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Construction and Applications of DNA Probes for Detection of Polychlorinated Biphenyl-Degrading Genotypes in Toxic Organic-Contaminated Soil Environments

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Several DNA probes for polychlorinated biphenyl (PCB)-degrading genotypes were constructed from PCB-degrading bacteria. These laboratory-engineered DNA probes were used for the detection, enumeration, and isolation of specific bacteria degrading PCBs. Dot blot analysis of purified DNA from toxic organic chemical-contaminated soil bacterial communities showed positive DNA-DNA hybridization with a ^{32}P-labeled DNA probe (pAW6194, *cbpABCD*). Less than 1% of bacterial colonies isolated from garden topsoil and >80% of bacteria isolated from PCB-contaminated soils showed DNA homologies with ^{32}P-labeled DNA probes. Some of the PCB-degrading bacterial isolates detected by the DNA probe method did not show biphenyl clearance. The DNA probe method was found to detect additional organisms with greater genetic potential to degrade PCBs than the biphenyl clearance method did. Results from this study demonstrate the usefulness of DNA probes in detecting specific PCB-degrading bacteria, abundance of PCB-degrading genotypes, and genotypic diversity among PCB-degrading bacteria in toxic chemical-polluted soil environments. We suggest that the DNA probe should be used with caution for accurate assessment of PCB-degradative capacity within soils and further recommend that a combination of DNA probe and biodegradation assay be used to determine the abundance of PCB-degrading bacteria in the soil bacterial community.

Current methods of detecting microorganisms by nucleic acid hybridization with DNA probes have been used as rapid, sensitive, and specific diagnostic techniques in infectious diseases (19, 24, 27). DNA-DNA hybridization have been successfully used to detect and identify enterohemorrhagic and enteroinvasive strains of *Escherichia coli* (19, 27) and *Shigella* spp. (27, 33) from diarrheal stools, *Salmonella typhi* from blood (24), and *Salmonella* spp. (9) and *Yersinia enterocolitica* (12, 14) from foods. Recently, this approach has been used for the detection of metal ion resistance (2), enterotoxigenic *E. coli* in water (8), catabolic genotypes in activated sludge (5), groundwater aquifer (15, 22, 28), and the soil microcosm (13), tracking of genetically engineered bacteria (1), and identification of bacteria in the environment (22, 28).

Isolation of bacteria degrading toxic organics (toluene, xylene, naphthalene, phenanthrene, anthracene, pentachlorophenol, trichloroethylene, and polychlorinated biphenyls [PCBs]) is most often accomplished by enrichment procedures (3, 4, 32). These procedures are slow and often gives false-positive growth due to medium contaminants. Sometimes organisms fail to grow because of the accumulation of toxic intermediary metabolites. Therefore, it is necessary to develop additional approaches to detect and isolate novel organisms with diverse metabolic pathways from the environment. Gene probes offer a promising solution to this problem.

Recently, bacterial genes involved in the catabolism of chlorinated biphenyls have been cloned in the cosmid vector pCP13 (17, 18). These catabolic genes (*cbpABCD*) can be used to construct gene probes for the detection and enumeration of PCB degradation genotypes in microbial communities and tracking genetically engineered bacteria degrading PCBs in the environment. The objective of this study was to construct DNA probes for the detection of organisms with efficient catabolic pathways for the degradation of chlorinated biphenyls. We have engineered several recombinant plasmids containing genes involved in the catabolism of PCBs. Two recombinant plasmids, pAW6194 and pAW313, were successfully used as DNA probes to detect PCB-degrading genotypes by colony hybridization and dot blot assays.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The sources and relevant characteristics of bacterial strains and plasmids used in this study were described previously (17, 18). The organisms were grown in phosphate-buffered basal synthetic medium (BSM) as described previously (18). D-Glucose, sodium succinate (0.1%), or biphenyl vapors were provided as sole source of carbon for bacterial growth. BSM was prepared by following the instructions of Bedard et al. (4). Luria broth (L broth) was 1% tryptone (Difco Laboratories, Detroit, Mich.), 0.5% yeast extract (Difco), 0.5% NaCl, and 0.1% D-glucose (Sigma Chemical Co., St. Louis, Mo.). For Luria agar medium (L agar), 1.5 g of agar (Difco) was added to 100 ml of L broth. Bacteria containing recombinant plasmids were grown in L broth amended with appropriate antibiotics at 30°C. Heterotrophic bacteria from 10 g each of garden topsoil (Bordines, Rochester, Mich.) and toxic organic-contaminated soils (Rasmussen landfill, Carter Electric, and J. E. Berger landfill site, Detroit, Mich.) were grown on L-agar plates amended with 300 μg of cycloheximide (Sigma) per ml and BSM agar supplemented with 0.1% succinate at 30°C for 5 to 7 days.

