APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1986, p. 761–768
0099-2240/86/040761-08$02.00/0

Vol. 51, No. 4

Rapid Assay for Screening and Characterizing Microorganisms for the Ability to Degrade Polychlorinated Biphenyls

DONNA L. BEDARD,* RONALD UNTERMAN, LAWRENCE H. BOPP, MICHAEL J. BRENNAN, MARIE L. HABERL, AND CARL JOHNSON

Biological Sciences Branch, Corporate Research and Development, General Electric Company, Schenectady, New York 12301

Received 25 October 1985/Accepted 22 January 1986

We designed a rapid assay that (i) assesses the polychlorinated biphenyl (PCB)-degradative competence and congener specificity of aerobic microorganisms, (ii) identifies strains capable of degrading highly chlorinated biphenyls, and (iii) distinguishes among those that degrade PCBs by alternative pathways. Prior attempts to assay PCB-degradative competence by measuring disappearance of Aroclors (commercial PCB mixtures) have frequently produced false-positive findings because of volatilization, adsorption, or absorption losses. Furthermore, these assays have generally left the chemical nature of the competence obscure because of incomplete gas chromatographic resolution and uncertain identification of Aroclor peaks. We avoided these problems by using defined mixtures of PCB congeners and by adopting incubation and extraction methods that prevent physical loss of PCBs. Our assay mixtures include PCB congeners ranging from dichloro- to hexachlorobiphenyls and representing various structural classes, e.g., congeners chlorinated on a single ring (2,3-dichlorobiphenyl), blocked at 2,3 sites (2,5,2′5′-tetrachlorobiphenyl), blocked at 3,4 sites (4,4′-dichlorobiphenyl), and lacking adjacent unchlorinated sites (2,4,5,2′,4′,5′-hexachlorobiphenyl). The PCB-degradative ability of microorganisms is assessed by packed-column gas chromatographic analysis of these defined congener mixtures following 24-h incubation with resting cells. When tested with 25 environmental isolates, this assay (i) revealed a broad range of PCB-degradative competence, (ii) highlighted differences in congener specificity and in the extent of degradation of individual congeners, (iii) predicted degradative competence on commercial PCBs, and (iv) identified strains with superior PCB-degradative ability.

Polychlorinated biphenyls (PCBs) were widely used in a number of industrial applications for nearly 50 years and, as a consequence, became widely distributed in the environment. Although PCBs can be at least partially degraded by biological, chemical, and photochemical means, concern has arisen over their persistence in the environment.

In the last 12 years, there have been numerous reports of bacterial degradation of chlorinated biphenyls containing one to three chlorines but few reports of degradation of the more highly chlorinated congeners (6, 10, 12; reviewed in reference 5). It is precisely the more recalcitrant PCBs, those with four or more chlorines, that are of greatest environmental concern. The report of Furukawa et al. (6) of two bacterial species that are capable of degrading tetrachloro- and pentachlorobiphenyls suggested that there might be other microorganisms with similar or better PCB-degradative competence. The discovery of such organisms would have a major impact on the development of effective biodegradative processes for cleanup of PCB-contaminated sites. We report here a novel assay specifically designed to rapidly identify microorganisms capable of degrading highly chlorinated biphenyls. Moreover, the assay distinguishes among organisms which may use alternative degradative pathways and highlights differences in congener specificity.

MATERIALS AND METHODS

Bacterial strains. H8 is a mixed culture derived from a landfill containing PCB-contaminated dredge spoils and enriched by repeated transfer on biphenyl medium. *Corynebacterium* sp. MB1 and *Alcaligenes eutrophus* H850 were isolated as previously described (3). All other bacterial strains were isolated from PCB-contaminated sites by enrichment on biphenyl medium and are described elsewhere (11; R. Unterman, D. L. Bedard, L. H. Bopp, M. J. Brennan, C. Johnson, and M. L. Haberl, submitted for publication).

