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Isolation of Adherent Polycyclic Aromatic Hydrocarbon (PAH)-Degrading Bacteria Using PAH-Sorbing Carriers

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Two different procedures were compared to isolate polycyclic aromatic hydrocarbon (PAH)-utilizing bacteria from PAH-contaminated soil and sludge samples, i.e., (i) shaken enrichment cultures in liquid mineral medium in which PAHs were supplied as crystals and (ii) a new method in which PAH degraders were enriched on and recovered from hydrophobic membranes containing sorbed PAHs. Both techniques were successful, but selected from the same source different bacterial strains able to grow on PAHs as the sole source of carbon and energy. The liquid enrichment mainly selected for *Sphingomonas* spp., whereas the membrane method exclusively led to the selection of *Mycobacterium* spp. Furthermore, in separate membrane enrichment set-ups with different membrane types, three repetitive extragenic palindromic PCR-related *Mycobacterium* strains were recovered. The new *Mycobacterium* isolates were strongly hydrophobic and displayed the capacity to adhere strongly to different surfaces. One strain, *Mycobacterium* sp. LB501T, displayed an unusual combination of high adhesion efficiency and an extremely high negative charge. This strain may represent a new bacterial species as suggested by 16S rRNA gene sequence analysis. These results indicate that the provision of hydrophobic sorbents containing sorbed PAHs in the enrichment procedure discriminated in favor of certain bacterial characteristics. The new isolation method is appropriate to select for adherent PAH-degrading bacteria, which might be useful to biodegrade sorbed PAHs in soils and sludge.

The fate of polycyclic aromatic hydrocarbons (PAHs) in nature is of great environmental concern due to their toxic, mutagenic, and carcinogenic properties. A major decomposition process of PAHs in the environment is microbial degradation. Lower-molecular-weight PAHs, such as naphthalene and phenanthrene (11), acenaphthene and acenaphthylene (36, 50), and fluorene (20, 40) are relatively easy to degrade, and a large number of strains able to metabolize or cometabolize these compounds has been described. Anthracene, although identical to phenanthrene in number of aromatic rings, seems much more difficult to degrade, which is probably due to its lower solubility in water (11, 32, 37, 59). Until 1990, there were no reports of axenic microbial cultures utilizing PAHs containing four or more fused rings as the sole source of carbon and energy. Since then, a number of pure cultures able to (co)metabolize fluoranthene (5, 7, 32, 41) and pyrene (5, 7, 12, 13, 28, 32, 55, 58) have been reported. Literature data describing microbial growth on chrysene and benz(a)anthracene are rather scarce (10, 58), whereas no microorganisms capable of utilizing five-ring PAHs, such as benzo(a)pyrene, as the sole carbon and energy source are known.

In soil environments, degradation of PAHs is strongly affected by the low bioavailability of the compounds, as they have only limited water solubility and tend to sorb strongly to particularly organic matter (11, 61). On the other hand, recent studies suggest that specific physiological properties of the microorganisms involved in the degradation of hydrophobic compounds might enhance the availability of the compound (22). These mechanisms promoting the transfer of hydrophobic substrate include (i) production of biosurfactants or the use of specific cell surface components with emulsifying properties (15); (ii) uptake systems with high substrate affinity, which efficiently reduce concentrations of the substrate close to the cell surface, thereby increasing the diffusive substrate flux; and (iii) reduction of the distance between cells and substrate by means of cell surface structures which promote adhesion to hydrophobic surfaces (23, 24, 46). The third of these mechanisms suggests that bacteria specialized in adhesion to PAH-sorbing, i.e., hydrophobic, surfaces in the soil have a selective advantage once they contain the necessary catabolic enzymes to use the compound. Bacteria in close contact with surfaces containing sorbed PAHs experience a microenvironment that is different from the surrounding bulk liquid. A higher PAH concentration near the sorbent surface may render PAHs more readily available for adhered bacteria than for bacteria present in the aqueous phase of the soil. Therefore, it can be suggested that bacteria with efficient adhesion capacities possess interesting PAH-degrading capacities.

However, in the laboratory, enrichment of bacteria able to utilize PAHs as the carbon source has mostly been done in shaken liquid media. As this method favors bacteria able to grow well in suspension, PAH-degrading bacteria that are strongly attached to soil particles and grow very slowly or not at all in suspension may be missed in the selection procedure (56). Moreover, these systems might be far from the situation which bacteria experience in natural soil environments, where the compounds are sorbed on soil particles. Therefore, in parallel with classical liquid enrichment cultures, a new isolation method in which PAHs were provided in a sorbed state was

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tested in order to enrich and isolate hydrophobic, adhering, and PAH-degrading bacteria, possibly with bioavailability promoting capacities. The main idea was to bring a contaminated slurry in contact with polymeric surfaces containing sorbed PAHs and to enrich and recover adhering PAH-degrading bacteria on the surface of the membranes. Both isolation methods were successful, and the isolates were characterized and compared.

(This work constitutes part of the Ph.D. thesis of Leen Bastiaens [4].)

MATERIALS AND METHODS

Bacterial strains and culture conditions. All strains used in this study were routinely grown at 28°C on L9 or Tris minimal medium agar plates supplemented with the appropriate carbon source or on 869 rich medium. The naphthalene-degrading strain *Pseudomonas putida* PpG7 and the biphenyl-degrader *Sphingomonas yanoikuyae* have been described previously (16, 33). *Sphingomonas* strains LH162, LH215, and LH227 were previously isolated on phenanthrene from the same soil samples used in the current study, using a classical liquid enrichment method similar as the one described below (4). Tris minimal medium has been described before (38). L9 minimal medium contained (per liter) 8.8 g of $Na_2HPO_4 \cdot 2H_2O$, 3.0 g of KH_2PO_4, 1.0 g of NH_4Cl, 0.5 g of NaCl, 1.0 ml of 1 M $MgSO_4$, and 2.5 ml of a trace element solution ([per liter] 23 mg of $MnCl_2 \cdot 2H_2O$, 30 mg of $MnCl_4 \cdot H_2O$, 31 mg of H_3BO_3, 36 mg of $CoCl_2 \cdot 6H_2O$, 10 mg of $CuCl_2 \cdot 2H_2O$, 20 mg of $NiCl_2 \cdot 6H_2O$, 30 mg of $Na_2MoO_4 \cdot 2H_2O$, and 50 mg $ZnCl_2$) (pH 7). If required, liquid minimal media were supplemented with 0.2% glucose. PAHs were provided as a carbon source by placing crystals on the agar after streaking of the culture. Fuel oil was spread onto the surface of the agar before streaking of the culture, whereas toluene was provided in the gas phase by incubating the petri dishes in closed jars containing a vial with toluene. Rich medium 869 contained (per liter) 10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, 1 g of D-glucose, 0.345 g of $CaCl_2 \cdot H_2O$ and 30 mg of cysteine (pH 7). Medium 3 contained 8 g of nutrient broth per liter (pH 7). Solid agar plates were prepared with 15 g of agar (Difco) per liter.

