APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 2002, p. 6106–6113 Vol. 68, No. 12
0099-2240/02/$04.00+0 DOI: 10.1128/AEM.68.12.6106–6113.2002

Linking of Microorganisms to Phenanthrene Metabolism in Soil by Analysis of ^{13}C-Labeled Cell Lipids

Anders R. Johnsen,[1]* Anne Winding,[1] Ulrich Karlson,[1] and Peter Roslev[2]

National Environmental Research Institute, Department of Environmental Chemistry and Microbiology, DK-4000 Roskilde,[1] and Department of Environmental Engineering, Aalborg University, DK-9000 Aalborg,[2] Denmark

Received 3 June 2002/Accepted 12 September 2002

Phenanthrene-metabolizing soil microbial communities were characterized by examining mineralization of [^{14}C]phenanthrene, by most-probable-number (MPN) counting, by 16S-23S spacer DNA analysis of the numerically dominant, culturable phenanthrene-degrading isolates, and by examining incorporation of [^{13}C]phenanthrene-derived carbon into sterols and polar lipid fatty acids (PLFAs). An unpolluted agricultural soil, a roadside soil diffusely polluted with polycyclic aromatic hydrocarbons (PAHs), and two highly PAH-polluted soils from industrial sites were analyzed. Microbial phenanthrene degraders were not detected by MPN counting in the agricultural soil and the roadside soil. In the industrial soils, phenanthrene degraders constituted 0.04 and 3.6% of the total number of CFU. 16S-23S spacer DNA analysis followed by partial 16S DNA sequencing of representative isolates from one of the industrial soils showed that one-half of the isolates belonged to the genus *Sphingomonas* and the other half were closely related to an unclassified beta-proteobacterium. The ^{13}C-PLFA profiles of the two industrial soils were relatively similar and resembled the profiles of phenanthrene-degrading *Sphingomonas* reference strains and unclassified beta-proteobacterium isolates but did not match the profiles of *Pseudomonas*, *Mycobacterium*, or *Nocardia* reference strains. The ^{13}C-PLFA profiles of phenanthrene degraders in the agricultural soil and the roadside soil were different from each other and different from the profiles of the highly polluted industrial soils. Only in the roadside soil were 10me/12me18:0 PLFAs enriched in ^{13}C, suggesting that actinomycetes metabolized phenanthrene in this soil. The ^{13}C-PLFA profiles of the unpolluted agricultural soil did not resemble the profiles of any of the reference strains. In all of the soils investigated, no excess ^{13}C was recovered in the 18:2ω6,9 PLFA, suggesting that fungi did not contribute significantly to assimilation of [^{13}C]phenanthrene.

Traditionally, the polycyclic aromatic hydrocarbon (PAH)-degrading microbial communities in PAH-polluted soils have been described by isolation of pure cultures on media containing PAHs as the major carbon sources, followed by taxonomic analysis of the isolates.

Generally, it seems that PAH mineralization in soil is dominated by bacterial strains belonging to a very limited number of taxonomic groups. Kästner et al. isolated PAH degraders from dilution series of five soils, and taxonomic determinations of representative PAH-degrading isolates led to the suggestion that nocardioform bacteria dominate the mineralization of PAHs in soil (23). Mueller et al. isolated PAH-degrading strains from four soils by successive enrichments in minimal medium supplemented with phenanthrene or fluoranthene; 27 of the 30 isolates were gram negative, and most isolates belonged to the genera *Sphingomonas*, *Burkholderia*, and *Pseudomonas*. Only three isolates were gram positive and grouped with the genus *Mycobacterium* (29). Ho et al. (18) isolated fluoranthene- and pyrene-degrading strains by liquid culture enrichment; 19 of 21 pyrene-degrading strains were gram positive, and 7 of these were mycobacteria. A total of 28 fluoranthene-degrading strains were all gram negative, and 4 of them belonged to the genus *Sphingomonas*. Bastiens et al. (3) compared traditional liquid enrichment and a new method in which PAH degraders were enriched on, and recovered from, hydrophobic membranes containing sorbed PAHs. The liquid enrichment mainly selected for sphingomonads, whereas the membrane method led to isolation of mycobacteria exclusively.

These methods suffer from four drawbacks: (i) the outcome depends on the choice of isolation protocol, especially if the protocol involves an enrichment step; (ii) the PAH degraders must be in a culturable state; (iii) PAH degradation genes on plasmids may be lost during isolation; and (iv) the isolation approach gives only indirect evidence of in situ activity.

Direct linking of in vivo metabolic processes to the taxonomic groups involved is sometimes feasible by using ^{14}C- or ^{13}C-labeled substrates in combination with lipid analysis (1, 6, 16, 31, 35, 36). Some taxonomic groups have specific lipid biomarkers (37, 38, 43), but for other groups it is necessary to compare the profiles of the lipid fatty acids with the profiles of reference strains in order to characterize the active organisms. The latter is possible only when few taxonomic groups are active in situ, as seems to be the case for the PAH degraders.

In this study, we characterized the phenanthrene-degrading microbial communities in four soils by adding ^{13}C-labeled phenanthrene to soil samples and then quantifying the ^{13}C labeling of sterols, glycolipid fatty acids (GLFAs), and polar lipid fatty acids (PLFAs) in the soil microbial communities. By using the ^{13}C approach to identify the bacteria that metabolized phenanthrene in situ, we avoided the possible biases associated with enrichment and isolation. The phenanthrene mineralization potential of the soil and the abundance of cul-

* Corresponding author. Mailing address: National Environmental Research Institute, Department of Environmental Chemistry and Microbiology, Frederiksborgvej 399, P.O. Box 358, DK-4000 Roskilde, Denmark. Phone: 45 46301386. Fax: 45 46301216. E-mail: arj@dmu.dk.

turable phenanthrene degraders were assayed with parallel samples. For comparison, the numerically dominant, culturable phenanthrene degraders were isolated from one of the soil samples. The isolates were grouped and identified to the genus level by 16S-23S spacer DNA PCR and partial 16S ribosomal DNA (rDNA) sequencing.

MATERIALS AND METHODS

Chemicals and reagents. All chemicals were analytical grade. [9-^{14}C]phenanthrene (purity, >98%) was obtained from Sigma-Aldrich, Copenhagen, Denmark. Side ring [$^{13}C_6$]phenanthrene (purity, 99%) was obtained from Cambridge Isotope Laboratories (Andover, Mass.). WST-1 cell proliferation reagent was obtained from Roche Molecular Biochemicals, Mannheim, Germany.