Detection of water-insoluble hydrocarbon-degrading bacteria. PCB-degrading bacteria were detected by spraying bacterial colonies grown on BSM agar plates with a 2.5% ethereal solution of biphenyls (Sigma). The biphenyl-degrading colonies were detected by a zone of clearance on the

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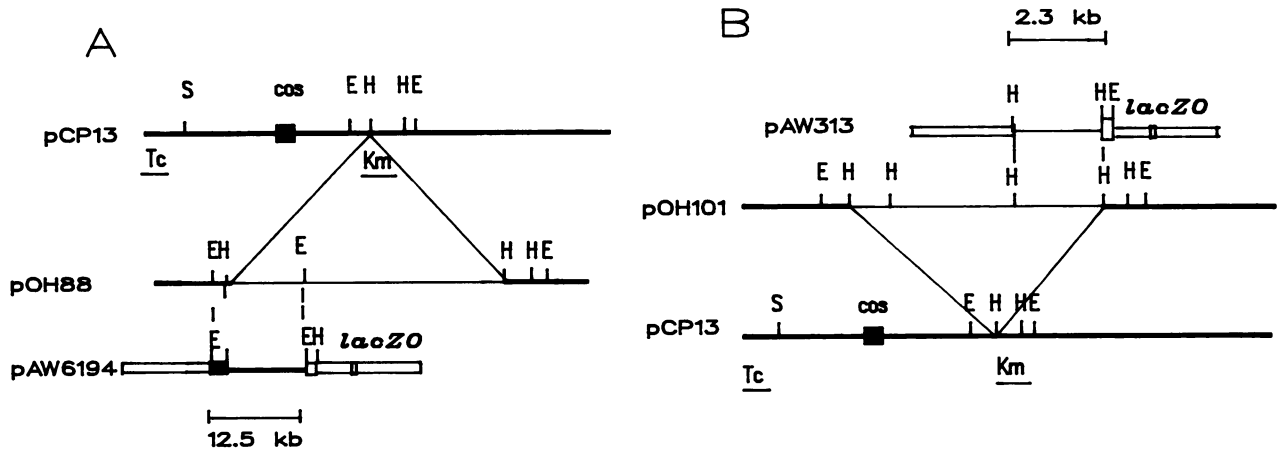

FIG. 1. Schematic of the construction of DNA probes and restriction maps of recombinant plasmids. E, *Eco*RI; H, *Hin*dIII. Tc, Tetracycline; Km, kanamycin; kb, kilobase pairs. Symbol: ■, multiple cloning sites of vector plasmids pUC18 and -19.

thin opaque biphenyl layer around bacterial colonies within 2 to 7 days at 30°C (32).

Transfer and lysis of bacterial colonies. Bacterial colonies from the appropriate dilution of L agar, BSM agar, and biphenyl-sprayed plates were transferred onto a nylon membrane (Gene Screen Plus; Dupont, NEN Research Products, Boston, Mass.) and lysed by placing the membrane (colony side up) on 0.5 N NaOH-saturated Whatman no. 1 filter paper for 15 min at room temperature. Alkali-treated membranes were neutralized by placing the membranes on Whatman no. 1 filter paper saturated with 1 M Tris hydrochloride–1 M NaCl, pH 7.0, for 15 min at room temperature. Membranes were washed with 100 ml of 10 mM Tris hydrochloride–1% sodium dodecyl sulfate (SDS), pH 8.0, solution and then prehybridized with 10 ml of prehybridization solution (10% dextran sulfate, 1 M NaCl, 1% SDS) in a heat-sealable plastic bag at 65°C.