Chemicals. Naphthalene, fluorene, phenanthrene, fluoranthene, chrysene, benz(a)anthracene, and benzo(a)pyrene were purchased from Janssen Chemica (Beerse, Belgium); dibenzothiophene and pyrene were purchased from Merck-Schuchart (Hohenbrunn bei München, Germany); anthracene was purchased from Pachard Instrument Company, Inc. (La Grange, Ill.); and acenaphthene and acenaphthylene were purchased from Aldrich-Chemie (Steinheim, Germany). The purity of the chemicals was 97 to 99%.

Isolation of PAH-degrading axenic cultures via classical shaken liquid medium enrichment. PAH-degrading bacteria were isolated from a mixture of historically contaminated soil samples suspended in L9. After addition of extra PAH compounds as crystals, the mixture was aerated to enrich in situ the PAH-degrading microflora. After several months, a sample of the slurry was shaken vigorously for 2 h to loosen the bacteria from the soil particles, and the largest ground particles were allowed to settle by gravity for 1 h. The upper aqueous phase was used to inoculate liquid enrichment cultures in 50 ml of L9 supplied with crystals of either fluorene, acenaphthene, acenaphthylene, phenanthrene, anthracene, fluoranthene, pyrene, chrysene, benzo(a)pyrene, or dibenzothiophene as the sole source of carbon and energy. The cultures were shaken (180 rpm) in the dark at 30°C. When turbidity increased, a part of the culture was transferred into fresh medium and further incubated. Axenic PAH-degrading strains were obtained by plating dilutions of the coculture on agar plates with 869 rich medium and isolation of colonies which could grow on L9 supplied with a PAH-compound as the sole carbon source (crystals).

Isolation of PAH-degrading axenic cultures using PAH-sorbing carriers. Flat-sheet Zirfon Z80 (Materials Division, Vlaamse Instelling voor Technologisch Onderzoek, Mol, Belgium), polysulfone, and Teflon membranes (Sartorius, Göttingen, Germany) were used as PAH-sorbing carriers. Teflon, polytetrafluoroethylene (PTFE) membranes (0.45-μm pore size and 100-μm thickness) are highly hydrophobic microfiltration membranes (contact angle > 100°). The Zirfon membrane, composed of polysulfone and zirconium oxide (18), was an ultrafiltration membrane with a thickness of less than 200 μm and a mean skin pore diameter of 12 nm. The hydrophobicity of Zirfon membranes (contact angle of about 78°) is lower than that of Teflon but rather comparable with the hydrophobicity of the polysulfone ultrafilters. The different flat-sheet membranes (squares of 10 cm^2) were separately incubated in 30 ml of L9 in the presence of 20 ml of the same PAH-contaminated soil suspension used in the shaken liquid enrichment procedure. PAHs (1 to 2 mg) were spiked on the membrane surfaces as a hexane or acetone solution. Time was given for the hexane and acetone to evaporate completely to avoid toxicity or utilization of these non-PAH organic compounds as the carbon source. Enrichments were started using membranes spiked with phenanthrene, anthracene, pyrene, fluoranthene, fluorene, benz(a)anthracene, benzo(a)pyrene, and dibenzothiophene. The slurry was gently shaken at 30°C and sheltered from light. After 3 to 4 weeks, the membranes were removed and rinsed gently with sterile water to remove soil particles and non-attached cells. The membranes were placed on L9 solid agar plates either with or without supplying extra PAH crystals on top of the membranes. Cycloheximide (50 μg ml^{-1}) was included in the medium to avoid the growth of fungi. After incubation, axenic PAH-degrading strains were purified from the developed cell mass on L9 agar plates.

Determination of PAH sorption efficiencies of the membranes. Sorption on and desorption from PAHs on the membranes was examined by spiking 0.2 to 1.0 mg of phenanthrene, fluoranthene, or pyrene, taken from a stock solution of hexane, on 10-cm^2 membranes. After evaporation of the hexane, the membranes were shaken in a glass tube with 5 ml of water for 4 to 7 days in the dark. For phenanthrene, tubes in which the PAH was spiked on the glass of the tube instead of directly on the membranes were also examined. The fate of the PAH was determined by calculating the amount recovered from the whole system and the amount sorbed on the membrane. PAHs were extracted with hexane and analyzed via reversed-phase high performance liquid chromatography (HPLC).

Determination of bacterial carbon source utilization patterns. A patch of each axenic culture was replica plated on L9 agar plates supplemented with a 3 mM concentration of one of the following carbon sources: D-glucose, D-gluconate, D-fructose, acetate, heptanoate, pelargonate, valerate, *iso*-valerate, fumarate, azelate, butyrate, pimelate, DL-lactate, L-malate, glycolate, DL-glycerate, trigoneline, itaconoate, pyruvate, citrate, formate, *trans*-aconitate, citraconate, glycerol, D-mannitol, gerianiol, ethanol, L-mandelate, D-mandelate, benzene, benzoate, salicylic acid, phenol, *p*-hydroxybenzoate, *m*-hydroxybenzoate, gallic acid, 3,4-dihydroxybenzoate, 2,5-dihydroxybenzoate, 3,4-dihydroxyphenylacetate, 2.5-dihydroxyphenylacetate, *m*-toluate, *p*-toluate, L-aspartate, tyrosine, threonine, or tryptophane.

DNA preparations. Crude preparations of plasmid DNA were obtained as described by Kado and Liu (31). Plasmid DNA from *Escherichia coli* was obtained as described by Ish-Horowicz and Burke (27) or using a plasmid extraction kit (QIAGEN Inc.). Total genomic DNAs were isolated as described by Bron and Venema (9).

REP-PCR fingerprint analysis. DNA regions between repetitive extragenic palindromic (REP) sequences were amplified by means of the oligonucleotide PCR primers 5′-IIIICGICGICATCIGGC-3′ (rep-1R-I) and 5′-ICGICTTATC IGGCCTAC-3′ (rep-2-1) (14). PCR amplification was performed in a final volume of 50 μl in which 1 to 7 μl of genomic DNA solution was mixed with a reaction buffer (75 mM Tris HCl [pH 9.0], 20 mM $(NH_4)_2SO_4$, 0.01% [wt/vol] Tween 20), $MgCl_2$ (2.5 mM), 1 μg of each primer, a 1 μM concentration of each deoxynucleoside triphosphate and 0.5 U of Red Goldstar polymerase (Eurogentec, Seraing, Belgium). Thermal cycling was carried out with an initial denaturation step at 95°C for 7 min followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 40°C for 1 min, and extension at 65°C for 8 min and a single final extension step at 65°C for 16 min. The PCR products were separated by agarose (1.5%) gel electrophoresis in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA). The REP patterns were analyzed and compared with Gelcompar software (Applied Maths, Kortrijk, Belgium) using a minimal profiling of 5.0%, a minimal area of 0.50, and a band position tolerance of 0.80%. Dendrograms were constructed using the unweighted pair group method using arithmetic averages (UPGMA) clustering algorithm (zone 85-395).

