APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 2002, p. 5826–5833 Vol. 68, No. 12
0099-2240/02/$04.00+0 DOI: 10.1128/AEM.68.12.5826–5833.2002

Rhodanobacter sp. Strain BPC1 in a Benzo[*a*]pyrene-Mineralizing Bacterial Consortium

Robert A. Kanaly,† Shigeaki Harayama,‡ and Kazuya Watanabe*

Marine Biotechnology Institute, 3-75-1 Heita, Kamaishi City, Iwate 026-0001, Japan

Received 3 May 2002/Accepted 6 September 2002

A bacterial consortium which rapidly mineralizes benzo[*a*]pyrene when it is grown on a high-boiling-point diesel fuel distillate (HBD) was recovered from soil and maintained for approximately 3 years. Previous studies have shown that mobilization of benzo[*a*]pyrene into the supernatant liquid precedes mineralization of this compound (R. Kanaly, R. Bartha, K. Watanabe, and S. Harayama, Appl. Environ. Microbiol. 66:4205-4211, 2000). In the present study, we found that sterilized supernatant liquid filtrate (SSLF) obtained from the growing consortium stimulated mineralization of benzo[*a*]pyrene when it was readministered to a consortium inoculum without HBD. Following this observation, eight bacterial strains were isolated from the consortium, and SSLF of each of them was assayed for the ability to stimulate benzo[*a*]pyrene mineralization by the original consortium. The SSLF obtained from one strain, designated BPC1, most vigorously stimulated benzo[*a*]pyrene mineralization by the original consortium; its effect was more than twofold greater than the effect of the SSLF obtained from the original consortium. A 16S rRNA gene sequence analysis and biochemical tests identified strain BPC1 as a member of the genus *Rhodanobacter*, whose type strain, *Rhodanobacter lindaniclasticus* RP5557, which was isolated for its ability to grow on the pesticide lindane, is not extant. Strain BPC1 could not grow on lindane, benzo[*a*]pyrene, simple hydrocarbons, and HBD in pure culture. In contrast, a competitive PCR assay indicated that strain BPC1 grew in the consortium fed only HBD and benzo[*a*]pyrene. This growth of BPC1 was concomitant with growth of the total bacterial consortium and preceded the initiation of benzo[*a*]pyrene mineralization. These results suggest that strain BPC1 has a specialized niche in the benzo[*a*]pyrene-mineralizing consortium; namely, it grows on metabolites produced by fellow members and contributes to benzo[*a*]pyrene mineralization by increasing the bioavailability of this compound.

Environmental pollution caused by the release of crude and refined petroleum products occurs in all areas of the world. These complex hydrocarbon mixtures may consist of hundreds of compounds that are highly variable in structure and in susceptibility to biodegradation (9). High-molecular-weight (HMW) polycyclic aromatic hydrocarbons (PAHs) are a class of compounds that are known for their environmental persistence (10, 26, 43) and are frequently encountered as constituents of multicompound non-aqueous-phase liquid (NAPL) mixtures, such as products of petroleum refining. The HMW PAH benzo[*a*]pyrene is a compound that has been intensely studied for many years due to its potent genotoxic (44) and immunotoxic (14) properties. Because of this, there is much interest in determining the fate of this compound in the natural environment in order to assess the potential exposure risk to humans (4) and to efficiently reduce elevated levels of the compound at polluted sites. Hence, benzo[*a*]pyrene is regulated by environmental agencies in countries throughout the world (12, 24, 31). Benzo[*a*]pyrene may be removed from the environment through the biodegradative actions of bacteria and fungi (10, 22, 26). The microbially induced biotransformations may be partial, as is the case for most fungi, or may lead to ring opening, as is the case for some bacteria. Although benzo[*a*]pyrene is a difficult compound for bacteria to biodegrade, a number of studies in the last 5 years have described benzo[*a*]pyrene biodegradation under various conditions (1, 7, 11, 19–21, 23, 37, 42, 47, 48). Extensive transformation of radiolabeled benzo[*a*]pyrene to carbon dioxide was shown to occur in some instances (8, 25–29, 54). Benzo[*a*]pyrene and other HMW PAHs are often biodegraded by fortuitous metabolism (26, 49), and under such circumstances an organism that grows on one compound biotransforms a second compound even though the organism derives neither energy nor carbon from transformation of the second compound.

We recovered from soil a bacterial consortium which rapidly mineralizes benzo[*a*]pyrene when it is grown on diesel fuel constituents, such as a high-boiling-point diesel fuel distillate (HBD) (25, 27–29). Due to the rapid nature of benzo[*a*]pyrene mineralization by this bacterial consortium, we were interested in examining more closely the microbial interactions that occur during the biodegradation process. Especially provocative was the possibility that benzo[*a*]pyrene mineralization could be stimulated by water-soluble supernatant compounds which appear during bacterial growth on mostly sparingly water-soluble NAPLs. Water-soluble compounds, such as biologically produced biosurfactant- and bioemulsifier-like compounds (7, 53) or synthetic surfactants (52), may act as contributing factors in facilitating HMW PAH biodegradation by bacteria. Our aims in this study were to investigate the potential role of bioproduced compounds and to continue to improve our understanding of the complex ecological interactions that occur within this bacterial consortium. Below we discuss a second member of

* Corresponding author. Mailing address: Marine Biotechnology Institute, 3-75-1 Heita, Kamaishi City, Iwate, 026-0001, Japan. Phone: 81-193-26-5781. Fax: 81-193-26-6584. E-mail: kazuya.watanabe@mbio.jp.

† Present address: Department of Asian Studies, Cornell University, Ithaca, NY 14853.

‡ Present address: National Institute of Technology and Evaluation, Shibuya, Tokyo, Japan.

the genus *Rhodanobacter* in the context of a complex bacterial consortium that rapidly mineralizes benzo[*a*]pyrene and grows on NAPLs.

MATERIALS AND METHODS

Chemicals. [7-^{14}C]benzo[*a*]pyrene (26.6 mCi mmol^{-1}) was purchased from Sigma Chemical Co., and the reported radiochemical purity was ≥98%. [^{14}C]sodium bicarbonate (50.0 mCi mmol^{-1}; purity, ≥98%) was purchased from New England Nuclear. Unlabeled benzo[*a*]pyrene (purity, 98%), diethyl ether, and chloroform were purchased from Wako Chemical Co. Diesel fuel was originally obtained from Exxon Corp. A diesel fuel distillation product (HBD) was prepared from diesel fuel by heat distillation to approximately >310°C, accounted for 42% of the diesel fuel by weight, and consisted of gas chromatography (GC)-analyzable hydrocarbons no lower than tetradecane (29). The straight-chain alkanes *n*-pentadecane through *n*-octadecane, naphthalene, phenanthrene, anthracene, fluoranthene, pyrene, and γ-hexachlorocyclohexane (lindane) were purchased from Wako Chemical Co.

Maintenance of the consortium. A bacterial consortium which grew on diesel fuel or diesel fuel distillation products as sole carbon sources and which mineralized benzo[*a*]pyrene was recovered from soil that had been used previously in hydrocarbon fuel bioremediation studies (25, 28). Some of the consortium members were preliminarily identified by sequencing major bands of 16S rRNA gene fragments appearing on denaturing gradient gel electrophoresis profiles of the benzo[*a*]pyrene-mineralizing consortium (27). The consortium was maintained in 3-liter flasks which contained 300 ml of Stanier's basal medium (SBM) (5), 10 mg of benzo[*a*]pyrene liter^{-1}, and 0.2% (wt/vol) diesel fuel with continuous rotary shaking at 100 rpm at 30°C in the dark. Approximately every 14 days, 10 ml of the culture suspension was transferred to fresh medium.