Phenanthrene-degrading reference strains. *Sphingomonas* sp. strain LH128, *Mycobacterium gilvum* VM552, and *Nocardia asteroides* VM451 were obtained from D. Springael (Vlaams Instituut voor Technologish Onderzoek, Mol, Belgium). *Sphingomonas paucimobilis* EPA505 (= DSM 7526) (28) was obtained from P. H. Pritchard (National Environmental Research Institute, Roskilde, Denmark). *Mycobacterium frederiksbergense* FAn9 (= DSM 44346) (41) was obtained from P. Willumsen (National Environmental Research Institute), and *Pseudomonas fluorescens* JAJ137 (22) was obtained from K. Johnsen (Geological Surveys, Copenhagen, Denmark). All strains were stored in 23% (vol/vol) glycerol at −80°C. Reference cultures used for lipid extraction were grown in the dark at 20°C and 180 rpm in 100-ml Erlenmeyer flasks containing 50 ml of phosphate minimal medium (21) supplemented with glucose (1 g liter^{-1}) and glycerol (1 g liter^{-1}). Phenanthrene-degrading isolate ARJ45 (this study) was grown in phosphate minimal medium supplemented with phenanthrene (400 mg liter^{-1}) because growth on glucose and glycerol was slow. The cultures were grown to an optical density at 450 nm of 0.4 to 0.8, and 25-ml subsamples were freeze-dried. The purity of a culture was confirmed by streaking 1 μl on tryptic soy agar (TSA) plates.

Soils. Four Danish soils with different levels of PAH contamination were used. Risø soil was an agricultural clay soil taken from a wheat field (depth, 0 to 6 cm) situated 400 m from the nearest road. The Amager soil, a humus-rich loam, was obtained from a grass-covered roadside (depth, 0 to 6 cm) in central Copenhagen, Denmark. The Amager site is exposed to heavy road traffic and is assumed to represent diffuse PAH pollution in urban areas. The Ringe soil originated from a former asphalt production plant. It was a clay soil heavily polluted with hydrocarbons. The B&W soil came from an old shipyard. It was a loam soil polluted with hydrocarbons and heavy metals and had a high content of humus and metal scrap. The B&W and Ringe soils were excavated from a depth of approximately 2 m and were stored in piles for 1 year before sampling. Approximately 400-g samples of the soils were obtained in late April, homogenized by sieving (3.5-mm mesh), and stored for 1 to 4 weeks at 4°C. The water content was determined gravimetrically by drying 10 g of soil overnight at 105°C.

Determination of soil PAH contents. The soil samples were spiked with a PAH recovery standard (containing 22 ^{13}C- or D (deuterium)-labeled PAHs), homogenized, and Soxhlet extracted with toluene for 24 h in the presence of activated copper. The extracts were concentrated under a stream of nitrogen, loaded onto SiO_2 columns, and eluted with pentane followed by dichloromethane. The dichloromethane fractions were loaded onto Al_2O_3-B columns and eluted with pentane followed by dichloromethane. The PAH content of the final dichloromethane fraction was quantified by adding a PAH standard (eight ^{13}C/D-labeled PAHs) and then analyzing the preparation with a Thermoquest Automass III gas chromatograph-mass spectrometer (Quadropole Instrument) in the single-ion monitoring mode. The controls included reagent blanks and a certified reference material sediment sample.

Determination of total CFU and MPN counts of phenanthrene degraders. Microbial cells were extracted by adding 50 ml of pyrophosphate buffer (1.2 mM $Na_4P_2O_7 \cdot 10H_2O$, pH 8.0) to 5 g (dry weight) of soil and then shaking the preparation with a wrist shaker for 10 min. The number of CFU was determined by plating a 10-fold dilution series on 10% TSA plates containing Delvocid (50 mg liter^{-1}). Delvocid is a commercial product containing natamycin to reduce the growth of fungi. The CFU were counted after 10 days of incubation at 30°C. The most probable number (MPN) of phenanthrene degraders in a soil extract was determined by using a microtiter plate method based on the respiratory reduction of a tetrazolium salt (WST-1) in positive wells (21).

Isolation of bacterial phenanthrene degraders. The numerically dominant, culturable phenanthrene degraders in the B&W soil were isolated from MPN microtiter plates by streaking 2-μl portions of the highest positive dilutions on 10% TSA plates. Only one to four phenanthrene-degrading cells were thought to have been inocula for the wells since the wells were the highest positive dilutions of fourfold dilution series. These one to four cells then proliferated in the wells during the 3 weeks of incubation. From each agar plate, two colonies representing the two most common morphologies were picked and pure streaked; i.e., two colonies were picked per microtiter plate well. The isolates were tested for growth on phenanthrene in liquid culture by using the WST-1 microtiter plate method (21). Only isolates that could grow with phenanthrene as the sole source of carbon were analyzed further.

Taxonomy of the bacterial isolates. The phenanthrene-degrading isolates were streaked on Luria-Bertani medium plates. DNA from colonies was extracted in Tris-EDTA buffer by using a freeze (−80°C)-thaw-boiling (102°C, 10 min)-centrifugation (10,000 × *g*, 10 min) cycle with final transfer of the supernatants to Eppendorf tubes. Primers were obtained from DNA Technology (Århus, Denmark) and MWG Biotech (Ebersberg, Germany). The 16S DNA primer Spf-190, specific for *Sphingomonas* sp. (26), and the universal 16S DNA primer Univ-16S-536-A-18 (P. A. Willumsen and B. M. Hansen, unpublished data) were used to identify isolates belonging to the genus *Sphingomonas* sensu lato. Identification of the *Sphingomonas* strains was further verified by PCR amplification with the 16S DNA primers Sphingo-108F and Sphingo-429R, which are specific for the genera *Sphingobium*, *Novosphingobium*, *Sphingopyxis*, and *Sphingomonas* belonging to the *Sphingomodaceae* family (N. Leys, A. Reyngaert, L. Bastiaens, E. Top, and D. Springael, unpublished data). The genetic similarity of all the isolates was further studied by PCR amplification of the highly variable spacer sequence between 16S and 23S rDNA (internally transcribed sequence [ITS]) by using the primers ITS-16S-1392-S-15 and ITS-23S-206-A-21 (Willumsen and Hansen, unpublished) targeting conserved sequences in 16S and 23S rDNA, respectively. The PCR products were analyzed by 2% agarose gel electrophoresis. Two strains belonging to 16S-23S spacer DNA similarity group 1, one strain belonging to group 3, one strain belonging to group 4, and one strain belonging to group 5 (see Table 3) were prepared for partial sequencing by PCR amplification of an approximately 500-bp fragment of 16S rDNA. The PCR was done by using universal primers S-D-Bact-0008-a-S-20 (5′-AGA GTT TGA TC[AC] TGG CTC AG-3′) modified from the primer described by Wilmotte et al. (42) and S-*-Univ-518-b-18 (15). The PCR products were purified by using QIAquick PCR purification kits (Qiagen, Hilden, Germany), dried, and sent to MWG Biotech for sequencing. The sequences were aligned with sequences in the GenBank database by using the software BLASTN 2.2.1 (2).

