Erb, R.W., Wagner-Dobler, I.
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(1993) Applied and Environmental Microbiology, 59 (12), pp. 4065-4073.

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1993, p. 4065–4073 Vol. 59, No. 12
0099-2240/93/124065-09$02.00/0

Detection of Polychlorinated Biphenyl Degradation Genes in Polluted Sediments by Direct DNA Extraction and Polymerase Chain Reaction

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Received 1 June 1993/Accepted 22 September 1993

It was the aim of this study to specifically detect the DNA sequences for the *bphC* gene, the *meta*-cleavage enzyme of the aerobic catabolic pathway for biphenyl and polychlorinated biphenyl degradation, in aquatic sediments without prior cultivation of microorganisms by using extraction of total DNA, PCR amplification of *bphC* sequences, and detection with specific gene probes. The direct DNA extraction protocol used was modified to enhance lysis efficiency. Crude extracts of DNA were further purified by gel filtration, which yielded DNA that could be used for the PCR. PCR primers were designed for conserved regions of the *bphC* gene from a sequence alignment of five known sequences. The specificity of PCR amplification was verified by using digoxigenin-labeled DNA probes which were located internal to the amplified gene sequence. The detection limit for the *bphC* gene of *Pseudomonas paucimobilis* Q1 and *Pseudomonas* sp. strain LB400 was 100 cells per g (wet weight) or approximately five copies of the target sequence per PCR reaction mixture. In total-DNA extracts of aerobic top layers of sediment samples obtained from three different sampling sites along the Elbe River, which has a long history of anthropogenic pollution, *Pseudomonas* sp. strain LB 400-like sequences for the *bphC* gene were detected, but *P. paucimobilis* Q1 sequences were not detected. No *bphC* sequences were detected in an unpolluted lake sediment. A restriction analysis did not reveal any heterogeneity in the PCR product, and the possibility that sequences highly related to the *bphC* gene (namely, *nahC* and *todE*) were present was excluded. Thus, for the first time it was possible to directly amplify and detect a chromosomally encoded, single-copy gene from a highly specialized subpopulation of the total microbial community in natural sediments.

It is one of the fundamental dilemmas in microbial ecology that only a fraction (0.01 to 10%) of the bacteria known to occur in natural habitats can be cultivated on laboratory media (2). While taxonomy has long been based mainly on phenotypic traits (e.g., the ability to use certain carbon sources), bacterial systematics is presently being revolutionized by the widespread use of sequence information. Analysis of rRNA sequences has proven to be especially useful, because RNA occurs in all cells and contains both highly conserved and variable regions (40). Thus, cultivating an organism is no longer a prerequisite for taxonomic identification. The direct extraction of total DNA from environmental samples and sequence analysis of rRNA genes have made it possible to investigate the diversity of microbial communities without prior cultivation. Thus, both bacterial systematics and the diversity of natural microbial assemblages are presently being reconsidered by using rRNA gene sequences (1, 11, 17, 24, 25, 37, 39).

When we intend to use microorganisms for human purposes (e.g., bioremediation of polluted environments), we of course deal only with bacteria from the culturable part of the community. Almost all of our knowledge about microbial physiology and genetics has come from pure cultures. What we do not know at present is how significant these organisms and their catabolic pathways are for xenobiotic compound degradation under natural conditions, how selective pressure influences pathway diversity, what role geographic heterogeneity plays, and how fast gene transfer and other evolutionary mechanisms work in nature. One approach to answering some of these questions is direct analysis of microbial communities by using molecular biology techniques.

The rRNA operon has the advantage that highly conserved regions can be used as PCR primers, and variable regions between the conserved regions can be amplified. Genes for catabolism of xenobiotic compounds, however, tend to be highly variable and appear to have evolved independently. For example, universal primers for dioxygenases cannot be developed. Rather, sets of primers for strongly related subgroups of genes are needed. In the case of aerobic polychlorinated biphenyl (PCB) degradation the best-characterized pathway is the chromosomally encoded *bph* pathway, which occurs in many pseudomonads and related genera (3, 7–10). This pathway is chromosomally encoded. The third enzymatic step, an extradiol *meta* cleavage, is performed by a 2,3-dihydroxybiphenyl dioxygenase, the product of the *bphC* gene. The five known *bphC* sequences exhibit a high degree of homology.

The objective of this study was to optimize the methods used for DNA extraction and purification and PCR amplification such that specific detection of *bph* genes in uninoculated sediment samples would be possible. The specificity of the PCR product obtained from natural sediment samples was verified by hybridization under high-stringency conditions with gene probes, and the diversity of the PCR products was tested by restriction analysis.

PCR and gene probes have been used previously to detect specific microorganisms in the environment. The samples were, however, inoculated with the target microorganisms (5, 21, 22, 26, 32–34). Recently, amplification of native environmental DNA has been accomplished for antibiotic

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resistance genes (16) and naphthalene degradation genes (13). An attempt was also made to study the occurrence of PCB degradation genotypes in the environment by using gene probes (36). The microaerophilic top layers of sediments in rivers and lakes are highly diverse and metabolically very active microbial ecosystems. Moreover, sediments act as the ultimate sinks for many man-made pollutants, including PCBs. The work described below was, to the best of our knowledge, the first amplification of a single-copy, chromosomally encoded catabolic gene from native river sediment DNA.

MATERIALS AND METHODS

Sampling sites and characteristics. Sediment samples were obtained from three polluted sites along the Elbe River and its tributaries and from Grumbacher Teich, a small, unpolluted lake in the Harz Mountains that has no history of anthropogenic chemical pollution (35).

The Elbe River has had a long history of PCB contamination. Some sediment samples were obtained from the Elbe River at Magdeburg (Elbe River kilometer 318). Other samples were obtained at Roßlau (Elbe River kilometer 259.5), which is where the Mulde River, a tributary of the Elbe River, flows into the Elbe River. The Mulde River carries the majority of the pollutant load of the Elbe River. Still other samples were obtained from the Spittelwasser River, a small river which directly received industrial effluents from the major industrial area of the former German Democratic Republic (Wolfen and Bitterfeld); the Spittelwasser River flows into the Mulde River close to the little town of Raguhn. Pollution increases upstream from Magdeburg to the Spittelwasser River.

The water contents were determined to be 25.6% in the Roßlau sediment, 52.2% in the Magdeburg sediment, and 90.6% in the Spittelwasser sediment.

Sediment cores were obtained by using a sampling procedure described previously (35). For direct DNA extraction only the top layer (0 to 5 mm) of each sediment core was used. This top slice was determined to be microaerophilic by measuring the vertical oxygen concentration gradient in steps of 100 μm with an oxygen microelectrode (Diamond, Inc., Ann Arbor, Mich.) mounted on a micromanipulator (Märzhauser, Wetzlar, Germany).