Cell growth. Cells were grown aerobically at 30°C in a gyratory shaker. The medium was a phosphate-buffered mineral salts medium (PAS) containing biphenyl as a carbon source and supplemented with 0.005% yeast extract. PAS was prepared by adding 77.5 ml of PA concentrate (see below) and 50 mg of yeast extract to 910 ml of glass-distilled water. After autoclaving and upon cooling, 10 ml of sterile PAS 100× salts was added. Biphenyl was supplied by the addition of sterile, molten biphenyl to the autoclaved medium. PA concentrate was composed of K_2HPO_4 (56.77 g/liter), KH_2PO_4 (21.94 g/liter), and NH_4Cl (27.61 g/liter). PAS 100× salts contained $MgSO_4$ (19.5 g/liter), $MnSO_4 \cdot H_2O$ (5 g/liter), $FeSO_4 \cdot 7H_2O$ (1 g/liter), and $CaCl_2 \cdot 2H_2O$ (0.3 g/liter) along with several drops of concentrated H_2SO_4 per liter to prevent precipitation of basic salts.

PCB congener mixtures. Pure PCB congeners were obtained from Foxboro Analabs, Inc. (North Haven, Conn.), or Ultra Scientific (Hope, R.I.). Stock solutions (100×) for our congener mixtures contained each of the desired congeners at a concentration of 0.5 mM in acetone. A stock solution of Aroclor 1248 in acetone was prepared at a concentration of 5 mg/ml.

Resting cell incubation. Cells were grown to an optical density at 615 nm of 1.0, then filtered through glass wool to remove biphenyl crystals, and washed twice with 0.05 M sodium phosphate buffer (pH 7.5). Cells were resuspended in the same buffer at an optical density at 615 nm of 1.0, and 1-ml portions were transferred to 2-dram (1 fluidram = 3.7

* Corresponding author.

TABLE 1. Recovery of PCB congeners from dead-cell controls

PCB congener[a]	% Recovery with:		
	Hexane	Triton-hexane	
	Aluminum cap	Aluminum cap	Polyethylene cap
2,4'	94	93	29
4,4'	91	97	28
2,4,4'	85	97	33
2,5,2',5'	79	95	35
2,3,2',5'	77	95	35
2,4,6,2',4'	ND[b]	95	41
2,3,2',3'	77	94	35
2,4,3',4'	70	94	41
2,4,5,2',3'	64	94	42
3,4,3',4'	69	97	63
2,4,5,2',4',5'	53	93	56

[a] PCB congeners are listed in order of increasing GC retention time.
[b] ND, Not determined.

ml) screw-cap glass vials. Control cells were inactivated with heat (70°C, 20 min) or by adding perchloric acid or mercuric chloride to final concentrations of 0.7% and 1 mM, respectively. Because PCBs are practically insoluble in water, the PCBs were added to the cell suspensions as concentrated acetone solutions. When added in this way, the PCBs immediately adsorbed to the cells. In defined congener mixture assays, the final concentration of each congener was 5 μM (1 to 2 ppm). In the Aroclor 1248 assay, the final concentration of PCB was 10 ppm. Cells were incubated for 24 h (defined congener assay) or 48 h (1248 assay) at 30°C in a gyratory shaker.

PCB extractions. Following incubation, each assay was stopped by the addition of perchloric acid (final concentration, 0.7%). Then PCBs were extracted by one of several methods (see Results and Discussion for an assessment of each). In the first method, 1/10 volume of 10% Triton X-100 was added to the cell suspension, followed by 4 volumes of hexane or a 1:1 hexane-ether mixture. Prior to mixing, a small amount of anhydrous sodium sulfate was added to prevent the formation of a stable emulsion. The samples were shaken vigorously for 20 to 30 min in a horizontal position on a reciprocating platform shaker. The phases separated rapidly without centrifugation, and the extracts were transferred to vials for gas chromatography (GC). Alternatively, cell suspensions were extracted with 4 volumes of hexane or ether without the addition of Triton X-100 or sodium sulfate.

GC analysis. Samples of defined congener mixtures were analyzed on either a Hewlett Packard 5880 or a Varian Vista 6000 GC equipped with an automatic sampler and an electron capture detector. A glass column (6ft [1.83 m] by 4 mm) packed with 1.5% SP-2250/1.95% SP-2401 on 100/120 Supelcoport (Supelco, Inc., Bellefonte, Pa.) was used. Samples were chromatographed isothermally at 190°C with nitrogen as the carrier gas at a flow rate of 60 ml/min. The injection and detector temperatures were 250 and 300°C, respectively. Extraction efficiencies of dead-cell controls were assessed by comparison with the appropriate congener mixture dissolved in hexane. A nondegradable PCB congener was used as an internal standard.

