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Occurrence and Phylogenetic Diversity of *Sphingomonas* Strains in Soils Contaminated with Polycyclic Aromatic Hydrocarbons

Natalie M. E. J. Leys,[1,2]† Annemie Ryngaert,[1] Leen Bastiaens,[1] Willy Verstraete,[2] Eva M. Top,[2]‡ and Dirk Springael[1,3]*

Environmental Technology, Flemish Institute for Technological Research, 2400 Mol,[1] Laboratory of Microbial Ecology and Technology, University of Ghent, 9000 Ghent,[2] and Laboratory for Soil and Water Management, Catholic University of Leuven, 3001 Heverlee,[3] Belgium

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Bacterial strains of the genus *Sphingomonas* are often isolated from contaminated soils for their ability to use polycyclic aromatic hydrocarbons (PAH) as the sole source of carbon and energy. The direct detection of *Sphingomonas* strains in contaminated soils, either indigenous or inoculated, is, as such, of interest for bioremediation purposes. In this study, a culture-independent PCR-based detection method using specific primers targeting the *Sphingomonas* 16S rRNA gene combined with denaturing gradient gel electrophoresis (DGGE) was developed to assess *Sphingomonas* diversity in PAH-contaminated soils. PCR using the new primer pair on a set of template DNAs of different bacterial genera showed that the method was selective for bacteria belonging to the family *Sphingomonadaceae*. Single-band DGGE profiles were obtained for most *Sphingomonas* strains tested. Strains belonging to the same species had identical DGGE fingerprints, and in most cases, these fingerprints were typical for one species. Inoculated strains could be detected at a cell concentration of 10^4 CFU g of soil^{-1}. The analysis of *Sphingomonas* population structures of several PAH-contaminated soils by the new PCR-DGGE method revealed that soils containing the highest phenanthrene concentrations showed the lowest *Sphingomonas* diversity. Sequence analysis of cloned PCR products amplified from soil DNA revealed new 16S rRNA gene *Sphingomonas* sequences significantly different from sequences from known cultivated isolates (i.e., sequences from environmental clones grouped phylogenetically with other environmental clone sequences available on the web and that possibly originated from several potential new species). In conclusion, the newly designed *Sphingomonas*-specific PCR-DGGE detection technique successfully analyzed the *Sphingomonas* communities from polluted soils at the species level and revealed different *Sphingomonas* members not previously detected by culture-dependent detection techniques.

The genus *Sphingomonas* was proposed in 1990 by Yabuuchi et al. (55) to describe a group of bacterial strains isolated from human clinical specimens and hospital environments. During the past 10 years, *Sphingomonas* strains have also been isolated from a variety of anthropogeneously contaminated environments—including terrestrial (subsurface) soil (1, 3, 5, 7, 17, 29, 33–35, 39, 43) and rhizosphere soil (12), sediment (river and subsurface sediments) (18, 19), or aquatic habitats, such as wastewater (10, 20, 33), groundwater (49), freshwater (42, 44, 45, 53), and marine water (21)—and were shown to possess unique abilities to degrade a variety of pollutants, including azo dyes (44), chlorinated phenols (7, 11), dibenzofurans (23, 52), insecticides (38), and herbicides (1, 26). In addition, *Sphingomonas* strains are often isolated from contaminated soils as degraders of polycyclic aromatic hydrocarbons (PAHs) (5, 24, 35, 39). PAHs are very hydrophobic toxic chemicals with low solubility in water, making them poorly available for natural bacterial degradation. Due to their ubiquitous distribution and their diverse catabolic capabilities towards recalcitrant organic pollutants, *Sphingomonas* strains can be considered as important biocatalysts for soil bioremediation.

Therefore, it is of major interest to be able to monitor the presence, biodiversity, and dynamics of *Sphingomonas* species in the environment. However, until today, only a limited number of studies have reported *Sphingomonas*-specific detection and monitoring techniques. The culture-independent molecular identification methods described so far had been based on the extraction of typical sphingolipids (27) or ribosomal DNA (rDNA) or rRNA as marker molecules (27, 42, 47, 50). Several rRNA gene-targeted fluorescence-labeled oligonucleotide probes were developed (i) by Thomas et al. (47) to specifically monitor the inoculated PAH-degrading *Sphingomonas* sp. strain 107 in soil via flow cytometry and (ii) by Schweitzer et al. (42) to analyze the composition of lake aggregate-associated *Sphingomonas* communities via fluorescent in situ hybridization (FISH). However, sphingolipid analysis gives no information on *Sphingomonas* diversity, and the currently available probes for detection of *Sphingomonas* by flow cytometry and FISH detect all species or only some species. Other researchers reported the application of specific PCR to detect *Sphingomonas* in environmental samples using the 16S rRNA gene as target molecule. van Elsas et al. (50) designed a specific primer set and internal probe targeting the ribosomal 16S rRNA genes to monitor by PCR *Sphingomonas chlorophenolica* RA2 (DSM8671) seeded in soil. Leung et al. (27) reported the need for two degenerate 16S rRNA gene primer

* Corresponding author. Present address: Catholic University of Leuven (KUL), Laboratory for Soil and Water Management, Kasteelpark Arenberg 20, 3001 Heverlee, Belgium. Phone: 32 (16) 321604. Fax: 32 (16) 321997. E-mail: dirk.springael@agr.kuleuven.ac.be.

† Present address: Belgian Nuclear Research Centre (SCK/CEN), Laboratory of Microbiology, 2400 Mol, Belgium.

‡ Present address: University of Idaho, Department of Biological Sciences, Moscow, ID 83844-3051.

sets (SPf-190/SPr1-852) for PCR detection of a spectrum of different *Sphingomonas* species in soil. Thus, none of the primer sets so far developed for PCR detection was designed to cover the total *Sphingomonas* genus, and degeneration made them unsuitable to directly assess the diversity of *Sphingomonas* species in soil by a fingerprinting method like denaturing gradient gel electrophoresis (DGGE).

This paper describes the design of a 16S rRNA gene-based nondegenerate primer set selective for specific PCR detection of all known *Sphingomonas* species and allowing subsequent differentiation between *Sphingomonas* species by DGGE analysis. The PCR-DGGE method was used to assess the phylogenetic diversity of the indigenous *Sphingomonas* strains in different PAH-contaminated soils.

MATERIALS AND METHODS

Bacterial strains and culture media. The bacterial strains used in this study are described in Table 1. For genomic DNA extraction, all strains were cultivated in 869 broth (32). For evaluation of the method's sensitivity, appropriate *Sphingomonas s*trains were cultivated in a phosphate-buffered minimal liquid medium described by Wick et al. (51), supplemented with 2 g of the appropriate PAH compound (ACROS Organics, Geel, Belgium) liter^{-1} provided as the sole carbon and energy source. All cultures were incubated in the dark on an orbital horizontal shaker at 200 rpm at a constant temperature of 30°C.

Soil samples. Soil samples were taken from different historically PAH-contaminated industrial sites, and their characteristics are summarized in Table 2. The methods applied for chemical and physical analysis have been reported previously (N. Leys, A. Ryngaert, L. Bastiaens, P. Wattiau, E. Top, and D. Springael, submitted for publication).

Design of a *Sphingomonas*-specific 16S rRNA gene primer set. Circa 215 sequences (minimum of 1,200 bp long) of both environmental and clinical *Sphingomonas* species available from the GenBank database (6) were selected and aligned by using the RPDII Hierarchy Browser program (9) and ClustalW software (48). The multiple alignment was further analyzed by TreeTop software for phylogenetic tree prediction and with the PLOTCON program (EMBOSS software, version 2.3.1) to identify variable gene regions. The sequence similarity was calculated by moving a window of 4 bp along the aligned sequences. Within the window, the similarity of any one position was taken to be the average of all possible pairwise scores (taken from the specified similarity matrix of the imported alignment) of the bases at that position. The average of the position similarities within the window was plotted, resulting in a similarity plot. The primers had to be located in a conserved region and had to amplify a variable region of a maximum of 500 bp to allow good DGGE analysis of the amplicons. Several possible primer combinations were visually selected from the constructed alignment of *rrn* genes of *Sphingomonas* species. The primer pairs were identified based on selectivity analysis using the Advanced BLAST Search program (GenBank, National Center for Biotechnology Information [NCBI]) (2) and the Sequence Match program (RDPII) (9). The final primer set consisted of the forward primer Sphingo108f (5′-GCGTAACGCGTGGGAATCTG-3′, *Escherichia coli* positions 108 to 128) and the reverse primer Sphingo420r (5′-TTAC AACCCTAAGGCCTTC-3′, *E. coli* positions 420 to 401). A 40-bp GC clamp (CGCGGGCGGCGCGCGGCGGGGCGGGGCGGGGGCGCGGGGGG) (37) was attached to the 5′ end of the reverse primer to allow DGGE analysis of the amplicons. This new primer pair Sphingo108f and GC40-Sphingo420r amplified a 312-bp sequence of the 16S rRNA gene, resulting in a PCR product 352 bp long.