Bacterial strains. *Pseudomonas* sp. strain LB400 (6), supplied by General Electric Research and Development, Schenectady, N.Y., and *Rhodococcus globerulus* P6 are both capable of degrading an exceptionally wide variety of PCB congeners. These strains were used as gram-negative and gram-positive seed organisms, respectively, to evaluate the lysis efficiency of the direct DNA extraction method.

In addition, DNA extracts from the PCB-degrading organisms *Pseudomonas* sp. strain LB 400, *Alcaligenes eutrophus* H850 (4) (also obtained from General Electric), and *Pseudomonas paucimobilis* Q1 (10) (supplied by K. Furukawa, Kyushu University, Fukuoka, Japan) were used as positive controls for PCR amplification of *bphC* genes and optimization of PCR conditions. DNA extracts from environmental isolates M1 and M2, which are capable of degrading 3- and 4-chlorobiphenyls (40a), were also used.

Sediment inoculation. To evaluate lysis efficiency and DNA recovery, 1-g sediment samples were inoculated with known densities of seed organisms. Prior to seeding, the sediments were sterilized by multiple autoclaving on 3 successive days. Sterility was checked by plating the samples on 0.1× Luria-Bertani medium (23) and determining direct microscopic counts by phase-contrast microscopy and fluorescence microscopy after 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining.

Free DNA in sterilized sediment samples was extracted with 2 ml of 120 mM sodium phosphate buffer (pH 8.0). After 15 min of shaking, the slurry was pelleted by centrifugation at 6,000 × *g* for 10 min. This procedure was repeated twice before the samples were seeded. Seed organisms were grown to the late exponential phase, harvested, washed, and resuspended in 10 mM phosphate buffer (pH 7.0). Bacterial density was determined by direct cell counts in a Thoma chamber by phase-contrast microscopy. Sediment samples were inoculated by adding 200 μl of a cell suspension containing 2.9×10^9 *Pseudomonas* sp. strain LB400 cells or 3.4×10^8 *R. globerulus* P6 cells and incubated at room temperature for 30 min prior to DNA extraction.

Direct extraction of DNA. DNA was extracted by the method of Tsai and Olson (31). We modified this method to increase lysis efficiency. Sediment samples (1 g, wet weight) were suspended in 2 ml of lysozyme lysis solution (0.15 M NaCl, 0.1 M Na_2EDTA [pH 8.0], 15 mg of lysozyme per ml) and incubated in a 37°C water bath for 2 h with agitation at 15-min intervals. Then 2 ml of sodium dodecyl sulfate (SDS) lysis solution (0.1 M NaCl, 0.5 M Tris-HCl [pH 8.0], 10% [wt/vol] SDS) was added, and five cycles of shock-freezing in liquid nitrogen and thawing in a 65°C water bath were conducted.

After the freeze-thaw cycles 2 ml of 0.1 M Tris-HCl (pH 8.0)-saturated phenol (23) was added, and each sample was emulsified by brief, gentle vortexing. The mixture was centrifuged at 6,000 × *g* for 10 min at 4°C (Sorvall SS-34 rotor). The sediment pellet was subjected to an additional lysis treatment as described above, whereas 3.5 ml of the top aqueous layer was collected, mixed with 1.75 ml of Tris-HCl-saturated phenol and 1.75 ml of a chloroform-isoamyl alcohol mixture (24:1), and centrifuged at 6,000 × *g* for 10 min. A 3-ml sample of the resulting extract was further extracted with an equal volume of the chloroform-isoamyl alcohol mixture.

Finally, the nucleic acids in the extracted aqueous phase (2.5 ml) were precipitated with 2.5 ml of cold isopropanol for 1 h or overnight. The crude nucleic acids were pelleted by centrifugation at 10,000 × *g* for 15 min and then vacuum dried with a Speedvac concentrator (Savant Instruments, Farmingdale, N.Y.). The pooled pellets from both lysis treatments, which were dark brown as a result of contaminating, coextracted humic materials, were resuspended in 100 μl of TE buffer (20 mM Tris-HCl, 1 mM EDTA; pH 8.0).

DNA purification by gel filtration. Crude extracts of DNA were purified by using Sephadex G-200 (Pharmacia AB, Molecular Biology Division, Uppsala, Sweden) spun columns (33). TE buffer-saturated Sephadex G-200 was packed into 2-ml disposable sterile syringes containing 0.2 to 0.3 cm of glass wool at the bottom. Excess TE buffer was removed by centrifuging the columns at 1,000 × *g* for 10 min in a swinging-bucket rotor (type H-6000A; Sorvall). Finally, each column contained a gel bed that was 1.0 to 1.5 cm high. The 100 μl of crude DNA extract was slowly loaded onto the center of each column, which was then centrifuged for 10-min periods at 1,000 × *g* until 100 μl of colorless DNA eluent was recovered. After this 1 μl of the pooled fraction was used as a PCR template to test for the presence of target DNA.

Primer design. PCR primers were selected on the basis of five previously published *bphC* sequences (Table 1). The open reading frames of these five sequences were aligned by

TABLE 1. *bphC* sequences used in this study

EMBL sequence name[a]	Bacterium	Reference
PP 23OH BP	*P. paucimobilis* Q1	29
PP BPH CDA	*P. pseudoalcaligenes* KF707	7
PP BPH CDB	*P. putida* KF715	12
M26433	*Pseudomonas* sp. strain KKS102	15
	Pseudomonas sp. strain LB400	14

[a] EMBL, European Molecular Biology Laboratory data base.

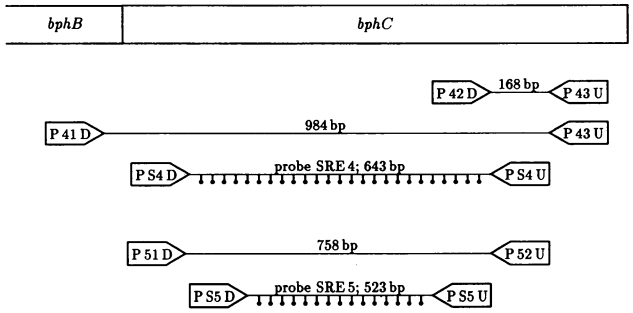

FIG. 1. Locations of primers and probes within the *bph* operon. The lengths of the PCR products and probes are indicated above the lines.

using the PC Gene software package (Intelli Genetics, Inc., Mountain View, Calif.) and were found to be 39.9% identical. The sequence alignment revealed a closer relationship among the *bphC* genes of *Pseudomonas pseudoalcaligenes* KF707, *Pseudomonas putida* KF715, *Pseudomonas* sp. strain KKS102, and *Pseudomonas* sp. strain LB400. The coding regions of these sequences were identical for 63.7% of all nucleotides. Taking this into account, we subdivided the five sequences into two groups; one group contained the *bphC* sequence of *P. paucimobilis* Q1 (the Q1 group), and the other group contained the other four sequences (the sequences of strains KF707, KF715, KKS102, and LB400) (the LB400 group).