Identification and phylogenetic characterization of the isolates. Isolates were identified by classical tests (Gram staining, oxidase and catalase test, aerobic and anaerobic growth on glucose). API2ONE test kits (BioMérieux, Marcy-l'Etoile, France), Fatty acid methyl ester (FAME) analysis, Biolog GN (Hayward, Calif.), and 16S rRNA gene sequence analysis. Test kits were used according to the manufacturer's procedures. To determine 16S rDNA sequences, the 16S rRNA gene was amplified from genomic DNA by PCR using a forward primer hybridizing at positions 8 to 27 and a reverse primer hybridizing at positions 1491 to 1512 relative to *E. coli* numbering. Sequences were determined directly from these PCR products using conserved bacterial 16S rDNA sequencing primers (60). The primers were labeled at the 5′ end with a [γ-^{33}P]ATP via a reaction catalyzed by T4 polynucleotide kinase (USB Amersham International plc, Little Chalfont, Buckinghamshire, England). The DNA was sequenced via the method of Sanger (47) using the Thermo Sequenase Cycle sequencing kit with 7-deaza-dGTP from USB Amersham International plc according to the recommendations of the manufacturer. The 16S rRNA gene sequences were aligned with published sequences from the GenBank database using NCBI BLAST comparison software (1). Phylogenetic trees were constructed as described previously (53).

PAH degradation assays. Metabolism and cometabolism of PAHs were studied in resting-cell assays. Cells were pregrown in L9 supplied with the PAH compound on which the strains has been initially isolated. Well-grown cultures were filtered through cotton wool in order to remove PAH crystals and washed twice with 0.01 M $MgSO_4$. The cells were resuspended and diluted in L9 to an optical density at 660 nm (OD_{660}) of 0.3. Aliquots of 0.5 ml of this cell suspension were added to glass tubes with screw caps lined with aluminum foil, which contained 2.5 ml of L9 and PAHs. The PAHs were spiked in the tubes from PAH stock solutions in hexane or acetone, using glass syringes, and medium and cells were added after the solvent was evaporated. The final absolute concentration of each PAH compound was 20 mg per liter. After 5 days of incubation on a shaker at 30°C and in the dark, remaining PAHs were extracted twice with 1 volume of hexane and quantified via HPLC analysis. Tubes containing dead cells (inactivated by heating or with 0.07% perchloric acid) and without cells were included as controls. Each assay was performed in duplicate.

TABLE 1. Gene probes used in this study

Probe	Encoded function	Organism of origin	Plasmid	Reference
bphA$_1$A$_2$A$_3$BEFG	Biphenyl degradation	*R. eutropha A5*	pECG333	39
bphC	2,3-Dihydroxylbiphenyl dioxygenase	*S. yanoikuyae* B1	pGJZ1513	34
nahAa	Naphthalene dioxygenase (reductase)	*P. putida* NCIB9216-4	pDTG150	51
nahG	Salicylate and phenol hydroxylase	*P. putida* PpG1064	pHF100	17
nahR/nahG	Regulator of naphthalene pathway	*P. putida* PpG7	pMS15	49
pheAbAaBAcAd	Naphthalene and phenanthrene dioxygenase and dehydrogenase	*Comamonas testosteroni* GZ39	pGJ21712	19
xylE	Catechol 2,3-dioxygenase	*S. yanoikuyae* B1	pGJZ1521	34

PAH analysis. PAHs in the hexane fraction were separated by reversed-phase HPLC analysis with an acetonitrile-water (75:25, vol/vol) eluent and a 5-μm LiChromospher 100RP-18 column (length, 125 mm; width, 4 mm; Merck). The flow rate was 1 ml min^{-1}, and PAHs were detected spectrophotometrically (254 nm). For integration of the chromatograms and quantification of the PAH amount, the software packet BORWIN (ATAS) was used.

Hydrocarbon degradation gene probes and DNA-DNA hybridization. A list of the gene probe used in this study is given in Table 1. The gene probes were separated from their cloning vectors by appropriate restriction enzyme digestion and agarose gel electrophoresis. The probes were purified from the agarose using the Gene Clean II purification kit Bio 101 (Westburg b.v., Leiden, The Netherlands). *Eco*RI-digested genomic DNA was separated by 0.8% agarose gel electrophoresis. The DNA was subsequently transferred to Hybond N$^+$ membranes (Amersham International plc) by Southern blotting (52). Labeling of the gene probes and hybridizations were performed by using the fluorescein gene image labeling and detection kit (Amersham International plc), and hybridization signals were visualized using Hyperfilm-MP (Amersham International plc) according to the manufacturer's instructions.

Determination of bacterial cell surface properties. To determine cell contact angles (θ_W), cell electrophoretic mobility (u), cell zeta potential (ζ), and cell adhesion properties, bacterial cells were resuspended in 10 mM phosphate-buffered saline (PBS) composed of 0.493 g of NaCl, 0.029 g of KH_2PO_4, and 0.119 g of K_2HPO_4 per liter (pH 7.2). The electrophoretic mobility and the hydrophobicity of the bacterial cells were determined as described by van Loosdrecht et al. (56). A Doppler electrophoretic light-scattering analyzer (Zetamaster; Malvern Instruments Ltd., Malvern, Worcestershire, United Kingdom) was used to measure the electrophoretic mobility. The applied voltage was 100 V. Zeta potentials were calculated from the electrophoretic mobility according to the method of Helmholtz-Smoluchowski (26). To determine cell surface hydrophobicity, bacterial cells were collected on 0.45-μm-pore-size Micropore filters (Schleicher & Schuell, Dassel, Germany). The filters were mounted on glass slides and dried for 2 h at room temperature. Cell surface hydrophobicity was quantified by measuring the contact angle between the cell layer and a drop of water with a microscope equipped with a goniometric eyepiece (Krůss GmbH, Hamburg, Germany). Hydrophobic Teflon 350 (a copolymer of perfluoroalkoxyheptafluoropropylene and PTFE) with a θ_W of 115 ± 2° and hydrophilic glass with a θ_W of 12 ± 2° were used as surfaces to study bacterial adhesion. Teflon granules with an average diameter of 375 μm were obtained from Dolder AG (Basel, Switzerland). Glass beads with diameters of 450 ± 50 μm were purchased from Roth AG (Reinach, Switzerland). Both materials were cleaned by submerging them in chromosulfuric acid for 24 h at 60°C and rinsing first with 0.5 M KCl and then with deionized water. Finally, they were air dried and stored in glass containers. Adhesion experiments in columns were performed according to the method described by Rijnaarts et al. (45). Glass columns (internal diameter, 1.0 cm; length, 9.5 cm) were filled with either glass or Teflon beads. The packing had a length of 9.3 ± 0.2 cm. The pore volumes (the volumes of liquid in the columns) were 2.54 and 2.46 ml per column for glass and Teflon columns, respectively (29). To determine breakthrough curves, the columns were first rinsed for 30 min with 10 mM PBS before the cell suspensions (C_0 = OD_{280} = 0.30 to 0.60) were applied. During cell loading phases (90 to 120 min) the cell concentration of the effluent (C) initially increased and reached a maximum (C_{max}/C_0). Afterwards nonadhering cells were removed with 10 mM PBS buffer (30 min), and the reversibility of adhesion of the remaining cells was determined by rinsing the columns with deionized water (30 to 60 min). During the whole experiment, effluent concentrations were measured every 10 min. Experiments were conducted in duplicate or triplicate. Adhesion efficiencies (α_0) were calculated as described by Jucker et al. (29).

