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Microcosm Enrichment of Biphenyl-Degrading Microbial Communities from Soils and Sediments

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A microcosm enrichment approach was employed to isolate bacteria which are representative of long-term biphenyl-adapted microbial communities. Growth of microorganisms was stimulated by incubating soil and sediment samples from polluted and nonpolluted sites with biphenyl crystals. After 6 months, stable population densities between 8×10^9 and 2×10^{11} CFU/ml were established in the microcosms, and a large percentage of the organisms were able to grow on biphenyl-containing minimal medium plates. A total of 177 biphenyl-degrading strains were subsequently isolated and characterized by their ability to grow on biphenyl in liquid culture and to accumulate a yellow *meta* cleavage product when they were sprayed with dihydroxybiphenyl. Isolates were identified by using a polyphasic approach, including fatty acid methyl ester (FAME) analysis, 16S rRNA gene sequence comparison, sodium dodecyl sulfate-polyacrylamide gel electrophoresis of whole-cell proteins, and genomic fingerprinting based on sequence variability in the 16S-23S ribosomal DNA intergenic spacer region. In all of the microcosms, isolates identified as *Rhodococcus opacus* dominated the cultivable microbial community, comprising a cluster of 137 isolates with very similar FAME profiles (Euclidean distances, <10) and identical 16S rRNA gene sequences. The *R. opacus* isolates from the different microcosms studied could not be distinguished from each other by any of the fingerprint methods used. In addition, three other FAME clusters were found in one or two of the microcosms analyzed; these clusters could be assigned to *Alcaligenes* sp., *Terrabacter* sp., and *Bacillus thuringiensis* on the basis of their FAME profiles and/or comparisons of the 16S rRNA gene sequences of representatives. Thus, the microcosm enrichments were strongly dominated by gram-positive bacteria, especially the species *R. opacus*, independent of the pollution history of the original sample. *R. opacus*, therefore, is a promising candidate for development of effective long-term inocula for polychlorinated biphenyl bioremediation.

Bacteria with the ability to mineralize biphenyl are widespread in soils and sediments. The enzymes involved in the biphenyl mineralization pathway are well studied and have broad substrate specificity, which allows the bacteria to simultaneously cometabolize polychlorinated biphenyls (PCBs) (27). Since the first isolation of two PCB-degrading bacteria by Ahmed and Focht (2), numerous *Pseudomonas* strains and related genera have been obtained from contaminated sites, and the biochemistry and genetics of PCB degradation have been studied extensively (20, 21, 27). Key enzymes of the biphenyl-PCB degradation pathway have been found to be ubiquitous in the environment (17, 51, 52). Plant-produced terpenes have been shown to be among the natural substrates for some biphenyl- and PCB-degrading bacteria in soil (3, 28) and to induce PCB cometabolism (23). Restructuring of the genus *Pseudomonas* and more sophisticated taxonomic analyses of PCB-degrading isolates have revealed that most of these organisms are members of the β subclass of the class *Proteobacteria* and few are true *Pseudomonas* strains (7, 35, 39). Recently, up to seven copies of the *bphC* gene were found in *Rhodococcus* strains, indicating the possible importance of this genus for PCB degradation (5, 6, 26, 32a).

Classical enrichment procedures select for strains with the highest growth rates under specified high-nutrient conditions, which are therefore often not representative of in situ communities. On the other hand, analysis of biphenyl-degrading communities without enrichment is difficult because the densities are usually too low. Moreover, the bacteria present in an environment that are able to degrade biphenyl might represent transiently imported strains and not necessarily those organisms which under long-term pollution conditions perform biphenyl degradation and PCB cometabolism in situ. Therefore, in this study we used a microcosm enrichment approach in which we added biphenyl crystals directly to microcosms containing environmental samples and incubated the microcosms for 6 months. The original samples from which microcosms were set up represented a diverse selection of environments, including contaminated and uncontaminated soils and sediments, as well as rotten wood, which is assumed to house microbial communities naturally adapted to degrade aromatic compounds (22). We hypothesized that different biphenyl-degrading microbial communities would develop within each microcosm as a result of different initial species composition, as well as habitat-specific physical, chemical, and biological factors, including the history of pollution.

However, the microorganisms isolated from the microcosms after 6 months of enrichment showed little taxonomic diversity. Moreover, isolates identified as *Rhodococcus opacus* were the dominant organisms in all samples and formed a tight fatty acid methyl ester (FAME) cluster. Representatives of this cluster from each of the seven environments investigated (core strains) were subsequently analyzed in more depth by comparing their 16S rRNA sequences and by using increasingly sensitive fingerprint methods.

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TABLE 1. Environmental samples

Matrix	Site	Strain designation prefix	Level of PCB pollution (mg of PCB/kg [dry wt])	No. of isolates
Soil	Loxstedt	LOX	ND[a]	10
	Berlin	BIE	60	63
Sediment	Grumbacher Teich	GT	ND	24
	Spittelwasser	SP	0.184	3
	Stepan	ST	0.836	27
	Georgswerder	GW	15	43
Rotten wood	Göttingen	H	ND	7

[a] ND, not determined.

MATERIALS AND METHODS

Environmental samples. The uncontaminated soil sample was taken from the top 5 mm of garden soil in the village of Loxstedt, Germany (Table 1). A contaminated soil sample was obtained from an airport in East Berlin, Germany, through the University of Bielefeld, and this sample contained 60 mg of PCB/kg (dry weight) (24b). Uncontaminated sediment was sampled from a small oligo- to mesotrophic lake in the Harz Mountains, the Grumbacher Teich; a layered sediment core was obtained at the lake bottom at a depth of 9 m by a diver, and the top 5 mm of the sediment was removed with a special slicing apparatus (50). Contaminated sediment samples were obtained from two sites along the Elbe River and a waste storage site. The Spittelwasser River is a small inflow into the Mulde River, which flows into the Elbe River at Roßlau; the pollution in this river is characterized by diverse industrial effluents from the chemical production sites in Bitterfeld (4), including 0.184 mg of PCB/kg (dry weight). Stepan is a little harbor behind a dam 420 km upstream of Magdeburg in the Czech Republic which has been exposed to long-term PCB pollution; the Stepan sediment sample was obtained from a research vessel with a sediment sampler and was stored at −20°C. It contained 0.836 mg of PCB/kg (dry weight) (24c). The sediment sample from the waste storage site at Georgswerder, close to Hamburg, Germany, was obtained manually from a seepage canal and contained 15 mg of PCB/kg (dry weight) (3a). A rotten wood sample was obtained from the center of an old beech tree in the area around Göttingen, Germany. The airport soil, river sediment, and waste storage site samples had a pollution history of several decades which included a broad array of xenobiotic compounds other than PCBs (4). All of the samples were maintained in sterile Falcon tubes and stored at 4°C with the exception of the Stepan sample, which was stored at −20°C.