DNA extraction. DNA was extracted from cultures and soil as described previously (Leys et al., submitted). The DNA concentrations in the 100-μl cell extracts and 50-μl soil extracts were measured spectroscopically. For PCR purposes, the concentration of pure strain DNA was adjusted to a final concentration of 100 ng μl^{-1}. For *Sphingomonas* cells, 100 ng of DNA corresponds to circa 2.9×10^7 cell equivalents and 2.9×10^7 copies of PCR targets, assuming a genomic molecular size of 3.2 Mb (i.e., ca. 2.1×10^9 Da = 3.5 fg of DNA) per cell (13) and only one 16S rRNA gene copy per genome (15, 49). To ensure that the soil DNA was of good quality for PCR, dilution series of all soil DNA extracts were tested in PCR with universal eubacterial 16S rRNA gene primer pair GC-63f and 518r with the forward primer linked to a 40-bp GC clamp (37). Dilutions of 1:10, 1:100, and 1:1,000 soil DNA extracts in water were further used as a template in a dilution-to-extinction PCR with the appropriate primer sets.

PCR. PCRs with universal eubacterial 16S rRNA gene primers were performed as previously described (31, 37). The PCR protocol used with the Sphingo108f/GC40-Sphingo420r primer pair consisted of a short denaturation of 15 s at 95°C, followed by 50 cycles of denaturation for 3 s at 95°C, annealing for 10 s at 62°C, and elongation for 30 s at 74°C. The last step included an extension for 2 min at 74°C. PCR was performed on Biometra (Göttingen, Germany) or Perkin-Elmer (Norwalk, Conn.) PCR machines. PCR mixtures contained 100 ng of pure strain DNA or dilutions of soil DNA as templates, 1 U of *Taq* polymerase, 25 pmol of the forward primer, 25 pmol of the reverse primer, 10 nmol of each deoxynucleoside triphosphate (dNTP), and 1× PCR buffer in a final volume of 50 μl. The *Taq* polymerase, dNTPs, and PCR buffer were purchased from TaKaRa.

DGGE analysis. The PCR products were checked on 1.5% agarose gels (MetaPhor, BioWhittaker, Labtrade, Inc., Miami, Fla.) and directly used for DGGE analysis on polyacrylamide gels as described by Muyzer et al. (36). Optimal denaturing conditions were defined based on the theoretical melting temperatures of amplification fragments produced with the Sphingo primer set as calculated with the DAN program (EMBOSS, version 2.3.1) and the Melt program (version 1.0.1; INGENY International BV, Goes, The Netherlands). A 6% polyacrylamide gel with a denaturing gradient of 40 to 75% (where 100% denaturant gels contain 7 M urea and 40% formamide) was used for DGGE analysis. Electrophoresis was performed at a constant voltage of 130 V for 16 h 40 min in 1× TAE (Tris-acetate-EDTA) running buffer at 60°C in the DGGE machine (INGENYphorU-2; INGENY International BV). After electrophoresis, the gels were stained with 1× SYBR Gold nucleic acid gel stain (Molecular Probes Europe BV, Leiden, The Netherlands) and photographed under UV light with a Pharmacia digital camera system with Liscap Image Capture software (Image Master VDS; Liscap Image Capture, version 1.0, Pharmacia Biotech, Cambridge, England). Photofiles were processed and analyzed with Bionumerics software (version 2.50; Applied Maths, Kortrijk, Belgium).

Sensitivity of PCR detection. To examine the sensitivity of the PCR method to detect *Sphingomonas* strains in soil, a standard made up of living cells of *Sphingomonas* sp. strain LB126 was added at different final cell concentrations (i.e., approximately 10^5, 10^4, 10^3, 10^1, and 10^0 CFU g^{-1}) to an uncontaminated model soil prior to DNA extraction. Before they were added to the soil samples, the cultures were filtered over glass wool to remove the excess of PAH crystals, washed twice, and finally appropriately diluted in an isotonic aqueous solution of 0.85% (wt/vol) NaCl. The total soil DNA extract was subsequently used as a template in PCR with the Sphingo primers, and PCR products were analyzed by DGGE.

PCR-DGGE analysis of *Sphingomonas* communities in PAH-contaminated soils. To assess the presence of *Sphingomonas* strains in a set of contaminated soils, soil DNA extracts were analyzed in PCR with the Sphingo primer set. To roughly estimate the concentration of the detected *Sphingomonas* cells, dilution series of noninoculated soil DNA extracts (1:1, 1:10, 1:100, and 1:1,000 dilutions in water) were tested in a dilution-to-extinction PCR approach, similar to the most probable number (MPN)-PCR approach. The final cell density within a soil was deduced from the highest template dilution for which a PCR product was still detected, taking into account that the highest dilution giving a signal contained a cell density approaching the determined detection limit. Parallel soil samples with added cells were regarded as positive PCR controls to ensure that negative PCR results with samples without added cells were not due to PCR inhibition effects. 16S rRNA gene amplicons resulting from PCR with the Sphingo primer set on the soil DNA extracts were cloned into plasmid vector pCR2.1-TOPO by using the TOPO cloning kit (N.V. Invitrogen SA, Merelbeke, Belgium) as described in the kit's protocol without prior concentration or purification. Clones containing recombinant vectors with the appropriate 16S rRNA gene fragment were compared with the soil *Sphingomonas* community fingerprints by using DGGE to identify which bands from the pattern were selected. A selection of clones with different DGGE patterns was sequenced by the Westburg Company. The 16S rRNA gene sequences obtained from the cloned PCR products were submitted to the Chimera Check program (RDPII) (9) to detect possible chimeras that could have been formed during PCR (30). A similarity analysis of the 16S rRNA gene sequences was obtained by using the Advanced Blast Search program (GenBank, NCBI) (2). To study the evolutionary relationships between the 16S rRNA gene sequences retrieved from PCR-amplified soil DNA and from known *Sphingomonas* species, clone sequences were imported into the alignment and edited manually to remove nucleotide positions of ambiguous alignment and gaps. Sequence similarities were calculated for the total length of the 16S rRNA gene sequences and corrected using Kimura's two-parameter algorithm to compensate for multiple nucleotide exchange, and a distance-based evolutionary tree was constructed using Kimura's corrected similarity values in the neighbor-joining algorithm of Saitou and Nei (40). The