Isolation of DNA from soil bacteria. Bacteria were isolated from the soil (50 g) sample by the procedure described by Holben et al. (13). Lysis of bacteria was accomplished by suspending the bacterial pellet in a freshly prepared solution of 50 mM Tris hydrochloride, 50 mM EDTA, 20% glucose, and 4 mg of lysozyme per ml, incubating at 37°C for 1 h, and adding appropriate amounts of an alkaline SDS solution to a final concentration of 0.2 N NaOH–2% SDS. The lysate was neutralized with 2 M Tris hydrochloride, pH 7.0. Proteins were extracted from the supernatant by phenol-chloroform treatment. The salt concentration of phenol-chloroform-extracted supernatant was adjusted to 1 M NaCl, and the DNA was precipitated by adding 2 volumes of ethanol followed by centrifugation at 23,000 × *g* for 30 min at 4°C. Further purification of the soil DNA preparations was done on cesium chloride-ethidium bromide equilibrium density gradients (20). Plasmid DNA was isolated by the alkaline lysis procedure (20) and purified on cesium chloride density gradients for the preparation of DNA probes.

Construction of DNA probes. Procedures for DNA ligation, transformation, and restriction enzyme analysis were described previously (17, 18, 22). Bacterial genes specifying degradation of PCBs into their corresponding chlorobenzoic acids were cloned in a *Hin*dIII site of cosmid pCP13 (18). A 12.5-kilobase *Eco*RI fragment specifying genes for the degradation of PCBs to chlorobenzoic acids was cloned in the *Eco*RI site of pUC19 downstream of the *lac* promoter from recombinant cosmid pOH88. The resulting hybrid plasmid, pAW6194 (*cbpABCD*), was used as a DNA probe to detect other PCB-degrading genotypes in the environment. The *cbpC* gene on pAW6194 was found to specify 3-phenylcatechol dioxygenase (3-PDase) with narrow substrate specificity (NSS) only for 3-phenylcatechol (18). A similar enzyme was reported by Furukawa and Arimura (11). Another DNA probe of the broad substrate specificity (BSS) enzyme 3-PDase was constructed by subcloning 2.3-kilobase *Hin*dIII DNA fragments into pUC18 from hybrid plasmid pOH101. The resulting plasmid was named pAW313. The BSS of 3-PDase (*cbpC*) on pOH101 has been described previously (18). Based on the substrate specificity of 3-PDase, a suffix NSS or BSS was attached with the recombinant plasmid used as DNA probe. Two recombinant plasmids, pAW313-BSS (broad substrate-specific 3-PDase) and pAW6194-NSS (*cbpABCD*; narrow substrate-specific 3-PDase), were used as DNA probes. The selection of these two recombinant plasmids was based on the size of the DNA fragment cloned and differences in the substrate specificities of 3-PDase on the cloned DNA fragments. The restriction maps of recombinant plasmids pAW313 and pAW6194 are shown in Fig. 1.

^{32}P labeling of DNA probe. Appropriate DNA (0.5 μg) was labeled with [α-^{32}P]dCTP as the sole labeling nucleotide by nick translation, following the instructions provided by the supplier (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). The specific activity of the DNA probes was about 10^8 cpm/μg of DNA.

Nylon membranes (Gene Screen Plus) for dot blot analysis were prepared by spotting appropriate amounts of denatured DNA. Denaturation of DNA was done with 0.3 M NaOH for 5 min. Denatured DNAs were chilled on ice and then applied to a nylon membrane with a dot blot apparatus (Hybridot; Bethesda Research Laboratories). The DNA spotted membranes were prehybridized in 10 ml of prehybridization solution in plastic sealable bags at 65°C for 16 h (29). Hybridizations were performed by using heat-denatured salmon sperm DNA (100 μg/ml) and radioactive ^{32}P-labeled DNA probe containing at least 5 × 10^6 dpm/ml in each plastic bag. The hybridization was carried out for 16 h at 65°C, and the membranes were washed twice with 2× SSC (0.3 M NaCl plus 0.03 M sodium citrate) at room temperature for 5 min with constant agitation, twice with 200 ml of a solution containing 2× SSC and 1% SDS at 65°C for 30 min with constant agitation, and twice with 0.1× SSC–0.5% SDS at 65°C for 30 min. The membranes were wrapped in plastic

wrap and exposed to X-ray film with a single intensifying screen at −70°C.