Batch adhesion experiments were conducted in 10 mM PBS as described previously (45). Transparent films of PFA-Teflon (a copolymer of perfluoroalkoxypropylene and PTFE; Fluoroplast, Raamsdonkveen, The Netherlands) and glass microscope coverslips were used as adhesion surfaces.

Nucleotide sequence accession numbers. The 16S rDNA sequences of the newly isolated *Mycobacterium* strains are available under the following accession numbers in the EMBL nucleotide sequence database: LB501T (AJ245702), LB307T (AJ245703), and LB208 (AJ245704).

RESULTS

Isolation of PAH-degrading bacteria via shaken aqueous enrichments providing the PAHs as crystals. Using the aqueous medium enrichment procedure in which the PAHs were provided as crystals (path A in Fig. 1), cultures that were able to utilize either fluorene, acenaphthene, acenaphthylene, phenanthrene, fluoranthene, pyrene, or dibenzothiophene as the sole carbon source were obtained (Table 2). The members of the coculture were separated by plating on rich medium agar plates. Colonies displaying different morphologies were purified and tested for growth in liquid and solid L9 minimal medium with PAHs as the sole source of carbon and energy. One fluoranthene-, five fluorene-, two phenanthrene-, one pyrene-, and six dibenzothiophene-utilizing axenic strains were isolated (Table 2). No axenic strains could be isolated using acenaphthene or acenaphthylene as the sole source of carbon. Further subcultivations and stability tests demonstrated that the strains isolated on fluorene and pyrene were the most stable ones, i.e., the ability to grow on PAHs was not lost after more than 100 generations of growth on nonselective rich medium 869 (Table 2).

Isolation of PAH degraders using PAH-sorbing carriers. An alternative isolation procedure with PAH-sorbing carriers was used to isolate hydrophobic and strongly adhering PAH degraders. In order to test their suitability as PAH-sorbing materials, different polymeric flat-sheet surfaces were tested for their PAH-sorbing properties, i.e., the very hydrophobic Teflon and the less hydrophobic polysulfone and Zirfon membranes, with Zirfon being most hydrophilic. The tests revealed that all three membranes displayed very good PAH sorption properties, although differences were observed in the reversibility of the sorption. The PAHs recovered from the membrane were in most cases near 100% for Teflon but were less for the two other carriers. Recovery from the whole system for polysulfone fluctuated between 52 and 99%, while the recovery from the Zirfon system did not exceed 24%. The amount of PAHs that was not recovered may be attributed to penetration into deeper layers of the carriers. Phenanthrene, fluoranthene, and pyrene acted in a similar way, although their water solubilities are different.

Teflon and polysulfone as well as Zirfon membranes were used as PAH-sorbing surfaces to select adhering PAH-degrading bacteria (path B in Fig. 1). After contact with the soil slurry for 3 to 4 weeks, the membranes were rinsed and placed on minimal medium agar plates with or without PAH crystals. A cell mass formed predominantly on the agar directly surrounding the membrane but also under and to a lesser extent, on top of the membranes. PAH-degrading bacteria were further purified from the cell mass within a week after incubation of the membrane on the agar plate. Several aliquots of cell mass from around, under, and on the membrane was streaked on L9 agar plates containing the single PAH crystals as the sole source of

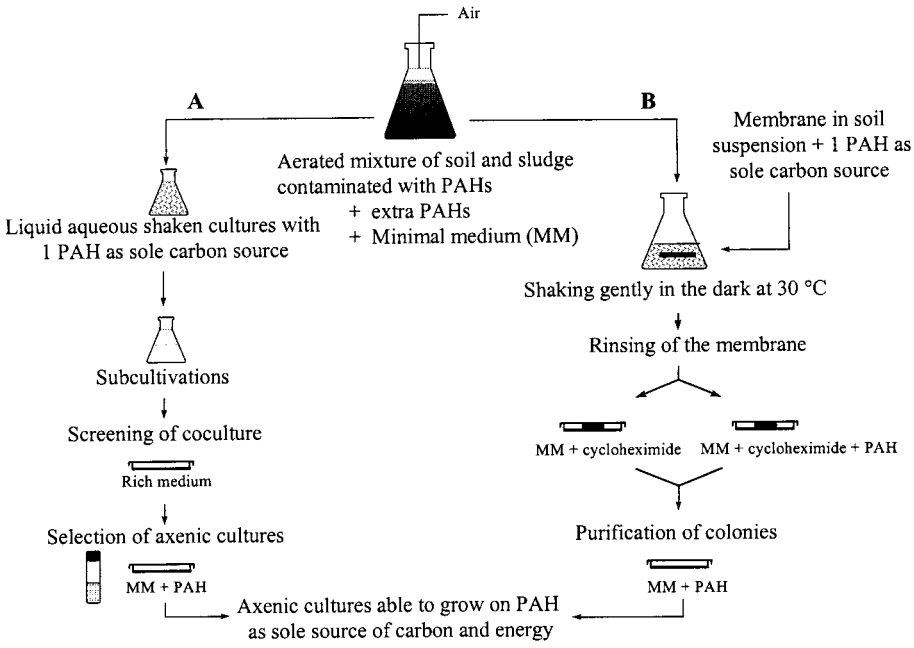

FIG. 1. Isolation of PAH-degrading bacteria using the classical liquid enrichment procedure (path A) and using PAH-sorbing membranes (path B).

carbon and energy. Pure strains were obtained after several purification steps on the same medium.

The use of Teflon membranes led to the isolation of one strain able to utilize anthracene as the sole carbon source (LB501T) and one strain able to grow on phenanthrene (LB307T). Two other phenanthrene-utilizing strains, LB300S and LB314Z, were isolated using polysulfone and Zirfon membranes, respectively (Table 2). No axenic PAH-degrading strains were obtained using the other tested PAH compounds.

Diversity of isolates. The diversity of the new PAH-degrading isolates was determined by comparing genotypic and phenotypic patterns obtained from REP-PCR analysis and carbon utilization tests, respectively. Cluster analysis of the REP-PCR fingerprints of the PAH-degrading isolates (Fig. 2) demonstrated different groups of REP-PCR-related strains, such as the five fluorene-utilizing strains LB100, LB110, LB117, LB126, and LB127 and the phenanthrene-degrading isolates LH128, LH162, and LH163. The three phenanthrene-degrading isolates, LB300S, LB307T, and LB314Z, although selected using different PAH-sorbing carriers, also showed very related REP-PCR patterns. REP-PCR-related strains also displayed nearly identical carbon source utilization patterns, suggesting that they represent highly similar strains. Interestingly, the REP-PCR patterns and carbon utilization patterns of all the bacterial strains recovered using PAH-sorbing carriers were significantly different from the patterns obtained for the strains isolated using liquid enrichment. This observation shows that different bacteria were isolated by the two isolation procedures and indicates that the presence of the PAH-sorbing membranes advantaged certain bacteria.