TABLE 1. Bacterial strains used in this study

Organism (origin or reference)	Compound catabolized[a]	Accession no. of 16S rRNA gene	PCR signal with Sphingo108f/Sphingo420r primers[b]
Proteobacteria phylum			
α-*Proteobacteria*, α-4 subclass			
Sphingomonadaceae family, *Sphingomonas* genus			
Sphingomonas adhaesiva Op-55 (DSM7418^T)	NR	D16146	+
Sphingomonas "agrestis" HV3 (57)	Nap	Y12803	+
Sphingomonas aromaticivorans F199 (DSM12444^T)	Nap, Tol, Xyl, Bip, Flu, Dibt, Cres	AB025012	+
Sphingomonas asaccharolytica Y-345 (DSM10564^T)	NR	Y09639	+
Sphingomonas capsulata 28 (DSM30196^T)	NR	D16147	+
Sphingomonas chlorophenolica (DSM7098^T)	PCP, TiCP	X87161	+
Sphingomonas chlorophenolica RA2 (DSM6824)	PCP	X87164	+
Sphingomonas sp. strain VM0440 (Springael, unpublished)	Phe	AY151392	+
Sphingomonas sp. strain LB126 (4, 5)	Flu	AF335501	+
Sphingomonas sp. strain VM0506 (Springael, unpublished)	Flu	AF335468	+
Sphingomonas sp. strain LH227 (5)	Phe	AY151393	+
Sphingomonas macrogolitabida 203 (DSM8826^T)	PEG	D13723	+
Sphingomonas mali Y-347 (DSM10565^T)	NR	Y09638	+
Sphingomonas natatoria UQM2507 (DSM3183^T)	NR	AB024288	+
Sphingomonas parapaucimobilis OH3607 (DSM7463^T)	NR	D13724	+
Sphingomonas paucimobilis KS0301 (LMG2239)	NR	D38420	+
Sphingomonas paucimobilis CL1/70 (DSM1098^T)	NR	D13725	+
Sphingomonas pruni Y-250 (DSM10566^T)	NR	Y09637	+
Sphingomonas rosa R135 (DSM7285^T)	NR	D13945	+
Sphingomonas sanguis KM2397 (LMG2240^T)	NR	D13726	+
Sphingomonas sp. strain EPA505 (DSM7526)	Flu, Nap, Phe, Ant, Bflu	U37341	+
Sphingomonas subarctica KF1 (DSM10700^T)	TeCP, TiCP	X94102	+
Sphingomonas subarctica KF3 (DSM10699)	TeCP, TiCP	X94103	+
Sphingomonas sp. strain LH128 (3)	Phe	AY151394	+
Sphingomonas suberifaciens CR-CA1 (DSM7465^T)	NR	D13737	+
Sphingomonas terrae (LMG10924)	NR	D38429	+
Sphingomonas terrae E-1-A (DSM8831^T)	PEG	D13727	+
Sphingomonas trueperi (DSM7225^T)	NR	X97776	+
Sphingomonas ursincola KR-99 (DSM9006^T)	NR	AB024289	+
Sphingomonas wittichii RW1 (DSM6014^T)	Dbf	AB021492	+
Sphingomonas xenophaga BN6 (DSM6383^T)	2-Nap-sulfonate	X94098	+
Sphingomonas yanoikuyae AB1105 (DSM7462^T)	NR	D16145	+
Sphingomonas yanoikuyae B1 (DSM6900)	Tol, Xyl, Bip, Nap, Phe	X94099	+
Sphingomonas yanoikuyae Pn4S (LMG3925)	NR	D13946	+
Other *Sphingomonadaceae* genera			
Porphyrobacter neustonensis (DSM9434^T)	NR	AB033327	+
Porphyrobacter tepidarius OT3 (DSM10594^T)	NR	AB033328	+
Erythrobacter litoralis T4 (DSM8509^T)	NR	AB013354	+
Erythromicrobium ramosum E5 (DSM8510^T)	NR	AB013355	+
Zymomonas mobilis subsp. *paniaceae* I (LMG448^T)	NR	AF281032	+
Other α-*Proteobacteria*			
Phyllobacterium rubiacearum (DSM5893^T)	NR	D12790	−
Agrobacterium luteum A61 (DSM5889^T)	NR	NR	−
Rhizobium radiobacter L624 (DSM30147^T)	NR	AJ389904	−
Rhizobium radiobacter B6 (DSM30205)	NR	D14500	(+)
Rhizobium radiobacter B2326 (DSM30203)	NR	D14506	(+)
Rhizobium rubi TR3 (DSM6772^T)	NR	D12787	(+)
Sinorhizobium meliloti 3DOa2 (DSM30135^T)	NR	D14509	−
Rhodobacter sphaeroides ATH2.4.1 (DSM158^T)	NR	D16425	−
Rhodobacter sphaeroides (DSM160)	NR	NR	−
Rhodobacter capsulatus (ATCC 23782)	NR	NR	−
Rhodospirillum rubrum B-280 (ATCC 19613)	NR	NR	−
Rhodospirillum rubrum S1H (ATCC 25903)	NR	NR	−
Brevundimonas diminuta 342 (DSM7234^T)	NR	AJ227778	−
Brevundimonas diminuta PC1818 (DSM1635)	NR	X87274	−
β-γ-δ-*Proteobacteria*			
Ralstonia metallidurans CH34 (DSM2839^T)	NR	Y10824	−

Continued on facing page

TABLE 1—*Continued*

Organism (origin or reference)	Compound catabolized[a]	Accession no. of 16S rRNA gene	PCR signal with Sphingo108f/Sphingo420r primers[b]
Burkholderia sp. strain JS150 (DSM8530)	Ben	AF262932	−
Aeromonas enteropelogenes J11 (DSM6394^T)	NR	X71121	−
Acinetobacter calcoaceticus 46 (DSM30006^T)	NR	AJ247199	−
Pseudomonas putida (DSM8368)	Nap, Phe, Flu, Fan	NR	−
Desulfobacter latus AcRS2 (DSM3381^T)	NR	AJ441315	−
Desulfonema magnum 4be13 (DSM2077^T)	NR	U45989	−
Desulfobulbus rhabdoformis M16 (DSM8777^T)	NR	U12253	−
Gram-positive bacteria			
Arthrobacter sulfureus 8-3 (DSM20167^T)	NR	X83409	−
Dietzia maris IMV 195 (DSM43627^T)	NR	X79290	−
Mycobacterium frederiksbergense FAn9 (DSM44346^T)	Fan, Phe, Pyr	AJ276274	−

[a] NR, not reported; Nap, naphthalene; Fan, fluoranthene; Pyr, pyrene; Flu, fluorene; Phe, phenanthrene; Ant, anthracene; Bflu, benzo(b)fluorene; Dibt, dibenzothiophene; Dibf, dibenzofurane; Bip, biphenyl; Ben, benzene; Tol, toluene; Xyl, xylene; TiCP, trichlorophenol; TeCP, tetrachlorophenol; PCP, pentachlorophenol; PEG, polyethylene glycol; Cres, cresol.
[b] Results of PCR with primers Sphingo108f and GC40-Sphingo420r on pure strain DNA extract are shown. +, high concentration of PCR product; (+), low concentration of PCR product; −, no detectable PCR product.

topography of the branching order within the dendrogram was evaluated by using the maximum-likelihood and maximum-parsimony character-based algorithms in parallel combined with bootstrap analysis with a round of 500 reassemblings. The 16S rRNA gene sequence from some closely related genera from the *Sphingomonadaceae* (*Zymomonas*, *Porphyrobacter*, *Erythrobacter*, *Sandaracinobacter*, etc.) and some more distantly related α-*Proteobacteria* (*Rhizobium*, *Rhodospirillum*, *Rhodobacter*, *Sinorhizobium*, etc.) were included as an out-group to root the tree.

Nucleotide sequence accession number. The 16S rRNA gene clone sequences retrieved from contaminated soils with the Sphingo primer set are available from GenBank under accession no. AY335445 to AY335484.

