<i>Gelidibacter algens</i> <i>Marinilabilia agarovorans</i> <i>(Cytophaga agarovorans)</i> <i>salmonicolor</i> <i>(Cytophaga salmonicolor)</i>	IFO 14957 ^T	(c) 199	0.01	-	0.70	-	-	-
	NCIMB 2217 ^T	(c) 199SW	0.01	-	0.50	-	-	-
	NCIMB 2216 ^T	(c) 199SW	0.05	-	1.25	-	-	-
<i>Myroides odoratus</i> <i>(Flavobacterium odoratum)</i> <i>odoratimimus</i> <i>(Flavobacterium odoratum)</i>	IAM 14199	NB	-	-	-	0.96	-	-
	JCM 7458 ^T	NB	-	-	-	0.77	-	-
	JCM 7459	NB	-	-	-	1.10	-	-
	JCM 7460 ^T	NB	-	-	-	1.23	-	-
<i>Pedobacter heparinum</i> <i>(Sphingobacterium heparinum)</i> <i>piscium</i> <i>(Sphingobacterium piscium)</i>	IAM 12655 ^T	(c) 199	0.07	-	0.04	2.56	-	-
	IFO 14984	(c) 199	-	-	-	1.00	-	-
	IFO 14985 ^T	(c) 199	-	-	-	2.50	-	-
<i>Persicobacter diffluens</i> <i>(Cytophaga diffluens)</i> <i>Psychroserpens burtonensis</i>	NCIMB 1402 ^T	(c) 199SW	0.20	-	2.30	-	-	-
	NCIMB 1430	MB	-	-	1.20	-	-	-
ATCC 700359 ^T	MB	-	-	0.02	-	0.60	-	0.88
<i>Salegendibacter salegens</i> <i>(Flavobacterium salegens)</i>	NCIMB 13461 ^T	MB	-	-	-	0.80	-	0.07
<i>Sphingobacterium faecium</i> <i>multivorum</i> <i>spirillum</i>	IFO 15299 ^T	(c) 199	-	-	-	2.66	-	-
	IFO 13310	(c) 199	-	-	-	2.60	-	-
	IFO 14087	(c) 199	-	-	-	3.25	-	-
	IFO 14947 ^T	(c) 199	-	-	-	2.55	-	-
	IAM 1493	(c) 199	-	-	-	2.23	-	-
	IFO 14986	(c) 199	-	-	-	1.85	-	-
	IFO 14987	(c) 199	-	-	-	3.24	-	-
	IFO 14948 ^T	(c) 199	-	-	-	2.75	-	-
IFO 14975	(c) 199	-	-	-	-	1.36	-	-

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Table 1 Continued

Organism	Strain number	Medium	Polyamines (µmol/g wet weight):					
			Put	Cad	Spd	HSpd	Spm	Agm
<i>thalpophilum</i>	IFO 14963 ^T	(c) 199	-	-	-	1.36	-	-
<i>mizutae</i>	GIFU 1203 ^T	(b) 199	0.01	-	-	3.60	-	0.02
(<i>Flavobacterium mizutai</i>)								
<i>antarcticum</i>	ATCC 61969 ^T	NB	-	-	-	1.26	-	-
(<i>antarcticus</i>)								

Put, putrescine; Cad, cadaverine; Spd, spermidine; HSpd, homospermidine; Spm, spermine; Agm, agmatine; ^T, Type strain; Med, culture medium; -, not detected (<0.005); IFO, Institute of Fermentation, Osaka, Japan; IAM, IAM Culture Collection, Institute of Molecular and Cellular Biosciences, The University of Tokyo, Japan; JCM, Japan Collection of Micro-organisms, RIKEN (Institute of Chemical and Physical Research), Saltama, Japan; GIFU, Gifu University, School of Medicine, Gifu, Japan; ATCC, American Type Culture Collection, Manassas, Virginia, U.S.A.; NCIMB, The National Collections of Industrial, Food and Marine Bacteria, Aberdeen, Scotland, U.K.; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany. Former names are shown in parentheses. Quotation marks indicate that the scientific name has not been validated. a, Cited from Hamana and Matsuzaki (1990); b, Hamana and Matsuzaki (1991); c, Hamana *et al.* (1995).