The Aroclor 1248 assay samples were analyzed on a Varian Vista GC 4600 equipped with an electron capture detector and splitter-injector (option 1070), both operated at 300°C, and a fused silica capillary column (J and W Scientific; 30 m by 0.25 mm [inside diameter]) coated with a 0.25-μm bonded liquid phase of Durabond-1. The carrier gas and makeup gas were helium (30 cm/s) and nitrogen (25 ml/min), respectively. Samples were chromatographed by using a temperature program which was held at 40°C for 2 min, then raised to 80°C at 10°C/min, then to 225°C at 6°C/min, and held at 225°C for 10 min. Injections (1 μl) were done by the splitless technique with a vent time of 0.9 min.

RESULTS AND DISCUSSION

Variables affecting quantitative recovery of PCBs. The volatility, hydrophobicity, and insolubility of PCBs pose particular difficulties in aqueous biodegradation assays and require special attention. Physical loss of PCBs due to evaporation or adsorption to the incubation vessel and the bacterial cells has often been mistakenly attributed to biodegradation. To minimize these problems, we designed an assay in which the incubation and extraction of each sample are carried out in a single 2-dram glass vial with a screw cap. This reduces the loss of PCBs by adsorption to glass.

Another source of extraction problems was attributed to absorption of PCBs by plastic containers and foam plugs. Table 1 (columns 2 and 3) compares the recovery of PCBs from two controls which differed only in the type of cap used on the incubation vial. The poor recovery from the sample with a polyethylene-lined cap could be mistaken for biodegradation when, in fact, it was due to absorption by the polyethylene. The use of aluminum-lined caps eliminated this problem.

We determined the amount of loss of PCBs by nonspecific adsorption to cells by monitoring the recovery of PCBs from dead-cell controls by using several extraction methods. Several bacterial species were used, including *Alcaligenes eutrophus*, a *Corynebacterium* sp., and *Pseudomonas putida*. Extraction with hexane frequently gave nonuniform recovery of PCBs. Nearly half of the samples exhibited progressively poorer extraction of those congeners with longer retention times (Table 1, column 1). We observed no differences in extraction efficiency for different bacterial species.

Since Triton X-100, a nonionic surfactant, increases the solubility of PCBs by a factor of 10^5 (T. Su, personal communication) and also disrupts cell membranes, we reasoned that addition of this surfactant to the cell suspension might improve the extraction of PCBs from cells. When Triton X-100 (final concentration, 1%) was added to the cell suspension prior to hexane extraction, all congeners except the first (2,4'- or 2,2'-dichlorobiphenyl) were uniformly and efficiently extracted for all species tested (Table 1, column 2); these two congeners were often extracted with 10 to 15% lower efficiency. Extraction of the surfactant-treated samples with a 1:1 hexane-ether mixture gave uniform recovery of all congeners with the *Corynebacterium* sp., but did not significantly improve the recovery of these dichlorobiphenyls with *A. eutrophus* and gave nonreproducible results with *P. putida*. Since 2,4'- and 2,2'-dichlorobiphenyl were completely degraded in nearly all the strains we tested, the slightly lower recovery of these congeners was not a significant problem.

Limitations of biodegradation assays with commercial PCB mixtures (Aroclors). There are several disadvantages to using Aroclor assays to measure degradation of PCBs. (i) Aroclor mixtures are produced by direct chlorination of biphenyl, and each consists of 40 to 60 detectable PCB congeners in widely differing proportions. The concentrations of individual congeners are often not known and cannot be inferred