Identification and phylogenetic distribution of the new PAH degrading isolates. The isolates were identified via Gram staining, API2ONE, FAME, and 16S rRNA gene sequence analysis. Most of the PAH-utilizing strains isolated from liquid enrichment cultures were found to be gram negative and to belong to the genus *Sphingomonas*. Only strain LB208 was gram variable and was identified by phenotypic and FAME analysis as belonging to the *Nocardia-Rhodococcus* group. All strains isolated using the PAH-sorbing carriers were gram vari-

TABLE 2. Isolated cocultures and axenic strains able to utilize PAHs as sole source of carbon

Isolation method	Coculture obtained on:	PAH-utilizing strain(s) obtained from coculture[a]	Stable PAH-utilizing strain
Liquid aqueous culture	Fluorene	LB100, LB110, LB117, LB126, LB127	LB100, LB110, LB117, LB126, LB127
	Acenaphthene	None	None
	Acenaphthylene	None	None
	Phenanthrene	LB309, LB310	None
	Fluoranthene	LB47	None
	Pyrene	LB208	LB208
	Dibenzothiophene	LB401, LB403, LB407, LB409, LB410, LB411	None
PAH-sorbing carrier	Phenanthrene	LB300S (Polysulfone)	LB300S
		LB307T (Teflon)	LB307T
		LB314Z (Zirfon)	LB314Z
	Anthracene	LB501T (Teflon)	LB501T

[a] The polymeric membrane used as PAH-sorbing carrier for the isolation of PAH degraders is indicated parenthetically.

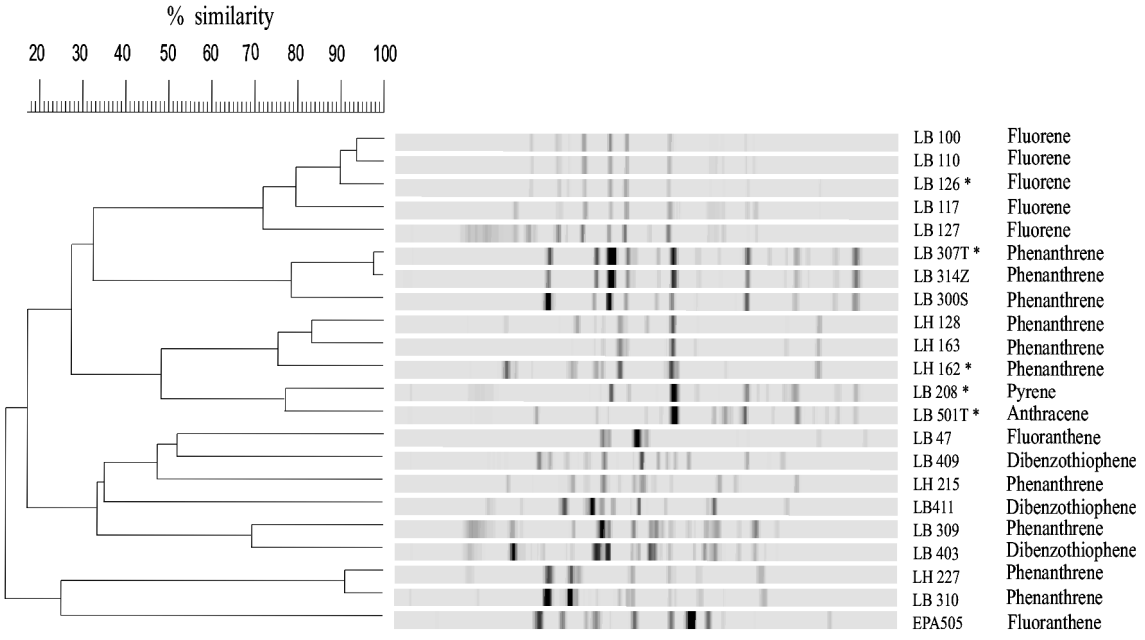

FIG. 2. Cluster analysis of REP-PCR patterns of the PAH-degrading isolates. The strains that were further characterized are marked with an asterisk.

able. Phenotypic analysis and FAME analysis suggested that the four strains belong to the *Nocardia-Rhodococcus* group.

LB208, LB307T, and LB501T were further classified by 16S rRNA gene sequence analysis as *Mycobacterium* species. The sequence of LB208 showed 100% similarity with the 16S rRNA gene sequence of *Mycobacterium gilvum* type strain ATCC 43909 and of the pyrene-degrading *Mycobacterium* sp. BB1 (5), which has been found to be, in all likelihood, identical to *M. gilvum* (6). The 16S rRNA gene sequence of LB501T showed highest similarity (99%) with the 16S rRNA gene sequence of the PAH-degrading strain *Mycobacterium* sp. CH-1 (12). The 16S rRNA gene sequence of LB307T was nearly identical to the 16S rRNA gene sequence of the type strain of *M. gilvum*, strain BB1 and strain LB208. However, LB208 and LB307T are not identical strains as shown in the REP-PCR assay. Figure 3 shows the newly isolated *Mycobacterium* sp. situated among *Mycobacterium* type strains in the phylogenetic dendrogram based on 16S rRNA gene sequences. Several members of the *Mycobacterium* genus that are known to degrade PAHs are included in the clustering (5, 12, 21, 25, 35; G. Lloyd-Jones and D. W. F. Hunter, unpublished results). As the other PAH degraders, the new isolates are members of the so-called fast-growing *Mycobacteria*. Both LB208 and LB307T clustered with the *M. gilvum* type strain ATCC 43909 and the PAH-degrading strain BB1. Strain LB501T clustered together with *Mycobacterium* sp. CH-1 (12). The closest well-identified *Mycobacterium* species to strain LB501T in the tree is *Mycobacterium neoaurum*, whose 16S rRNA gene sequence shows 97% identity with LB501T. The phylogenetic distribution of the PAH-degrading *Sphingomonas* sp. is the subject of a separate study.

PAH metabolism and cometabolism capacities of the new PAH-degrading isolates. The ability of the isolates to utilize PAHs other than those used for the isolation was tested both in liquid mineral aqueous medium and on solid agar plates (Table 3). Strain LB208, isolated on pyrene, also grew on phenanthrene and fluoranthene, and the phenanthrene-utilizing strain LB307T was able to grow additionally on dibenzothiophene and fluoranthene. Most strains transformed dibenzothiophene into red metabolites, but a clear appearance of cell mass was not observed. Strains LB208, LB307T, and LB501T were also able to grow on diesel fuel as the sole carbon source. The capacity of the stains to cometabolize PAHs was examined in resting cell assays over a period of 5 days (Table 4). All strains were able to extensively cometabolize several three- and four-ring PAHs. Benz(a)anthracene was only cometabolized to a limited extent by strains LB307T and LB501T. No significant degradation of chrysene or benzo(a) pyrene was observed.

Plasmid content of the PAH-degrading isolates and DNA-DNA hybridization of genomic DNA with xenobiotic catabolic gene probes. Plasmids could only be detected in the Kado and Liu extracts (31) of most PAH-degrading *Sphingomonas* strains. The number of plasmid bands appearing after gel electrophoresis was high (one to eight), but neither the patterns nor the number was reproducible, indicating high instability of the plasmids (data not shown).