Analytical procedures. The degradation of 4-chlorobiphenyl (4-CBP) was determined by the formation of 4-chlorobenzoic acid and the disappearance of 4-CBP in culture medium by using high-pressure liquid chromatography (The Perkin-Elmer Corp., Norwalk, Conn.) and a gas chromatograph-mass spectrometer (QP-1000; Shimadzu). Bacterial colonies showing DNA hybridization, clearance of biphenyl alone, or both were grown in BSM broth with 4-CBP (20 μg/ml) at 30°C for 5 days. Nonbiological degradation of 4-CBP was determined by using a heat-killed bacterial cell suspension in BSM broth with 4-CBP (20 μg/ml). An additional control of uninoculated BSM broth with 4-CBP (20 μg/ml) was included. In all experiments, controls were treated in the same manner as experimental samples. The degradation products from the culture medium containing 4-CBP were acidified to pH 1.0 with H_2SO_4, and the metabolites were extracted twice with equal volumes of high-pressure liquid chromatography-grade ethyl acetate. Anhydrous Na_2SO_4 was added to the combined ethyl acetate fractions and air dried. The remaining residue was dissolved in methanol (high-pressure liquid chromatography grade), and appropriate (20-μl) aliquots of methanol were analyzed by high-pressure liquid chromatography (Perkin-Elmer) with a C_{18} reverse-phase column, using methanol-water (80:20) as the solvent system with a flow rate of 2 ml/min. The UV absorbance of the column effluent was monitored with a diode array detector (200- to 350-nm wavelength range; LC235; Perkin-Elmer). Identification and quantitation of the substrate and metabolite peaks were done by high-pressure liquid chromatography–co-chromatography, UV spectrum, and peak areas of known standards. For gas chromatographic-mass spectral analysis, the extracted metabolites were derivatized with *N,O*-bis(trimethylsilyl)trifluoroacetamide (Pierce Chemical Co., Rockford, Ill.). Analysis was carried out on a gas chromatography-mass spectrum system (QP-1000; Shimadzu) as described previously (18).

RESULTS AND DISCUSSION

Detection of PCB-degrading genotypes. Composite soils from PCB-contaminated landfill sites (Rasmussen landfill and J. E. Berger) and garden topsoils (Bordines) were used to detect and isolate PCB-degrading bacteria in the soil bacterial community. Presence of PCB-degrading genotypes in the purified DNAs isolated from the bacterial communities of the landfill and garden topsoils were detected with ^{32}P-labeled DNA probes pAW6194-NSS and pAW313-BSS by dot blot hybridization assay. Figure 2 shows the results of DNA-DNA hybridization with landfill and garden topsoil bacterial DNA, using ^{32}P-labeled pAW6194-NSS as DNA probe. Only the Carter Electric site DNA showed positive DNA-DNA hybridization. No evidence of *cpb* DNA was found with garden topsoil or Rasmussen landfill DNAs. The DNA detection limit of our probe was 10 pg (Fig. 2, lane D) (1, 3–5).

Detection of PCB-degrading genotypes in polluted soils was also done by colony hybridization (12). Bacterial colonies were isolated by direct plating of the bacterial suspension from the PCB-contaminated and garden topsoils. Figure 3 shows the results of the bacterial colony hybridization with ^{32}P-labeled pAW6194-NSS. Several bacterial colonies from Rasmussen landfill soil showed DNA-DNA hybridization (Fig. 3Bb), and only 1 of 108 bacterial colonies showed specific DNA hybridization in garden topsoil (Fig. 3Ab).