Monitoring [7-^{14}C]benzo[*a*]pyrene mineralization in liquid culture. [7-^{14}C]benzo[*a*]pyrene mineralization was monitored in 300-ml Erlenmeyer flasks. For each flask two 16-gauge stainless steel syringe needles (lengths, 13 and 7 cm) were inserted through a silicone stopper, and the stopper was wrapped in Teflon tape. The 7-cm-long syringe needle was fitted with a one-way valve (Sigma), and the 13-cm-long syringe needle was fitted with a plastic plug. Experimental treatments were prepared by adding [7-^{14}C]benzo[*a*]pyrene plus other compounds (depending on the experiment), and then the flasks were sealed with the needle-containing silicone stoppers described above.

Addition of [7-^{14}C]benzo[*a*]pyrene to the flasks and monitoring of $^{14}CO_2$ evolution were performed as follows. A solution containing a known mass of benzo[*a*]pyrene and [7-^{14}C]benzo[*a*]pyrene was prepared by dissolving the components in a known volume of diethyl ether in a 10-ml glass vial sealed with a Teflon septum and aluminum crimp top. A known volume of this mixture was added to each of the flasks by using a gas-tight Hamilton microsyringe. After the diethyl ether was evaporated under a gentle N_2 stream, all remaining flask contents were added. Aqueous-phase additions and inocula were typically added to the flask after all other components were added. The flasks were incubated at 28°C in the dark with rotary shaking (150 rpm). The radiolabeled carbon dioxide released during [7-^{14}C]benzo[*a*]pyrene mineralization was trapped by flushing the flasks with air through a series of vials which contained Oxosol C^{14} (National Diagnostics). Radioactivity was measured with a model 1900CA Tri-Carb liquid scintillation analyzer (Packard Instrument Co.), and counts were corrected for background by using pure Oxosol C^{14}. The efficiency of the CO_2-trapping apparatus was checked routinely by transferring aliquots of an aqueous $NaH^{14}CO_3$ (pH 10) solution to sealed Erlenmeyer flasks and then adding HCl; the amount of the resulting $^{14}CO_2$ was measured.

Preparation of consortium supernatant liquid fractions for [7-^{14}C]benzo[*a*]pyrene radiorespirometry assays. Stimulation of benzo[*a*]pyrene mineralization by the consortium was tested after addition of sterilized supernatant liquid fractions that were obtained from the original consortium. Three fractions were prepared following consortium growth for 7 days in SBM which contained 0.1% (wt/vol) diesel fuel plus 10 mg of benzo[*a*]pyrene liter^{-1} as follows. Twelve-milliliter aliquots of the culture suspension were centrifuged at 10,000 × *g* for 20 min. The resultant supernatant liquid was collected and designated the raw supernatant liquid. Other 12-ml aliquots were filtered through Whatman no. 1 filter paper (Whatman, Maidstone, England) following centrifugation. Both the clear filtrate and the filter residue were recovered, and they were designated the supernatant liquid filtrate and the supernatant liquid filter residue, respectively. The supernatant liquid residue was obtained by carefully rinsing the filter paper with SBM, after which the recovered material was resuspended in 12 ml of SBM. The three supernatant liquid fractions (raw supernatant liquid, supernatant liquid filtrate, and supernatant liquid filter residue) were sterilized by autoclaving them at 121°C for 30 min. After this each fraction was added to flasks that were prepared for [7-^{14}C]benzo[*a*]pyrene radiorespirometry studies as described above.

Isolation of bacteria. Bacterial strains were isolated from the consortium grown on diesel fuel and benzo[*a*]pyrene by serial dilution followed by streaking on agar plates under various conditions. The agar plates (containing 1.5% Bacto Agar [Difco]) used for isolation contained one of the following media: SBM supplemented with phenanthrene or chrysene crystals, SBM supplemented with diesel fuel volatile compounds (0.8%, wt/vol), nutrient broth (Difco), or dCGY medium (composed of 0.1% [wt/vol] Casamino Acids, 0.1% [wt/vol] glycerol, and 0.1% [wt/vol] yeast extract, all obtained from Difco). The plates were incubated at 30°C. Colonies that formed on the plates were picked and purified by restreaking. Following isolation, all strains were maintained in liquid culture in SBM containing 0.1% (wt/vol) HBD, 10 mg of benzo[*a*]pyrene liter^{-1}, and 120 mg of PCY (40 mg of peptone [Difco] liter^{-1}, 40 mg of yeast extract liter^{-1}, and 40 mg of Casamino Acids liter^{-1}) liter^{-1}.

Benzo[*a*]pyrene biotransformation, mineralization, and mobilization assays with isolated bacteria. Bacterial inocula for benzo[*a*]pyrene biotransformation assays were cultured for 1 week on a medium containing 0.1% (wt/vol) HBD, 120 mg of PCY liter^{-1}, and 10 mg of benzo[*a*]pyrene liter^{-1}. A known mass of benzo[*a*]pyrene was dissolved in diethyl ether, and the resulting solution was added to the bottoms of 200-ml Erlenmeyer flasks with a microsyringe under aseptic conditions. After evaporation of the diethyl ether, 2 ml of inoculum, SBM, HBD, and PCY were aseptically added, and the flasks were incubated with continuous rotary shaking at 100 rpm at 30°C in the dark. After 14 days, the flasks were removed from the shaker, tetracosane (internal extraction standard) was added, and the culture fluids were extracted with 2 volumes of chloroform in separatory funnels. The chloroform extracts were each filtered through anhydrous sodium sulfate, concentrated to approximately 2 ml in a rotary evaporator, transferred to a volumetric flask, and resuspended in a known volume of chloroform. Analyses of culture extracts by GC-mass spectrometry (MS) were performed by using a QP-5000 instrument (Shimadzu) fitted with a fused silica capillary column (DB-5; 30 m by 0.25 mm; J & W Scientific). The temperature program was as follows: 50°C for 2 min, followed by an increase at a rate of 6°C per min to 300°C. The injection volume was 1 μl, and the carrier gas was helium (flow rate, 1.7 ml min^{-1}). The mass selective detector was operated in the scan mode to obtain spectral data for compound identification (benzo[*a*]pyrene molecular ion at *m/z* 252 and characteristic fragments at *m/z* 126).

In radiolabeled benzo[*a*]pyrene mineralization and mobilization assays, bacterial strains were grown for 1 week as described above, after which 3-ml portions each of culture suspension were transferred to 300-ml flasks which contained HBD (0.1%, wt/vol), PCY (120 mg liter^{-1}), and [7-^{14}C]benzo[*a*]pyrene (10 mg liter^{-1}). Each culture was grown under the conditions described above and was subjected to a radiorespirometry assay as described above. A set of abiotic controls were also prepared. After the flasks were monitored for $^{14}CO_2$ evolution for 2 weeks, the culture medium in each flask was decanted, and the inside of the flask was gently rinsed with SBM and air dried. The residue adhering to the inner glass surface of the flask was extracted twice with 100 ml of chloroform. The chloroform extract was concentrated in a water bath under a gentle nitrogen stream to known volumes in 20-ml graduated cylinders. Aliquots of the resulting concentrated extracts were transferred to liquid scintillation vials (Wheaton) in triplicate and combined with Scintiverse BD (Fisher Scientific). Radioactivity was measured with the scintillation analyzer, and the values were corrected by using background measurements.