Experimental setup and isolation of strains. Duplicate slurry microcosms were prepared from each sample by mixing 2 g of sample and 18 ml of M9 minimal medium (7 g of Na_2HPO_4 per liter, 3 g of KH_2PO_4 per liter, 0.5 g of NaCl per liter, 1 g of NH_4Cl per liter; pH adjusted to 7.0 to 7.2) in Erlenmeyer flasks. Biphenyl crystals were added to one microcosm of each pair to yield a concentration of approximately 650 μM in the liquid phase. The other microcosm was left untreated as a control. The microcosms were shaken gently with a rotary shaker for 6 months at room temperature. Evaporated water and consumed biphenyl were periodically replaced. As a control, biphenyl crystals were incubated in liquid minimal medium for 1 week, and then an undiluted aliquot was plated onto biphenyl-containing minimal medium plates. In addition, biphenyl crystals were exposed to Luria-Bertani (LB) medium plates. In both cases no growth occurred.

The total numbers of cultivable bacteria in the slurries were determined by the plate count method by using 0.1× Luria-Bertani medium (1 g of tryptone per liter, 0.5 g of yeast extract per liter, 1 g of NaCl per liter; pH 7.0 to 7.2). Biphenyl-degrading microorganisms were selectively enumerated on M9 minimal medium agar plates supplemented with 0.05 g of yeast extract per liter; biphenyl crystals were placed in the lid of each petri dish. To enumerate bacteria, 1-ml aliquots of the slurries were serially diluted (in 0.85% [wt/vol] NaCl), and two appropriate dilutions of each sample were plated in triplicate. All of the plates were incubated at 30°C for 1 week (0.1× LB medium) or 2 weeks (minimal medium).

Colonies from the highest sample dilutions from biphenyl-enriched microcosms were assayed for the activity of the 2,3-dihydroxybiphenyl dioxygenase enzyme. To this end, plates were sprayed with an aqueous solution containing 0.1% (wt/vol) dihydroxybiphenyl and 10% (vol/vol) acetone. Appearance of the yellow metabolite 2-hydroxy-6-oxo-6-phenyl-hexa-2,4-dienoic acid, which is formed by *meta* cleavage of 2,3-dihydroxybiphenyl, indicated that 2,3-dihydroxybiphenyl dioxygenase was present and was observed within several minutes. As many single yellow colonies as possible were picked. Isolates were purified by several transfers to 0.1× LB medium plates. If two or more morphological types of colonies were visible, both were subsequently analyzed.

Purified strains were tested for their ability to degrade biphenyl by (i) growing the organisms in liquid M9 minimal medium containing biphenyl as the sole source of carbon and energy and (ii) assaying for the presence of 2,3-dihydroxybiphenyl dioxygenase.

To preserve isolates, 1.5-ml portions of cultures in liquid minimal medium were centrifuged (5 min, 5,000 × *g*), the pellets were resuspended in 1 ml of fresh LB medium, and 200 μl of sterile glycerol was added to each preparation. Stock cultures were stored at −70°C.

FAME analysis. Cultures were streaked onto plates containing 30 g of Trypticase soy broth (BBL, Becton Dickinson Microbiology Systems, Cockeysville, Md.) per liter supplemented with 15 g of Bacto Agar (Difco Laboratories, Detroit, Mich.) per liter. The plates were incubated for 24 h at 28°C. A loopful of cells from the overlap area of the second and third series of streaks on each plate was harvested. The cells were saponified, methylated to FAMEs, and extracted as described in the standardized protocol of the Microbial Identification System (MIDI) (Microbial ID, Inc., Newark, Del.). FAMEs were analyzed with a gas chromatograph (model HP5890A; Hewlett-Packard, Avondale, Pa.) equipped with a flame ionization detector, an autosampler, an integrator, and a personal computer (38). The whole-cell FAME profiles were identified and quantified by using the Microbial Identification System software package (MIS, version 3.9). Principal-component analysis (24a) of the quantitative fatty acid data was performed with the MIS software package, and the results were plotted graphically in two dimensions.

DNA isolation and determination of nucleotide base composition. Genomic DNAs were isolated by the protocol of Wilson (54) from 2-ml portions of cultures grown in LB medium or from colonies scraped off agar plates, followed by treatment with RNase A (Sigma) (50°C for 2 h) and additional phenol-chloroform-isoamyl alcohol extractions. Appropriate amounts of DNA were digested enzymatically, and the average guanine-plus-cytosine (G+C) contents were determined by high-performance liquid chromatography (45) in triplicate. Calculations were carried out as described by Mesbah et al. (34) by using nonmethylated phage λ DNA as the standard.

16S rRNA sequencing. Colonies were scraped off agar plates and DNA was extracted as described above. Nearly complete 16S rRNA genes were amplified by PCR by using a forward primer hybridizing at positions 8 to 27 and a reverse primer hybridizing at the complement of positions 1525 to 1541 (*Escherichia coli* 16S rRNA gene sequence numbering). PCR was carried out with a GeneAmp model 9600 thermocycler (Perkin-Elmer, Weiterstadt, Germany) under conditions described previously (30). Amplified DNA was purified by using Microcon 100 microconcentrators (Amicon GmbH, Witten, Germany), and quality was controlled by gel electrophoresis on a 1% agarose gel with TAE buffer and subsequent ethidium bromide staining (43). The sequence of the amplified 16S ribosomal DNA (rDNA) was determined directly by using a model 373A DNA sequencer (Perkin-Elmer, Applied Biosystems GmbH, Weiterstadt, Germany) and the protocols recommended by the manufacturer for *Taq* polymerase-initiated cycle sequencing with fluorescent dye-labeled dideoxynucleotides and standard 16S rRNA sequencing primers (33). The resulting sequences were aligned with reference 16S rRNA and 16S rRNA gene sequences (14, 48) by using the evolutionarily conserved primary sequence and secondary structure as references. Evolutionary distances (29) were calculated from complete sequence pair similarity values by using only homologous, unambiguously determined nucleotide positions. A phylogenetic tree was constructed by using the DNADIST and FITCH programs of the PHYLIP package (18).