RESULTS

Design of a *Sphingomonas* genus-specific primer set. The *rrn* gene is moderately conserved within the *Sphingomonas* genus, as was indicated by a similarity plot created from an alignment of *Sphingomonas* 16S RNA gene sequences (minimum of 1,300 bp). The alignment showed a minimum similarity of ca. 89% over the total length of the *rrn* gene within the *Sphingomonas* genus (data not shown). From the alignment, we selected a new nondegenerate primer set that would anneal to 16S rRNA gene sequences and that spanned a region between 200 and 600 bp long with high variability in order to allow differentiation of the various species by DGGE analysis of the PCR-products. Blast (NCBI) and Sequence Match (RPDII) analyses (April 2003) were used to check primer selectivity. Of the six different primers selected and tested in different appropriate combinations (data not shown), the primer pair Sphingo108f/Sphingo420r was the best combination possible, targeting as many *Sphingomonas* species as possible and as few as possible non-*Sphingomonas* sequences. The forward primer Sphingo108f was highly selective for the *Sphingomonas* genus (Table 3). Of all sequences available in the NCBI database (9), which currently holds circa 375 *Sphingomonas* genus sequences of all lengths, ca. 350 sequences were found 100% homologous to the Sphingo108f primer sequence by using the Sequence Match software (RDPII). Besides, within *Sphingomonas* strains, the forward primer was also 100% conserved in 16S rRNA gene sequences of *Sandaracinobacter*, *Zymomonas*, *Porphyrobacter*, *Erythrobacter*, or *Erythromicrobium* strains, which like *Sphingomonas* belong to the family *Sphingomonadaceae* (Table 3). Only a few of the sequences with 100% homology to primer Sphingo108f (ca. 20 sequences) corresponded to some *Caulobacter*, *Pseudomonas*, or *Rhizobium* strains. At least two mismatches were found between the primers in 16S rRNA gene sequences of other strains not belonging to the family *Sphingomonadaceae* (Table 3). The reverse primer Sphingo420r proved to be more conserved (i.e., at least 1,600 sequences in the bacterial ribosomal database showed 100% similarity to the primer sequence). Sequences of all genera of

TABLE 2. Characteristics of soil samples used in this study

Soil	Origin	Soil type	pH	Total organic carbon (%)	PAH concn (mg kg^{-1})	Mineral oil concn (mg kg^{-1})	DNA concn (μg g^{-1})[a]	Highest PCR-positive template dilution[b]	Estimated cell concn (cells g^{-1})[c]
K3840	Gasoline station site (Denmark)	Sand	8.2	0.50	20	98	2.75	1/100	10^6
B101	Coal gasification plant (Belgium)	Sand	7.0	2.63	107	70	27.25	1/100	10^5
TM	Coal gasification plant (Belgium)	Sand	8.0	3.85	506	4,600	4.75	1/100	10^6
BarI	Coal gasification plant (Germany)	Gravel	8.9	4.63	1,029	109	6.15	1/100	10^6
AndE	Railway station site (Spain)	Clay	8.1	2.35	3,022	2,700	ND[d]	1/100	10^6

[a] DNA recovery per gram of soil (mean value of two parallel extractions of one soil sample).
[b] Product of PCR with Sphingo108f and GC40-Sphingo420r on soil DNA extract.
[c] Roughly estimated *Sphingomonas* cell concentration based on a dilution-to-extinction PCR approach.
[d] ND, not determined.

TABLE 3. DNA sequence homology between the *Sphingomonas* genus-specific primers and the 16S rRNA gene sequences of different bacterial genera and species

Organism (accession no.)[a]	Primer sequence[b]	
	Sphingo108f (*E. coli* positions 108–128)	Sphingo420r (*E. coli* positions 420–401)
Sphingomonas genus strains	5′-GCGTAACGCGTGGGAATCTG-3′	5′-TTACAACCCTAAGGCCTTC-3′
S. wittichii DSM6014^T (AB021492)	--------------------	--------------------
S. pituitosa DSM13101^T (AJ243751)	--------------------	--------------------
S. trueperi DSM7225^T (X97776)	--------------------	--------------------
S. paucimobilis DSM10987^T (U37337)	--------------------	----------G----------
S. parapaucimobilis DSM7463^T (D13724)	--------------------	----------G----------
S. sanguinis LMG17325^T (D13726)	--------------------	----------G----------
S. aquatilis IFO16772^T (AF131295)	--------------------	--------------------
S. echinoides DSM1805^T (AB021370)	--------------------	--------------------
S. adhaesiva DSM7418^T (D16146)	--------------------	----------G----------
S. pruni DSM10566^T (Y09637)	--------------------	--------------------
S. mali DSM10565^T (Y096368)	--------------------	--------------------
S. asaccharolytica DSM10564^T (Y09639)	--------------------	--------------------
S. suberifaciens DSM7465^T (D13737)	--------------------	--------------------
S. yanoikuyae DSM7462^T (D16145)	--------------------	--------------------
S. xenophaga DSM6383^T (X94098)	--------------------	--------------------
S. chlorophenolicum DSM7098^T (X87161)	--------------------	--------------------
S. chungbukensis JCM11454^T (AF159257)	--------------------	--------------------
S. herbicidivorans DSM11019^T (AB042233)	--------------------	--------------------
S. cloacae JCM10874^T (AB040739)	--------------------	--------------------
S. rosa DSM7285^T (D13945)	--------------------	--------------------
S. stygia CIP10514^T (AB025013)	--------------------	--------------------
S. subterranea CIP105153^T (AB025014)	--------------------	--------------------
S. aromaticivorans DSM12444^T (AB025012)	--------------------	--------------------
S. capsulatum DSM30196^T (D16147)	--------------------	--------------------
S. terrae DSM8831^T (D13727)	--------------------	----------G----------
S. macrogolitabida DSM8826^T (D13723)	--------------------	----------G----------
S. alaskensis DSM13593^T (Z73631)	--------------------	--------------------
S. taejonensis JCM11457^T (AF131297)	--------------------	----------G----------
S. subarctica DSM10700^T (X941025)	--------------------	--------------------
Other *Sphingomonadaceae* family strains		
Sandaracinobacter sibericus RB16–17 (Y10678)	--------------------	---------G---------
Porphyrobacter tepidarius DSM10594^T (AB033328)	--------------------	--------------------
Porphyrobacter neustonensis DSM9434^T (AB033327)	--------------------	--------------------
Erythrobacter longus DSM6997^T (M59062)	--------------------	--------------------
Erythromicrobium ramosum DSM8510^T (AB013355)	--------------------	--------------------
Non–*Sphingomonadaceae* strains		
Rhizobium rubi IFO13261 (D14503)	A------------------A	--------------------
Rhizobium rubi DSM9772^T (X67228)	A------------------A	--------------------
Rhodobacter sphaeroides 2.4.1^T (X53853)	A---------------CG--	--------------------
Methylobacterium radiotolerans JCM2831^T (D32227)	A---------------CG--	--------------------
Rhizobium radiobacter DSM30147^T (AJ389904)	A---------------CA-A	--------------------
Methylobacterium organophilum JCM2833^T (D32226)	A-----A---------CG-A	--------------------
Rickettsia massiliae Mtu1^T (L36214)	A-----A-----------A	--------------------
Rickettsia honei RB^T (U17645)	A-----A-----------A	--------------------
Bradyrhizobium japonicum DSM30131^T (U69638)	A---------------CG-A	---------G---------
Rhodospirillum rubrum ATCC11170^T (D30778)	A-----A----------G-A	---------G---------
Caulobacter vibroides CB2A^T (M83799)	A-----A---------CG--	------T----A-------
Pseudomonas aeruginosa LMG1242^T (Z76651)	A----T-C-A----------	C-----T----A-------
Pseudomonas putida DSM291^T (Z76667)	A----T-C-A----------	------T----A-------

[a] Accession no. of 16S rRNA gene sequence in GenBank (NCBI).
[b] Results are presented in a consensus table of matches. Dashes indicate identical nucleotides.

the family *Sphingomonadaceae* (i.e., *Sphingomonas*, *Zymomonas*, *Porphyrobacter*, *Erythrobacter*, and *Erythromicrobium*) aligned perfectly with the reverse primer sequence. Some *Sphingomonas* and *Sandaracinobacter* species had a single mismatch with the reverse primer. Most non-*Sphingomonadaceae* sequences with 100% homology to the Sphingo420r primer belonged to some strains of the genera *Rhizobium*, *Methylobacterium*, and *Rickettsia*. The newly developed Sphingo108f/GC40-Sphingo420r primer pair produced only products of the appropriate size and only with the DNA obtained from all 34 tested *Sphingomonas* strains representing different species (Table 1), while the other tested primer combinations did not. As expected, positive PCR results also were obtained for most of the test strains belonging to the other *Sphingomonadaceae*