Screening supernatant liquid filtrates of bacterial isolates for stimulation of benzo[*a*]pyrene mineralization. Bacterial isolates were cultivated in 200 ml of SBM which contained 0.1% (wt/vol) HBD, 10 mg of benzo[*a*]pyrene liter^{-1}, and 120 mg of PCY liter^{-1} in 2-liter baffled flasks at 30°C with rotary shaking (100 rpm). After 1 week of growth, the culture suspensions were centrifuged for 20 min at 13,100 × *g* and 4°C. The supernatant liquid was recovered and filtered through Whatman no. 1 filter paper to remove floating aggregates. This was followed by second filtration through 0.2-μm-pore-size nitrocellulose membranes (Millipore). The resulting supernatant liquid filtrate was autoclaved at 121°C for 30 min. In duplicate 300-ml flasks, 15 ml of supernatant liquid filtrate from each strain was added to an equal volume of SBM containing 120 mg of PCY liter^{-1}, 10 mg of benzo[*a*]pyrene liter^{-1}, and 1 ml of consortium inoculum. Positive and negative controls were also prepared. The positive control consisted of sterile supernatant liquid filtrate from a 1-week-old consortium growing on 0.1% (wt/vol) HBD and 120 mg of PCY liter^{-1} and exposed to 10 mg of benzo[*a*]pyrene liter^{-1}, and the negative control consisted of sterile supernatant liquid filtrate prepared abiotically from flasks that were incubated with 0.1% (wt/vol) HBD, 10 mg of benzo[*a*]pyrene liter^{-1}, and 120 mg of PCY liter^{-1} for 1 week without inoculum.

16S rRNA gene sequence analysis. Isolated strains were grown in liquid SBM cultures containing PCY, diesel fuel, and benzo[*a*]pyrene. Cells were collected by centrifugation (15,000 × *g*, 10 min), suspended in Tris-EDTA buffer, and extracted for recovery of total DNA as described previously (51). The DNA was subjected to PCR to amplify almost full-length 16S rRNA gene (16S rDNA) fragments by using the following primers: 5′-AGAGTTTGATCCTGGCTCA G-3′ (*Escherichia coli* 16S rDNA positions 8 to 27) and 5′-CAKAAAGGAGG TGATCC-3′ (*E. coli* 16S rDNA positions 1529 to 1546). Amplification was performed with a Progene thermal cycler (Techne) by using a 50-μl mixture containing 1.25 U of *Taq* DNA polymerase (Amplitaq Gold; Applied Biosystems), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (wt/vol) gelatin, each deoxynucleoside triphosphate at a concentration of 200 mM, 50 pmol of each primer, and 50 ng of template DNA. The PCR conditions used were as follows: 10 min of activation of the polymerase at 94°C, followed by 35 cycles consisting of 1 min at 94°C, 1 min at 52°C, and 2 min at 72°C, and finally 10 min of extension at 72°C. The PCR products were electrophoresed and then purified with a QIAquick gel extraction kit (QIAGEN). The nucleotide sequences of the PCR products were determined as described previously (15) by using a Dye terminator cycle DNA sequencing kit (Applied Biosystems) and a model 377 DNA sequencer (Applied Biosystems).

Database searches were conducted by using the BLAST program (2, 3) with the GenBank database. 16S rDNA sequences of strains BPC1 through BPC8 and sequences retrieved from the database were aligned by using the ClustalW software, version 1.7 (46), and alignments were refined by visual inspection. A neighbor-joining tree (41) was constructed by using the njplot software in ClustalW, version 1.7.

Substrate utilization, phenotypic, and taxonomic tests. The following carbon sources were tested as carbon and energy sources; *n*-pentadecane through *n*-octadecane, naphthalene, phenanthrene, anthracene, fluoranthene, pyrene, lindane, HBD, and PCY. In duplicate 300-ml flasks, SBM was inoculated with 100 μl of bacterial cells grown on PCY and one of the substrates at a concentration of 100 mg $liter^{-1}$ and incubated at 28°C in the dark with rotary shaking at 100 rpm. Culture samples were taken at regular intervals, and optical density was measured at 600 nm. Two weeks after cultivation was started, culture suspensions were extracted with chloroform and analyzed by GC-MS by using the procedures described above.

To test for the ability to utilize (oxidize) various carbon sources, a 96-well BIOLOG GN microplate (Biolog Inc.) was used as recommended by the manufacturer. The cells used for the Biolog tests were grown on 0.8% (wt/vol) PCY for approximately 48 h, harvested by centrifugation, washed, and resuspended in 0.85% sterile saline buffer. Phenotypic characteristics were also determined by using the API 20NE microtube system (API bioMérieux) as recommended by the manufacturer.

The guanine-plus-cytosine (G+C) content of the DNA of strain BPC1 was determined by high-performance liquid chromatography after the strain DNA was extracted, purified, and digested by nuclease P1 by using the method of Katayama-Fujimura et al. (30).

For microscopic characterization, cells were grown on PCY medium containing diesel fuel, and the morphology and dimensions of the organism were determined from photomicrographs obtained by using scanning electron microscopy and phase microscopy. For electron microscopy, cells were collected by centrifugation at approximately 10,000 × *g*, resuspended in water, and fixed on an aluminum plate by using a standard procedure (6). Cells were visualized with an S-2500 scanning electron microscope (Hitachi). The width and length values given below are the averages of several cell measurements.

cPCR. The abundance of strain BPC1 in the benzo[*a*]pyrene-mineralizing consortium was determined by competitive PCR (cPCR) by using the method described previously (51). The primers used for cPCR, 444-F (5′-CAGGAACG AAATCTGCATGC-3′) and 865-R (5′-TGCTTCGACACTGATCTCCG-3′), were designed by comparing the 16S rDNA sequence of strain BPC1 with the 16S rDNA sequences of closely related strains (see Fig. 5). The specificity of these primers was checked by using the probe match program in the Ribosomal Database Project (RDP) database (36). For comparison, the total bacterial abundance was determined by cPCR performed with primers P1 and P2 (38). Competitor fragments were produced by using a competitive DNA construction kit (Takara Shuzo). Amplification was performed as described above, except that an appropriate amount of the competitor fragment was added. The thermal cycle was as follows: 10 min of activation of the polymerase at 94°C, followed by 40 cycles consisting of 1 min at 94°C, 1 min at 55°C (for the total bacteria) or 60°C (for the strain BPC1 population), and 2 min at 72°C, and finally 10 min of extension at 72°C. Two microliters of the PCR product was electrophoresed through a 1.5% (wt/vol) agarose gel with Tris-borate-EDTA buffer, and the gel was photographed after it was stained with SYBR Gold I. Band intensity was quantified by using the Multianalyst software supplied with Gel Doc 2000 (Bio-Rad). The copy number was estimated by a method described elsewhere (34).

Nucleotide sequence accession numbers. The nucleotide sequences reported in this paper have been deposited in the GSDB, DDBJ, EMBL, and NCBI nucleotide sequence databases under accession no. AF494537 to AF494544.

RESULTS AND DISCUSSION

Effects of supernatant liquid fractions on benzo[*a*]pyrene mineralization. Rapid benzo[*a*]pyrene mineralization by the consortium occurs only when the consortium is supplied with a hydrocarbon NAPL, such as diesel fuel or HBD (27, 29). It was determined in a previous study that benzo[*a*]pyrene was mobilized into culture supernatant at levels greater than its aqueous solubility prior to its mineralization (27); more than 2 mg of benzo[*a*]pyrene $liter^{-1}$ was detected in suspension by radiotracer mass balances by using [7-^{14}C]benzo[*a*]pyrene (the aqueous solubility of benzo[*a*]pyrene is approximately 0.0038 mg $liter^{-1}$). It was hypothesized that solubilization of benzo[*a*]pyrene was a necessary step in the overall biodegradation process because benzo[*a*]pyrene bioavailability to the consortium organisms was increased. Increased bioavailability of target hydrophobic compounds to degrading organisms is an integral part of the biodegradation of these compounds, especially PAHs (18, 32).