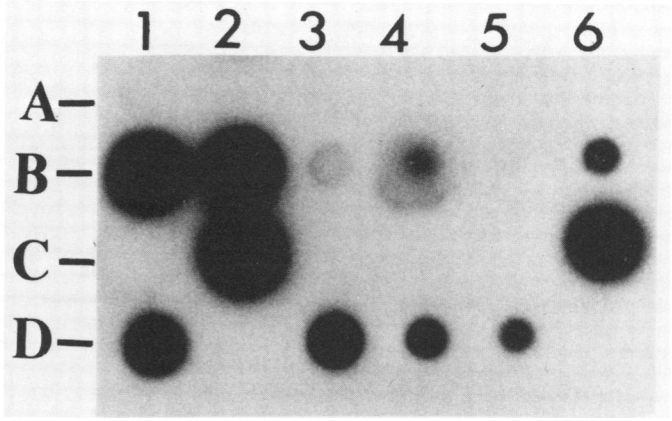

FIG. 2. Dot blot analysis of purified DNA from garden topsoil and toxic organic chemical-contaminated soils. Lanes: A1 to A6, garden topsoil; B1, garden topsoil plus OU83, pAW313, and pOH101; B2, pAW6194 plus garden topsoil; B3, pOH88 plus garden topsoil; B4, pAW6194, pOH88, OU83, and garden topsoil; B5, mixed toxic organic-contaminated soils from the Rasmussen landfill site; B6, mixed toxic organic-contaminated soils from Carter Electric site in Detroit, Mich.; C1, saline; C2, pOH88 DNA; C3 and C5, blank; C4, pOH101 DNA; C6, pUC19 DNA; D1, pAW6194 DNA (10^5 pg); D2, blank; D3, pAW6194 DNA (10^3 pg); D4, pAW6194 (10^2 pg); D5, pAW6194 DNA (10 pg); D6, pAW6194 DNA (1 pg).

This bacterial colony from garden topsoil might have common DNA sequences with cloned DNA insert or vector DNAs. No DNA-DNA hybridization was observed when DNA probe pAW313-BSS was used on bacterial colonies from garden topsoils. These results eliminate a remote possibility of plasmid vector (pUC19) DNA sequences in the natural soil bacterial communities tested in our experiments. Bacterial colonies showing homologous DNA sequences with one probe (pAW 6194-NSS) did not hybridize to the second probe (pAW313-BSS). These observations indicate the high specificity of the DNA hybridization method and the presence of a higher number of specific PCB-degrading genotypes in the PCB-contaminated soils as compared with unpolluted garden topsoils. This difference is probably from the selection of PCB-degrading genotypes in a PCB-polluted environment. The number of PCB-degrading genotypes varied among different PCB-polluted soil samples. Additional (20%) PCB-degrading organisms were detected by using two different DNA probes from toxic chemical-polluted soils.

Metabolic and genotypic diversity among PCB-degrading bacteria was determined by isolating bacterial colonies showing biphenyl clearance and then probing these colonies with radiolabeled DNA probes. Figure 4 shows the results of bacterial colonies showing biphenyl clearance and DNA-DNA colony hybridization with pAW6194-NSS. Several bacterial colonies (from the J. E. Berger site) showing biphenyl clearance (Fig. 4Aa) did not hybridize with the ^{32}P-labeled pAW6194-NSS DNA probe (Fig. 4Ab). Bacterial colonies hybridized with pAW6194-NSS did not hybridize with pAW313-BSS and vice versa, indicating an abundance of genetic diversity among PCB-degrading genotypes in the environment. Similar results have been shown by Yates and Mondello (34) in Southern blotting experiments and Taira et al. in DNA sequence homology studies (30). Recently, genotypic heterogeneity among 4-CBP-degrading bacteria have been shown by Darakhshan et al. (A. Darakhshan, B. Guilbault, and M. Sylvestre, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, Q71, p. 341) by using a DNA probe speci-