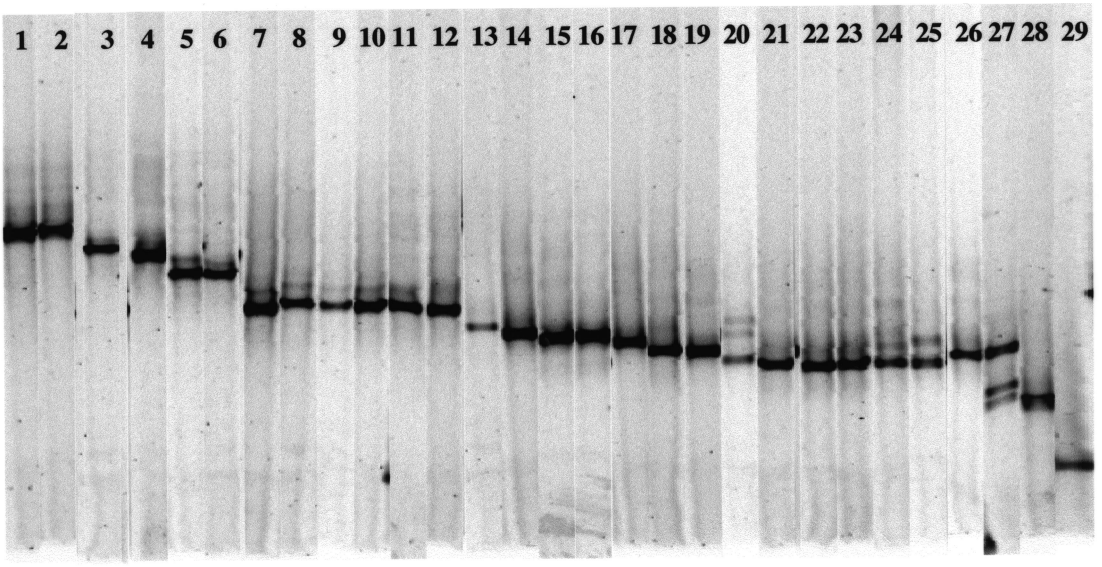

FIG. 1. *Sphingomonas* species differentiation by DGGE analysis of DNA fragments amplified with primers Sphingo108f/GC40 and Sphingo420r. The separate lanes represent the different species-specific DGGE melting profiles of different tested *Sphingomonas* strains. Lanes: 1, *Sphingomonas* sp. strain VM0506; 2, *Sphingomonas* sp. strain LB126; 3, *S. macrogolitabida* DSM8826^T; 4, *S. natatoria* DSM3183^T; 5, *S. mali* DSM10565^T; 6, *S. terrae* DSM8831^T; 7, *S. yanoikuyae* DSM7462^T; 8, *S. suberifaciens* DSM7465^T; 9, *S. asaccharolytica* DSM10564^T; 10, *S. pruni* DSM10566^T; 11, *S. capsulata* DSM30196^T; 12, *S. rosa* DSM7285^T; 13, *S. aromaticivorans* DSM12444^T; 14, *S. xenophaga* DSM6383^T; 15, *Zymomonas mobilis* LMG448^T; 16, *Erythrobacter litoralis* DSM8509^T; 17, *Sphingomonas* sp. strain LH227; 18, *S. wittichii* DSM6014^T; 19, *Sphingomonas* sp. strain EPA505; 20, *S. paucimobilis* DSM1098^T; 21, *Sphingomonas* sp. strain LH128; 22, *S. subarctica* DSM10700^T; 23, *S. subarctica* DSM10699; 24, *S. paucimobilis* LMG2239; 25, *S. parapaucimobilis* DSM7463^T; 26, *S. sanguis* LMG2240; 27, *S. trueperi* DSM7225^T; 28, *S. flava* DSM6824; 29, *S. adhaesiva* DSM7418^T. Lanes were ordered with Bionumerics software to group and compare several DGGE profiles.

genera (i.e., *Porphyrobacter*, *Erythrobacter*, *Zymomonas*, and *Erythromicrobium*) and faint signals were obtained for some *Rhizobium* strains. In PCR with the DNA of the 11 tested non-α-*Proteobacteria* genera (Table 1), no products were detected. It can thus be concluded that the newly designed primer set Sphingo108f/Sphingo420r is selective for the detection of *Sphingomonas* strains and probably all bacteria belonging to the family *Sphingomonadaceae*.

DGGE analysis of pure strain PCR fragments amplified with the Sphingo primer set. In order to examine if DGGE analysis would allow direct differentiation of *Sphingomonas* species in mixed environmental communities, a GC40 clamp was attached to the reverse primer Sphingo420r and the PCR-obtained 16S rRNA gene fragments were loaded on a DGGE gel (Fig. 1). All tested *Sphingomonas* strains were characterized by a DGGE profile consisting of a single band, except for *S. trueperi* DSM7225^T (lane 27) and *S. paucimobilis* DSM7463^T (lane 20), which showed two less-intense additional bands. Strains which are very closely related based on the 16S rRNA gene, most likely belonging to the same species, showed identical DGGE fingerprints, as indicated for strains VM0506 and LB126, closely related to *Sphingomonas chungbukensis* (lanes 1 and 2), or three *S. subarctica* strains (lanes 21 to 23). Different species showed mostly different DGGE fingerprints. However, some very closely related species (amplicon similarity of >97%) displayed similar DGGE fingerprints, like, for example, *S. paucimobilis* and *S. parapaucimobilis* (lanes 24 and 25) or *Sphingomonas asaccharolytica* and *Sphingomonas pruni* (lanes 9 and 10). Similar DGGE fingerprints were also found for two more distantly related species, such as *Sphingomonas mali* and *Sphingomonas terrae* (lanes 5 and 6).

Limit of detection of *Sphingomonas* in soil using the PCR protocol with primers Sphingo108f and GC40-Sphingo420r. An inoculated soil experiment was set up to investigate the amplification sensitivity of the new primer set Sphingo108f/GC40-Sphingo420r. Living cells of *Sphingomonas* sp. strain LB126 were added at different final cell concentrations to an uncontaminated model soil prior to DNA extraction. *Sphingomonas* strain LB126 could be detected down to a cell concentration of 2×10^4 CFU g^{-1}.

Analysis of *Sphingomonas* soil populations with primer set Sphingo108f/GC40-Sphingo420r. Different PAH-contaminated soil samples with different contamination records from different European sites (Table 2) were screened for the presence of *Sphingomonas* species by PCR with the Sphingo primer set on total soil DNA extracts followed by DGGE analysis of the resulting 16S rRNA gene amplicons for diversity analysis. The DNA concentration in the soil extract indicated an approximate DNA recovery of 0.135 to 1.375 μg of DNA g of $soil^{-1}$. Assuming that 100% of the in situ biomass represents bacteria and a bacterial cell contains in general 5 fg of DNA per cell (8), this would theoretically be equivalent to 2.7×10^7 to 2.8×10^8 cells g of $soil^{-1}$. Indigenous *Sphingomonas* could be detected in all tested soils (Fig. 2). The dilution-to-extinction PCR method roughly estimated the total *Sphingomonas* cell concentration to be between 10^5 and 10^6 cells per g of soil (Table 2).