To obtain primers that were both highly specific for the known sequences and at the same time allowed the detection of related, but unknown, *bphC* genes, two group-specific primer sets were designed. The first primer set, designated primer set P5, consisted of two primers, P51D and P52U. For these primers two regions that exhibited nucleotide conservation in all five *bphC* sequences were chosen, substituting nonidentical nucleotides with the respective nucleotide of the strain Q1 sequence. Thus, this primer set was optimized for the *bphC* sequence of strain Q1 and closely related sequences, but in principle should have allowed amplification of all five sequences.

The second primer set, designated primer set P4, consisted of primers P41D, P42D, and P43U. The construction of these primers was based on regions conserved in the four more closely related *bphC* sequences (the sequences of strains KF707, KF715, KKS102, and LB400); these primers allowed specific detection not only of these four sequences but also of other related sequences. All primers contained restriction sites for cloning that were synthesized as 5′ parts of the primers but were not used in this study. These restriction site linkers are indicated below in lowercase letters. Primers P41D, P42D, and P51D contained the recognition sequence for *Bam*HI, and primers P43U and P52U contained the recognition sequence for *Hin*dIII.

The sequence of PCR primer P51D was 5′-cgggatcc cgGGTTACCTCGGGTTGACCGT-3′, corresponding to nucleotides 72 to 91 of the PP 23OH BP sequence, and the sequence of PCR primer P52U was 5′-cccaagcttgggTAGA AGGTCAGCGCCTGGTC-3′, corresponding to nucleotides 808 to 789. PCR amplification of strain Q1 DNA with primers P51D and P52U resulted in a 758-bp product (the entire *bphC* gene of strain Q1 is 900 bp long). Primer P41D was located in the *bphB* gene, 103 nucleotides upstream of the *bphC* start codon; its sequence was 5′-cgggatcccgACGACGGTGGCT-TGGGCGT-3′, corresponding to nucleotides 6384 to 6402 of the strain LB400 sequence. The sequence of primer P42D was 5′-cgcggatccgcgGGGCGCCACACCAATGACCA-3′, corresponding to nucleotides 7202 to 7221 of the strain LB400 sequence, and the sequence of primer P43U was 5′-cccaagcttggGACTTGTGGCCCCACATG-3′, corresponding to nucleotides 7347 to 7330. PCR amplification of LB400 DNA with primers P41D and P43U resulted in a 984-bp product, whereas amplification with primers P42D and P43U generated a 168-bp PCR product. The length of the entire *bphC* gene ranged from 879 bp (strain KF715) to 897 bp (strain LB400).

PCR amplification. PCR amplification was performed in a total volume of 50 μl in 0.5-ml Eppendorf tubes under a layer of light mineral oil by using a programmable DNA thermal cycler (Landgraf, Langenhagen, Germany). Each reaction mixture contained 0.25 mM dATP, 0.25 mM dCTP, 0.25 mM dGTP, 0.25 mM dTTP, each primer at a concentration of 0.5 μM, and 1.5 U of *Taq* DNA polymerase (Boehringer, Mannheim, Germany) in a buffer containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 0.01% (wt/vol) gelatin.

To amplify *bphC* genes from uninoculated sediments, 1 μl of undiluted, purified DNA extract was used as the template for the PCR. The PCR was performed by using an optimized PCR protocol consisting of denaturation at 95°C for 30 s, primer annealing at 35°C for 1 min, and primer extension at 72°C for 3 min, with a 10-min final extension step at 72°C in the last cycle. The low annealing temperature was selected to allow amplification of *bphC* genes that may have divergent base pair compositions. At the end of 35 cycles, 1 μl of the amplified product was transferred to a fresh reaction mixture and amplified for another 35 cycles; this resulted in "double PCR" amplification.

PCR products were electrophoresed in 1% agarose gels and were stained with ethidium bromide by using standard techniques (23).

Probes and DNA hybridization. Two gene probes, designated SRE4 and SRE5, were used to detect and to verify the identities of the PCR products. Probe SRE4 was designed for hybridization with PCR products obtained with primer set P4, and probe SRE5 was designed to detect products of primer set P5 (Fig. 1). Both probes were labeled with digoxigenin-11-dUTP (Boehringer).

Nonradioactive label was synthesized and incorporated into the probes by using a modified PCR protocol. Primers PS4D and PS4U (used to generate probe SRE4) and primers PS5D and PS5U (used for synthesis of probe SRE5) were located internal to the respective PCR products to be detected, thus avoiding hybridization of probes to their targets because of primer identity (Fig. 1). Each PCR mixture contained 0.07 mM digoxigenin-11-dUTP, 0.13 mM dTTP, 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, each primer at a concentration of 0.5 μM, and 1.5 U of *Taq* DNA poly-

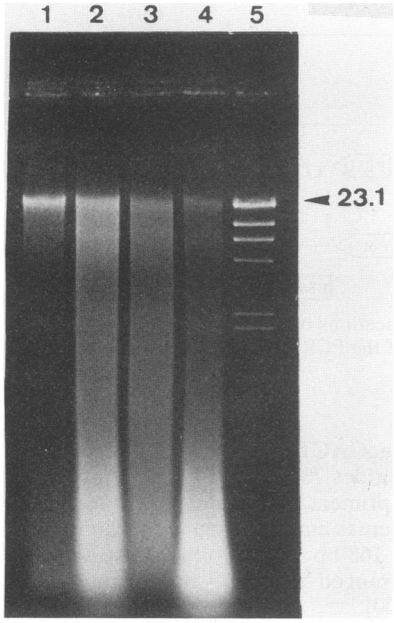

FIG. 2. Crude extracts of DNA obtained from four sediment samples. Lane 1, Grumbacher Teich sample; lane 2, Roßlau sample; lane 3, Magdeburg sample; lane 4, Spittelwasser River sample; lane 5, *Hin*dIII-digested λ DNA size marker.