Genus *Cytophaga* and *Cytophaga* subgroup

Two *Cytophaga* clades, one of *C. arvensicola* and another of *C. hutchinsonii*/*C. aurantiaca*, were phylogenetically located in the two divergent clusters (Figure 1). However, the type species of the genus *Cytophaga* is *C. hutchinsonii*, and so the definition of the *Cytophaga* subgroup is conflicting. These two *Cytophaga* groups, as well as *C. marinoflava* and *C. latercula* belonging to the *Flavobacter* subgroup, contained homospermidine as the major polyamine.

Saprospira subgroup (family Saprospiraceae)

A phylogenetic cluster represented by *Saprospira*, the *Saprospira* subgroup, was heterogeneous in their polyamine profiles. *Saprospira grandis* contained agmatine, *Lewinella* spermidine, *Chitinophaga* homospermidine, *Haliscomenobacter* spermidine, *Persicobacter* spermidine, *Flammeovirga* cadaverine, as previously described (Hamana *et al.*, 1995; Hamana and Nakagawa, 2001). *Flexibacter elegans*, *F. filiformis* and *F. sancti* belonging to a phylogenetic cluster of the subgroup contained homospermidine as the major polyamine, whereas *Flexibacter litoralis*, *F. polymorphus* and *F. aggregans* belonging to another phylogenetic cluster of this subgroup contained spermidine as the major polyamine.

Distribution of spermidine, homospermidine, cadaverine and agmatine

Putrescine is produced by ornithine decarboxylase from L-ornithine, and spermidine is synthesized from putrescine by aminopropyltransferase. Homospermidine is synthesized from putrescine by aminobutyl transfer mediated by Schiff-base complex formation with γ -aminobutyraldehyde. Within the *Flavobacterium*-*Flexibacter*-*Cytophaga*-*Sphingobacterium* complex, except for *Flammeovirga aprica*, *Saprospira grandis* and '*Microscilla arenaria*', contain one of the two triamines, spermidine and homospermidine. Distribution of the two triamines roughly correlated with their phylogenetic positions, as shown in Figure 1. Homospermidine was distributed ubiquitously in *Flavobacter* and *Sphingobacter* subgroups, but sporadically in other outside subgroups. Distribution of spermidine was limited in the outside subgroups. The occurrence of spermidine or homospermidine as the major polyamine in many *Flavobacterium*, *Flexibacter* and *Cytophaga* species phylogenetically widespread within this complex, and some *Cytophaga* species belonging to the Bacteroidaceae, serve as a chemotaxonomic marker for the reclassification of the species.

Cadaverine is produced by lysine decarboxylase from L-lysine. Cadaverine was found as the major polyamine in *Flammeovirga aprica* (Nakagawa *et al.*, 1997) and an organism classified as *Flexibacter aurantiacus* NCIMB 1394. This diamine was detected as a minor polyamine in *Microscilla marina*, *Flexibacter maritimus* and *Flexibacter roseolus*, containing spermidine or homospermidine, but the cellular concentrations of the diamine in them was altered by culture conditions.

Agmatine was the major polyamine of *Saprospira grandis* which lacked triamines. Within the others containing spermidine or homospermidine, agmatine which is produced by arginine decarboxylase from L-arginine, was detected in some strains of *Cellulophaga lytica*, *Flavobacterium xanthum*, *Flexibacter aggregans*, *Flexibacter litolis*, *Flexibacter maritimus*, *Flexibacter tractuosus* and *Psychroserpens burtonensis*. The distribution of agmatine is not a good chemotaxonomic characteristic because its cellular concentration can be altered by culture conditions.

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