The DGGE profiles of the *Sphingomonas* community in the

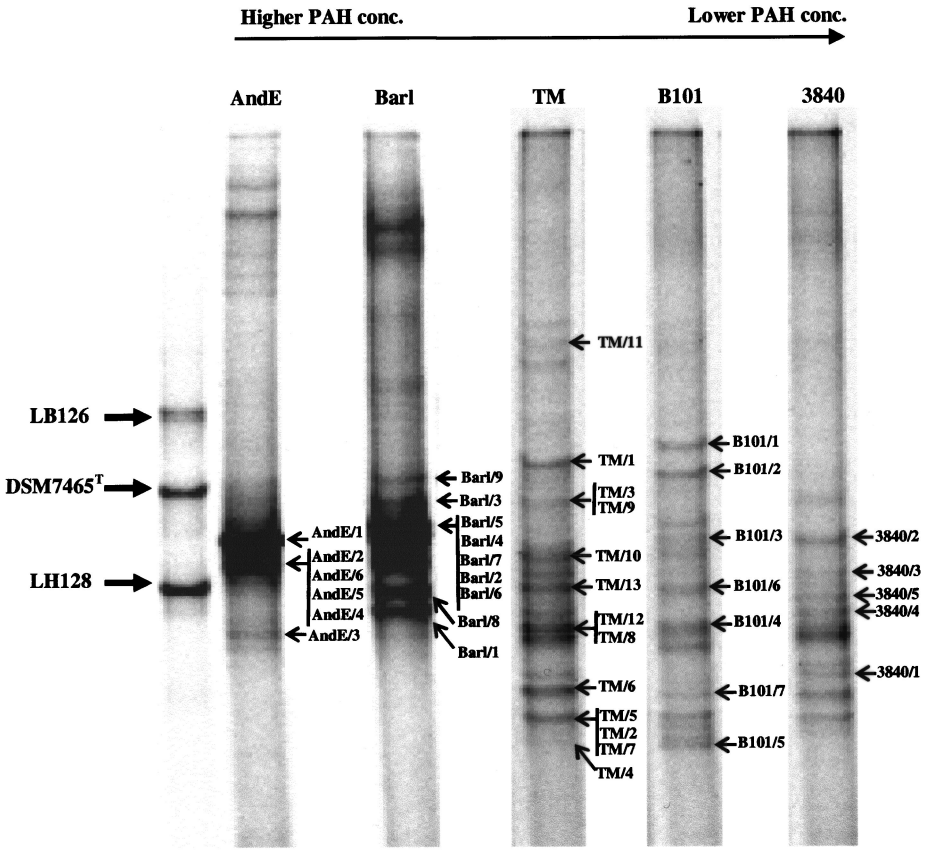

FIG. 2. DGGE analyses of indigenous *Sphingomonas* communities in natural soil samples using primers Sphingo108f and GC40-Sphingo420r in PCR. The separate lanes indicate the DGGE fingerprints of the indigenous *Sphingomonas* community of PAH-contaminated soils AndE, Barl, TM, B101, and K3840. Cloned bands are indicated within the soil fingerprint based on the comparison of migration profiles of pure clones and the soil profile. A mixture of six strains was used as a marker during DGGE analysis.

soil samples retrieved by PCR with primer set Sphingo108f/GC40-Sphingo420r were relatively complex, comprising several bands for each sample (Fig. 2). Soils containing highest concentrations of PAHs showed the lowest number of *Sphingomonas* 16S rRNA gene bands, while less-contaminated soils showed a significantly higher number of bands in DGGE fingerprinting. The diversity differences among the samples were further analyzed by random cloning of 16S rRNA gene PCR products and sequencing of clones showing diverse DGGE patterns. A comparison of the soil DGGE profiles and the DGGE profiles obtained with the soil clones allowed presumptive identification of some bands (Fig. 2). Most cloned sequences matched significantly (93 to 99% similarity) with 16S rRNA gene *Sphingomonas* sequences from the databases by Blast analysis (Table 4). However, 60% of the Blast results were sequences from "uncultured" α-*Proteobacteria* and *Sphingomonas* isolates with unknown phylogenetic positions within the *Sphingomonas* genus. To further identify the species lineation, the 40 cloned 16S rRNA gene sequences were aligned with ca. 200 database sequences and a phylogenetic tree was constructed. Phylogenic analysis revealed that all clone sequences exhibited high levels of similarity to sequences typical of the family *Sphingomonadaceae*, except one (clone Barl/9) that was more related to other α-*Proteobacteria* (Table 4 and Fig. 3). Only a few clone sequences were placed in groups with *Sphingomonadaceae* genera different from *Sphingomonas*, like *Sandaracinobacter* (clone TM/2) or *Erythrobacter* (clone TM/3), which are intermixed with the clusters of the *Sphingomonas* genus in the phylogenetic tree (Fig. 3). Thus, most cloned sequences were affiliated with true *Sphingomonas* sequences, confirming the specificity of the newly designed Sphingo primer set. However, only a very small percentage of cloned sequences (5 of 40) seemed to be related to cultured PAH-degrading identified *Sphingomonas* species, such as *S. wittichii* (Barl/1 and TM/1), *S. yanoikuyae* and *S. xenophaga* (Barl/8), *S. chilensis* (3840/2), and *S. subarctica* (Barl/8). These culturable PAH-degrading *Sphingomonas* isolates are exclusively connected to strains found in the former "*Sphingobium*," "*Sphingopyxis*," and "*Novosphingobium*" genera proposed in 2001 by Takeuchi et al. (46). There were no PAH-degrading isolates or cloned sequences from PAH-contaminated soil found to be related to any of the species of the former "*Sphingomonas sensu stricto*" genus. Most clone sequences isolated in this study were rather grouped in clusters with other uncultured *Sphingomonas* 16S rRNA gene sequences and a few unidentified *Sphingomonas* sp. 16S rRNA gene sequences. Thus, these

TABLE 4. Results of analysis of BLAST 16S rRNA gene cloned sequences retrieved from different soil samples

Soil	Clone (accession no.)	Best match in BLAST analysis (2)	Closest species match
K3840	3840/1 (AY335480)	91% to uncultured *Sphingomonas* clone CEA (AF392653)	
	3840/2 (AY335481)	95% to *S. witflariensis* W-50 (AJ416410)	*S. witflariensis*
	3840/3 (AY335482)	98% to uncultured *Sphingomonas* clone D104 (AF337854)	Putative new *Sphingomonas* species 2
	3840/4 (AY335483)	98% to uncultured *Sphingomonas* clone 367-2 (AF423253)	Putative new genus
	3840/5 (AY335484)	98% to uncultured *Sphingomonas* clone 739-2 (AF42389)	Putative new *Sphingomonas* species 2
B101	B101/1 (AY335454)	97% to *Afipia* genospecies 11 (U87782)	putative new *Sphingomonas* species 2
	B101/2 (AY335455)	99% to uncultured *Sphingomonas* clone 768-2 (AF423293)	Putative new genus
	B101/3 (AY335456)	96% to *Sphingomonas* sp. strain K6 (AJ000918)	Putative new *Sphingomonas* species 2
	B101/4 (AY335459)	95% to *Sphingomonas* sp. strain SIA181-1A1 (AF395032)	
	B101/5 (AY335460)	95% to uncultured *Sphingomonas* clone 739-2 (AF42389)	Putative new *Sphingomonas* species 2
	B101/6 (AY335457)	97% to uncultured *Sphingomonas* clone Blccii3 (AJ318120)	Putative new *Sphingomonas* species 3
	B101/7 (AY335458)	98% to *Sphingomonas* sp. strain RSI-28 (AJ252595)	Putative new *Sphingomonas* species 2
TM	TM/1 (AY335468)	96% to uncultured *Sphingomonas* clone WD290 (AF058299)	*S. wittichii*
	TM/2 (AY335476)	96% to uncultured *Sphingomonas* clone TRS1 (AJ006014)	*Sandaracinobacter sibericus*
	TM/3 (AY335470)	98% to *Porphyrobacter* sp. strain MBIC3936 (AF058299)	*Erythrobacter longus*
	TM/4 (AY335479)	96% to uncultured *Sphingomonas* clone 739-2 (AF42389)	Putative new *Sphingomonas* species 2
	TM/5 (AY335477)	97% to uncultured *Sphingomonas* clone WD249 (AJ292599)	Putative new *Sphingomonas* species 2
	TM/6 (AY335475)	98% to uncultured *Sphingomonas* clone saf2-409 (AF078258)	Putative new *Sphingomonas* species 2
	TM/7 (AY335478)	96% to uncultured *Sphingomonas* clone 739-2 (AF42389)	Putative new *Sphingomonas* species 2
	TM/8 (AY335474)	96% to *Sphingomonas* sp. strain KA1 (AB064271)	*S. subarctica*
	TM/9 (AY335469)	99% to *Afipia* genospecies 13 (U87784)	Putative new genus
	TM/10 (AY335471)	97% to uncultured *Sphingomonas* clone t008 (AF422583)	*S. hassiacum*
	TM/11 (AY335467)	97% to uncultured *Sphingomonas* clone S23435 (D84626)	*S. hassiacum*
	TM/12 (AY335473)	98% to uncultured *Sphingomonas* clone a13104 (AY103311)	Putative new *Sphingomonas* species 2
	TM/13 (AY335472)	97% to uncultured *Sphingomonas* clone D104 (AF337854)	Putative new *Sphingomonas* species 2
Barl	Barl/1 (AY335453)	98% to *Sphingomonas* sp. strain SRS2 (AJ251638)	*S. wittichii*
	Barl/2 (AY335450)	98% to uncultured *Sphingomonas* clone AW030 (AF385533)	Putative new *Sphingomonas* species 1
	Barl/3 (AY335446)	98% to *S. suberifaciens* (D13737)	*S. suberifaciens*
	Barl/4 (AY335448)	99% to uncultured *Sphingomonas* clone AW030 (AF385533)	Putative new *Sphingomonas* species 1
	Barl/5 (AY335447)	97% to uncultured *Sphingomonas* clone IAFR401 (AF270954)	*S. suberifaciens*
	Barl/6 (AY335451)	96% to *Sphingomonas* sp. strain K6 (AJ000918)	*S. suberifaciens*
	Barl/7 (AY335449)	97% to uncultured *Sphingomonas* clone IAFR401 (AF270954)	*S. suberifaciens*
	Barl/8 (AY335452)	97% to *S. xenophaga* UN1F2 (U37346)	*S. xenophaga*
	Barl/9 (AY335445)	93% to uncultured *Sphingomonas* clone WD2107 (AJ292610)	α-*Proteobacteria*
And	AndE/1 (AY335461)	95% to uncultured *Sphingomonas* clone BIccii3 (AJ318120)	Putative new *Sphingomonas* species 3
	AndE/2 (AY335462)	99% to *Sphingomonas* sp. strain GTIN11 (AY056468)	*S. cloacae*
	AndE/3 (AY335466)	98% to *S. xenophaga* UN1F2 (U37346)	*S. cloacae*
	AndE/4 (AY335465)	99% to *Sphingomonas* sp. strain GTIN11 (AY056468)	*S. cloacae*
	AndE/5 (AY335464)	99% to *Sphingomonas* sp. strain GTIN11 (AY056468)	*S. cloacae*
	AndE/6 (AY335463)	99% to *Sphingomonas* sp. strain GTIN11 (AY056468)	*S. cloacae*