In the present study, we first investigated the possibility that water-soluble compounds in the supernatant liquid might contribute to benzo[*a*]pyrene biodegradation by testing three supernatant liquid fractions from the growing consortium. The supernatant liquid fractions were obtained from a 1-week-old consortium culture which was fed diesel fuel and benzo[*a*]pyrene. Methylene chloride- and chloroform-extractable GC-analyzable diesel fuel compounds were mostly undetected at this time (data not shown). Our aim was to examine the supernatant liquid filtrate which contained neither consortium cells, polymer-like matrix, nor diesel fuel residues. Some undegraded diesel fuel compounds did not settle during the centrifugation step, and therefore the filtration step was included. When the consortium inoculum was added to SBM which contained [7-^{14}C]benzo[*a*]pyrene and the recovered supernatant liquid fractions (neither diesel fuel nor HBD was added), it was found that benzo[*a*]pyrene mineralization was stimulated in all cases and to various extents (Fig. 1). After 3 weeks, the sterile raw supernatant liquid and the sterile filter residue stimulated approximately 35 and 17% conversion of [7-^{14}C]benzo[*a*]pyrene to carbon dioxide, respectively. However, the sterile supernatant liquid filtrate also stimulated approximately 17% conversion of [7-^{14}C]benzo[*a*]pyrene. The fact that diesel fuel components were unable to pass through the filter indicated that the supernatant liquid filtrate contained soluble stimulatory compounds produced by the consortium. The stimulatory effect of the sterile filter residue was most likely due to the presence of metabolites or undegraded diesel fuel components or both. The negative control, which consisted of inoculum only without additional treatment, stimulated less than 10% mineralization.

Isolation of consortium members and their effects. Following the detection of benzo[*a*]pyrene mineralization stimulation by the sterile supernatant liquid filtrate, we were interested in examining specific consortium strains to determine which strains were instrumental in benzo[*a*]pyrene biodegradation.

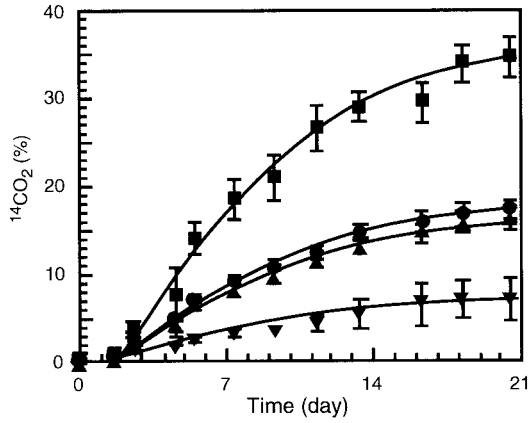

FIG. 1. Mineralization of 10 mg of [7-^{14}C]benzo[*a*]pyrene liter^{-1} by a bacterial consortium treated with different supernatant liquid fractions. The treatments consisted of sterile raw supernatant liquid from the parent consortium (■), sterile supernatant liquid filtrate from the parent consortium (●), sterile supernatant liquid filter residue from the parent consortium (▲), and inoculum only without treatment (negative control) (▼). Each point represents the average for triplicate flasks, and the error bars indicate the standard deviations; error bars smaller than the symbols are not shown.

Eight bacterial isolates were selected for further investigation based on (i) the similarity of their 16S rDNA sequences to the sequences of denaturing gradient gel electrophoresis bands that represented major members of the benzo[*a*]pyrene-mineralizing consortium (27) or (ii) the similarity of their sequences to the sequences of organisms that are known to have properties relevant to benzo[*a*]pyrene biodegradation (e.g., strains known to produce biosurfactant or biodegrade persistent compounds). Information regarding the eight strains, designated BPC1 (benzo[*a*]pyrene consortium 1) through BPC8, is given in Table 1. The strains were isolated on various carbon sources, as indicated in Table 1, and the 16S rDNA of each strain was partially sequenced. Two strains were isolated by using chrysene, and although we have not yet examined the catabolic capabilities of these strains in detail, we have found that the consortium is capable of mineralizing radiolabeled chrysene to $^{14}CO_2$ (unpublished data). We found that the 16S rDNA sequences of three of the eight strains were 100% identical to the sequences of major bands appearing in denaturing gradient gel electrophoresis profiles for the benzo[*a*]pyrene-mineralizing consortium (27), *Ralstonia eutropha*, *Sphingomonas paucimobilis*, and *Mycobacterium ratisbonense.* Two of the other strains isolated were found to be members of the same genera of organisms previously identified by denaturing gradient gel electrophoresis, namely, *Burkholderia* and *Sphingobacterium*. All strains were tested individually in radiorespirometry studies for the ability to cometabolically mineralize radiolabeled benzo[*a*]pyrene when they were incubated for 3 weeks with 0.1% HBD. HBD was used as a carbon source throughout these studies because it was previously found to facilitate mineralization of benzo[*a*]pyrene by the consortium at a higher rate than the rate facilitated by diesel fuel (29). The radiorespirometry assays showed that none of the individual strains could mineralize benzo[*a*]pyrene singly or when it was combined into an eight-member gnotobiotic consortium (data not shown). Additionally, we tested each strain individually for the ability to biotransform nonradiolabeled benzo[*a*]pyrene when it was incubated with 0.1% HBD for 2 weeks. Our aim was to identify an organism that might be instrumental in initiating the benzo[*a*]pyrene biodegradation process, at least. GC-MS analyses following culture medium extraction indicated that none of the strains was able to significantly biotransform benzo[*a*]pyrene singly or when it was recombined (the level of benzo[*a*]pyrene recovery for each strain is shown in Table 1). Indeed, it is difficult to isolate benzo[*a*]pyrene-degrading organisms by serial dilution or plating techniques, presumably because benzo[*a*]pyrene biodegradation most likely occurs by a cooperative mechanism involving at least several consortium members.

After we understood that the strains did not possess benzo[*a*]pyrene mineralization or biotransformation capabilities, we were interested to know if any of the strains was more or less capable of contributing to the mobilization of benzo[*a*]pyrene into the culture supernatant. Therefore, in addition to the radiorespirometry assays, we also measured [7-^{14}C]benzo[*a*]pyrene mobilization from the inner surfaces of glass flasks to the culture medium. As shown in Fig. 2, all strains mobilized benzo[*a*]pyrene into the culture medium compared to the abiotic control, but some strains were capable of mobilizing more than 75% of the benzo[*a*]pyrene. The results of this assay suggested that individual strains produced compounds during growth on HBD hydrocarbons which contributed to the overall

TABLE 1. Strains isolated from the consortium

Strain	Carbon source isolated from[a]	No. of base pairs sequenced	% Similarity of isolated strain and database sequences	Most closely related organism	Accession no.	Taxonomic group	% of benzo[*a*]pyrene recovered[b]
BPC1	dCGY	1,504	98	*Rhodanobacter lindanoclasticus*	AF039167	γ-*Proteobacteria*	93.7 ± 4.2
BPC2	Phenanthrene	1,459	99	*Burkholderia cepacia*	AF311971	β-*Proteobacteria*	89.6 ± 10
BPC3	Nutrient	1,435	99	*Ralstonia eutropha*	M32021	β-*Proteobacteria*	93.3 ± 3.7
BPC4	Diesel fuel vapor	1,331	97	*Sphingomonas paucimobilis*	X94100	α-*Proteobacteria*	95.4 ± 4.2
BPC5	Chrysene	1,450	100	*Mycobacterium ratisbonense*	AF055331	Actinomycetes	93.4 ± 1.9
BPC6	Chrysene	1,339	99	*Ochrobactrum anthropi*	U88441	α-*Proteobacteria*	92.6 ± 3.7
BPC7	Nutrient	1,468	98	*Achromobacter xylosoxidans*	AF225979	β-*Proteobacteria*	88.3 ± 0.3
BPC8	dCGY	1,030	91	*Sphingobacterium comitans*	X91814	*Cytophagales*	93.8 ± 3.6

[a] Strains from the diesel fuel-maintained consortium were isolated after serial dilution and streaking onto agar plates. See text for details.