SDS-PAGE of whole-cell proteins. As described previously (40), whole-cell proteins were solubilized by treatment with 2% sodium dodecyl sulfate (SDS) and separated by polyacrylamide gel electrophoresis (PAGE) in a 12% (wt/vol) polyacrylamide slab gel. Stained protein electrophoretic patterns were scanned with a model 2202 laser densitometer (LKB, Bromma, Sweden) linked to a personal computer. Normalization of the patterns, further processing, and numerical analysis were performed by using the GelCompar program, version 3.1 (Applied Maths, Kortrijk, Belgium) (49).

16S-23S rDNA ISR fingerprint analysis. DNA was isolated from late-exponential-phase cells as described previously (54). DNA concentrations were determined by measuring the absorbance at 260 nm and were adjusted to 1 μg/μl with sterile deionized water. Alternatively, a rapid DNA isolation method was used to isolate DNA from bacterial colonies. Colonies that were at least 1 mm in diameter were individually picked from the culture plates with an inoculating loop and suspended in 100 μl of 5% Chelex 100 (sodium form; 100 to 200 mesh) in sterile TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]). Samples were vortex mixed, boiled for 15 min, and then centrifuged for 5 min at 12,000 × *g*. The supernatant was stored at 4°C until it was analyzed. A 1-μl aliquot was added to each PCR mixture. The 16S-23S rDNA intergenic spacer region (ISR) was amplified by PCR with oligonucleotide primers designed to anneal to conserved positions in the 3′ and 5′ regions of the bacterial 16S rRNA and 23S rRNA genes, respectively. The forward primer used, 16f945 (5′-GGGCCCGCACAAG CGGTGG), corresponds to positions 927 to 945 of the *E. coli* 16S rRNA gene (9), and the reverse primer used, 23r458 (5′-CTTTCCCTCACGGTAC), corresponds to the complement of positions 458 to 473 of the *E. coli* 23S rRNA gene (8). The PCRs were carried out in a 100-μl reaction volume containing 10 mM Tris-HCl [pH 8.3], 1.5 mM $MgCl_2$, 50 mM KCl, 0.001% gelatin, each of the four deoxyribonucleoside triphosphates (Pharmacia, Biotech Europe GmbH, Freiburg, Germany) at a concentration of 200 μM, 0.5 μM forward primer, 0.5 μM reverse primer, template DNA, and 2.5 U of Amplitaq DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.) by using a Perkin-Elmer Cetus GeneAmp

model 9600 PCR system. After denaturation at 96°C for 2 min, amplifications were performed for 30 cycles consisting of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, and DNA extension at 72°C for 2 min. A final elongation step at 72°C for 10 min was included. Five microliters of amplified DNA was analyzed by 1.5% (wt/vol) NuSieve 3:1 (FMC Bioproducts, Rockland, Maine) agarose gel electrophoresis.

The ISR PCR products were digested by using the tetrameric restriction endonuclease *Taq*I (Boehringer, Mannheim, Germany) at 65°C according to the supplier's recommendations. The resulting fragments were separated by 3.0% (wt/vol) NuSieve 3:1 agarose (FMC Bioproducts) gel electrophoresis, stained with ethidium bromide, and visualized by UV excitation.

SSCP analysis of ISR restriction fragments. The procedure used for single-strand conformational polymorphism (SSCP) analysis of ISR restriction fragments was essentially the procedure described by Orita et al. (37). Briefly, 1 μl of a *Taq*I-digested PCR mixture was diluted with 10 μl of TE buffer, boiled for 5 min, quenched on ice, and mixed with 5 μl of SSCP loading mixture (95% deionized formamide, 0.05% tracking dyes [bromophenol blue and xylene cyanol]). The samples were electrophoresed on an 8% acrylamide-bisacrylamide (29:1) gel in TBE buffer (90 mM Tris-borate, 2 mM EDTA [pH 8.0]) for 5 h at 80 V and 4°C. After electrophoresis, the polyacrylamide gels were stained with ethidium bromide and visualized under UV light or stained with a Plus One silver staining kit (Pharmacia Biotech, Heidelberg, Germany).

Nucleotide sequence accession number. The 16S rRNA gene sequence of strain GW-86a has been deposited in the EMBL nucleotide database under accession no. RSPAJ2749.

RESULTS

Bacterial densities in the microcosms. The initial densities of cultivable bacteria in the microcosms were between 2×10^7 and 9×10^7 CFU/ml. Bacterial densities were subsequently determined at monthly intervals both on complex medium (0.1× LB medium) and on minimal medium. In all microcosms, both with and without biphenyl amendment, the bacterial densities increased during the first few weeks of incubation, probably due to oxygenation of the sample and solubilization of organic carbon. After this, the bacterial densities in the microcosms incubated with biphenyl increased further by approximately 2 orders of magnitude compared to the control microcosms. After 6 months of enrichment, total bacterial densities in the biphenyl-enriched microcosms had reached stable levels that ranged from 8×10^9 CFU/ml (rotten wood sample) to 2×10^{11} CFU/ml (Stepan sample). The plate counts for bacteria on biphenyl-containing minimal medium agar plates ranged from 2×10^9 CFU/ml (rotten wood sample) to 2×10^{11} CFU/ml (Stepan sample).

FAMEs and cluster analysis. A two-dimensional plot of the principal-component analysis performed on FAMEs from all of the biphenyl-degrading isolates is shown in Fig. 1. FAME cluster 1 contained the bulk of the strains analyzed, namely, 137 strains isolated independently from microcosms generated with samples from seven different environments. The Euclidean distances between these isolates were <10, indicating possible identity at the species level. However, the MIDI system was not able to identify these organisms. Subsequently, the following seven core strains representing cluster 1 isolates from the seven environments were studied: LOX-158b, BIE-17, H-131, GT-159, SP-184, ST-215, and GW-86a. These strains were analyzed further by performing 16S rRNA gene sequencing and by using typing methods based on the fingerprint patterns obtained for the 16S-23S rDNA ISR (ISR-PCR, SSCP) and whole-cell protein profiles (SDS-PAGE).

FAME cluster 2 from the two-dimensional principal-component cluster analysis contained 12 strains which were identified by their FAME profiles as *Yersinia fredriksenii* and had match values between 0.5 and 0.9. Again, the Euclidean distances between the isolates were <10, indicating possible identity at the species level. All 12 strains were isolated from the Georgswerder waste storage sediment sample.

FAME cluster 3 was comprised of eight *Bacillus* strains identified as *Bacillus thuringiensis* on the basis of their FAME profiles; these strains had match values between 0.2 and 0.5. All eight strains were obtained from the nonpolluted lake sediment (Grumbacher Teich) sample.