groups could represent 16S rRNA gene sequences of new (uncultivable) species within the *Sphingomonas* genus. The cluster with isolate "*Sphingomonas* sp. strain Ellin4265" could even represent a new genus within the *Sphingomonadaceae* different from *Sphingomonas* because of its organization in the phylogenetic tree in a separate branch together with *Sandaracinobacter*. Other 16S rRNA gene clones were grouped in possibly new *Sphingomonas* species with (i) isolate *Sphingomonas* sp. strain AW030 (species 1), (ii) isolates *Sphingomonas* sp. strain SIA181-1A1 and RSI-28 (species 2), or (iii) isolate *Sphingomonas* sp. strain SI-15 (species 3). An especially high fraction of cloned sequences (12 of 40 clones) was found in the clusters of possible new species 2. Most sequences originating from one soil were relatively taxonomically spread across the total *Sphingomonas* genus, except for the sequences originating from soil AndE, the most heavily contaminated soil tested, for which 5 of 6 sequences grouped together in the cluster with *S. cloacae* IAM14885^T.

DISCUSSION

To analyze and monitor the diversity and dynamics of the *Sphingomonas* population during bioremediation processes, a detection method allowing simultaneous detection of several *Sphingomonas* species was developed. Up to now, the available primer combinations based on 16S rRNA gene were relatively strain and/or species specific (27, 50) and were not suited for simultaneous detection of all PAH-degrading *Sphingomonas* species. Therefore, we developed a new set of *Sphingomonas* genus-specific 16S rRNA gene primers: primer set Sphingo108f/Sphingo-420r. As the primer set had to target the whole *Sphingomonas* genus, we were not able to exclude the detection of other *Sphingomonadaceae* genera, such as *Zymomonas*, *Porphyrobacter*, *Erythrobacter*, and *Erythromicrobium*, intermixed with the *Sphingomonas* genus branches in the 16S rRNA gene-based phylogenetic tree of the *Sphingomonadaceae* and some *Rhizobium* strains.

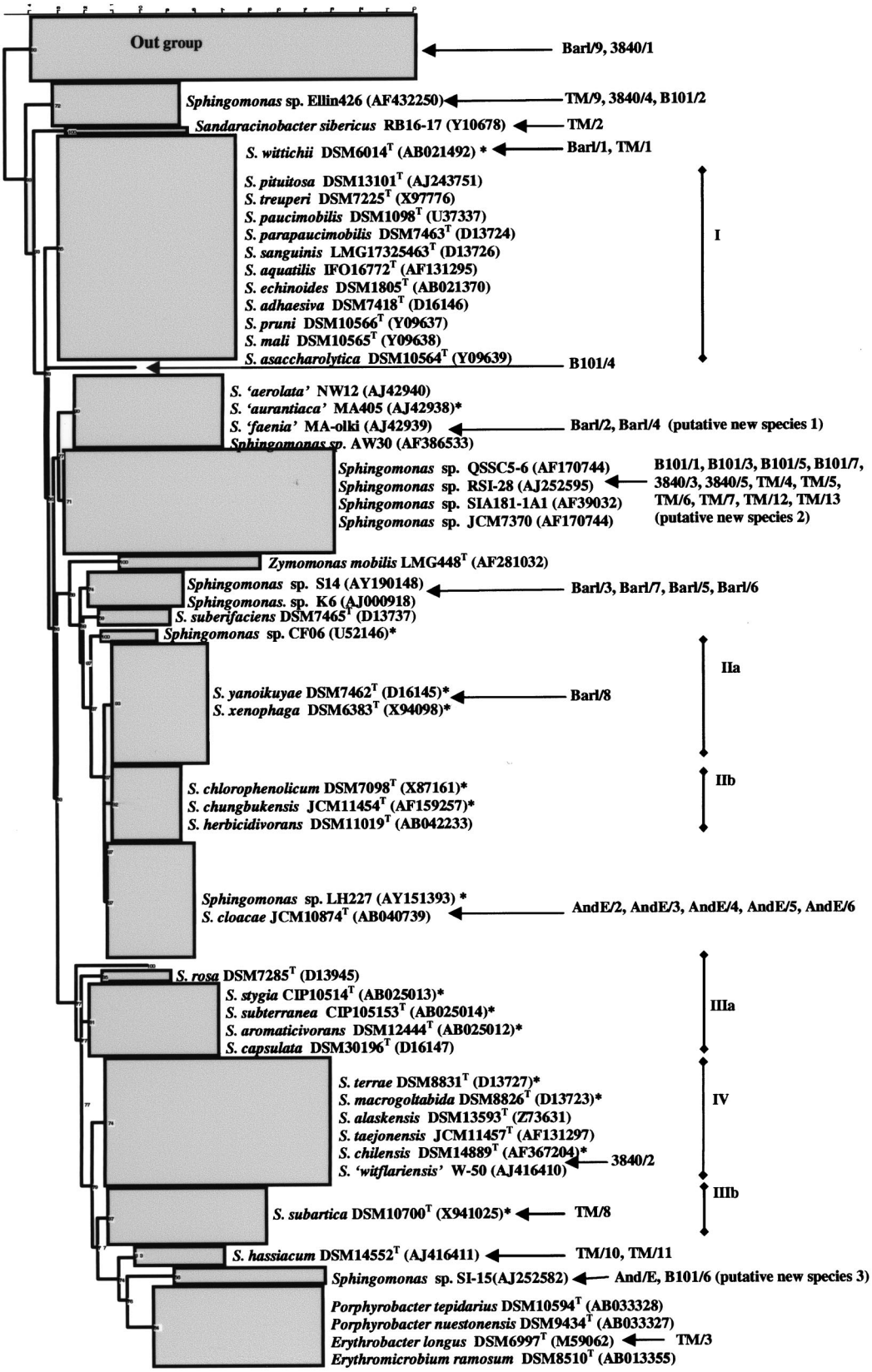

FIG. 3. Phylogenetic analyses of *Sphingomonas* sequences retrieved from soil DNA extract with primers Sphingo108f and GC40-Sphingo420r in PCR. The phylogenetic relationships of cloned sequences are indicated in a character-based evolutionary tree based on the total length of the 16S rRNA gene sequences and constructed using the neighbor-joining algorithm. An out-group of the closely related genera *Rhizobium* and