[^{14}C]phenanthrene mineralization by soil bacteria. One-gram (dry weight) portions of soil were placed in 10-ml sterile glass scintillation vials. One hundred microliters of ^{14}C-labeled phenanthrene in an acetone solution (30 μg ml^{-1}; 197 Bq ml^{-1}) was added to each vial, and the acetone was evaporated for 2 h. Two grams (dry weight) of fresh soil was added to the vial and mixed with the ^{14}C-contaminated soil to give a final concentration of added phenanthrene of 1 mg kg of soil^{-1}. Two subsamples from each soil were spiked with phenanthrene. Filter paper was placed in the bottoms of sterile 250-ml Blue Cap flasks, and 2 ml of NaH_2PO_4 buffer (0.1 M, pH 5) was added to each flask to keep the air water saturated. The vials were placed in the flasks; the flasks were closed with silicone stoppers and incubated at room temperature in the dark to limit fixation of $^{14}CO_2$ by photoautotrophs. The $^{14}CO_2$ produced was collected in 400 μl of 1.0 M KOH contained in glass vials suspended from the silicone stoppers by steel hooks. The $^{14}CO_2$ was quantified by mixing the KOH with 1 ml of water and 1.5 ml of Ready Gel scintillation cocktail (Beckman) and counting with an LS1801 Beckman scintillation counter.

^{13}C labeling of phenanthrene-metabolizing soil bacteria. The ^{13}C-labeling procedure was identical to and done in parallel with the procedure used in the [^{14}C]phenanthrene mineralization assays. Three micrograms of side ring [$^{13}C_6$]phenanthrene dissolved in acetone (100 μl) was added to each sample. Soil samples spiked with acetone alone were used as controls. The soil samples were incubated until the parallel [^{14}C]phenanthrene experiments showed 40 to 45% mineralization of the added phenanthrene (9 to 18 days) (Fig. 1). Samples were incubated in the dark with CO_2 traps containing KOH to limit fixation of $^{13}CO_2$ by autotrophs. Two subsamples of each soil were spiked with labeled phenanthrene.

Lipid extraction. Lipids were extracted from pure cultures and soil samples by using a mixture of dichloromethane and methanol as described previously (10, 35). The extractable lipids were separated into four fractions by silicic acid column chromatography (10). One of the four fractions contained PAH contaminants, one contained neutral lipids, one contained glycolipids, and one contained polar lipids. Polar lipids and glycolipids were dried under N_2 and then subjected to mild alkaline methanolysis to form fatty acid methyl esters (FAMEs). Sterols present in the neutral lipid fraction were recovered in hexane after alkaline saponification and then converted to the corresponding trimethylsilyl ethers by derivatization with bis(trimethylsilyl)trifluoroacetamide (25).

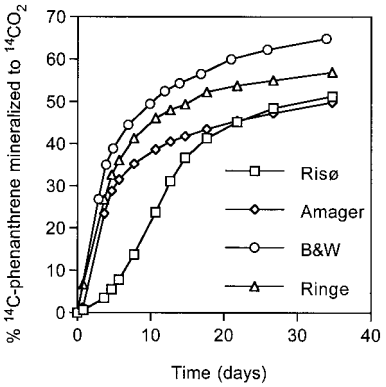

FIG. 1. Mineralization of ^{14}C-labeled phenanthrene (1 mg kg^{-1}) by four Danish soil samples with different histories of PAH preexposure. Phenanthrene mineralization was estimated by using the recovery of $^{14}CO_2$. Each soil was tested in duplicate.

Lipid analysis. ^{13}C-labeled sterols and FAMEs were analyzed with a Finnigan Delta Plus XL gas chromatograph-combustion isotope ratio mass spectrometer (GC-C-IRMS). The gas chromatograph (Hewlett-Packard 6890) was equipped with an HP-5MS column (length, 60 m; inside diameter, 0.25 mm) and a GC/C III combustion interface. He was used as the carrier gas. Nonlabeled sterols and FAMEs were analyzed with a Hewlett-Packard 5890 series II gas chromatograph equipped with a flame ionization detector and an HP Ultra 2 capillary column (length, 50 m; inside diameter, 0.2 mm). Samples (1 to 5 μl) were analyzed in the splitless mode. H_2 was used as the carrier gas. Fatty acids were identified based on a retention time index calculated relative to the retention times of the internal standards 13:0 and 19:0. The retention time indices were compared with those of reference standards and results obtained previously by Microbial Insights (Knoxville, Tenn.). GLC 87 and Supelco 37 standard mixtures were obtained from Nu Check Prep Inc. (Elysian, Minn.) and Supelco (Bellefonte, Pa.), respectively. Fatty acids were named by using the nomenclature described previously (34). The percentages of ^{13}C incorporated into individual fatty acids were calculated as described by Boschker et al. (6).

Statistics. Principal-component analysis (PCA) of the PLFA distributions was carried out by using the computer program Unscrambler (CAMO A/S, Trondheim, Norway). In the PCA, the data were log transformed to decrease the importance of minor differences in the relative amounts of individual PLFAs. A value of 1 was added to all numbers before log transformation.

RESULTS AND DISCUSSION

Soil PAHs. The total PAH contents of the soils (Table 1) ranged from 0.3 mg kg^{-1} in the noncontaminated agricultural soil to 234 mg kg^{-1} in the highly polluted Ringe soil (3 orders of magnitude). The diffusely polluted roadside soil (Amager) had PAH concentrations that were approximately 10-fold higher than those of the agricultural soil (Risø). The agricultural soil contained only trace amounts of phenanthrene (19 μg kg^{-1}). The phenanthrene concentrations were 20-fold higher in the roadside soil (Amager) and 740- and 380-fold higher in the highly polluted soils (B&W and Ringe, respectively). The amount of ^{13}C- or ^{14}C-labeled phenanthrene added in the experiments (1 mg kg^{-1}) was 50 times higher than the background level (Risø), twice the amount found in the roadside soil (Amager), and only 1/8 and 1/15 the amounts found in the highly polluted soils (Ringe and B&W, respectively).

Bacterial numbers and mineralization of [^{14}C]phenanthrene. The total numbers of CFU in the four soils, determined by counting the heterotrophic colonies growing on 10% TSA, ranged from 10^7 to 10^8 CFU per g (Table 2). The numbers of

TABLE 1. PAH contents of the four soils examined

Soil	Concn (mg kg^{-1}) of:														
	Naphthalene	Acenaphthylene	Acenaphthene	Fluorene	Phenanthrene	Anthracene	Fluoranthene	Pyrene	Benzo[*a*]anthracene	Triphenylene-chrysene	Benzo[*b* + *k*]fluoranthene	Benzo[*a*]pyrene	Indeno[1,2,3-*cd*]pyrene	Benzo[*ghi*]perylene	Dibenzo[*a*, *h*]antheracene	Total
Risø	6	21	1	19	19	54	43	20	9	44	24	ND[a]	10	ND	ND	270
Amager	520	130	ND	100	410	87	540	520	360	730	140	51	59	480	ND	4,100
B&W	5,900	870	360	2,800	15,000	4,400	29,000	21,000	15,000	21,000	31,000	1,300	9,500	11,000	1,800	170,000
Ringe	4,600	4,700	130	1,500	7,600	5,800	33,000	29,000	22,000	27,000	56,000	2,000	26,000	22,000	3,400	230,000

[a] ND, not detected.