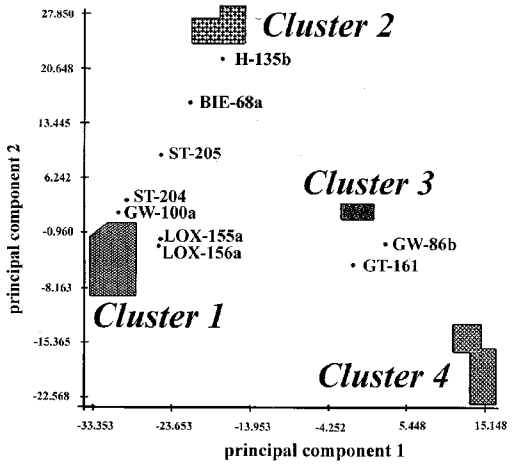

FIG. 1. Two-dimensional plot of 177 biphenyl-degrading isolates from microcosm enrichments from soils and sediments based on a principal-component analysis of their FAME profiles. The original two-dimensional plot obtained with the MIS software (version 3.9) was scanned by using Photoshop (4.0); strain labels assigned by the MIS software were replaced by original strain numbers for isolates outside clusters; cluster borders were manually drawn. Cluster 1 (*Rhodococcus* sp.; match values, 0.1 to 0.3) contains the following 137 strains: BIE-1 to BIE-5, BIE-7 to BIE-10, BIE-15 to BIE-22, BIE-24 to BIE-59, BIE-61 to BIE-64, BIE-66, BIE-67, BIE-68b, BIE-71, BIE-72a, GW-75a, GW-77b, GW-78, GW-80, GW-82, GW-85, GW-86a, GW-89, GW-90b, GW-91a, GW-94b, GW-96 to GW-98, GW-101a, GW-102a, GW-104, GW-105a, H-119, H-126, H-127, H-131, H-135a, H-135d, LOX-151 to LOX-154, LOX-156b, LOX-157a, LOX-158a, LOX-158b, GT-159, GT-163a, GT-165, GT-166, GT-169 to GT-177, GT-181, GT-182, SP-183, SP-184, SP-185a, ST-186 to ST-203, ST-209a, ST-209b, ST-211 to ST-213, ST-214b, and ST-215. Cluster 2 (*Y. fredriksenii*; match values, 0.5 to 0.9) contains the following 12 strains: GW-75b, GW-76a, GW-76b, GW-77a, GW-81, GW-91a, GW-91b, GW-94a, GW-99, GW-100b, GW-103a, and GW-114a. Cluster 3 (*B. thuringiensis*; match values, 0.2 to 0.5) contains the following eight strains: GT-160, GT-162, GT-163b, GT-164, GT-167, GT-168, GT-178, and GT-179. Cluster 4 (*Nocardiopsis* sp.; match values, 0 to 0.2) contains the following 11 strains: GW-73, GW-90a, GW-93a, GW-93b, GW-93c, GW-101b, GW-102b, GW-107a, GW-107b, GW-115a, and GW-115b. The following additional strains were included in the analysis: LOX-155a and LOX-166a (*R. erythropolis*; match value, 0.9); GW-100a, ST-204, and ST-205 (*R. rhodochrous*; match values, 0.3 to 0.4); BIE-68a and H-135b (*A. eutrophus*; match value, 0.4); GW-86b (*B. coagulans*; match value, 0.4); and GT-161 (*R. equi*; match value, 0.1).

FAME cluster 4 consisted of 11 coryneform strains (designated as *Nocardiopsis* strains by the MIDI system) which could not be identified on the basis of their FAME profiles. All of these strains were isolated from the waste storage sediment (Georgswerder) sample and formed a tight cluster (Euclidean distances, <10).

Finally, several strains which did not fall into one of the clusters described above were found. *Rhodococcus erythropolis* was isolated twice from polluted soil (LOX-155a, LOX-156a) and was identified with a match value of 0.9; *Rhodococcus rhodochrous* (match values, 0.3 to 0.4) was isolated twice from polluted river sediment (ST-204, ST-205) and once from waste storage sediment (GW-100a); *Rhodococcus equi* (match value, 0.1) was isolated from nonpolluted lake sediment (GT-101); *Bacillus coagulans* (match value, 0.4) was isolated from waste storage sediment (GW-86b); and *Alcaligenes eutrophus* (match value, 0.4) was isolated from polluted soil (BIE-68a) and rotten wood (H-135b). The identities of the latter two strains were confirmed by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM).

16S rRNA gene sequence analysis. (i) FAME cluster 1. Nearly complete PCR-amplified 16S rDNA sequences were deter-

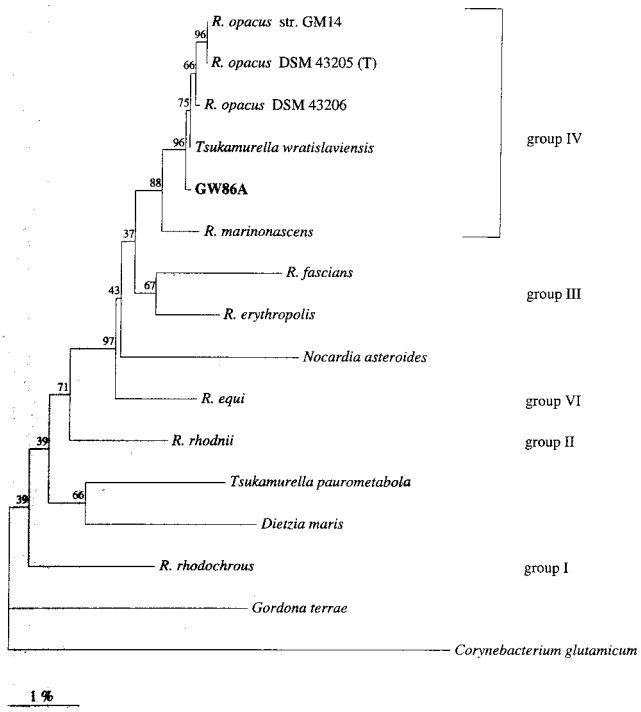

FIG. 2. Unrooted dendrogram showing the estimated phylogenetic position of isolate GW-86a in the genus *Rhodococcus* based on nearly complete 16S rRNA gene sequences. The positions of representative members of RNA groups described by Rainey et al. (41) are shown as references. Bar = 1% inferred nucleotide substitutions. The dendrogram was constructed by using the algorithm of Fitch and Margoliash (18) for evolutionary distances (29). The numbers at the branch points are bootstrap values determined by using a distance matrix with 100 replicates.

mined for the seven core strains, and these sequences were identical. The phylogenetic position of strain GW-86a is shown in Fig. 2. Strain GW-86a exhibited the highest similarity (99.9%) (Table 2) with *Tsukamurella wratislaviensis* (24). Table 2 shows that strain GW-86a is also very similar to *R. opacus* (level of similarity, 99.6%), a facultative chemolithoautotroph (1) described in 1994 (32) which is characterized by its ability to degrade aromatic carboxylic acids (15), substituted phenols and catechols (42), and phthalic acid esters (16).