Most tested *Sphingomonas* species were characterized by a single-band DGGE fingerprint of the amplicon obtained after PCR with the Sphingo108f/GC40-Sphingo420r primer set. A multiple-band DGGE pattern was found for only 2 of 40 tested strains. A multiple-band DGGE fingerprint for a pure strain could indicate multiple 16S rRNA gene copies with sequence divergence. So far, only two references could be found that report on the rRNA gene copy number in *Sphingomonas* species. Both reports show only 1 *rrn* gene copy number for *Sphingomonas* strains MT1 (DSM13663) (49) and *S. alaskensis* RB2256 (DSM 13593^T) (15). In addition, also in the draft genome sequence of *S. aromaticivorans* DSM12444, available at the Joint Genome Institute web site (http://www.jgi.doe.gov/), so far only one 16S rRNA gene copy has been identified in one contig. However, one *rrn* gene copy is relatively exceptional in the bacterial world: in most prokaryotes, the rDNA consists of tandem repeated arrays of the *rrn* genes (25). The closely related organism *Zymomonas mobilis* ZM4 (ATCC 31821), for example, contains four gene copies (22). Further molecular analysis is needed to confirm that the tested *S. trueperi* and *S. paucimobilis* species type strains indeed contain multiple *rrn* gene copies that could explain the multiple-band DGGE pattern.

Pure strain DGGE fingerprints were mostly inter- and intraspecies specific: i.e., strains officially belonging to the same species showed identical DGGE fingerprints and different species showed different DGGE fingerprints. Overlapping fingerprints were found for some strains and species. Similarly, temperature gradient gel electrophoresis (TGGE) and DGGE analyses of 16S rRNA gene fragments could not discriminate between several species of *Burkolderia* (14) and *Bifidobacterium* (41) or *Arthrobacter* and *Nocardioides* (16), due to the high levels of conservation of the amplified 16S rRNA gene fragments. It is clear that the practical resolution limit of the DGGE technique is at the species or genus level or intermediate between the two, depending on the gene conservation level within the taxonomic group under investigation. However, all currently known species grouping-related PAH-degrading *Sphingomonas* strains could be well separated on a DGGE gel, indicating that the newly developed PCR-DGGE technique was suitable to assess the diversity and dynamics of currently known PAH-degrading *Sphingomonas* populations in soil. These results suggest that each band in a *Sphingomonas* community DGGE fingerprint of environmental samples produced by the Sphingo primer set would mostly indicate only one species or very closely related species.

It has been proven that the new *Sphingomonas*-specific primer set was still amplifying 16S rRNA genes from different species at cell concentrations of 10^4 CFU g^{-1} in different soil types. This detection limit could be expected for all *Sphingomonas* species, since most *Sphingomonas* species seem to contain only one 16S rRNA gene copy. The same cell concentrations for different species would lead to the same template target concentrations (16S rRNA gene concentration) and thus the same detection levels. The detection limit of 10^4 CFU g^{-1} is lower than other reported detection sensitivities for similar direct PCR methods, such as, for example, those for *Burkholderia* species (5×10^5 CFU g^{-1}) (14) or *Mycobacterium* species (ca. 10^6 CFU g^{-1}) (Leys et al., submitted), especially since *Sphingomonas* species seem to contain only one target copy in their DNA in comparison with most other soil bacteria, which can contain many copies of the *rrn* genes per cell (e.g., five to six copies for *Burkholderia*), which in the latter case will improve the cell detection limit.

Finally, the newly developed PCR-DGGE method using the new Sphingo primer set allowed us to analyze the indigenous *Sphingomonas* population in five different PAH-contaminated soils. *Sphingomonas* species were present in all tested soils, originating from very different locations and characterized by very different geological and chemical properties. Their relatively high cell concentrations of 10^5 to 10^6 cells per g of soil and their frequent isolation from contaminated soils during enrichment on PAHs as carbon sources (5, 24, 35, 39) indicate that *Sphingomonas* strains seem to be important colonizers and possibly endemic pollutant degraders in PAH-contaminated soils.

Sequence analysis of DGGE band patterns revealed the presences of "new" 16S rRNA gene sequences grouped in possibly four new *Sphingomonas* species and one new *Sphingomonadaceae* genus. Most soil-extracted *Sphingomonas* sequences had only a limited relationship with identified species and cultivated PAH-degrading isolates. These results were compared with the results obtained with a culture-dependent *Sphingomonas* detection method: i.e., a selective plating technique based on the intrinsic streptomycin resistance and the typical yellow morphotype of *Sphingomonas*, tested on the same soil samples (K. Vanboekhoven, unpublished data). The dominant cultivable *Sphingomonas* strains isolated in that work were very different from the dominant *Sphingomonas* strains detected by our molecular method. Based on 16S rRNA gene sequence, the isolates were mostly grouped in an unidentified cluster—possibly a new species—with *Sphingomonas* sp. strain LH227 (5) (9 of 22 isolates) or in a cluster with *S. taejonensis*, *S. chilensis*, and *S. witflariensis* (5 of 22 isolates). Only a very few of our clone sequences were related to 16S rRNA genes of the isolates, and if there was a relationship, clones and isolates seldom originated from the same PAH-contaminated soil. It might be that the dominant strains detected by the PCR-based

Rhodospirillum was included to root the tree. The bar at the top indicates the percent similarity, with 1% indicating 1 nucleotide substitution per 100 positions. The tree was tested for branching order confidence by maximum-parsimony analysis and a round of 500 bootstraps. Bootstrap values are indicated at branch points, and values above 70% indicate reliable branches. Extended branches were collapsed to form smaller blocks. Most important representative strains are indicated per block, with the accession numbers of the sequences indicated between parentheses. Species harboring PAH-degrading isolates are indicated with an asterisk. The positions of the clone sequences retrieved from soil are indicated on the right of the tree. Species are grouped based on their 16S rRNA gene sequence similarity. Species groups resembled the clustering previously described by Takeuchi et al. (46), who divided the *Sphingomonas* genus into four new genera based on the 16S rRNA gene dendrogram. Later, this division of the *Sphingomonas* genus was reconsidered by Yabuuchi et al. (54) due to the lack of phenotypic and biochemical evidence. The clusters in the figure indicated as I to IV represent the phylogenetic clusters previously assigned to the genera "*Sphingomonas sensu stricto*," "*Sphingobium*," "*Novosphingobium*," and "*Sphingopyxis*," respectively (46).

method are streptomycin sensitive and therefore were excluded from the population detected by the culture-dependent approach. However, this is unlikely, since all *Sphingomonas* species tested so far have been streptomycin resistant. Moreover, most of our cloned sequences were most similar to sequences of other uncultured *Sphingomonas* strains. Thus, based on the nature of the new sequences detected using the culture-independent technique, these sequences most likely represent truly nonculturable *Sphingomanas* strains present in soil.

A diverse group of *Sphingomonas* strains belonging to different species clusters in the genus were present at relatively equal cell concentrations in low and moderately contaminated soils. Soils containing high concentrations of PAHs (mainly phenanthrene) were characterized with less-complex DGGE band patterns than less-contaminated soils and hence seem to be dominated by a less-diverse group of *Sphingomonas* species. Our results may suggest that high PAH concentrations have enriched a few *Sphingomonas* strains in a very high concentration, which possibly masked the detection of other species present in lower concentrations. The soil DGGE fingerprinting technique did clearly show some additional community information (noncloned fainter bands in the fingerprints) that simple cloning procedures could not reveal. Pure cloning strategies did not allow a complete qualitative or accurate quantitative determination of the microbial population presented by the gene pool extracted from the habitat under study as previously concluded by Liesack et al. (28). More intense bands within the DGGE fingerprint were clearly cloned more easily.

In conclusion, the PCR-DGGE detection method described in this study, based on newly developed *Sphingomonas*-specific primers, proved to be a powerful tool for analyzing *Sphingomonas* population diversity and dynamics in environmental samples. Furthermore, the primers developed in this study could be useful in a reverse transcription-PCR approach targeting rRNA in order to identify the active *Sphingomonas* strains involved in PAH biodegradation in the environment.

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