TABLE 2. DNA yields and recovery rates as determined by the direct DNA extraction method

Source of sediment	Sample[a]	Concn of bacteria (no. of cells/g [wet wt])[b]	DNA yield (μg/g [wet wt])[c]	Recovery rate (%)[d]
Spittelwasser River	NS	6.4×10^9	34.3	
	LB	2.9×10^9	15.5	98
	P6	3.4×10^8	1.80	97
Roßlau	NS	1.2×10^{10}	59.4	
	LB	2.9×10^9	15.3	97
	P6	3.4×10^8	1.79	97
Magdeburg	NS	1.0×10^{10}	51.7	
	LB	2.9×10^9	15.0	95
	P6	3.4×10^8	1.72	93
Grumbacher Teich	NS	1.5×10^8	0.82	
	LB	2.9×10^9	15.6	99
	P6	3.4×10^8	1.81	98

[a] NS, nonsterile, uninoculated sediment sample; LB, sterile sediment sample seeded with *Pseudomonas* sp. strain LB400; P6, sterile sediment sample seeded with *R. globerulus* P6.
[b] Determined by direct microscopic enumeration.
[c] Determined spectrophotometrically after Sephadex G-200 gel filtration.
[d] Calculated by comparing the amount of DNA extracted from sterile, seeded sediment samples with the amount of DNA recovered from pure culture controls having the same density.

merase in the reaction buffer described above. The PCR was performed for 40 cycles according to the following protocol: denaturation at 95°C for 30 s; primer annealing at 50°C for 30 s; and primer extension at 72°C for 3 min, with a 10-min final extension step at 72°C in the last cycle. DNA from pure cultures of *P. paucimobilis* Q1 and *Pseudomonas* sp. strain LB400 was used as a template for synthesis of probes SRE5 and SRE4, respectively. Therefore, probe SRE4 was identical to nucleotides 6635 to 7278 of the strain LB400 sequence, whereas probe SRE5 corresponded to nucleotides 105 to 628 of the PP 23OH BP sequence.

For detection and verification purposes the amplified DNA was transferred onto positively charged Hybond N^+ nylon membranes (Amersham, Braunschweig, Germany) either by dot blotting, using a Bio-Dot apparatus (Bio-Rad Laboratories), or by Southern blotting (23). Prehybridization (at 60°C), hybridization (at 60°C), posthybridization washes, and subsequent chemiluminescent detection were performed under high-stringency conditions by using a dig luminescence detection kit (Boehringer) according to the manufacturer's instructions.

Restriction digestion of PCR products. Prior to digestion, PCR products were concentrated and purified with Centricon 100 microconcentrators (Amicon, Beverly, Mass.) as specified by the manufacturer. Restriction digestions were performed by using a standard protocol (23).

RESULTS AND DISCUSSION

Direct DNA extraction. Figure 2 shows the crude DNA extracted from four different sediments visualized in an ethidium bromide-stained agarose gel. The largest DNA was more than 23 kb long, and most DNA was in the size range around 20 kb, indicating that owing to the "soft lysis" conditions used the extracted DNA had high molecular weight. Harsher lysis conditions cause severe DNA shearing, and thus extracted DNA is more fragmented. For example, the direct lysis procedure of Ogram et al. (19), which included mechanical disruption in a bead beater, produced DNA fragments smaller than 10 kb, and the combined lysis protocol of Picard et al. (21), which included sonication, microwave heating, and thermal shocks, resulted in DNA fragments that ranged in size from 100 to 500 bp. The less prominent shearing effects of the protocol used in this study are an important prerequisite for the in situ detection of catabolic single-copy genes by the PCR. If the genes to be detected are fragmented, they do not provide good templates for the PCR, and thus the sensitivity of detection might be drastically reduced, especially when only a few copies of the gene are present initially in the sample.

Therefore, the lysis conditions represent a critical step in the direct DNA extraction procedure. On the one hand, the conditions should be as quantitative as possible to ensure maximum sensitivity; on the other hand, they have to be gentle enough to reduce DNA shearing to a minimum.

Lysis efficiency and DNA recovery. Sediment samples were inoculated with gram-negative or gram-positive seed organisms to evaluate lysis efficiency and DNA recovery. The cell numbers in the seeding experiments were similar to the direct microscopic counts in the sediments investigated (Table 2).

After the first lysis treatment the lysis efficiency was more than 99%, as determined by direct microscopic enumeration after lysis. In agreement with the original study (31), no difference was detected between the gram-negative and gram-positive seed organisms.

The lysis efficiency was increased further by subjecting each sediment pellet to a second lysis treatment. After this treatment less than 100 cells per g of sediment were detected. Thus, our modified lysis procedure with an increased number of freeze-thaw cycles and an additional lysis treatment is an excellent method for combination with the very

sensitive PCR detection procedure, since on the part of lysis no limitation on detection sensitivity is to be expected.

The efficiency of DNA recovery from sediments was determined by comparing the amounts of DNA extracted from seeded sterile sediment samples with the amounts of DNA recovered from pure culture controls having the same density (Table 2). The DNA recovery rates ranged from 93 to 99%, depending on the sample; these values were consistent with results obtained by other investigators (21, 28, 31). The absolute yields were specific for the sediments tested, which differed with regard to texture and the number of microorganisms present; however, they were within the range of values (12 to 50 μg/g) determined by other workers (19, 21, 31).

Purification of extracted DNA. A significant problem associated with the direct lysis procedure is that a large amount of contaminating humic material is coextracted with the DNA. Particularly if the DNA is to be subjected to enzymatic treatments, such as restriction digestion or PCR, a high level of purity of the DNA preparation is required for successful PCR amplification.

Crude DNA extracts obtained from all four sediments sampled were very dark brown. No PCR products were obtained when these crude extracts were used directly for the PCR, even when they were diluted 1,000-fold. This failure could have been due either to humic material that interfered with the PCR or to a lack of template DNA as a result of the high level of dilution. When template DNA was not a limiting factor (i.e., when 100 amol of a cloned *bphABC* fragment was added to the PCR mixture), inhibition could be attenuated by diluting the crude extract at least 100-fold. However, as little as 1 μl of undiluted crude DNA extract or 1 μl of crude DNA extract diluted 1:10 was sufficient to completely inhibit the PCR regardless of the amount of template DNA present. Similar results have been reported previously by Tsai and Olson (32).

A method used for further purification should be effective, simple, and rapid, should allow the processing of many samples in parallel, and should minimize losses of DNA. In this study we adapted a gel filtration approach to separate humic substances from crude DNA (33). Using Sephadex G-200 column purification, we effectively cleaned the dark brown crude DNA extracts. Humic compounds were retarded selectively without any additional fragmentation or significant losses of DNA (between 5 and 15%). Prior to purification A_{260} values do not provide true measurements of the amount of DNA (28), as shown by spectrophotometric analysis. Therefore, losses were determined by gel electrophoresis. After Sephadex purification A_{260}/A_{280} ratios ranged between 1.6 and 1.9. However, a component absorbing at 230 nm persisted in the recovered DNA, indicating that the preparation was still contaminated. Nevertheless, the level of purity was sufficient to allow subsequent PCR amplification of undiluted DNA extracts or DNA extracts diluted 1:10.