[b] All strains were screened for the ability to biotransform benzo[*a*]pyrene cometabolically when they were grown on HBD. Benzo[*a*]pyrene was recovered by chloroform extraction and was quantified by GC-MS. The level of recovery of benzo[*a*]pyrene for abiotic controls was 95.8% ± 2.3%. The level of recovery of benzo[*a*]pyrene for the original consortium (positive control) was 33.5% ± 0.5%.

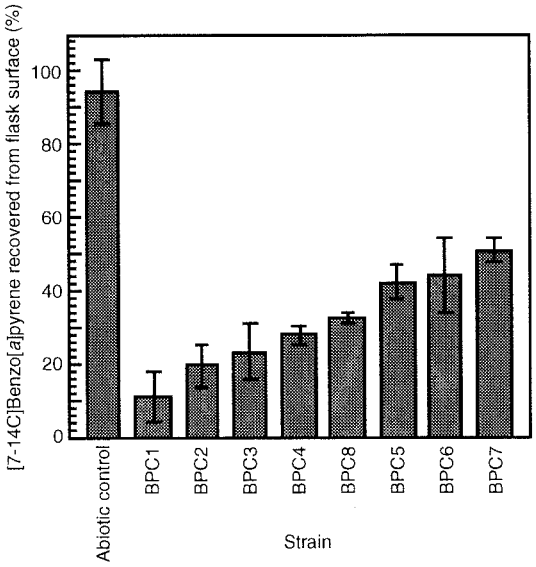

FIG. 2. [7-^{14}C]benzo[*a*]pyrene recovered from flask surfaces after incubation with strains BPC1 through BPC8. The error bars indicate the ranges for duplicates.

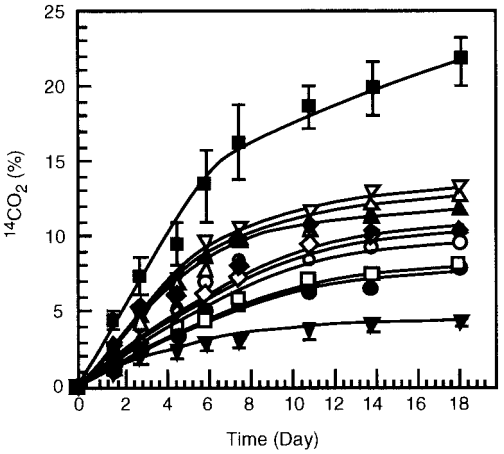

FIG. 3. Mineralization of 10 mg of [7-^{14}C]benzo[*a*]pyrene liter^{-1} by a bacterial consortium treated with sterile supernatant liquid filtrates obtained from individual bacterial strains. The supernatants tested were obtained from cultures of strain BPC1 (■), BPC2 (▽), BPC3 (▲), BPC4 (●), BPC5 (△), BPC6 (♦), BPC7 (○), and BPC8 (□). For comparison, sterile supernatant liquid filtrates obtained from the original consortium (◇) and from an uninoculated control (▼) were also assayed. Each point represents the average for duplicate treatments. The error bars indicate the ranges and are shown only for strain BPC1 supernatant liquid filtrate and the uninoculated control supernatant liquid filtrate for clarity.

benzo[*a*]pyrene mineralization process by mobilizing benzo[*a*]pyrene into the culture medium. Considering these results, we next examined the supernatant liquid filtrates from the individual strains for the ability to facilitate benzo[*a*]pyrene mineralization by the original consortium in the absence of diesel fuel fractions.

Effects of supernatant liquid filtrates from individual strains on benzo[*a*]pyrene mineralization by the consortium. The results of the radiorespirometry assays used to examine the ability of the supernatant liquid filtrates from individual strains to stimulate benzo[*a*]pyrene mineralization by the consortium inoculum without diesel fuel fractions are shown in Fig. 3. Figure 3 shows that the sterile supernatant liquid filtrate of strain BPC1 stimulated benzo[*a*]pyrene mineralization most vigorously; it stimulated mineralization approximately twofold compared to the mineralization in the presence of sterile supernatant liquid filtrates obtained from the original consortium or from the other strains. This large stimulation effect of strain BPC1 compared to the stimulation effects of the other seven strains from the consortium resulted in an effort to further elucidate the role of strain BPC1 in the consortium.

When the consortium culture was grown on a hydrocarbon NAPL such as HBD, benzo[*a*]pyrene mineralization began only after a lag period and then occurred exponentially, as shown in Fig. 4. The overall effect was a characteristic sigmoid curve which represented conversion of carbon 7 in the benzo[*a*]pyrene molecule to carbon dioxide. Such a pattern of benzo[*a*]pyrene mineralization must have occurred because the mineralization step was linked to the growth of some population(s) in the consortium (29). Exponential growth of cells in the consortium occurs during the utilization of NAPL constituent compounds, and during this process some type of fortuitous or cooperative metabolism of benzo[*a*]pyrene occurs. However, when the rate of benzo[*a*]pyrene mineralization is governed by the solubilization rate, mineralization must occur in a linear fashion with little or no lag time (29). Figure 4 shows that this was the case for the effect of each of the supernatant liquid filtrates. In these cases, benzo[*a*]pyrene became more bioavailable to the benzo[*a*]pyrene-mineralizing but weakly growing population (no additional carbon source was added) via a physical effect that was most likely facilitated by the action of surfactant-like water-soluble compounds present in the culture supernatant. Generally, surfactants and nonsubstrate NAPLs may facilitate biodegradation of PAHs through an

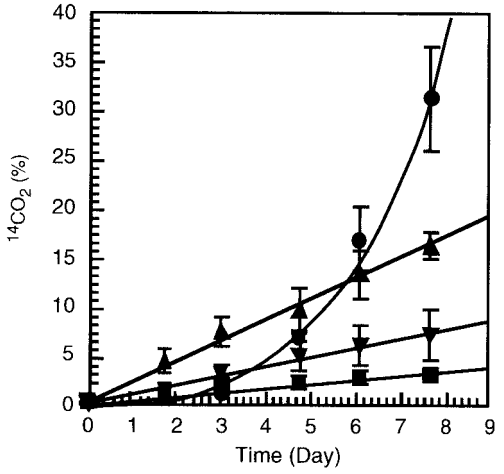

FIG. 4. Mineralization of 10 mg of [7-^{14}C]benzo[*a*]pyrene liter^{-1} by the bacterial consortium treated with HBD (●), supernatant liquid filtrate from strain BPC1 (▲), supernatant liquid filtrate from the original consortium (▼), and supernatant liquid filtrate from an uninoculated control (abiotic preculture) (■). Each point represents the average for duplicate treatments, and the error bars indicate the ranges.

increase in the PAH-bacterium contact area. NAPL effects on PAH biodegradation have been shown in various studies (16, 17, 33), including a study of the consortium examined in this study (29).