Of the tested catabolic probes (Table 1), only probes containing *bphC* and *xylE* of *S. yanoikuyae* B1 hybridized strongly, and this only with the genomic DNA of the tested phenanthrene-degrading *Sphingomonas* strains. For these strains, the same hybridization pattern was obtained as for strain B1 (34), indicating that the genes were very conserved (data not shown). The $bphA_1A_2A_3BEFG$ probe, encoding biphenyl degradation in *Ralstonia eutropha* A5 showed weak hybridization with all four tested *Sphingomonas* spp. (LH128, LH162, LH227, and LB117).

Cell surface properties of newly isolated PAH-degrading isolates. The cell surface properties of PAH-degrading isolates derived by either isolation method—i.e., strains LH162, LB126, and LB208, isolated via aqueous enrichment, and strains LB307T and LB501T selected using PAH-sorbing carriers—were compared. The well-studied naphthalene-degrading *P. putida* strain PpG7 (16) was also included in the study. Both cells pregrown on L9 with glucose and cell pregrown on the relevant PAH compound as the sole source of carbon and

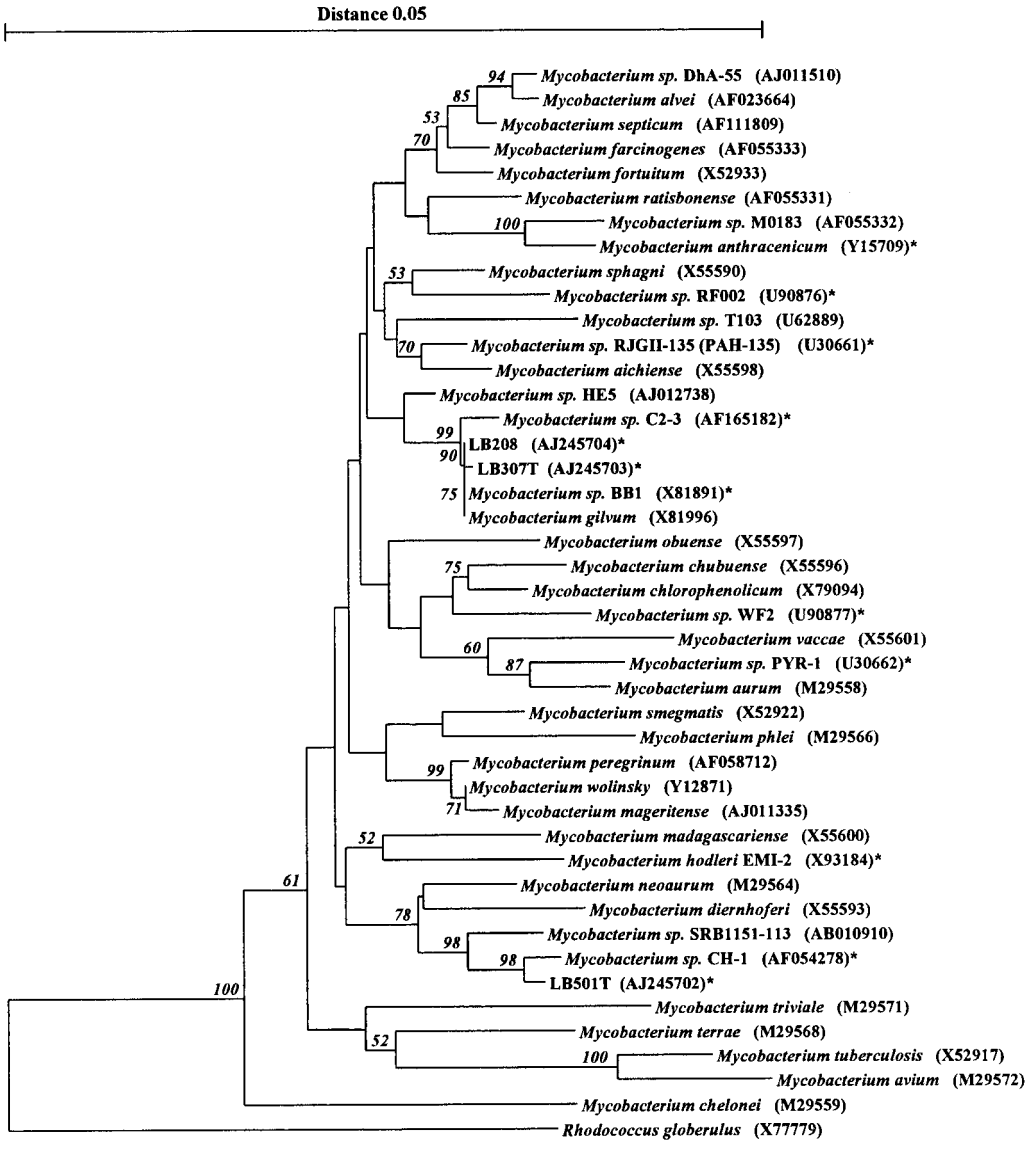

FIG. 3. Position of the new isolated PAH-degrading *Mycobacterium* spp. in the distance tree of predominantly fast-growing *Mycobacterium* species, based on 16S rRNA gene sequence analysis. Bootstrap values are indicated at the corresponding nodes (expressed as percentages). The distance between two species is obtained by summing the lengths of the connecting horizontal branches using the scale at the top. PAH-degrading strains are marked with an asterisk. Accession numbers for the 16S rRNA gene sequences are indicated between brackets.

energy were tested in order to examine whether the used carbon source affected the cell surface properties.

Hydrophobicity of the strains was examined by contact angle measurements (Table 5). As strains with θ_W values of <30 to 35° are considered hydrophilic and those with θ_W values of $>70°$ are considered hydrophobic, PpG7 and LB126 were rather hydrophilic. LH162 displayed an intermediate hydrophobicity, and the three *Mycobacterium* species displayed very hydrophobic properties. Variations in the θ_W values for a strain can be explained by different growth phase conditions in which the cells were harvested and by the formation of stable aggregates which inhibited the formation of a smooth cell layer on the filters used for contact angle measurements. This occurred with all three *Mycobacterium* species. Especially LB307T and LB501T, isolated via PAH-sorbing carriers, formed very stable aggregates in PBS buffer and even in distilled water.

Zeta potential measurements revealed that all strains were

TABLE 3. Growth of the isolates on PAHs as sole source of carbon and energy

Strain	Growth on[a]:							
	Naph	Acene	Fluor	Dib	Anthr	Phen	Flu	Pyr
LH162	−	−	−	− (r)	−	+	−	−
LH215	−	−	−	− (r)	−	+	− (r-b)	−
LH227	−	−	−	− (r)	−	+	− (r-b)	−
LB126	−	−	+	−	− (y)	−	−	−
LB208	−	−	−	− (r)	−	+	+	+
LB307T	−	ND	−	+	−	+	+	?
LB501T	−	−	−	− (r)	+	?	− (r-b)	?

[a] Naph, naphthalene; Acene, acenaphthene; Fluor, fluorene; Dib, dibenzothiophene; Anthr, anthracene; Phen, phenanthrene; Flu, fluoranthene; Pyr, pyrene; +, growth; ?, only a few very small colonies; −, no growth; ND, not determined. For letters in parentheses, r, y, b, and r-b stand for red, yellow, brown, and red-brown coloration of the medium, respectively.