The average G+C content was determined for one of the seven core strains (ST-215) and was found to be 65.5 ± 1%, which is in the range typical for *Rhodococcus* species (63 to 73%) but lower than the values described for true *Tsukamurella* species (67 to 68%) (32).

(ii) FAME cluster 2. Sequencing of the nearly complete PCR-amplified 16S rDNA (1,472 bp) of a representative cluster 2 strain (GW-75b) revealed that this organism was most closely related to *Alcaligenes denitrificans* (similarity, 97.5%) and isolate RW21a, a nitrobenzene degrader (similarity, 97.1%), followed by various strains of *Ralstonia eutropha* (similarity, 92.7 to 92.4%).

(iii) FAME cluster 4. Partial 16S rRNA gene sequences of two representative cluster 4 strains, GW-90a and GW-93b, which were 472 and 469 bp long, respectively, were identical and most similar to the sequence of *Terrabacter* sp. (similarity, 98.73 and 98.514%, respectively); the genus *Terrabacter* is a coryneform genus (formerly *Pimelobacter*) which was described in 1989 (11) and contains a single species, *Terrabacter tumescens.*

16S-23S ISR fingerprints. The 16S-23S rDNA ISR was used as a rapid bacterial identification and typing system for *Rhodococcus* strains at the species level. Recommended primers (25) that bind to highly conserved regions in the 5′ part of the 23S rRNA gene and the 3′ part of the 16S rRNA gene were used. A total of 17 strains were analyzed, including nine strains of different *Rhodococcus* species, a *T. wratislaviensis* strain, and the seven core strains. ISR variations in the *Rhodococcus* strains were successfully detected (Fig. 3A). All of the strains produced two clear dominant bands at positions between 1.4 and 1.7 kb. Approximately 0.6 kb of the amplification product belonged to the 16S rRNA gene fragment, and approximately 0.5 kb belonged to the 23S rRNA gene; the difference (~0.5 kb) represented the 16S-23S rDNA ISR. In addition, two species, *R. erythropolis* and *R. equi*, produced a third band at >0.5 kb that was not present in any other strain analyzed. This band could represent a third rRNA operon without any tRNA coding regions in the interspacer region. The ISR-PCR products of the seven core strains showed identical mobilities, which were most similar to those observed for *R. opacus* and *T. wratislaviensis*, the most closely related species based on the 16S rDNA sequence analysis results.

Restriction digestion of the amplified ISR fragments was performed in order to obtain a resolution higher than the resolution possible when 16S rRNA gene sequences were compared and to confirm the assignment of each strain to a molecular typing group. After *Taq*I digestion, a more complex fingerprint pattern was apparent. Again, different species were easily distinguishable with a 3% agarose gel (Fig. 3B). One band at ~0.35 kb was present in all of the strains studied. The seven core strains produced identical patterns, which were most similar to the *R. opacus* and *T. wratislaviensis* patterns. These patterns were comprised of the band common to all strains of the *Rhodococcus* species analyzed, a second band at ~0.20 kb, and a third band at ~0.7 kb. However, the *R. opacus* 0.7-kb band exhibited a slightly greater mobility than the corresponding bands of the seven core strains and *T. wratislaviensis*. Moreover, *T. wratislaviensis* produced one extra band at ~0.21 kb which distinguished it from the other eight strains,

TABLE 2. Levels of sequence similarity between strain GW-86a and related organisms in the genus *Rhodococcus*, representatives of other *Rhodococcus* groups, and related coryneforms[a]

Reference strain	Accession no.	% Similarity to strain GW-86a
Tsukamurella wratislaviensis NCIMB 13082T[b]	Z37138	99.9
Rhodococcus opacus DSM 43205T	X80630	99.6
Rhodococcus opacus DSM 43206	X80631	99.7
Rhodococcus opacus GM 14	X89710	99.6
Rhodococcus marinonascens DSM 43752T	X80617	99.0
Rhodococcus erythropolis DSM 43066T	X79289	97.9
Rhodococcus equi DSM 20307T	X80614	97.7
Rhodococcus fascians DSM 20669T	X79186	97.0
Rhodococcus rhodnii DSM 43335T	X80621	96.7
Rhodococcus rhodochrous DSM 43241T	X79288	95.8
Gordona terrae DSM 43249T	X79286	93.5
Nocardia asteroides DSM 43757T	X80606	96.3
Dietzia maris DSM 43672T	X79290	94.8
Tsukamurella paurometabola DSM 20162T	X80628	95.4
Corynebacterium glutamicum ATCC 13032T	Z46753	90.9

[a] The 16S rRNA gene sequences of reference organisms were obtained from the EMBL nucleotide database.
[b] T = type strain.