Specificity and sensitivity of primers and probes. In most previously characterized PCB degraders the catabolic genes are located on the bacterial chromosome and are clustered in the *bph* operon. For some PCB-degrading strains this operon has been extensively characterized, cloned, and sequenced (7, 12, 15, 18, 29, 41). Some of the *bph* genes which encode the 2,3-dihydroxybiphenyl dioxygenase are highly conserved (8, 9). The specificity of the PCR primers was evaluated by amplifying *bphC* genes from pure cultures of *A. eutrophus* H850, *Pseudomonas* sp. strain LB400, and *P. paucimobilis* Q1 by using a simplified PCR protocol consisting of only 35 cycles, a shorter annealing time (30 s), and a higher annealing temperature (40°C).

DNA from *Pseudomonas* sp. strain LB400 and *P. paucimobilis* Q1 was used as a positive control for primer sets P4 and P5, respectively. Moreover, DNA from *A. eutrophus* H850 was used because its *bphC* gene is very similar to the *bphC* gene from *Pseudomonas* sp. strain LB400 (41).

As expected, primer set P4 allowed amplification of the closely related *bphC* genes of *Pseudomonas* sp. strain LB400 and *A. eutrophus* H850, whereas the *bphC* sequence of *P. paucimobilis* Q1 was too divergent to be amplified with the P4 primers at an annealing temperature of 40°C.

Because of choice and design, the P5 primers amplified the strain Q1 sequence as well as the *bphC* sequences of *Pseudomonas* sp. strain LB400 and *A. eutrophus* H850, although at a lower efficiency. These results demonstrate that sequence alignments based on only five known sequences should allow the design of specific primers having predictable performance under the conditions described above.

The sensitivity and specificity of gene probes SRE4 and SRE5 were determined by performing a dot blot analysis with pure culture DNA from *Pseudomonas* sp. strain LB400 and *P. paucimobilis* Q1. With both probes the *bphC* gene could be detected when 0.05 μg of genomic DNA was used. Assuming a genome size of 5×10^6 bp, 0.05 μg of strain LB400 DNA corresponded to 6.4 pg or 9×10^6 copies of the 643-bp target sequence, whereas 0.05 μg of strain Q1 DNA corresponded to 5.2 pg or 9×10^6 copies of the 523-bp target sequence.

When less than 10 μg of genomic DNA (1.8×10^9 target copies) was used, the probes were able to differentiate between the *bphC* genes of *Pseudomonas* sp. strain LB400 and *P. paucimobilis* Q1; i.e., SRE4 hybridized with the target sequence of *Pseudomonas* sp. strain LB400 but not with the *bphC* sequence of *P. paucimobilis* Q1 and vice versa. However, after PCR amplification of genomic DNA neither probe could differentiate between the two *bphC* genes. This was due to the level of sequence homology of the two *bphC* genes and the high concentration of target sequences after PCR amplification.

Detection limit. Sediment samples were inoculated with various cell densities ranging from 0 to 1.0×10^6 cells per g. After DNA extraction and purification 5-μl portions of purified extracts were used as templates for double PCR in a reaction volume scaled up to 100 μl.

The sensitivity of detection was determined to be 100 cells per g (wet weight) by dot blot detection of amplification products (Fig. 3). This detection limit was found for both *Pseudomonas* sp. strain LB400 and *P. paucimobilis* Q1 in all sediment samples. Since only 5 μl of the 100 μl (5%) of the purified DNA extract obtained from sediments initially seeded with 100 cells per g could be used for the PCR without inhibitory effects, the detection limit can also be interpreted as five *bphC* copies per PCR mixture, which is close to the theoretical detection limit of one copy of template DNA per PCR mixture.

PCR amplification of *bphC* sequences from uninoculated sediments. Total-DNA extracts of the top layers of sediment obtained from three contaminated sites along the Elbe River (Magdeburg, Roßlau, Spittelwasser River) yielded a PCR product after double PCR amplification with primer set P4 which was specific for the LB400 group of PCB degraders (Table 3). The double PCR selectively diluted inhibitory contaminants, whereas dilution of the target was compensated for by amplification (even if it was not very efficient)

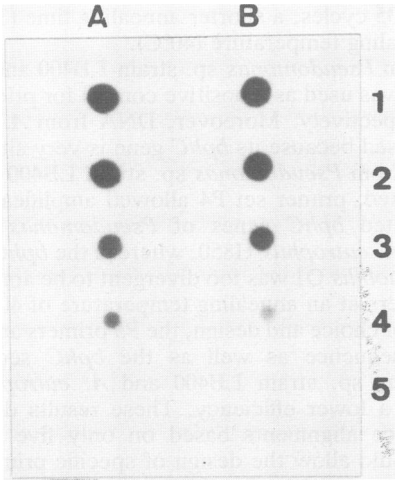

FIG. 3. Detection limit for *P. paucimobilis* Q1 cells in seeded sterile sediment samples (1 g [wet weight]) as determined by total DNA extraction, Sephadex G-200 purification, and PCR amplification. PCR products were detected by hybridization with gene probe SRE5. Lane A, Grumbacher Teich sediment; lane B, Spittelwasser River sediment. Row 1, 1.0×10^4 cells per g; row 2, 1.0×10^3 cells per g; row 3, 5.0×10^2 cells per g; row 4, 1.0×10^2 cells per g; row 5, 5.0×10^1 cells per g.

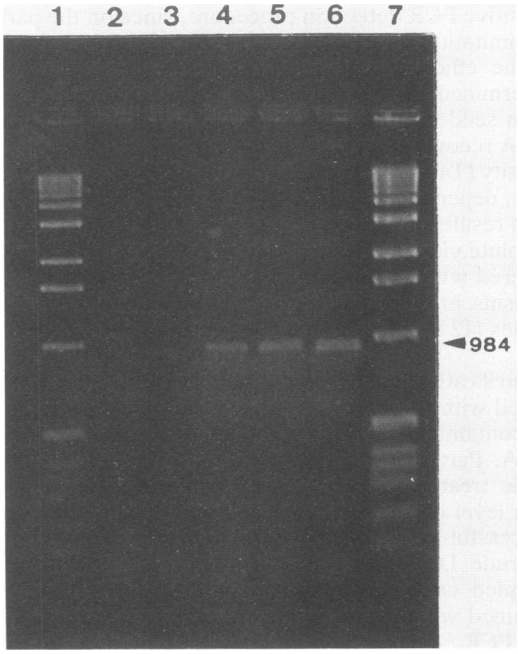

FIG. 4. Products obtained after double PCR amplification with primers P41D and P43U by using purified DNA extracts obtained from different uninoculated sediment samples as templates. Lanes 1 and 7, 1-kb ladder marker; lane 2, negative control; lane 3, Grumbacher Teich sample; lane 4, Magdeburg sample; lane 5, Roßlau sample; lane 6, Spittelwasser River sample.

during the first round of the PCR. Thus, the template/inhibitor ratio increased, enhancing the probability of successful amplification. When primers P42D and P43U were used, the amplified 168-bp product could in most cases be detected on an agarose gel, in contrast to the 948-bp product obtained after amplification with primers P41D and P43U (Fig. 4).