TABLE 4. (Co)metabolization of PAHs as determined in resting cell assays

Strain	(Co)metabolism of[a]:								
	Fluor	Dib	Anthr	Phen	Flu	Pyr	BaA	Chrys	BaP
LH162	+++	+++	?	+++	−	−	−	−	−
LH215	ND	ND	ND	+++	ND	ND	ND	ND	ND
LH227	++	+++	+	+++	+	+	−	−	−
LB126	+++	++	?	++	?	?	−	−	−
LB208	+++	+	−	+++	++	+++	−	ND	−
LB307T	+++	++	+	+++	+	+++	+	−	−
LB501T	+++	+++	+++	+++	+++	−	+	−	−

[a] Fluor, fluorene; Dib, dibenzothiophene; Anthr, anthracene; Phen, phenanthrene; Flu, fluoranthene; Pyr, pyrene; BaA, benz(a)anthracene; Chrys, chrysene; BaP, benzo(a)pyrene; +++, >80% (co)metabolized; ++, >50% (co)metabolized; +, <50% (co)metabolized; ?, <20% (co)metabolized; −, no (co)metabolization; ND, not determined. For each strain, the result for the PAH compound used for isolation is underlined.

negatively charged at a neutral pH (Table 5). Especially LB501T showed an extreme negative charge. In Fig. 4 the new isolates were plotted together with more than 150 other strains (gram positive, gram negative, clinical, and environmental isolates) based on contact angle and electrophoretic mobility measurements. The data for the 150 strains have been published previously by Jucker et al. (29). The figure clearly shows the exceptional wall characteristics of LB501T. *Mycobacterium* sp. LB307T, the other strain isolated via PAH-sorbing carriers, was situated near the dashed line, and represents another extreme case, since no bacteria situated above this line have yet been described.

All six strains were further examined for their adhesion to glass (hydrophilic) and Teflon (hydrophobic) beads in column adhesion experiments. The maximum C/C_0 values differed considerably for the different strains. Adhesion efficiencies, by definition, the experimental deposition rate divided by the theoretical transport rate, and adhesion reversibility were calculated based on the obtained C/C_0 values (Table 5). In all cases, adhesion of the cells to Teflon was higher than that to glass. PpG7 and LB126 adhered only to Teflon, while LH162 stuck to neither glass nor Teflon. The *Mycobacterium* sp. strains LB208, LB307T, and LB501T adhered to glass and very strongly to Teflon. Due to the rapid formation of stable aggregates, OD measurements and consequently breakthrough curves for the *Mycobacterium* sp. strains were less stable, and a shorter cell-loading time was used for strains LB307T and LB501T. For all strains, the cells pregrown on glucose exhibited a higher adhesion capacity than cells pregrown on PAHs.

Adhesion of LB208, LB307T, and LB501T to glass and Teflon was also tested in batch adhesion experiments. However, quantification of adhered cells or aggregates was impossible after even a few hours due to the formation of high amounts of different sized and dense aggregates. The percentage of membrane surface covered with aggregates was higher for Teflon than for glass and was the highest for LB307T.

DISCUSSION

A new method was introduced to isolate PAH-degrading bacteria from contaminated soils and sludge and was compared with commonly used liquid aqueous one-phase suspension enrichment methods. In the new method, PAHs were supplied sorbed to a solid phase and hydrophobic and adhering PAH-degrading bacteria were enriched on the sorbing carrier. The method differs from the liquid biphasic aqueous-organic isolation system described by Ascon-Cabrera and Lebeault (2) in the fact that PAHs are sorbed onto solid surfaces that imitate the static state of the solid matrix of the soil instead of the nonaqueous liquid phase.

In our study, although the same soil sample was used as the bacterial source in both isolation procedures, different bacterial strains were obtained depending on the procedure. Moreover, liquid enrichment cultures mainly led to the isolation of *Sphingomonas* sp. while isolates obtained on membranes enriched were all *Mycobacterium* species. Furthermore, the isolates obtained with the membrane procedure strongly adhered to different substrates.

Members of the *Sphingomonas* and *Mycobacterium* genus have been isolated previously as degraders of PAHs and degraders of other pollutants with low solubility in water (3, 35,

TABLE 5. Surface characteristics of PAH-degrading bacteria

Strain (carbon source on which it was pregrown)	θ_W (°)[a]	u (10^{-8} m^2 V^{-1} s^{-1})[a]	ζ (mV)[a]	Value for column adhesion with beads of:					
				Glass			Teflon		
				C_{max}/C_0	α_0	Rev.[b] (%)	C_{max}/C_0	α_0	Rev. (%)
PpG7 (glucose)	31 ± 5	−2.46 ± 0.39	−32.0 ± 5.2	0.958	0.088	3.5	0.312	0.546	3.0
PpG7 (naphthalene)	29 ± 2	−2.52 ± 0.49	−32.7 ± 6.1	0.988	0.065	3.4	0.668	0.227	4.9
LH162 (glucose)	60 ± 16	−3.01 ± 0.53	−38.9 ± 6.8	0.986	0.003	1.0	0.989	0.074	5.7
LH162 (phenanthrene)	59 ± 12	−2.78 ± 0.45	−36.2 ± 6.0	0.981	0.028	1.0	0.977	0.029	4.9
LB126 (glucose)	34 ± 10	−0.59 ± 0.11	−7.7 ± 1.4	0.992	0.026	5.8	0.578	0.337	1.6
LB126 (fluorene)	36 ± 3	−0.84 ± 0.12	−11.0 ± 1.6	0.989	0.024	4.9	0.631	0.467	1.6
LB208 (glucose)	85 ± 4	−3.43 ± 0.92	−44.7 ± 12.1	0.327	0.561	3.1	0.147	0.921	2.1
LB208 (pyrene)	88 ± 2	−3.39 ± 0.37	−44.2 ± 3.4	0.578	0.559	7.0	0.319	0.773	4.6
LB307T (glucose)	101 ± 8	−1.75 ± 0.33	−21.6 ± 2.6	0.256	0.513	10.8	0.078	1.233	0.8
LB307T (phenanthrene)	103 ± 6	−2.21 ± 0.41	−28.7 ± 5.4	0.368	0.454	10.0	0.173	0.846	1.2
LB501T (glucose)	89 ± 10	−5.04 ± 0.40	−65.5 ± 5.8	0.328	0.488	11.0	0.19	0.820	1.7
LB501T (anthracene)	ND	ND	ND	ND	ND	ND	ND	ND	ND

[a] Mean values were calculated from four to twelve independent measurements.
[b] Rev., reversibility of adhesion.