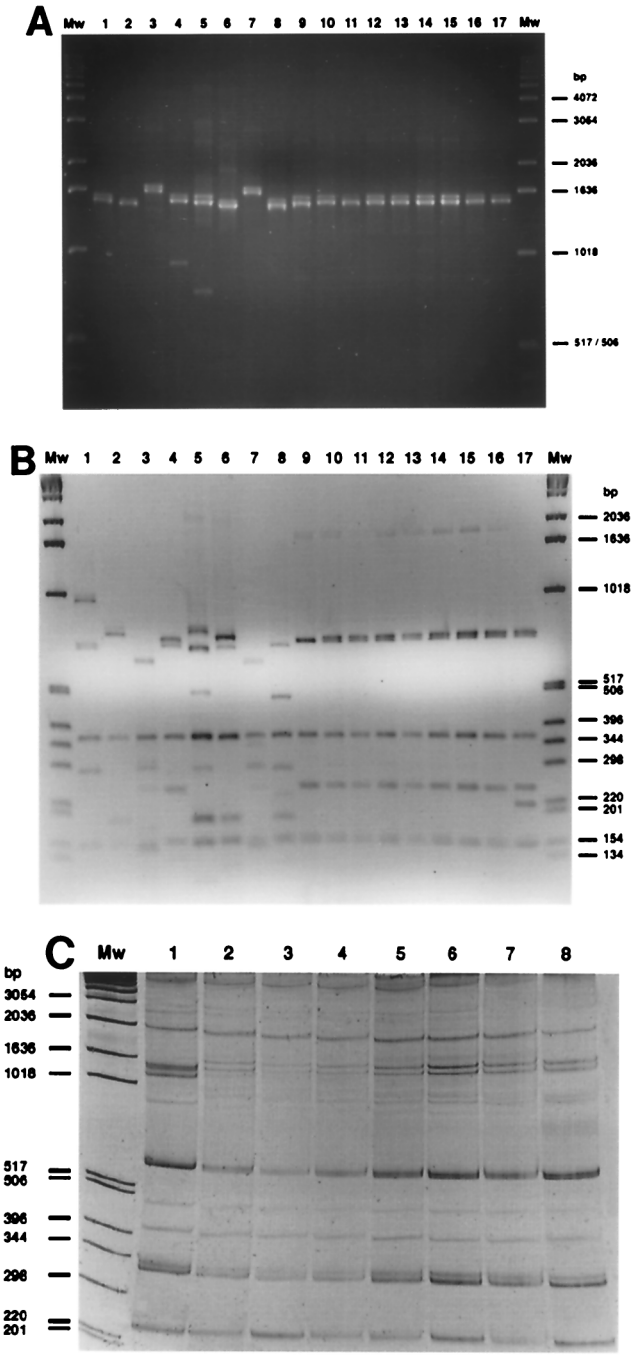

FIG. 3. Fingerprint analysis of *Rhodococcus* cluster 1 core strains and *Rhodococcus* type strains based on 16S-23S ISR polymorphism. (A) PCR amplified 16S-23S rRNA ISR of cluster 1 core strains (*Rhodococcus* isolates) and related strains separated in a 1.5% agarose gel. Lane Mw, 1-kb DNA ladder molecular size marker; lane 1, *R. aurantiacus* NCB 9557; lane 2, *R. coprophilus* DSM 43339; lane 3, *R. corallinus* DSM 43248; lane 4, *R. erythropolis* DSM 311; lane 5, *R. equi* DSM 777; lane 6, *R. rhodochrous* ATCC 2976; lane 7, *R. rubropertinctus* DSM 43179; lane 8, *R. ruber* DSM 43232; lane 9, *R. opacus* DSM 427; lane 10, LOX-158b; lane 11, H-131; lane 12, SP-184; lane 13, GT-159; lane 14, ST-215; lane 15, BIE-17; lane 16, GW-86a; lane 17, *T. wratislaviensis* DSM 44107. (B) RFLP analysis of *Taq*I-digested PCR-amplified ISR of cluster 1 core strains and related strains in a 3.0% agarose gel. Lane Mw, 1-kb DNA ladder molecular size marker; lane 1, *R. aurantiacus* NCB 9557; lane 2, *R. coprophilus* DSM 43339; lane 3, *R. corallinus* DSM 43248; lane 4, *R. erythropolis* DSM 311; lane 5, *R. equi* DSM 777; lane 6, *R. rhodochrous* ATCC 2976; lane 7, *R. rubropertinctus* DSM 43179; lane 8, *R. ruber* DSM 43232; lane 9, *R. opacus* DSM 427; lane 10, LOX-158b; lane 11, H-131; lane 12, SP-184; lane 13, GT-159; lane 14, ST-215; lane 15, BIE-17; lane 16, GW-86a; lane 17, *T. wratislaviensis* DSM 44107. (C) SSCP analysis of PCR-amplified 16S-23S rRNA ISR after *Taq*I digestion for cluster 1 core strains (*Rhodococcus* isolates) and their closest relative (*R. opacus*), showing identical RFLP fingerprints. Lane Mw, 1-kb DNA ladder molecular weight marker; lane 1, *R. opacus* DSM 427; lane 2, LOX-158b; lane 3, H-131; lane 4, SP-184; lane 5, GT-159; lane 6, ST-215; lane 7, BIE-17; lane 8, GW-86a.

including the *R. opacus* strain. Thus, on the basis of ISR fingerprints the seven core strains were shown to be identical and very similar to both *R. opacus* and *T. wratislaviensis*.

SSCP of 16S-23S ISR. To obtain a higher resolution, the nucleic acid sequence polymorphism of the amplified ISRs was analyzed by electrophoresing single strands of the *Taq*I-digested fragments on a polyacrylamide gel (Fig. 3C). After SSCP analysis of *R. opacus* and the seven core strains, the large restriction enzyme-digested fragment observed on agarose gels at 0.7 kb could be resolved into two bands. Moreover, a mobility shift for these bands was clearly detected in *R. opacus*. Again, the seven environmental isolates produced identical patterns, confirming that all of these strains constitute a group of closely related organisms, probably a *R. opacus* subspecies or a group of *R. opacus* strains.

SDS-PAGE analysis. A comparison of the normalized protein patterns shown in Fig. 4 revealed that all of the representative core isolates were very similar to each other and also nearly identical to the type strain of *R. opacus*, strain LMG 18000 ($r = 0.95$). The type strain of *T. wratislaviensis*, LMG 17999, was also included in Fig. 4, and it grouped at a correlation level of 0.87. This lower level of similarity was mainly due to one dense band at a molecular weight of approximately 60,000. When this single dense band was omitted from the cluster analysis, the type strain of *T. wratislaviensis* grouped with all of the other strains, including the *R. opacus* strains, at a correlation level higher than 0.94 (data not shown).

DISCUSSION

Phylogenetic classification of isolates. Determination of the FAME profiles of the isolates was a fast and relatively easy way to obtain an overview of the taxonomic clusters present. The following four main clusters were identified by this analysis: cluster 1 (*Rhodococcus* sp.), which was isolated from all seven microcosm enrichments; cluster 2 (*Yersinia* sp.), which was found only at the waste storage site Georgswerder; cluster 3 (*Bacillus thuringiensis*), which was isolated from the nonpolluted lake sediment sample obtained from Grumbacher Teich; and cluster 4 (*Nocardiopsis* sp.), which was found only at the waste storage site Georgswerder. The majority of the isolates (168 of 177 strains) belonged to these four clusters. In addition, nine isolates that did not belong to the above clusters were obtained; they were identified by the MIDI system as *R. equi*, *R. erythropolis*, *R. rhodochrous*, and *A. eutrophus*. Thus, all sites had a predominance of cluster 1 isolates. Apart from this, very little taxonomic diversity was present, composed of coryneform bacteria (*Rhodococcus* and *Nocardiopsis* spp.), several *Bacillus* isolates, members of the β subclass of the class *Proteobacteria* (*Alcaligenes* spp.), and representatives of the *Enterobacteriaceae* (*Yersinia* spp.). The greatest microbial diversity was observed in the waste storage sediment sample from Georgswerder. Cluster 2 (*Yersinia* sp.) and cluster 4 (*Nocardiopsis* sp.) strains were isolated from this site exclusively. Conversely, the nonpolluted lake sediment site (Grumbacher Teich) differed from the other sites because of the occurrence of *B. thuringiensis*, which was not observed elsewhere.