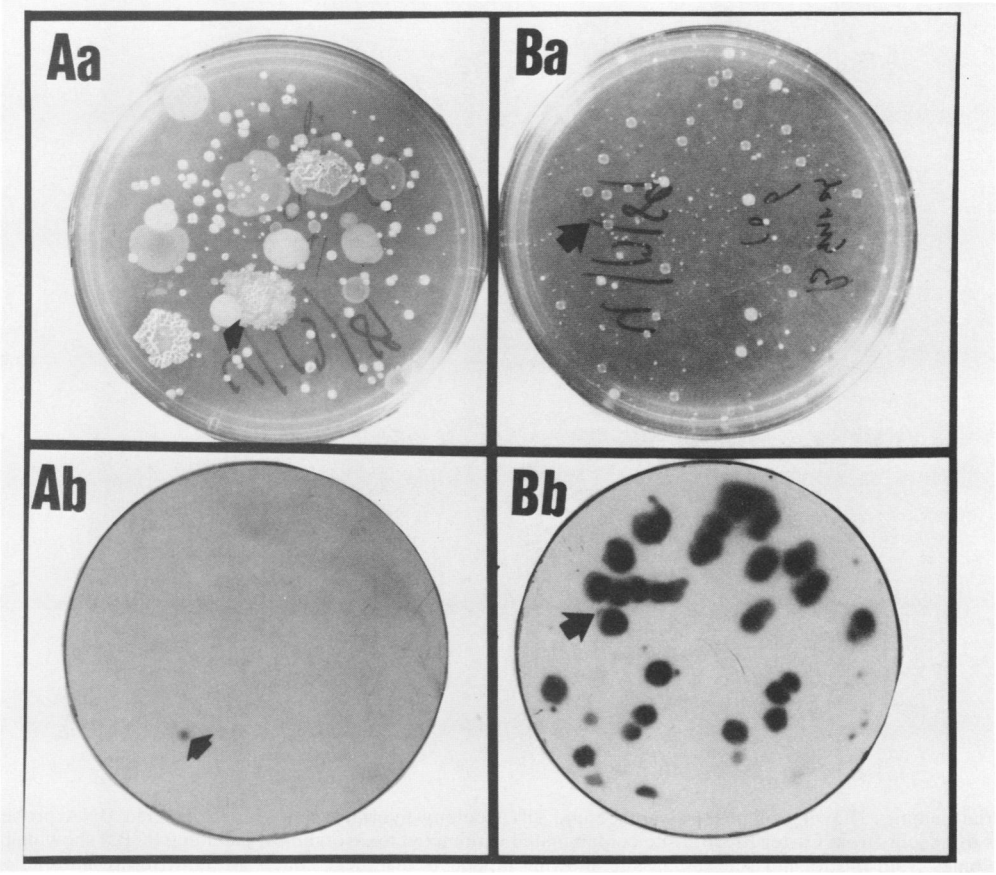

FIG. 3. Bacterial colony hybridization with ^{32}P-labeled DNA probe pAW6194. (Aa) Bacterial colonies from garden topsoil. (Ba) Bacterial colonies from the Rasmussen landfill site. Panels Ab and Bb are autoradiograms of bacterial colonies transferred from panels Aa and Ba, respectively, onto nylon membranes (Gene Screen Plus).

fying the 4-CBP degradation pathway. The dissimilarity of other catabolic genotypes such as xylene degradation has also been demonstrated (16). These results suggest that PCB-contaminated soil has a bacterial community with diverse PCB-degrading genotypes. The diversity among the PCB-degrading genotypes may well be responsible for the different metabolic activities and catabolic pathways for the degradation of PCBs. Metabolic diversity among PCB-degrading bacteria has recently been shown in *Alcaligenes eutrophus* (4), *Pseudomonas* sp. (3) and *Pseudomonas putida* OU83 (18).

Gene expression of bacterial colonies containing PCB-degrading genotypes was determined by assaying the biodegradation of 4-CBP and biphenyl clearance methods. Twenty percent of the colonies in PCB-contaminated soils from the Rasmussen landfill showed biphenyl clearance, and none of the bacterial colonies isolated from the PCB-contaminated in J. E. Berger site were positive for biphenyl clearance. Specific DNA hybridization with 60% of bacterial colonies from the Rasmussen landfill and 20% of bacterial isolates from the J. E. Berger site was detected by the DNA probe method (Table 1). Incidentally, some of the bacterial colonies isolated by the DNA probe method showed increased production of enzymes as compared with the parent *P. putida* OU83 from which the DNA probe was originally constructed. For example, an unidentified bacterium, AW-4, containing DNA sequences homologous to the pAW6194-NSS probe produced 15-fold-increased amounts of 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienote (HOPDA) hydrolase activity (*cbpD* gene product) compared with *P. putida* OU83 (data not shown). These results suggest that the DNA probe method is helpful in detecting organisms which can produce increased amounts of enzymes involved in the degradation of xenobiotics. All bacterial isolates showing biphenyl clearance were found to degrade 4-CBP into 4-chlorobenzoic acid. However, 6 of 10 bacterial colonies isolated by the DNA probe method showed degradation of 4-CBP into 4-chlorobenzoic acid. This observation suggests that the bacterial community in the environment has more PCB-degrading genotypic potential than phenotypically expressed degradative ability. This difference could be attributed to the presence of repressed DNA sequences of PCB-degrading