TABLE 2. Estimates of the populations of the culturable heterotrophic bacteria (CFU) and the phenanthrene-degrading bacteria (MPN) in the four soils examined

Soil	No. of culturable heterotrophic bacteria (10^7 CFU g^{-1})[a]	MPN count (MPN g^{-1})[b]	% of phenanthrene degraders
Risø	1.91 ± 0.42	$<3.1 \times 10^1$ ($0\text{-}15.0 \times 10^1$)	0
Amager	9.00 ± 0.91	$<3.5 \times 10^1$ ($0\text{-}17.0 \times 10^1$)	0
B&W	2.46 ± 0.15	1.1×10^4 ($0.11 \times 10^4\text{-}3.4 \times 10^4$)	0.04
Ringe	4.88 ± 0.21	1.8×10^6 ($0.33 \times 10^6\text{-}6.2 \times 10^6$)	3.6

[a] Mean ± standard deviation.
[b] The values in parentheses are 95% confidence intervals.

phenanthrene degraders were below the detection limit in the agricultural soil (Risø) and in the roadside soil (Amager). In contrast, the two highly polluted soils contained large populations of phenanthrene degraders, which accounted for 0.04% (B&W) and 3.6% (Ringe) of the total number of CFU (Table 2).

In the PAH-contaminated Ringe and B&W soils, mineralization of the added [^{14}C]phenanthrene started at the maximum rate immediately after [^{14}C]phenanthrene addition, with no lag phase (Fig. 1). This suggests that the phenanthrene mineralization was limited by the bioavailability of phenanthrene rather than by the number of phenanthrene degraders in the soil, which agrees with the high numbers of phenanthrene degraders found in these two soils. Thus, the phenanthrene mineralization rate was likely controlled by the rate of dissolution of the phenanthrene crystals and not by the mineralization potential of the degrader population (17, 39, 40). If the degradation potential had been the initially controlling factor, we would have observed a lag phase. In the roadside soil (Amager) the rate of mineralization reached the maximum value after 1 day, whereas in the agricultural soil the mineralization rate increased over the first 8 days. This suggests that there was an initial increase in the number or activity of phenanthrene degraders in the agricultural soil, in which the amount of phenanthrene added was 50 times the background level, and to a lesser extent in the roadside soil. The results demonstrated that there was potential for phenanthrene mineralization not only in the polluted soils but also in the agricultural soil containing only background levels of PAHs.

The MPN method likely underestimated the number of phenanthrene degraders by detecting only those cells able to grow in liquid mineral medium with phenanthrene as the sole source of carbon and energy. In addition, the MPN method does not take into account cometabolism in which phenanthrene is mineralized by consortia of microorganisms. Therefore, the absence of phenanthrene degraders in the Risø and Amager soils as determined by the MPN method (Table 2) is not in conflict with the observed phenanthrene mineralization rates. Probably, a low number of phenanthrene degraders were present in the Risø soil and they were enriched by the addition of phenanthrene, as indicated by the increasing mineralization rates during the first 8 days of incubation (Fig. 1).

Taxonomy of phenanthrene-degrading isolates from the B&W soil. The numerically dominant, culturable phenanthrene degraders belonged to two genera. 16S-23S spacer DNA analysis placed 10 of the isolates in one group (group 1), three isolates in separate groups (groups 2 to 4), and the remaining 14 isolates in group 5 (Table 3). The isolates in each similarity group had identical 16S-23S spacer DNA bands based on the positions of the bands on the gels. Isolates with identical bands are generally assumed to be similar at the species or subspecies level (7, 14, 20). When growing on 10% TSA, the isolates in groups 1 to 4 all formed yellow colonies, while those in group 5 formed small grey colonies. The members of groups 1 to 4 belonged to the genus *Sphingomonas* sensu lato, as determined by *Sphingomonas*-specific PCR amplification of 16S rDNA with two separate primer sets and as confirmed by partial sequencing of representatives of the groups with universal primers. *Sphingomonas paucimobilis* EPA505 is a well-known PAH degrader (29), *Sphingomonas subarctica* is reported to degrade chlorophenol (30), and *Sphingomonas* sp. strain BAL5 was isolated from the Baltic Sea without any report of degradation of xenobiotic compounds (33). There is no published information concerning *Sphingomonas* sp. strain MBIC3020. The two *Sphingomonas*-specific primer sets did not amplify ARJ45 DNA (group 5). The closest relative of group 5, based on the partial 16S sequence obtained with universal primers, was an unclassified beta-proteobacterium (ultramicrobacterium strain ND5) isolated from polluted urban soil in Tokyo, Japan (19).

Lipid biomarkers. ^{13}C enrichment of cell lipids in soil incubated with [^{13}C]phenanthrene indicated that there was metabolism of phenanthrene, which led to assimilation of phenanthrene-derived carbon into the microbial biomass. ^{13}C enrichment of cell lipids was measured relative to the ^{13}C in controls to which only the acetone solvent was added. Only lipid fatty acids containing 1% or more of the recovered ^{13}C

TABLE 3. Characterization of the 27 culturable, numerically dominant, phenanthrene-degrading bacterial isolates from the B&W soil

16S-23S spacer DNA similarity group	No. of isolates	Spf190, Sphingo-108F, and Sphingo-429R positive[a]	Strain(s) sequenced	Closest relative based on partial 16S rDNA sequence		
				Taxon	% Homology	Accession no.
1	10	Yes	ARJ7 and ARJ19	*Sphingomonas paucimobilis* EPA505	100	437341
				Sphingomonas subarctica	100	X99100
2	1	Yes	ND[b]	ND		
3	1	Yes	ARJ81	*Sphingomonas* sp. strain BAL5	100	463937
4	1	Yes	ARJ13	*Sphingomonas subarctica*	98	X94098
				Sphingomonas sp. strain MBIC3020	98	AB025279
5	14	No	ARJ45	Unclassified beta-proteobacterium strain ND5	100	AB008506

[a] Spf90, Sphingo-108F, and Sphingo-429R are *Sphingomonas*-specific 16S DNA primers.
[b] ND, not determined.