Substrate utilization patterns of isolated strains, including BPC1. Strains BPC1 through BPC8 were incubated with HBD as a sole carbon source for 2 weeks, and this was followed by a GC-MS analysis of extracted culture suspensions. Most of the strains degraded the GC-analyzable fraction of HBD (data not shown); the only exception was strain BPC1, and the results for this strain were similar to the results for the abiotic controls. Indeed, the results for strain BPC1 were examined further by subjecting the organism to growth requirement assays with single hydrocarbon compounds. The results of these assays indicated that strain BPC1 was unable to utilize any of the single hydrocarbon compounds tested for growth, which included straight-chain alkanes (pentadecane through octadecane), naphthalene, phenanthrene, anthracene, fluoranthene, and pyrene. Growth occurred only in a PCY positive control culture. In addition, strain BPC1 failed to grow on the same hydrocarbon compounds when the culture was supplemented with PCY as there was no detectable difference between the growth that occurred in the PCY positive control and the growth that occurred in the hydrocarbon-containing cultures supplemented with PCY. Interestingly, strain BPC1 cells also failed to grow when they were fed HBD. In experiments in which BPC1 was incubated with HBD and PCY and the culture suspensions were extracted and analyzed by GC-MS, the data were inconclusive. We could not detect a noticeable difference in the biodegradation patterns between the BPC1 treatments and the abiotic controls.

Growth of BPC1 in the benzo[*a*]pyrene-mineralizing consortium. cPCR performed with primers 444-F and 865-R was used to quantify the BPC1 population in the benzo[*a*]pyrene-mineralizing consortium in order to determine if it actually grew and played any role in the consortium fed only HBD and benzo[*a*]pyrene (Fig. 5). The RDP search with the probe match program (36) indicated that there was no sequence which completely matched either 444-F or 865-R in the database. One 16S rDNA sequence (that of *Rhodanobacter lindaniclasticus* RP5557) exhibited one mismatch with 444-F, while another sequence (that of *Frateuria aurantia* IFO 3245) exhibited one mismatch with 865-F. Other sequences in the RDP database exhibited at least two mismatches with 444-F and 865-R. This database analysis demonstrated the high specificity of these primers for BPC1. The utility of cPCR for analyzing the population dynamics of specific microorganisms in microbial communities has been demonstrated and discussed in several studies (35, 50).

Figure 5 shows the population dynamics of BPC1 over time compared to the dynamics of the total bacterial populations and to the benzo[*a*]pyrene mineralization pattern. The data indicate that BPC1 actually grew in the consortium, which occurred concomitant with the total growth of bacteria in the consortium. In addition, the data show that the growth of BPC1 preceded benzo[*a*]pyrene mineralization. Understanding that strain BPC1 was incapable of growth on HBD and benzo[*a*]pyrene, we surmised that strain BPC1 required metabolites produced during the growth of other consortium members to facilitate its growth in the consortium. This idea is supported by the recent observation that there are active exchanges of intermediate metabolites in bacterial consortia involved in hydrocarbon degradation (40). It is likely that the growth of BPC1 in the consortium was beneficial to benzo[*a*]pyrene mineralization, as was the supernatant liquid filtrate of the BPC1 pure culture.

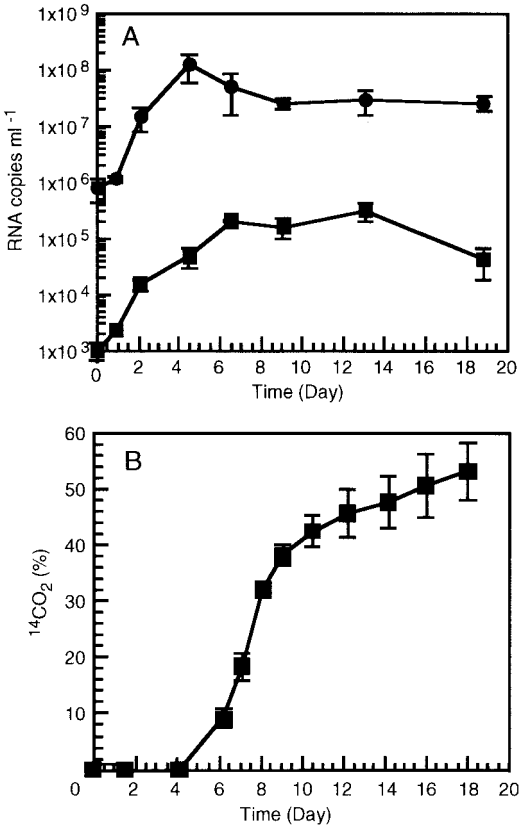

FIG. 5. (A) Growth of strain BPC1 (■) compared to the total growth of bacterial populations (●) in the benzo[*a*]pyrene-mineralizing consortium as determined by cPCR. (B) Time course of benzo[*a*]pyrene mineralization ($^{14}CO_2$ evolution). The points represent the averages for triplicate determinations, and the error bars indicate the standard deviations; error bars smaller than the symbols are not shown.

Characteristics of strain BPC1. A phylogenetic analysis performed with 16S rDNA sequences indicated that strain BPC1 was most closely related to *R. lindaniclasticus* strain RP5557 (98% identity). *R. lindaniclasticus* was isolated because of its ability to degrade the environmentally persistent pesticide lindane (39, 45); unfortunately, however, this strain is not extant (D. Janssens, BCCM/LMG Bacteria Collection, personal communication). The total DNA of strain BPC1 had a G+C content of 67.9 mol%, a value that differed slightly from the 63.0 mol% G+C content described for *R. lindaniclasticus* (39). Physiological comparisons of strain BPC1 and *R. lindaniclasticus* indicated that there were some differences between the two organisms. Strain BPC1 was capable of utilizing some compounds that *R. lindaniclasticus* was reported to be unable to utilize (39), including D-fructose, D-mannose, maltose, lactose, D-raffinose, glycogen, xylitol, *N*-acetylglucosamine, *B*-gentiobiose, methyl alpha-glucoside, D-turanose, glucanate, and mal-

onate. In addition, strain BPC1 was positive for arginine dihydrolase in phenotypic tests. Strain BPC1 was a gram-negative, rod-shaped, nonmotile organism that was originally isolated by directly spreading a dilution of the consortium culture on dCGY agar plates (50). On dCGY agar plates, colonies of strain BPC1 were translucent whitish yellow, smoothly circular, 0.2 to 2 mm in diameter, and slightly raised with regular edges. The cells were approximately 0.8 to 1.2 μm long and 0.3 to 0.6 μm wide. Flagella were not observed by scanning electron microscopy, and the cells divided by binary fission. In contrast to strain RP5557, strain BPC1 failed to grow on nutrient agar plates after 4 weeks of incubation at 30°C and, most notably, was unable to utilize lindane in liquid culture growth assays. Based on these results, we tentatively identified strain BPC1 as a *Rhodanobacter* sp. strain. Further studies are needed to propose a new species for strain BPC1.

Conclusions. Rapid mineralization of environmentally relevant concentrations of benzo[*a*]pyrene was stimulated by water-soluble products obtained from a growing bacterial consortium. Previous discussions regarding the biodegradation of benzo[*a*]pyrene and most HMW PAHs (PAHs with more than three rings) in nature have focused on the degradation of these compounds by fortuitous metabolism. Typically, benzo[*a*]pyrene biodegradation by bacteria occurs through fortuitous metabolism, in which benzo[*a*]pyrene-degrading organisms grow on a primary PAH or hydrocarbon cosubstrate having relatively low water solubility as a source of carbon and energy. During growth on the cosubstrate, benzo[*a*]pyrene is biodegraded. In a previous study (27), it was shown that benzo[*a*]pyrene mineralization occurred only when the consortium was supplied with a suitable hydrocarbon cosubstrate. In this study, we determined that biologically produced aqueous-phase compounds may stimulate benzo[*a*]pyrene mineralization by a consortium.