The difficulty of reproducibly generating enough PCR product from environmental DNA to visualize it directly on an ethidium bromide-stained agarose gel has been described previously (20, 22, 27). This problem is probably caused by both the low copy number of the target DNA and the remaining impurities in the DNA extract, which reduce the efficiency of amplification. However, the PCR products could be detected reproducibly after Southern blotting and hybridization with gene probe SRE4 (Fig. 5). Thus, we could amplify and detect a chromosomally encoded catabolic gene of a highly specialized subpopulation from uninoculated environmental samples and thereby detect biphenyl degradation genes in environmental samples. Since primers were designed for conserved regions of the known *bphC* sequences, they in principle allowed detection of strongly related yet unknown sequences as well.

TABLE 3. PCR amplification of *bphC* genes from uninoculated sediment samples and environmental isolates

Purified DNA extract from:	PCR results with the following primers:		
	P41D and P43U	P42D and P43U	P51D and P52U
Spittelwasser River	+	+	−
Roßlau	+	+	−
Magdeburg	+	+	−
Grumbacher Teich	−	−	−
M1	−	−	+
M2	−	−	+

There was no difference among the three sampling sites located along the Elbe River (Fig. 4 and 5); i.e., the occurrence of the *bphC* gene sequences was not correlated with the present level of PCB contamination (data not shown). In the DNA extract obtained from the unpolluted site at Grumbacher Teich, however, none of the primer sets generated a PCR product. Therefore, the Elbe River sediment community can be viewed as adapted to PCBs, in contrast to the microbial community of a nonpolluted site. When primer set P5 was used, no *bphC* genes were detected; either sequences similar to the *P. paucimobilis* Q1 sequence were not present, or their levels were below the detection limit.

In contrast, when primers P51D and P52U were used, *bphC*-specific sequences were amplified with template DNA from Elbe River isolates M1 and M2, which had been enriched on chlorobiphenyls in flow-through columns (40a), indicating that these strains may contain a gene similar to the *bphC* gene of *P. paucimobilis* Q1.

Since *bphC* sequences from the LB400 group but not *bphC* sequences from the Q1 group were detected in total DNA extracts from the Elbe River sediments, we expected that strain LB400-like sequences would be present in randomly picked isolates. However, strains M1 and M2 were subjected to prolonged enrichment and may represent less common components of the Elbe River sediment microbial community.

Restriction analysis of amplified sequences. A "shotgun experiment" was performed to examine sequence variability within the amplified *bphC* genes. The PCR products from the Elbe River sediment DNA were digested with seven restriction enzymes which do not cut the known *bphC* sequences;

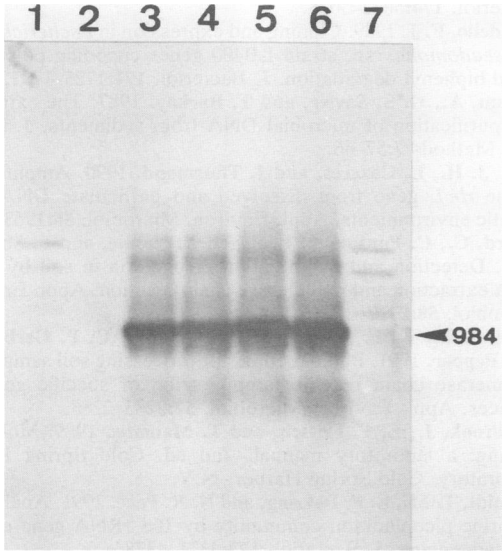

FIG. 5. Southern blot hybridization of products obtained after double PCR amplification with primers P41D and P43U by using template DNA from uninoculated sediment samples. Signals were detected by hybridization with digoxigenin-labeled gene probe SRE4. Lanes 1 and 7, digoxigenin-labeled size marker IV (Boehringer); lane 2, Grumbacher Teich sample; lane 3, Magdeburg sample; lane 4, Roßlau sample; lane 5, Spittelwasser River sample; lane 6, positive control (*Pseudomonas* sp. strain LB400 DNA).

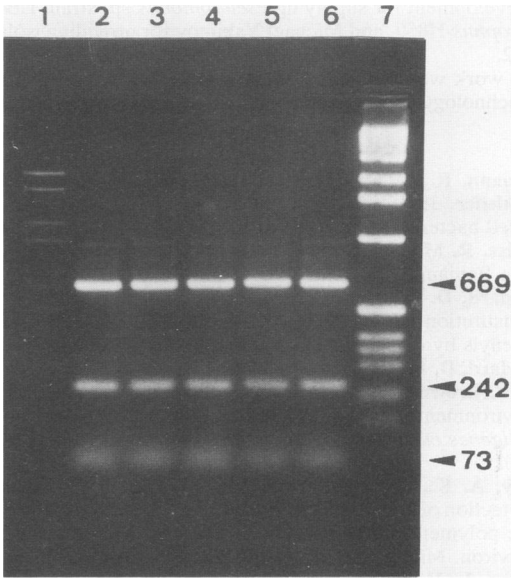

FIG. 6. Restriction analysis of PCR products obtained with purified DNA extracts of uninoculated sediment samples. Amplified products were digested with *Rsa*I. Lane 1, digoxigenin-labeled size marker IV (Boehringer); lane 2, amplified *bphABC* fragment; lane 3 amplified *bphC* gene from *Pseudomonas* sp. strain LB400; lane 4, amplified products from Spittelwasser River sediment; lane 5, amplified products from Roßlau sediment; lane 6, amplified product from Magdeburg sediment; lane 7, 1-kb ladder marker.

these enzymes failed to cut the amplified products (data not shown). Thus, the diversity of *bphC* genes may be low in the sediment microbial community in the Elbe River or undetectable with the method which we used. Small amounts of nondominant sequences may have been undetectable after restriction digestion, or recognition sites for the restriction enzymes used may not have varied in different sequences. To obtain a better picture, high-resolution gel electrophoresis or cloning and sequencing should be used (38).