TABLE 4. ^{13}C-PLFA profiles of phenanthrene-degrading soil organisms in Risø, Amager, B&W, and Ringe soils and PLFA compositions of phenanthrene-degrading laboratory reference strains

Fatty acid	^{13}C-PLFA abundance (%) in soils[a]				PLFA abundance (%) in reference strains[a,b]						
	Risø	Amager	B&W	Ringe	LH128	EPA505	JAJ137	FAn9	VM552	VM451	ARJ45
Unidentified 12					3.5	1.0					
14:0	4.7	3.0		0.7[c]				4.7	4.6	3.2	
br15:0	2.2	1.4	1.0	0.8[c]	1.0						
Unidentified					1.0						
16:1ω9										1.9	
16:1ω7/ω5	16.1	12.0	31.3	36.1	20.6	12.7	48.4	4.8	19.5	16.9	15.6
16:0	42.4	44.6	24.7	26.5	2.8	6.2	35.2	20.5	23.2	24.3	34.0
me16:0	1.8										
Unidentified 17					2.4						
17:1			14.8	14.1							46.2
18:1ω9	11.1	18.0						17.4	32.7	26.7	1.9
18:1ω7/ω5	21.0	5.4	17.1	20.2	65.7	71.9	15.3				
18:0		1.1						29.4	1.5		
br19:1						4.3					2.0
10/12me18:0		7.4							18.4	27.1	
Unidentified					1.0						
19:1			7.6	1.2		3.0					
20:0								23.3			
Other	0.6	5.8	3.6	0.4	2.2	1.1	1.1				0.3

[a] The PLFA abundance is the relative amount of ^{13}C (soils) or total C (reference strains) in each fatty acid.
[b] The identities of the reference strains are as follows: LH128, *Sphingomonas* sp.; EPA505, *S. paucimobilis*; JAJ137, *P. fluorescens*; FAn9, *M. frederiksbergense*; VM552, *M. gilvum*; VM451, *N. asteroides*; and ARJ45, unclassified beta-proteobacterium isolated in this study.
[c] PLFAs highly enriched in ^{13}C but containing less than 1% of the total amount of ^{13}C due to small amounts of the PLFAs.

were considered significant, as small variations in the measured $^{13}C/^{12}C$ ratio might give false positives for compounds found at high concentrations. Two exceptions were the PLFAs 14:0 and br15:0 enriched in ^{13}C in the Ringe soil (Table 4). These compounds contained less than 1% of the ^{13}C due to small amounts of the two fatty acids, but they were clearly enriched (increases in the δ ^{13}C values of 9.0 and 6.3‰, respectively).

Sterols. The sterol fraction from the polluted soils (Amager, Ringe, and B&W) was ink black and contained numerous interfering compounds that made GC-C-IRMS analysis impossible. GC-C-IRMS analysis of the sterol extract from the agricultural soil (Risø) showed no ^{13}C enrichment of any of the sterols, including cholesterol and ergosterol (data not shown). Sterols are generally considered indicative of eukaryotic organisms, since only a very limited number of bacteria (e.g., methanotrophic bacteria and cyanobacteria) contain these lipid compounds. Ergosterol is the predominant sterol in most fungi (27). The lack of ^{13}C enrichment of ergosterol suggests that soil fungi did not significantly metabolize phenanthrene in the Risø soil. The lack of ^{13}C enrichment of cholesterol and other sterols further indicates that the added phenanthrene was not metabolized by eukaryotes. These findings also indicate that ^{13}C originating from phenanthrene metabolized by prokaryotes did not enter higher trophic levels; i.e., phenanthrene-degrading bacteria were not consumed to any detectable extent by nematodes, microarthropods, ciliates, or other microeukaryotes. This may have been due in part to reductions in the microeukaryote populations caused by sieving of the soil samples, mixing of the samples, etc.

^{13}C is expected to spread through the trophic levels with time as the primary consumers are eaten by predators or detritivores. Since the agricultural Risø soil was incubated longer than the other soils and since it was probably more biologically active than the subsoils, we suggest that the ^{13}C also was not passed through the trophic levels in these samples (i.e., that ^{13}C enrichment of cell lipids was indicative of the organisms that initially metabolized the added [^{13}C]phenanthrene).

Polar lipids (PLFAs). The distribution of ^{13}C in the soil PLFAs (^{13}C-PLFA profiles) may indicate which phylogenetic groups metabolized phenanthrene in the four soils investigated. The PLFAs 10me18:0 and 12me18:0 are indicative of actinomycetes (37, 38, 43). In the Amager soil, 10me18:0 and 12me18:0 were enriched in ^{13}C (7.2% of the total ^{13}C assimilated into the PLFAs), suggesting that actinomycetes were among the dominant phenanthrene degraders in this soil. The lack of ^{13}C enrichment of 10me18:0 and 12me18:0 in the Risø, Ringe, and B&W soils suggests that actinomycetes were of minor importance in these soils.

It may be speculated that soil fungi metabolize PAHs or PAH metabolites, contributing to the overall mineralization of PAHs. For instance, the white rot fungus *Bjerkandera* sp. strain BOS55 has been shown to mineralize [^{14}C]benzo[*a*]pyrene to $^{14}CO_2$ in pure culture (24). The PLFAs 18:2ω6, 18:3ω6, and 18:3ω3 are considered fungal PLFA biomarkers (37, 38). ^{13}C enrichment of the combined 18:2-18:3 pool was not observed in any of the soils investigated, suggesting that soil fungi did not metabolize the added phenanthrene. This agrees well with the lack of ^{13}C enrichment of ergosterol in the Risø soil, as described above. However, the most common effect of fungi on PAH degradation may be activation and solubilization of PAHs by nonspecific fungal enzymes rather than complete mineralization (4, 5, 24, 32). In this study, incorporation of ^{13}C from phenanthrene into bacterial lipids indicated that there was bacterial metabolism and assimilation of phenanthrene-derived carbon, but this did not exclude the possibility that there was initial nonspecific activation of the phenanthrene molecules by fungal extracellular enzymes.