TABLE 1. Comparison of DNA probe and biphenyl clearance methods for detecting PCB-degrading bacteria in soils contaminated with toxic organic chemicals

Composite soil samples (PCBs, 100–10,000 ppm)	Bacterial CFU/g of soil (pAW6194)[a]	Positive bacterial colonies (%)	
		DNA probe	Biphenyl clearance
Rasmussen landfill	$4.2 \times 10^8 \pm 0.6$	60	21
J. E. Berger dump site	$8.6 \times 10^8 \pm 0.9$	20	0

[a] Soil (10 g) was mixed with 100 ml of 10 mM potassium phosphate buffer (pH 7.0), and the soil slurry was shaken on an orbital shaker for 6 h at room temperature before appropriate plating.

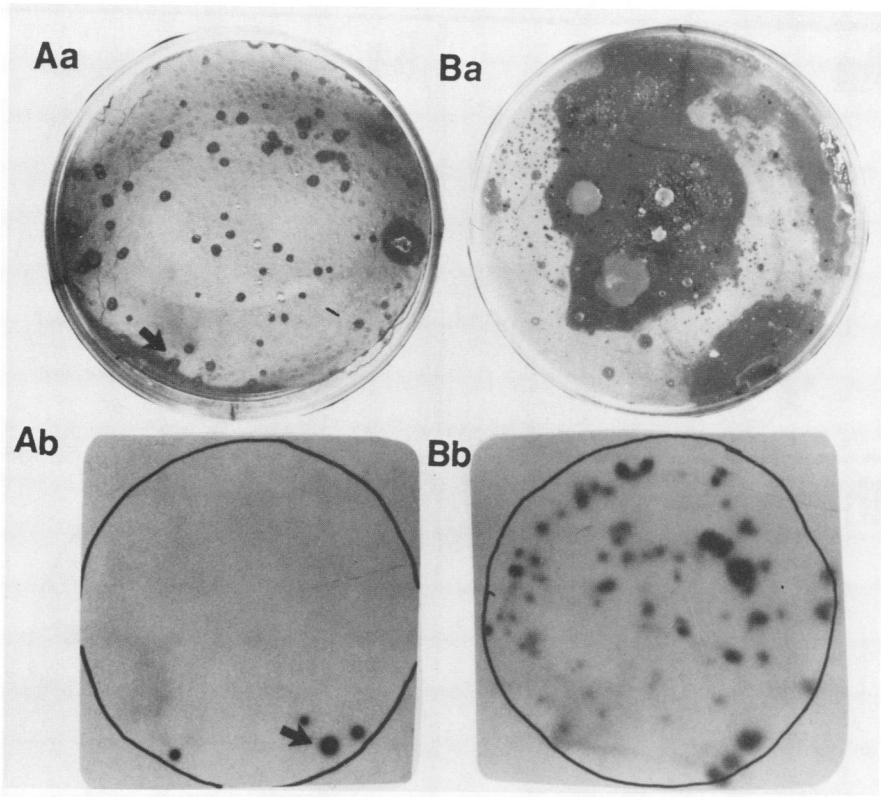

FIG. 4. Bacterial colonies showing biphenyl clearance and DNA colony hybridization with ^{32}P-labeled DNA probe (pAW6194). (Aa) Bacterial colonies from soils (from Carter Electric site contaminated with mixed toxic organics, including PCBs) showing biphenyl clearance. (Ba) Bacterial colonies from the Rasmussen landfill site showing biphenyl clearance. Panels Ab and Bb are autoradiograms of bacterial colonies transferred from panels Aa and Bb, respectively, onto nylon membranes (Gene Screen Plus).

bacterial genotype homologous to our DNA probe, mutation in the regulatory elements, or lack of genes specifying the first few enzymes of the PCB catabolic pathways. Because of the diversity of PCB-degrading genotypes and lack of sufficient data on DNA probes, it seems difficult to assess accurately the PCB-degradative potential within soils. In our opinion, more detailed studies are needed with different DNA probes to evaluate their usefulness in determining the PCB-degradative capacity of different soils. We suggest that the DNA probe method be used with caution to enumerate PCB-degrading bacteria in the environment and further recommend that a combination of DNA probe, biodegradation assay, and biphenyl clearance methods be used to study the abundance of PCB-degrading bacteria and isolation of native soil organisms with faster degradative abilities.

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