A second restriction digestion experiment was performed to specifically search for dioxygenases with highly related sequences but different substrate specificities. The nucleotide sequences of the 1,2-dihydroxynaphthalene dioxygenase (*nahC*) encoded on plasmid NAH7 of *P. putida* and the 3-methylcatechol-2,3-dioxygenase (*todE*) of *P. putida* F1 are related to the *bphC* sequences more closely than some of the *bphC* sequences are related to each other. It has even been speculated that *bphC* and *todE* may share a common ancestry (30). Primer set P4 was most probably able to amplify *todE*. Therefore, the PCR products from Elbe River sediments were digested with *Rsa*I, which yielded clearly different fragments for *bphC*, *nahC*, and *todE*. The restriction pattern obtained was specific for *bphC* (Fig. 6). Again, this seems to indicate that there is low diversity in the sediment microbial community.

Because of the long history of pollution of the Elbe River (including pollution with naphthalene and toluene), adaptation to the polluting compounds can be expected. However, the majority of toluene and naphthalene degraders may use other catabolic pathways.

The isolates obtained from the Elbe River which we investigated contained a *bphC* sequence from the Q1 group of PCB degraders. A PCR with the sediment DNA, however, yielded LB400-like sequences, which revealed no diversity as determined by a crude restriction enzyme analysis. Thus, the genetic diversity of natural microbial populations with respect to PCB degradation genes needs to be analyzed with more sensitive techniques (e.g., high-resolution gel electrophoresis, cloning, and sequencing).

Conclusions. The data presented above may have applications for practical bioremediation approaches. In the future it may be possible, by using standard protocols for DNA extraction and the PCR, to rapidly evaluate the degradative potential of a microbial community directly, thus obviating the need for prior cultivation or adding ^{14}C-labeled substrate. By using mRNA instead of DNA as the target sequence, not only the potential but the actual degradative activity of a microbial community may be assessed directly.

Other possible applications are oriented more toward elucidation of fundamental questions in microbial ecology. Unfortunately, quantification of PCR products and thus quantification of gene frequencies are not feasible at present. Moreover, different sequences may have different amplification efficiencies in the PCR. However, a qualitative picture of diversity of PCB degradation genes in environmental samples could be obtained by analyzing the PCR products by denaturing gradient gel electrophoresis. Cloning and sequencing would then yield the necessary high-resolution information. The importance of catabolic sequences in total-DNA extracts from the environment could then be analyzed and compared with the diversity of the culturable part of the microbial community for the genes in question.

ACKNOWLEDGMENTS

We are grateful to Bernd Hofer for his advice on primer design and to the scientific diving group of the University of Clausthal-Zellerfeld for their support in obtaining samples of the sediments from Grumbacher Teich. Furthermore, we thank Kensuke Furukawa for supplying *P. paucimobilis* Q1, General Electric Research

and Development for supplying *Pseudomonas* sp. strain LB400 and *A. eutrophus* H850, and Michael Yakimov for providing isolates M1 and M2.

This work was supported by the German Ministry of Research and Technology (BMFT Vorhaben 0319-433A).