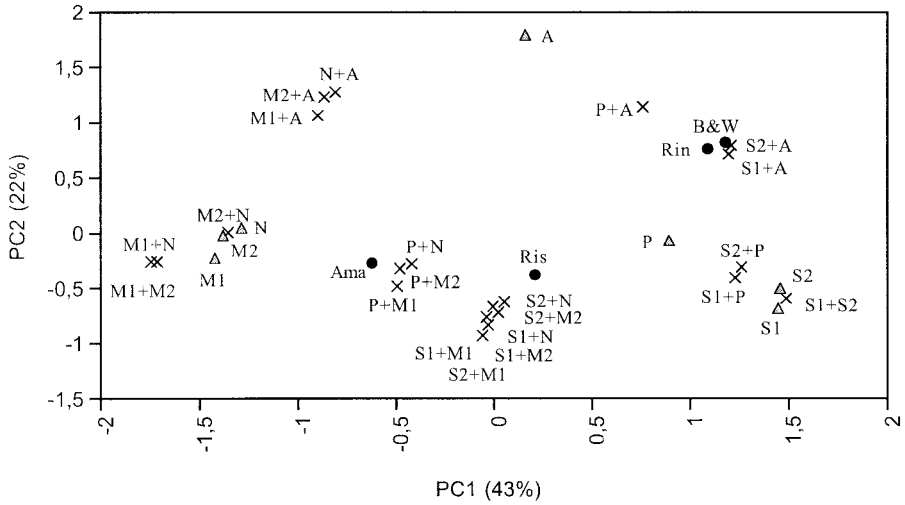

FIG. 2. PCA of log-transformed ^{13}C-PLFA profiles for soil samples and log-transformed PLFA profiles of reference strains and combinations of two reference strains. Symbols: ●, soils; ▲, reference strains; ×, combinations of reference strains. Ama, Amager soil; Ris, Risø soil; B&W, B&W shipyard soil; Rin, Ringe soil; S1, *Sphingomonas* sp. strain LH128; S2, *S. paucimobilis* EPA505; P, *P. fluorescens* JAJ137; M1, *Mycobacterium frederiksbergense* FAn9; M2, *M. gilvum* VM552; N, *Nocardia asteroides* VM451; A, ARJ45.

By using PCA, soil ^{13}C-PLFA profiles were compared to the PLFA profiles of bacterial strains (known to degrade phenanthrene) representing the genera *Sphingomonas* sensu lato, *Pseudomonas*, *Mycobacterium*, and *Nocardia.* Combinations of two strains were included in order to examine the possibility that phenanthrene metabolism in the soils was dominated by two genera, as indicated by the taxonomic analysis of the isolates from the B&W soil. The PLFA profile for a combination of two strains was obtained by calculating the average of the two PLFA profiles. The amounts of PLFAs were log transformed to make the relative concentration of each PLFA less significant, as the relative proportions of PLFAs may depend on the carbon source, nutritional status, and environmental parameters (11–13). In soil, the PAH degraders probably grow on many different carbon sources, with PAHs contributing only a minor fraction of the assimilated carbon (8, 9). We therefore grew the reference strains on carbohydrates instead of phenanthrene.

In the PCA, the gram-positive genera (*Mycobacterium* and *Nocardia*) and the gram-negative genera (*Sphingomonas*, *Pseudomonas*, and ARJ45) were well separated (Fig. 2). The two sphingomonads were almost identical and were located closer to *P. fluorescens* JAJ137 than to the gram-positive strains. Isolate ARJ45 was separated from the other gram-negative strains due to large amounts of a PLFA tentatively identified as 17:1. The actinomycetes (*M. gilvum* VM552, *M. frederiksbergense* FAn9, and *N. asteroides* VM451) clustered tightly together, mainly due to the fatty acids 10/12me18:0, 18:1ω9, and 18:0.

The ^{13}C-PLFA profiles of the highly polluted B&W and Ringe soils were very similar in the PCA, indicating that the dominant phenanthrene degraders in the two soils were comparable at the PLFA level (Fig. 2). The profiles of the B&W and Ringe soils were located very close to the combined *Sphingomonas* and ARJ45 profiles, suggesting that *Sphingomonas*-like and ARJ45-like bacteria played a dominant role in mineralization of phenanthrene in the Ringe and B&W soils. Interestingly, this agrees with the taxonomic characterization of the phenanthrene-degrading isolates from the B&W soil (Table 3). A comparison of the B&W and Ringe ^{13}C-PLFA profiles and the combined *Sphingomonas*-ARJ45 PLFA profiles is presented in Fig. 3. The PLFA profile of *M. gilvum* VM552 is included for comparison.

In the PCA, the profile of the diffusely polluted Amager soil was located close to the combinations (1:1) of actinomycete and *Pseudomonas* profiles, suggesting that actinomycetes and possibly *Pseudomonas*-like bacteria were involved in phenanthrene metabolism in this soil. *P. fluorescens* JAJ137 was described by 16:1ω5/ω7, 16:0, and 18:1ω7/ω5 PLFAs (Table 4). This combination of PLFAs contained only limited information. Two of the PLFAs, 16:1ω5 and 18:1ω7, are common signatures of eubacteria (37, 38), and 16:0 was found in all the reference strains (Table 4), as observed in other studies (1).

Cross-validation of the data showed that the model was robust. The grouping of the B&W, Ringe, and Amager soils with specific reference strains in the PC1-PC2 plot was also seen for PC3 (explaining 20% of the variation) (data not shown), supporting the conclusions.

The ^{13}C-PLFA profile of the nonpolluted agricultural soil (Risø) resembled neither the profiles of the other soils nor the profile of any of the reference strains. This indicates that the population of active phenanthrene degraders in this soil was different from the populations in the more polluted soils and also different from the reference strains.

Glycolipids (GLFAs). The ^{13}C-GLFA profile of the agricultural soil was different from the GLFA profiles of the reference cultures, supporting the conclusions from the PLFA analysis (data not shown). The GLFA fractions of the Amager, Ringe, and B&W soils contained large amounts of interfering organic substances that prohibited a GC-C-IRMS analysis.

^{13}C-lipid profiling in combination with reference strains may in some cases identify the microbial taxa involved in degradation of soil contaminants at a particular site. This method is especially suitable for PCA when a limited number of degrad-