Solubilization of benzo[*a*]pyrene into the culture suspension seems to be an essential step for rapid mineralization of the compound by the consortium. *Rhodanobacter* sp. strain BPC1 was identified as a bacterial strain in a population involved in this solubilization step. Interestingly, although strain BPC1 was unable to grow on diesel fuel constituents and benzo[*a*]pyrene in pure culture, it grew in the consortium fed HBD and benzo[*a*]pyrene, probably by utilizing metabolites produced by fellow members. We therefore believe that mineralization of benzo[*a*]pyrene is a cooperative task involving bacteria with specialized niches, and this may be a reason why we have failed to isolate bacteria capable of degrading and mineralizing benzo[*a*]pyrene.

Although benzo[*a*]pyrene mobilization and mineralization were also influenced by other isolates in the consortium, strain BPC1 was the most active organism. Ten organisms belonging to the consortium were preliminarily identified previously by molecular community analyses (27). Five of the eight isolates that were recovered from the consortium in the present study were members of the same genera that contain these previously identified organisms. However, when the isolates were recombined into an eight-member gnotobiotic consortium, benzo[*a*]pyrene was not biodegraded. Indeed, it seems that a key strain that is needed to support benzo[*a*]pyrene mineralization by providing either cofactors or metabolites to strain BPC1 or by acting directly in the benzo[*a*]pyrene biotransformation process has yet to be recovered.

Research developments over the past 10 years have helped shape the idea that microbial consortia as entities may be more versatile and effective in cleaning up environmental pollution. Indeed, microbial cooperation may promote broader and more efficient in situ degradation of complex pollutant mixtures (49). Recently, the use of microbial consortia has gained widespread support due to advances in molecular biology and the resulting ability of microbiologists to study and monitor such populations (13). Microbial consortia may possess more potential for cleaning up polluting waste because the organisms complement each other through interdependence. Unraveling the microbial consortium black box is a difficult task, especially when the organisms are growing on complex substrates, such as fuel products. However, knowledge can be garnered from bioconsortium studies and can contribute to our understanding of these microecosystems. Future investigations of the types and properties of the compound(s) produced by *Rhodanobacter* strain BPC1 during the NAPL-benzo[*a*]pyrene hydrocarbon biodegradation process are planned.

ACKNOWLEDGMENTS

We gratefully acknowledge Fusako Numazaki for her expert microbiological and molecular technical assistance. We also acknowledge Atsuko Katsuta for assistance with isolate characterization and Miyuki Nishijima for performing G+C content analyses.

This work was supported by the New Energy and Industrial Technology Development Organization (NEDO).

REFERENCES

1. **Aitken, M. D., W. T. Stringfellow, R. D. Nagel, C. Kazunga, and S.-H. Chen.** 1998. Characteristics of phenanthrene-degrading bacteria isolated from soils contaminated with polycyclic aromatic hydrocarbons. Can. J. Microbiol. **44:** 743–752.
2. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
3. **Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
4. **Angerer, J., C. Mannschreck, and J. Gündel.** 1997. Biological monitoring and biochemical effect monitoring of exposure to polycyclic aromatic hydrocarbons. Int. Arch. Occup. Environ. Health **70:**365–377.
5. **Atlas, R. M.** 1993. Handbook of microbiological media. CRC Press, Inc., Boca Raton, Fla.
6. **Beveridge, T. J., T. J. Popkin, and R. M. Cole.** 1994. Electron microscopy, p. 42–71. *In* P. Gerhardt, R. G. E. Murray, W. A. Wood, and N. R. Krieg (ed.), Methods for general and molecular bacteriology. American Society for Microbiology, Washington, D.C.
7. **Boonchan, S., M. L. Britz, and G. A. Stanley.** 1998. Surfactant-enhanced biodegradation of high molecular weight polycyclic aromatic hydrocarbons by *Stenotrophomonas maltophila*. Biotechnol. Bioeng. **59:**482–494.
8. **Boonchan, S., M. L. Britz, and G. A. Stanley.** 2000. Degradation and mineralization of high-molecular-weight polycyclic aromatic hydrocarbons by defined fungal-bacterial cocultures. Appl. Environ. Microbiol. **66**:1007–1019.
9. **Bossert, I. D., and G. Compeau.** 1995. Cleanup of petroleum hydrocarbon contamination in soil, p. 77–126. *In* L. Y. Young and C. E. Cerniglia (ed.), Microbial transformation and degradation of toxic organic chemicals. Wiley-Liss, New York, N.Y.
10. **Cerniglia, C. E.** 1992. Biodegradation of polycyclic aromatic hydrocarbons. Biodegradation **3:**351–368.
11. **Chen, S.-H., and M. D. Aitken.** 1999. Salicylate stimulates the degradation of high-molecular weight polycyclic aromatic hydrocarbons by *Pseudomonas saccharophila* P15. Environ. Sci. Technol. **33:**435–439.
12. **Collins, J. F., J. P. Brown, G. V. Alexeeff, and A. G. Salmon.** 1998. Potency equivalency factors for some polycyclic aromatic hydrocarbons and polycyclic aromatic hydrocarbon derivatives. Regul. Toxicol. Pharmacol. **28:**45–54.
13. **Davey, M. E., and G. A. O'Toole.** 2000. Microbial biofilms: from ecology to molecular genetics. Microbiol. Mol. Biol. Rev. **64:**847–867.
14. **Davila, D. R., D. P. Davis, K. Campbell, J. C. Cambier, L. A. Zigmond, and S. C. Burchiel.** 1995. Role of alterations in Ca^{2+}-associated signaling pathways in the immunotoxicity of polycyclic aromatic hydrocarbons. J. Toxicol. Environ. Health **45:**101–126.