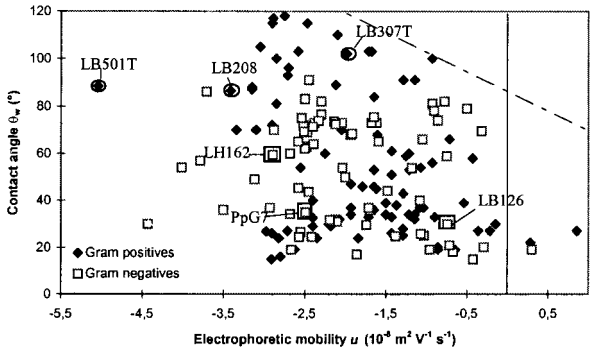

FIG. 4. Contact angles plotted against the electrophoretic mobilities of several gram-positive and gram-negative isolates, showing the position of the newly isolated PAH degraders. All *u* values were measured at an ionic strength of 10 mM (or 8.1 mM) and pH 7.0 to 7.2. Contact angles were measured in distilled water or at an ionic strength of 10 mM (29).

43). Interestingly, a relation can be observed between the complexity and recalcitrance of PAH compounds and the number of *Sphingomonas* and gram-positive bacterial isolates which have been described to biodegrade these PAHs. Most bacteria selected on naphthalene, a two-ring PAH, belong to the fluorescent pseudomonads (4, 16). Many phenanthrene- and anthracene-utilizing isolates are *Pseudomonas* sp. (37), *Sphingomonas* sp. (32), or gram positive organisms of the *Nocardia-Rhodococcus-Mycobacterium* group (12). At more than three aromatic rings, mainly gram-positive PAH-degrading bacteria (5, 7, 13, 28, 55, 58) but also some *Sphingomonas* sp. (41, 59) have been reported. The genera *Sphingomonas* and especially *Mycobacterium* therefore seem to be specialized in degrading such less-bioavailable compounds. They both have a particular outer cell wall layer, i.e., glycosphingolipids for *Sphingomonas* (62) and glycolipids such as mycolic acids for *Mycobacteria* (48), which may be important for the interaction with or uptake of hydrophobic compounds (44).

In our tests, the membrane procedure exclusively led to the isolation of *Mycobacterium* species. As shown by REP-PCR and C source utilization profiles, three different membranes spiked with phenanthrene even selected highly similar *Mycobacterium* strains, i.e., LB300S, LB307T, and LB314Z. The fact that the membrane procedure seems to favor the isolation of *Mycobacterium* species can be explained by the hydrophobic cell wall of members of this genus. Hydrophobic bacteria may be easily expelled from the water phase due to unfavorable energetic effects and seek contact with the hydrophobic membrane. Further, additional specific cell wall characteristics may play a role in efficient attachment to and colonization of the PAH-sorbing carriers. The PAHs sorbed on the membrane, if available for the bacterium, may further enable the attached strains to form cell mass, which is important to finally recover them from the membrane.

It is of particular interest that an anthracene-utilizing *Mycobacterium* sp. (i.e., LB501T) was isolated from a PAH-sorbing membrane while no anthracene-utilizing strains were isolated form the liquid enrichment cultures. This can possibly be explained by the high hydrophobicity and low water solubility of anthracene in comparison with phenanthrene and even pyrene. Anthracene may therefore not be bioavailable or be only limitedly bioavailable for less hydrophobic strains. In liquid minimum medium as well as on solid agar plates extensive growth of LB501T occurred predominantly around the PAH crystals, indicating that a close contact between solid PAH and the bacterium was an advantage. Anthracene might be utilized directly and solubilized at the level of the cell wall. Since the 16S rRNA gene sequence of *Mycobacterium* sp. LB501T did not show more than 97% similarity to the 16S rRNA gene sequence of any well-identified *Mycobacterium* sp. LB501T might represent a new species within the genus *Mycobacterium*.

Using the one-phase aqueous enrichment method, only one *Mycobacterium* species (i.e., LB208) was isolated, which, however, was a strain different from the membrane-enriched *Mycobacterium* sp. strain LB208 and other previously reported PAH-degrading *Mycobacterium* strains might have different PAH-degrading strategies or other cell surface properties than the strains isolated via the PAH-sorbing surfaces. Based on hydrophobicity and cell surface charge, LB208 was indeed less extreme than LB501T and LB307T (Fig. 4). The difference in cell wall properties between strain LB208 and LB307T indicates that, although both strains belong to the same species, LB307T might contain additional functions for adhesion.

Contact angle, electrophoretic mobility, zeta potential measurements, and adhesion experiments indeed indicated that the membrane isolation method selected very hydrophobic and strongly adhering bacteria. The three *Mycobacterium* sp. strains, LB208, LB307T, and LB501T, were found to be much more hydrophobic than strain LH162, which displayed an intermediate hydrophobicity, and the rather hydrophilic strains PpG7 and LB126. The strains isolated via the membrane method (LB501T and LB307T) were found to be the most adherent and formed the most stable aggregates. It has been discussed that an extreme adhesion tendency may be ecologically unfavorable and that such bacteria might not exist. However, strongly adhering bacteria may tend to escape conventional isolation (56). According to the so-called extended DLVO or colloid stability theory (57), in which electrostatic repulsion, van der Waals attraction, and acid-base (hydrophobic) interactions are considered, the adhesion capacity is inversely correlated with a more negative surface charge and favored by the hydrophobicity of the bacterial cells. If only DLVO forces were influencing adhesion, one would expect LB307T to be the most adhering strain, due to its hydrophobicity and its low charge, and LB501T to be the least adhering strain, due to its extremely negative charge. Adhesion experiments confirmed that *Mycobacterium* sp. LB307T, isolated via a Teflon flat-sheet membrane, was the most adhering strain. However, LB501T also displayed strong adhering capacity to both glass and Teflon, indicating that adhesion of the strain was influenced by strong non-DLVO attractive forces, such as contributions of cell wall polymers (30). Also the formation of very stable aggregates is in obvious contradiction with the high electrostatic repulsion expected to occur between the highly negatively charged cells and may be explained by favorable polymer interactions. Nevertheless, the study shows that *Mycobacterium* sp. LB501T displays an unusable combination of a very high adhesion efficiency and an extreme highly negative charge (−66 mV). It has been reported that relatively hydrophobic strains tend to possess highly negative electrophoretic potentials and that the influence of the electrophoretic mobility on adhesion may be limited, especially for hydrophobic cells (54, 56). In contrast with LB501T, *Sphingomonas* sp. LH162, although rather hydrophobic and moderately charged, did not adhere at all to Teflon. Steric hindrance by cell wall polymers may have inhibited a close approach of the cells to the surface. This may also explain why the strain was not selected using the hydrophobic carriers.

In conclusion, materials on which PAHs sorb were proven to be useful to select and isolate new pollutant-degrading, hydrophobic, and adhering bacteria, which may escape classical microbiological isolation techniques. The selection of bacteria

with hydrophobic cell surfaces from leaves and larch needles via plastic materials and Teflon tubes has been reported before (42), but the isolation of hydrophobic adhering PAH degraders from soils and sludges with PAH-sorbing flat-sheet membranes is new. The method should be considered rather as a complementary system and not a replacement of the liquid cell suspension method, since both methods seem to select for different kinds of bacteria. It should be noted that also the membrane method theoretically requires the detachment of some individual bacteria or release of daughter cells from one sorbent (soil) to move to another (PAH-sorbing carrier). The answer to whether the strains isolated via the membranes are actually able to degrade sorbed PAHs more efficiently awaits further research.

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