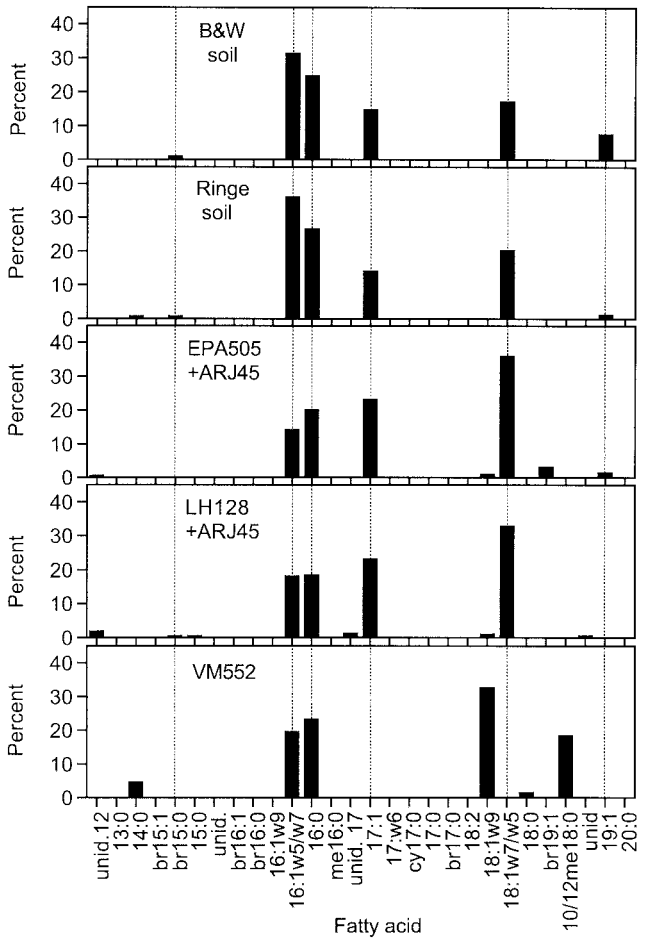

FIG. 3. Comparison of ^{13}C-PLFA profiles for PAH-polluted B&W and Ringe soils with PLFA profiles of PAH-degrading reference strains (LH128+ARJ45, combination of *Sphingomonas* sp. strain LH128 and beta-proteobacterium strain ARJ45 profiles; EPA505+ARJ45, combination of *Sphingomonas* sp. strain EPA505 and beta-proteobacterium strain ARJ45 profiles; VM552, *M. gilvum* VM552 profile). unid., unidentified fatty acid.

ing genera are active in the soil. Comparison of ^{13}C-lipid analysis and the traditional isolation technique based on dilution series (B&W soil) is interesting, as the two methods yielded similar results. The ^{13}C-PLFA method detects only those cells that are metabolically active in situ, so it seems that the strains isolated by the traditional method actually represented the active population in the soil. It is also interesting that similar ^{13}C-PLFA profiles, dominated by sphingomonads and ARJ45-like bacteria, were obtained for the two highly contaminated soils (B&W and Ringe), since the two sampling sites are separated by 160 km and represent two different soil types. Based on sequence similarity, the closest relative of ARJ45 was a beta-proteobacterium isolated from diffusely polluted soil in Tokyo. This suggests that ARJ45-like bacteria may be widely distributed and may constitute an overlooked group of PAH degraders.

In conclusion, the ^{13}C-lipid profile of the active phenanthrene degraders in a nonpolluted soil was different from the profiles in polluted soils. Actinomycetes were among the dominant phenanthrene degraders in a diffusely polluted soil. The profiles of the two highly contaminated soils were similar and were dominated by organisms related to *Sphingomonas* and novel isolate ARJ45. These organisms were also found to be the numerically dominant culturable soil phenanthrene degraders. Fungi did not contribute significantly to phenanthrene metabolism, as measured by assimilation of phenanthrene-derived carbon into the microbial biomass.

ACKNOWLEDGMENTS

This work was supported by the European Commission (contract BIO4-CT97-2015) and the Danish Technical Research Council framework program "Activity and Diversity in Complex Microbial Systems."

REFERENCES

1. **Abraham, W.-R., C. Hesse, and O. Peltz.** 1998. Ratios of carbon isotopes in microbial lipids as an indicator of substrate usage. Appl. Environ. Microbiol. **64:**4202–4209.
2. **Altschul, S. F., T. L. Madden, A. A. Schäfer, 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.
3. **Bastiaens, L., D. Springael, P. Wattiau, H. Harms, R. deWachter, H. Verachtert, and L. Diels.** 2000. Isolation of adherent polycyclic aromatic hydrocarbon (PAH)-degrading bacteria using PAH-sorbing carriers. Appl. Environ. Microbiol. **66:**1834–1843.
4. **Bogan, B. W., and R. T. Lamar.** 1996. Polycyclic aromatic hydrocarbon-degrading capabilities of *Phanerochaete laevis* HHB-1625 and its extracellular ligninolytic enzymes. Appl. Environ. Microbiol. **62:**1597–1603.
5. **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.
6. **Boschker, H. T. S., S. C. Nold, P. Wellsbury, D. Bos, W. deGraaf, R. Pel, R. J. Parkes, and T. E. Cappenberg.** 1998. Direct linking of microbial populations to specific biogeochemical processes by ^{13}C-labelling of biomarkers. Nature **392:**801–805.
7. **Boyer, S. L., V. R. Flechtner, and J. R. Johansen.** 2001. Is the 16S-23S rRNA internal transcribed spacer region a good tool for use in molecular systematics and population genetics? A case study in cyanobacteria. Mol. Biol. Evol. **18:**1057–1069.
8. **Egli, T.** 1995. The ecological and physiological significance of the growth of heterotrophic microorganisms with mixtures of substrates. Adv. Microb. Ecol. **14:**305–386.
9. **Egli, T.** 2002. Microbial degradation of pollutants at low concentrations and in the presence of alternative carbon substrates: emerging patterns, p. 131–139. *In* S. N. Agathos and W. Reineke (ed.), Biotechnology for the environment: strategy and fundamentals. Focus on biotechnology, vol. 3A. Kluwer Academic Publishers, Dordrecht, The Netherlands.
10. **Fang, J., and R. H. Findlay.** 1996. The use of a classic lipid extraction method for simultaneous recovery of organic pollutants and microbial lipids from sediments. J. Microbiol. Methods **27:**63–71.
11. **Fukanaga, N., and N. J. Russel.** 1990. Membrane lipid composition and glucose uptake in two psycrotolerant bacteria from Antarctica. J. Gen. Microbiol. **136:**1669–1673.
12. **Gounot, A.-M.** 1991. Bacterial life at low temperatures: physiological aspects and biotechnological aspects. J. Appl. Bacteriol. **71:**386–397.
13. **Grau, R., and D. Demandoza.** 1993. Regulation of the synthesis of unsaturated fatty-acids by growth temperature in *Bacillus subtilis*. Mol. Microbiol. **8:**535–542.
14. **Gürtler, V., and V. A. Stanisich.** 1996. New approaches to typing and identification of bacteria using the 16S-23S rDNA spacer region. Microbiology **142:**3–16.
15. **Hansen, B. M., T. D. Leser, and N. B. Hendriksen.** 2001. Polymerase chain reaction assay for the detection of *Bacillus cereus* group cells. FEMS Microbiol. Lett. **202:**209–213.
16. **Hanson, J., J. Macalady, D. Harris, and K. Scow.** 1999. Linking toluene degradation with specific microbial populations in soil. Appl. Environ. Microbiol. **65:**5403–5408.
17. **Harms, H., and T. N. P. Bosma.** 1997. Mass transfer limitation of microbial growth and pollutant degradation. J. Ind. Microbiol. **18:**97–105.
18. **Ho, Y., M. Jackson, Y. Yang, J. G. Mueller, and P. H. Pritchard.** 2000. Characterization of fluoranthene- and pyrene-degrading bacteria isolated from PAH-contaminated soils and sediments and comparison of several *Sphingomonas* spp. J. Ind. Microbiol. Biotechnol. **2:**100–112.
19. **Iizuka, T., S. Yamanaka, T. Nishiyama, and A. Hiraishi.** 1998. Isolation and phylogenetic analysis of aerobic copiotrophic ultramicrobacteria from urban soil. J. Gen. Appl. Microbiol. **44:**75–84.
20. **Jensen, M. A., J. A. Webster, and N. Strauss.** 1993. Rapid identification of

bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms. Appl. Environ. Microbiol. **59:**945–952.
21. **Johnsen, A. R., K. Bendixen, and U. Karlson.** 2002. Detection of microbial growth on polycyclic aromatic hydrocarbons in microtiter plates by using the respiration indicator WST-1. Appl. Environ. Microbiol. **68:**2683–2689.
22. **Johnsen, K., S. Andersen, and C. S. Jacobsen.** 1996. Phenotypic and genotypic characterization of phenanthrene-degrading fluorescent *Pseudononas* biovars. Appl. Environ. Microbiol. **62:**3818–3825.
23. **Kästner, M., M. Breuer-Jammali, and B. Mahro.** 1994. Enumeration and characterization of the soil microflora from hydrocarbon-contaminated soil sites able to mineralize polycyclic hydrocarbons (PAH). Appl. Microbiol. Biotechnol. **41:**267–273.
24. **Kotterman, M. J. J., E. H. Vis, and J. A. Field.** 1998. Successive mineralization and detoxification of benzo[*a*]pyrene by the white rot fungus *Bjergkandera* sp. strain BOS55 and indigenous microflora. Appl. Environ. Microbiol. **64:**2853–2858.
25. **Leeming, R.** 1996. Coprostanol and related sterols as tracers for fecal contamination in Australian aquatic environments. Ph.D. thesis. Canberra University, Canberra, Australia.
26. **Leung, K. T., Y. J. Chang, Y. D. Gan, A. Peacock, S. J. Macnaughton, J. R. Stephen, and R. S. Burkhalter.** 1999. Detection of *Sphingomonas* spp. in soil by PCR and sphingolipid biomarker analysis. J. Ind. Microbiol. Biotechnol. **23:**252–260.
27. **Logel, D. M.** 1988. Fungal lipids, p. 699–806. *In* S. G. Wilkinson (ed.), Microbial lipids, vol. 7. Academic Press, London, United Kingdom.
28. **Mueller, J. G., P. J. Chapman, B. O. Blattmann, and P. H. Pritchard.** 1990. Isolation and characterization of a fluoranthene-utilizing strain of *Pseudomonas paucimobilis*. Appl. Environ. Microbiol. **56:**1079–1086.
29. **Mueller, J. G., R. Devereux, D. L. Santavy, S. E. Lantz, S. G. Willis, and P. H. Pritchard.** 1997. Phylogenetic and physiological comparisons of PAH-degrading bacteria from geographically diverse soils. Antonie Leeuwenhoek **71:**329–343.
30. **Nohynek, L. J., E.-L. Numiarho-Lassila, E.-L. Suhonon, H.-J. Busse, M. Mohammed, J. Hantula, F. Rainey, and M. S. Salkinoja-Salonen.** 1996. Description of chlorophenol-degrading *Pseudomonas* sp. strains KF1^{T}, KF3, and NKF1 as a new species of the genus *Sphingomonas*, *Sphingomonas subarctica* sp. nov. Int. J. Syst. Bacteriol. **46:**1042–1055.
31. **Pel, R., R. Oldenhuis, W. Brand, A. Vos, J. C. Gottschal, and K. B. Zwart.** 1997. Stable-isotope analysis of a combined nitrification-denitrification sustained by thermophilic methanotrophs under low-oxygen conditions. Appl. Environ. Microbiol. **63:**474–481.
32. **Pickard, M. A., R. Roman, R. Tinoco, and R. Vasquez-Duhalt.** 1999. Polycyclic aromatic hydrocarbon metabolism by white rot fungi and oxidation by *Coriolopsis gallica* UAMH 8260 laccase. Appl. Environ. Microbiol. **65:**3805–3809.
33. **Pinhassi, J., U. Zweifel, and Å. Hagström.** 1997. Dominant marine bacterioplankton species found among colony-forming bacteria. Appl. Environ. Microbiol. **63:**3359–3366.
34. **Roslev, P., and N. Iversen.** 1999. Radioactive fingerprinting of microorganisms that oxidize atmospheric methane in different soils. Appl. Environ. Microbiol. **65:**4064–4070.
35. **Roslev, P., N. Iversen, and K. Henriksen.** 1998. Direct fingerprinting of metabolically active bacteria in environmental samples by substrate specific radiolabelling and lipid analysis. J. Microbiol. Methods **31:**99–111.
36. **Tunlid, A., H. G. Ek, G. Westerdahl, and G. Odham.** 1987. Determination of ^{13}C-enrichment in bacterial fatty acids using chemical ionization mass spectrometry with negative ion detection. J. Microbiol. Methods **7:**77–89.
37. **Tunlid, A., and D. C. White.** 1992. Biochemical analysis of biomass, community structure, nutritional status and metabolic activity of microbial communities in soil, p. 229–262. *In* J.-M. Bollag (ed.), Soil biochemistry, vol. 7. Marcel Dekker Inc., New York, N.Y.
38. **White, D. C., H. C. Pinkart, and D. B. Ringelberg.** 1997. Biomass measurements: biochemical approaches, p. 91–101. *In* C. J. Hurst, G. R. Knudsen, M. J. McInerney, L. D. Stetzenbach, and M. V. Walter (ed.), Manual of environmental microbiology. ASM Press, Washington, D.C.
39. **Wick, L. Y., T. Colangelo, and H. Harms.** 2001. Kinetics of mass-transfer limited bacterial growth on solid PAHs. Environ. Sci. Technol. **35:**354–361.
40. **Wick, L. Y., D. Springael, and H. Harms.** 2001. Bacterial strategies to improve the bioavailability of hydrophobic organic pollutants, p. 203–217. *In* G. Matz (ed.), Treatment of contaminated soil. Springer-Verlag, Berlin, Germany.
41. **Willumsen, P., U. Karlson, E. Stakebrandt, and R. M. Kroppenstedt.** 2001. *Mycobacterium frederiksbergense* sp. nov., a novel polycyclic aromatic hydrocarbon-degrading *Mycobacterium* species. Int. J. Syst. Evol. Microbiol. **51:**1715–1722.
42. **Wilmotte, A., G. van der Auwera, and R. De Wachter.** 1993. Structure of the 16S ribosomal RNA of the thermophilic cyanobacterium *Chlorogloeopsis* HTF (*Mastigocladus laminosus* HTF) strain PCC7518, and phylogenetic analysis. FEBS Lett. **37:**96–100.
43. **Zelles, L.** 1999. Fatty acid patterns of phospholipid and lipopolysaccharides in the characterization of microbial communities in soil: a review. Biol. Fertil. Soils **29:**111–129.