The species identities proposed by the MIDI system for cluster 1, 2, and 4 isolates had very low match values or were

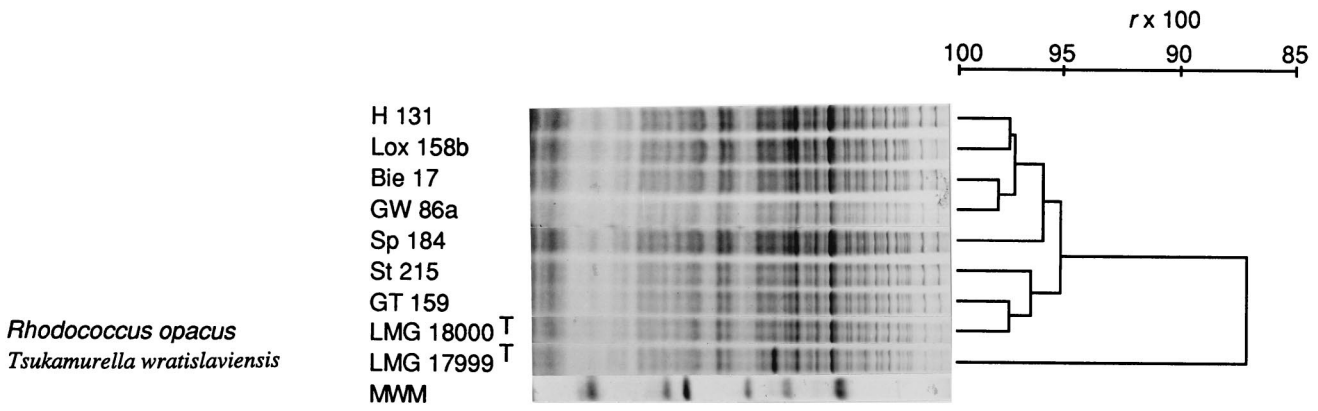

FIG. 4. SDS-PAGE proteins electropherograms for cluster 1 core strains and the phylogenetically most closely related type strains (*R. opacus*, *T. wratislaviensis*) and corresponding dendrogram based on an average linkage cluster analysis of correlation coefficients (*r* values). The molecular weight markers used (lane MWM) were (from left to right) trypsin inhibitor (molecular weight, 20,100), carbonic anhydrase (29,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), egg albumin (45,000), bovine albumin (66,000), and β-galactosidase (116,000).

not consistent with the results of physiological tests (Biolog). By sequencing the 16S rRNA genes of representatives of these clusters, the phylogenetic positions of the isolates could clearly be identified.

The FAME cluster 1 isolates included 137 strains of *Rhodococcus* sp., which were dominant in all seven environments studied. Therefore, seven FAME cluster 1 isolates, one from each environment, were defined as core strains and analyzed further. The seven FAME cluster 1 core strains were shown to have identical 16S rRNA gene sequences, which were most similar to the sequences of *T. wratislaviensis* (99.9% similarity) and *R. opacus* (99.6% similarity). The genus *Tsukamurella* was described by Collins et al. (12), and *Tsukamurella paurometabola* (formerly *Corynebacterium paurometabolum* and *Rhodococcus aurantiacus*) is the type species. Additional species were subsequently isolated from immunocompromised patients; these species were *Tsukamurella inchonensis* (55) and *Tsukamurella pulmonis* (56). In 1991, *T. wratislaviensis* was described (24). On the basis of 16S rRNA gene sequence comparisons, however, *T. wratislaviensis* appears to be a misclassified *Rhodococcus* species (Fig. 2). This has been confirmed by a chemotaxonomic analysis, which failed to detect the mucolates with 64 to 68 carbon atoms typical of the genus *Tsukamurella* (32b).

The following interesting phenomenon was observed during direct sequencing of the PCR-amplified 16S rDNAs of the seven *Rhodococcus* core strains: after a certain point in the sequence in helix 27 (36) corresponding to *E. coli* 16S rRNA gene sequence position 839, the sequences were not readable. This effect was observed regardless of the sequence primers employed and the template strand (i.e., forward or reverse). In this sequence region, the sequence was determined to be GGGTT(T)VV. The third T (or A in the case of the complementary sequence) (in parentheses) always produced a signal with lower intensity which was overlaid by the next C (or G) signal. For the reference organisms, the sequence was determined to be either GGGTTCC (*Rhodococcus marinascens*, *R. erythropolis*, *Rhodococcus fascians*) or GGGTTTCC (*R. opacus*, *R. equi*, *Rhodococcus rhodnii*, *Rhodococcus rhodochrous*, *Nocardia asteroides*, *Tsukamurella* sp.), and no problems in sequencing were observed (40a). We can exclude the possibility that a faulty primer caused this effect, which is known as the n-1 effect, because an array of different sequencing primers which had performed perfectly in other cases (same lot) were employed. In addition, this effect was found to begin at a specific nucleotide position in all strains. We therefore concluded that these *Rhodococcus* strains possess more than one rDNA operon with identical sequences except for the addition of one nucleotide (T).

The 16S rRNA gene sequence of the FAME cluster 2 isolate exhibited a high level of similarity (97.5%) to the *A. denitrificans* 16S rRNA gene sequence. The cluster 2 isolates probably represent a new *Alcaligenes* species which was misclassified as a *Yersinia* species because of the high levels of similarity between the FAMEs of *Alcaligenes* and *Yersinia* species, which differ only in the amounts of the less important fatty acids.

The 16S rRNA gene sequences of FAME cluster 4 isolates were most similar to the 16S rRNA gene sequence of *Terrabacter* sp. (98.7% identity). Probably, the cluster 4 isolates represent a new species of the genus *Terrabacter*, considering the level of 16S rRNA gene sequence difference and the significant differences from *T. tumescens* in the FAMEs.