15. **Edwards, U., T. Rogall, H. Blöcker, M. Emde, and E. C. Bőttger.** 1989. Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. Nucleic Acids Res. **17:**7843–7853.
16. **Efroymson, R. A., and M. Alexander.** 1991. Biodegradation by an *Arthrobacter* species of hydrocarbons partitioned into an organic solvent. Appl. Environ. Microbiol. **57:**1441–1447.
17. **Efroymson, R. A., and M. Alexander.** 1994. Biodegradation in soil of hydrophobic pollutants in nonaqueous-phase liquids (NAPLs). Environ. Toxicol. Chem. **13:**405–411.
18. **Harms, H., and T. N. P. Bosma.** 1997. Mass transfer limitation of microbial growth and pollutant degradation. J. Ind. Microbiol. Biotechnol. **18:**97–105.
19. **Juhasz, A. L., M. L. Britz, and G. A. Stanley.** 1996. Degradation of high molecular weight polycyclic aromatic hydrocarbons by *Pseudomonas cepacia*. Biotechnol. Lett. **18:**577–582.
20. **Juhasz, A. L., M. L. Britz, and G. A. Stanley.** 1997. Degradation of fluoranthene, pyrene, benz[*a*]anthracene and dibenz[*a,h*]anthracene by *Burkholderia cepacia*. J. Appl. Microbiol. **83:**189–198.
21. **Juhasz, A. L., M. L. Britz, and G. A. Stanley.** 2000. Evaluation of a creosote-based medium for the growth and preparation of a PAH-degrading bacterial community for bioaugmentation. J. Ind. Microbiol. Biotechnol. **24:**277–284.
22. **Juhasz, A. L., and R. Naidu.** 2000. Bioremediation of high molecular weight polycyclic aromatic hydrocarbons: a review of the microbial degradation of benzo[*a*]pyrene. Int. Biodeterior. Biodeg. **45:**57–88.
23. **Juhasz, A. L., G. A. Stanley, and M. L. Britz.** 2000. Microbial degradation and detoxification of high molecular weight polycyclic aromatic hydrocarbons by *Stenotrophomonas maltophila* strain VUN 10,003. Lett. Appl. Microbiol. **30:**396–401.
24. **Kalf, D. F., T. Crommentuijn, and E. J. van de Plassche.** 1997. Environmental quality objectives for 10 polycyclic aromatic hydrocarbons (PAHs). Ecotoxicol. Environ. Safety **36:**89–97.
25. **Kanaly, R., R. Bartha, S. Fogel, and M. Findlay.** 1997. Biodegradation of [^{14}C]benzo[*a*]pyrene added in crude oil to uncontaminated soil. Appl. Environ. Microbiol. **63:**4511–4515.
26. **Kanaly, R., and S. Harayama.** 2000. Biodegradation of high-molecular-weight polycyclic aromatic hydrocarbons by bacteria. J. Bacteriol. **182:**2059–2067.
27. **Kanaly, R. A., R. Bartha, K. Watanabe, and S. Harayama.** 2000. Rapid mineralization of benzo[*a*]pyrene by a microbial consortium growing on diesel fuel. Appl. Environ. Microbiol. **66:**4205–4211.
28. **Kanaly, R. A., and R. Bartha.** 1999. Cometabolic mineralization of benzo-[*a*]pyrene caused by hydrocarbon additions to soil. Environ. Toxicol. Chem. **18:**2186–2190.
29. **Kanaly, R. A., R. Bartha, K. Watanabe, and S. Harayama.** 2001. Enhanced mineralization of benzo[*a*]pyrene in the presence of nonaqueous phase liquids. Environ. Toxicol. Chem. **20:**498–501.
30. **Katayama-Fujimura, Y., Y. Komatsu, H. Kuraishi, and T. Kaneko.** 1984. Estimation of DNA base composition by high performance liquid chromatography of its nuclease P1 hydrolysate. Agric. Biol. Chem. **48:**3169–3172.
31. **Keith, L. H., and W. A. Telliard.** 1979. Priority pollutants I—a perspective view. Environ. Sci. Technol. **13:**416–423.
32. **Köhler, A., M. Schüttoff, D. Bryniok, and H.-J. Knackmuß.** 1994. Enhanced biodegradation of phenanthrene in a biphasic culture system. Biodegradation **5:**93–103.
33. **Labare, M. P., and M. Alexander.** 1995. Enhanced mineralization of organic compounds in nonaqueous-phase liquids. Environ. Toxicol. Chem. **14:**257–265.
34. **Lee, S. Y., J. Bollinger, D. Bezdicek, and A. Ogram.** 1996. Estimation of the abundance of an uncultured soil bacterial strain by a competitive quantitative PCR method. Appl. Environ. Microbiol. **62:**3787–3793.
35. **Leser, T. D., M. Boye, and N. Hendriksen.** 1995. Survival and activity of *Pseudomonas* sp. strain B13 (FR1) in a marine microcosm determined by quantitative PCR and an rRNA-targeting probe and its effect on indigenous bacterioplankton. Appl. Environ. Microbiol. **61:**1201–1207.
36. **Maidak, B. L., J. R. Cole, C. T. Parker, Jr., G. M. Garrity, N. Larsen, B. Li, T. G. Lilburn, M. J. McCaughey, G. J. Olsen, R. Overbeek, S. Pramanik, T. M. Schmidt, J. M. Tiedje, and C. R. Woese.** 1999. A new version of the RDP (Ribosomal Database Project). Nucleic Acids Res. **27:**171–173.
37. **Marcoux, J., E. Déziel, R. Villemur, F. Lépine, J.-G. Bisaillon, and R. Beaudet.** 2000. Optimization of high-molecular-weight polycyclic aromatic hydrocarbons' degradation in a two-liquid-phase bioreactor. J. Appl. Microbiol. **88:**655–662.
38. **Muyzer, G., E. C. de Waal, and A. G. Uitterlinden.** 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. **59:**695–700.
39. **Nalin, R., P. Simonet, T. M. Vogel, and P. Normand.** 1999. *Rhodanobacter lindaniclasticus* gen. nov., sp. nov., a lindane-degrading bacterium. Int. J. Syst. Bacteriol. **49:**19–23.
40. **Pelz, O., M. Tesar, R.-M. Wittich, E. R. B. Moore, K. N. Timmis, and W.-R. Abraham.** 1999. Towards elucidation of microbial community metabolic pathways: unravelling the network of carbon sharing in a pollutant-degrading bacterial consortium by immunocapture and isotopic ratio mass spectrometry. Environ. Microbiol. **1:**167–174.
41. **Saitou, N., and M. Nei.** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4:**406–425.
42. **Schneider, J., R. Grosser, K. Jayasimhulu, W. Xue, and D. Warshawsky.** 1996. Degradation of pyrene, benz[*a*]anthracene, and benzo[*a*]pyrene by *Mycobacterium* sp. strain RJGII-135, isolated from a former coal gasification site. Appl. Environ. Microbiol. **62:**13–19.
43. **Shuttleworth, K. L., and C. E. Cerniglia.** 1995. Environmental aspects of PAH biodegradation. Appl. Biochem. Biotechnol. **54:**291–302.
44. **Sutherland, J. B., F. Rafii, A. A. Khan, and C. E. Cerniglia.** 1995. Mechanisms of polycyclic aromatic hydrocarbon degradation, p. 269–306. *In* L. Y. Young and C. E. Cerniglia (ed.), Microbial transformation and degradation of toxic organic chemicals. Wiley-Liss, New York, N.Y.
45. **Thomas, J.-C., F. Berger, M. Jacquier, D. Bernillon, F. Baud-Grasset, N. Truffaut, P. Normand, T. M. Vogel, and P. Simonet.** 1996. Isolation and characterization of a novel gamma-hexachlorocyclohexane-degrading bacterium. J. Bacteriol. **178:**6049–6055.
46. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:**4673–4680.
47. **Trzesicka-Mlynarz, D., and O. P. Ward.** 1995. Degradation of polycyclic aromatic hydrocarbons (PAHs) by a mixed culture and its component pure cultures, obtained from PAH-contaminated soil. Can. J. Microbiol. **41:**470–476.
48. **Villemur, R., E. Déziel, A. Benachenhou, J. Marcoux, E. Gauthier, F. Lépine, R. Beaudet, and Y. Comeau.** 2000. Two-liquid-phase slurry bioreactors to enhance the degradation of high-molecular-weight polycyclic aromatic hydrocarbons in soil. Biotechnol. Prog. **16:**966–972.
49. **Wackett, L. P., and C. D. Hershberger.** 2001. Biocatalysis and biodegradation: microbial transformation of organic compounds. ASM Press, Washington, D.C.
50. **Watanabe, K., M. Teramoto, H. Futamata, and S. Harayama.** 1998. Molecular detection, isolation, and physiological characterization of functionally dominant phenol-degrading bacteria in activated sludge. Appl. Environ. Microbiol. **64:**4396–4402.
51. **Watanabe, K., M. Teramoto, and S. Harayama.** 1999. An outbreak of non-flocculating catabolic populations caused the breakdown of a phenol-digesting activated-sludge process. Appl. Environ. Microbiol. **65:**2813–2819.
52. **Willumsen, P. A., and E. Arvin.** 1999. Kinetics of degradation of surfactant-solubilized fluoranthene by a *Sphingomonas paucimobilis*. Environ. Sci. Technol. **33:**2571–2578.
53. **Willumsen, P. A., and U. Karlson.** 1997. Screening of bacteria, isolated from PAH-contaminated soils, for production of biosurfactants and bioemulsifiers. Biodegradation **7:**415–423.
54. **Ye, D., M. A. Siddiqi, A. E. Maccubbin, S. Kumar, and H. C. Sikka.** 1996. Degradation of polynuclear aromatic hydrocarbons by *Sphingomonas paucimobilis*. Environ. Sci. Technol. **30:**137–142.