REFERENCES

1. **Amann, R., N. Springer, W. Ludwig, H.-D. Gortz, and K.-H. Schleifer.** 1991. Identification *in situ* and phylogeny of uncultured bacterial endosymbionts. Nature (London) **351:**161–164.
2. **Atlas, R. M., and R. Bartha.** 1987. Microbial ecology, 2nd ed. The Benjamin Cummings Publishing Co., Inc., Reading, Mass.
3. **Bedard, D. L., and M. L. Haberl.** 1990. Influence of chlorine substitution pattern on the degradation of polychlorinated biphenyls by eight bacterial strains. Microb. Ecol. **20:**87–102.
4. **Bedard, D. L., R. E. Wagner, M. J. Brennan, M. L. Haberl, and J. F. Brown, Jr.** 1987. Extensive degradation of aroclors and environmentally transformed polychlorinated biphenyls by *Alcaligenes eutrophus* H850. Appl. Environ. Microbiol. **53:**1094–1102.
5. **Bey, A. K., J. L. DiCesare, L. Haff, and R. M. Atlas.** 1991. Detection of *Escherichia coli* and *Shigella* spp. in water by using the polymerase chain reaction and gene probes for *uid*. Appl. Environ. Microbiol. **57:**1013–1017.
6. **Bopp, L. H.** 1986. Degradation of highly chlorinated PCBs by *Pseudomonas* strain LB400. J. Ind. Microbiol. **1:**23–29.
7. **Furukawa, K., N. Arimura, and T. Miyazaki.** 1987. Nucleotide sequence of the 2,3-dihydroxybiphenyl dioxygenase gene of *Pseudomonas pseudoalcaligenes*. J. Bacteriol. **169:**427–429.
8. **Furukawa, K., N. Hayase, and K. Taira.** 1989. Biphenyl/polychlorinated biphenyl catabolic gene (*bph* operon): organization, function, and molecular relationship in various pseudomonads, p. 111–120. *In* S. Silver, A. M. Chakrabarty, B. Iglewski, and S. Kaplan (ed.), *Pseudomonas*: biotransformations, pathogenesis, and evolving biotechnology. American Society for Microbiology, Washington, D.C.
9. **Furukawa, K., N. Hayase, K. Taira, and N. Tomizuka.** 1989. Molecular relationship of chromosomal genes encoding biphenyl/polychlorinated biphenyl catabolism: some soil bacteria possess a highly conserved *bph* operon. J. Bacteriol. **171:**5467–5472.
10. **Furukawa, K., J. R. Simon, and A. M. Chakrabarty.** 1983. Common induction and regulation of biphenyl, xylene/toluene, and salicylate catabolism in *Pseudomonas paucimobilis* Q1. J. Bacteriol. **154:**1356–1362.
11. **Giovannoni, S. J., T. B. Britschgi, C. L. Moyer, and K. G. Field.** 1990. Genetic diversity in Sargasso Sea bacterioplankton. Nature (London) **345:**60–63.
12. **Hayase, N., K. Taira, and K. Furukawa.** 1990. *Pseudomonas putida* KF715 *bphABCD* operon encoding biphenyl and polychlorinated biphenyl degradation: cloning, analysis, and expression in soil bacteria. J. Bacteriol. **172:**1160–1164.
13. **Herrick, J. B., E. L. Madsen, C. A. Batt, and W. C. Ghiorse.** 1993. Polymerase chain reaction amplification of naphthalene-catabolic and 16S rRNA gene sequences from indigenous sediment bacteria. Appl. Environ. Microbiol. **59:**687–694.
14. **Hofer, B., L. D. Eltis, D. N. Dowling, and K. N. Timmis.** 1993. Genetic analysis of a *Pseudomonas* locus encoding a pathway for biphenyl/polychlorinated biphenyl (PCB) degradation. Gene **130:**47–55.
15. **Kimbara, K., T. Hashimoto, M. Fukuda, T. Koana, M. Takagi, M. Oishi, and K. Yano.** 1989. Cloning and sequencing of two tandem genes involved in degradation of 2,3-dihydroxybiphenyl to benzoic acid in the polychlorinated biphenyl-degrading soil bacterium *Pseudomonas* sp. strain KKS 102. J. Bacteriol. **171:**2740–2747.
16. **Leff, L. G., J. R. Dana, J. V. McArthur, and L. J. Shimkets.** 1993. Detection of Tn*5*-like sequences in kanamycin-resistant stream bacteria and environmental DNA. Appl. Environ. Microbiol. **59:**417–421.
17. **Liesack, W., and E. Stackebrandt.** 1992. Occurrence of novel groups of the domain *Bacteria* as revealed by analysis of genetic material isolated from an Australian terrestrial environment. J. Bacteriol. **174:**5072–5078.
18. **Mondello, F. J.** 1989. Cloning and expression in *Escherichia coli* of *Pseudomonas* sp. strain LB400 genes encoding polychlorinated biphenyl degradation. J. Bacteriol. **171:**1725–1732.
19. **Ogram, A., G. S. Sayler, and T. Barkay.** 1987. The extraction and purification of microbial DNA from sediments. J. Microbiol. Methods **7:**57–66.
20. **Paul, J. H., L. Cazares, and J. Thurmond.** 1990. Amplification of the *rbcL* gene from dissolved and particulate DNA from aquatic environments. Appl. Environ. Microbiol. **56:**1963–1966.
21. **Picard, C., C. Ponsonnet, E. Paget, X. Nesme, and P. Simonet.** 1992. Detection and enumeration of bacteria in soil by direct DNA extraction and polymerase chain reaction. Appl. Environ. Microbiol. **58:**2717–2722.
22. **Pillai, S. D., K. L. Josephson, R. L. Bailey, C. P. Gerba, and I. L. Pepper.** 1991. Rapid method for processing soil samples for polymerase chain reaction amplification of specific gene sequences. Appl. Environ. Microbiol. **57:**2283–2286.
23. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **Schmidt, T. M., E. F. DeLong, and N. R. Pace.** 1991. Analysis of a marine picoplankton community by 16S rRNA gene cloning and sequencing. J. Bacteriol. **173:**4371–4378.
25. **Stahl, D. A., D. J. Lane, G. J. Olsen, and N. R. Pace.** 1985. Characterization of a Yellowstone hot spring microbial community by 5S rRNA sequences. Appl. Environ. Microbiol. **49:**1379–1384.
26. **Steffan, R. J., and R. M. Atlas.** 1988. DNA amplification to enhance detection of genetically engineered bacteria in environmental samples. Appl. Environ. Microbiol. **54:**2185–2191.
27. **Steffan, R. J., and R. M. Atlas.** 1990. Solution hybridization assay for detecting genetically engineered bacteria in environmental samples. BioTechniques **8:**316–318.
28. **Steffan, R. J., J. Goksøyr, A. K. Bey, and R. M. Atlas.** 1988. Recovery of DNA from soils and sediments. Appl. Environ. Microbiol. **54:**2908–2915.
29. **Taira, K., N. Hayase, N. Arimura, S. Yamashita, T. Miyazaki, and K. Furukawa.** 1988. Cloning and nucleotide sequence of the 2,3-dihydroxybiphenyl dioxygenase gene from the PCB-degrading strain *Pseudomonas paucimobilis* Q1. Biochemistry **27:**3990–3996.
30. **Taira, K., J. Hirose, S. Hayashida, and K. Furukawa.** 1992. Analysis of *bph* operon from the polychlorinated biphenyl-degrading strain *Pseudomonas pseudoalcaligenes* KF 707. J. Biol. Chem. **267:**4844–4853.
31. **Tsai, Y.-L., and B. H. Olson.** 1991. Rapid method for direct extraction of DNA from soil and sediments. Appl. Environ. Microbiol. **57:**1070–1074.
32. **Tsai, Y.-L., and B. H. Olson.** 1992. Detection of low numbers of bacterial cells in soils and sediments by polymerase chain reaction. Appl. Environ. Microbiol. **58:**754–757.
33. **Tsai, Y.-L., and B. H. Olson.** 1992. Rapid method for separation of bacterial DNA from humic substances in sediments for polymerase chain reaction. Appl. Environ. Microbiol. **58:**2292–2295.
34. **van Elsas, J. D., L. S. van Overbeek, and R. Fouchier.** 1991. A specific marker, *pat*, for studying the fate of introduced bacteria and their DNA in soil using a combination of detection techniques. Plant Soil **138:**49–60.
35. **Wagner-Döbler, I., R. Pipke, K. N. Timmis, and D. F. Dwyer.** 1992. Evaluation of aquatic sediment microcosms and their use in assessing possible effects of introduced microorganisms on ecosystem parameters. Appl. Environ. Microbiol. **58:**1249–1258.
36. **Walia, S., A. Khan, and N. Rosenthal.** 1990. Construction and applications of DNA probes for detection of polychlorinated biphenyl-degrading genotypes in toxic organic-contaminated soil environments. Appl. Environ. Microbiol. **56:**254–259.
37. **Ward, D. M., R. Weller, and M. M. Bateson.** 1990. 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. Nature (London) **345:**63–65.
38. **Wartell, R. M., S. H. Hosseini, and C. P. Moran, Jr.** 1990.

Detecting base pair substitutions in DNA fragments by temperature-gradient gel electrophoresis. Nucleic Acids Res. **18**:2699–2705.

39. **Weller, R., J. D. Weller, and D. Ward.** 1991. 16S rRNA sequences of uncultivated hot spring cyanobacterial mat inhabitants retrieved as randomly primed cDNA. Appl. Environ. Microbiol. **57**:1146–1151.
40. **Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. **51**:221–271.

40a. **Yakimov, M.** Personal communication.

41. **Yates, J. R., and F. J. Mondello.** 1989. Sequence similarities in the genes encoding polychlorinated biphenyl degradation by *Pseudomonas* sp. strain LB400 and *Alcaligenes eutrophus* H850. J. Bacteriol. **171**:1733–1735.