Identification and typing of *Rhodococcus* isolates and related reference strains. (i) 16S-23S rDNA ISR polymorphism. A fingerprint method, based on PCR amplification of the 16S-23S rDNA ISR (25), allowed observation of spacer regions of different lengths in different *Rhodococcus* species. Variations in the sequence, length, number, and composition of the 16S-23S rDNA spacer DNAs are due, in part, to the number and type of tRNA genes that they contain. The considerable heterogeneity in this region provides a basis for identification and typing of the organisms. Heterogeneity has been found in both the number and the length of the PCR fragments obtained and thus demonstrates that this method is applicable as a means for differentiation, typing, and rapid identification of isolates of *Rhodococcus* species.

A second level of discrimination was used to confirm the identities of the strains, particularly when ISR fragments of the same size or very similar sizes were obtained. A restriction fragment length polymorphism (RFLP) analysis was carried out with the ISR fragments obtained from isolates that produced similar or identical ISR-PCR patterns following tetrameric site-specific restriction endonuclease treatment with *Taq*I. The resulting patterns allowed differentiation at the species level between the *Rhodococcus* strains analyzed.

In order to distinguish between fragments of the same size obtained after digestion with *Taq*I, a third level of discrimination was achieved by application of a high-resolution gel electrophoresis method, the SSCP method (37). Here, double-

stranded DNA is denatured to single-stranded DNA, and the products are separated by nondenaturing PAGE. Point mutations in the sequences cause minor changes in secondary-structure conformations, resulting in mobility differences. This method has been used extensively to detect single point mutations in human genes related to inherited diseases (10). This makes it an attractive tool for ISR analysis, where it is sufficient to detect multiple sequence changes without the need for sequencing. Analysis of the electrophoretic mobilities of the products obtained from amplified *Taq*I-digested ISR fragments of the seven core strains and the most closely related type strain (*R. opacus*) allowed us to confirm clearly that the seven core strains were identical and to detect minor differences between them and the type strain examined. The SSCP method was the only fingerprint method used in this study which was able to differentiate between the reference organisms and the environmental isolates of *R. opacus*. The three typing methods based on 16S-23S rRNA ISR polymorphism exhibited successively increasing levels of sensitivity and have proven to be fast, sensitive, and reliable methods for determining relationships among the *Rhodococcus* species and strains studied.

(ii) SDS-PAGE of whole-cell proteins. Protein electrophoresis of whole-cell proteins has been proven to be a sensitive technique for providing information on the similarity of strains of the same species in various genera of gram-negative and gram-positive bacteria (13, 31, 44, 46, 47, 53). In general, there is a good correlation between groups obtained by determining protein patterns and DNA-DNA homology values. A comparison of the normalized protein patterns shown in Fig. 4 revealed that all of the representative core isolates, the type strain of *R. opacus*, and the type strain of the misclassified species *T. wratislaviensis* are visually very similar. From these data, we concluded that the seven isolates studied belong to a single species, *R. opacus*.

After numerical analysis, the type strain of *T. wratislaviensis*, LMG 17999, grouped at a significantly lower correlation level (0.87) with *R. opacus* and the core strains than those strains grouped among each other ($r = 0.95$). This lower similarity value was mainly due to one dense band at a molecular weight of approximately 60,000. Variable dense bands that influence the clustering sequences of strains within species have been found in several other genera. For these taxa, the variable band region indicates interstrain variability and is omitted in order to obtain congruence between protein pattern similarity and DNA homology (14, 47). When the single dense band at a molecular weight of approximately 60,000 was omitted from the cluster analysis, the type strain of *T. wratislaviensis* grouped with all of the other strains, including the *R. opacus* strains, at a correlation level higher than 0.94 (data not shown).

All of the data collectively suggest that the seven *Rhodococcus* environmental isolates belong to the species *R. opacus*. Moreover, *T. wratislaviensis* should be recognized as misclassified *R. opacus*. The ultimate proof of this, however, should come from DNA-DNA hybridization experiments performed with the type strains of the two species.

Microcosm enrichment of microbial communities. Contrary to our initial hypothesis, very little taxonomic diversity was observed in the biphenyl-mineralizing bacteria which were isolated after microcosm enrichment from diverse environmental samples. By contrast, all of the biphenyl-mineralizing communities were dominated by FAME cluster 1 strains, which were identified as *R. opacus* on the basis of 16S rRNA gene sequence information, genomic fingerprints, and SDS-PAGE patterns of whole-cell proteins with none of the typing methods used here. Differences between cluster 1 isolates from the different environments were detected. However, the reference strains of *R. opacus* and *T. wratislaviensis* could be differentiated from the environmental isolates of *R. opacus* on the basis of the SSCP gels of *Taq*I-digested amplified interspacer region fragments.

The FAME cluster 1 strain of *R. opacus* was convergently selected for in all seven environments studied. It was the dominant member of the cultivable microbial communities in the microcosms when long-term biphenyl amendment was used, both in soils and in sediments and independent of the level and history of PCB pollution. Since it is generally assumed that in microbial communities everything is (almost) everywhere (19), the initial presence of *R. opacus* in such diverse habitats as river, lake, and waste storage sediments and various types of soils is not surprising. However, *R. opacus* must have outcompeted other species of biphenyl-mineralizing bacteria in the microcosms during the enrichment experiment, thereby demonstrating an ecological competence which cannot be inferred from biochemical and physiological data. Its ability to maintain high population densities in diverse habitats following specific carbon amendment might make this organism a promising candidate for use in bioremediation requiring long-term survival of inocula (e.g., for recalcitrant xenobiotic compounds like PCBs).

The metabolic versatility of *R. opacus* is known to be very high (32). It would be interesting to know if similar results would have been obtained with microcosm enrichments with other carbon sources which can be mineralized by *R. opacus* (e.g., aromatic carboxylic acids, substituted phenols and catechols, and phthalic acid esters) (15, 16, 42).

It is interesting that mainly *R. opacus* but also other gram-positive bacteria (*R. equi*, *R. erythropolis*, *R. rhodochrous*, *Bacillus* sp.) became predominant after 6 months of microcosm enrichment. Some representatives of the β subclass of the class *Proteobacteria* (*Alcaligenes* sp., *A. eutrophus*) were also found, while *Pseudomonas* strains were not isolated. Classical enrichment tends to yield *Pseudomonas* strains and related gram-negative strains because of their high growth rates. In this study, however, gram-positive bacteria became established at high population densities in heterogeneous matrices following prolonged selection for biphenyl degradation. The microcosm enrichment approach used here might be valuable for isolating bacteria more representative of naturally degrading microbial communities than standard enrichment procedures.

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