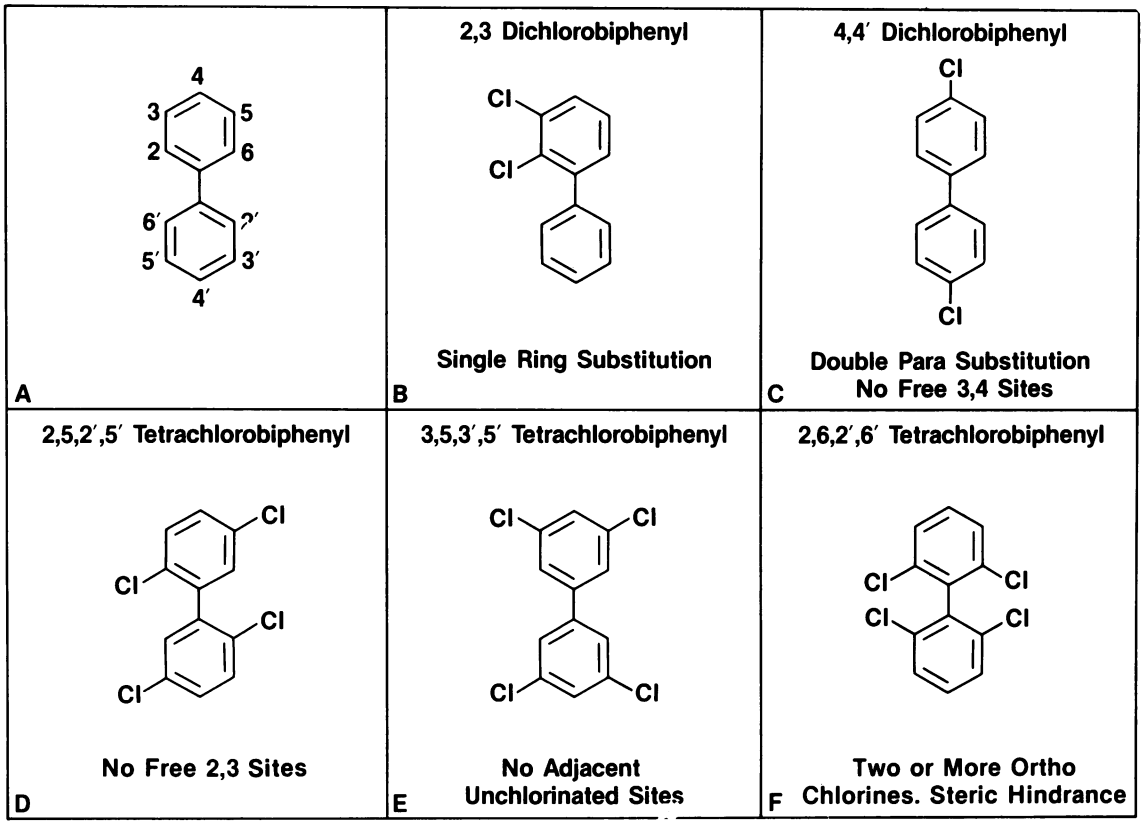


FIG. 1. Structural classes of PCB congeners. Panel A demonstrates the numbering system for PCBs. Panels B through F show examples of congeners that represent different structural challenges for PCB-degradative enzymes.

from visual inspection of a GC profile obtained with an electron capture detector, because response factors for congeners with the same number of chlorines can vary as much as eightfold (9). (ii) Congeners are poorly resolved by packed-column GC analysis, so identification of individual degraded congeners is not possible. (iii) Capillary GC affords better resolution, but in the many instances in which PCB homologs coelute, the congener composition of Aroclor peaks is still uncertain, since it is impossible to know whether a particular peak is composed of only one or two of several coeluting homologs or all of them. (iv) In Aroclor 1248, two-thirds (22 of 33) of the largest peaks resolved by capillary GC contain two or more congeners, making identification of degraded congeners ambiguous (J. F. Brown, Jr., and R. E. Wagner, personal communication). Although much information can be obtained from capillary GC analysis of biodegradation of Aroclors, we found that the limitations described above made this substrate unsuitable for rapid screening of cultures for PCB-degradative ability.

Designing congener mixtures for rapid assay of PCB-degradative competence. (i) Seven-congener mixture. We sought to develop a simpler assay mixture that would overcome the limitations of Aroclor assays and which would screen microorganisms for the ability to degrade PCBs. The congeners selected for our first defined mixture satisfied several criteria. (i) The chosen congeners were present in commercial mixtures of PCBs (with one exception); (ii) they were completely resolved by packed-column GC, and (iii) they represented different structural classes of PCB (Fig. 1). Several investigators (2, 4, 7) have noted that the pattern of chlorination may influence biodegradation, but no comprehensive study of this phenomenon has been done. Furukawa and co-workers (6, 7) noted that 2,4,4'- and 2,3,2',3'-chlorobiphenyls were rapidly degraded by *Acinetobacter* sp. P6, whereas 2,5;2',5'- and 2,3,5,6-tetrachlorobiphenyls were not. We included these congeners to identify cultures which resembled P6 as well as those with ability to degrade congeners that were not readily degraded by P6.

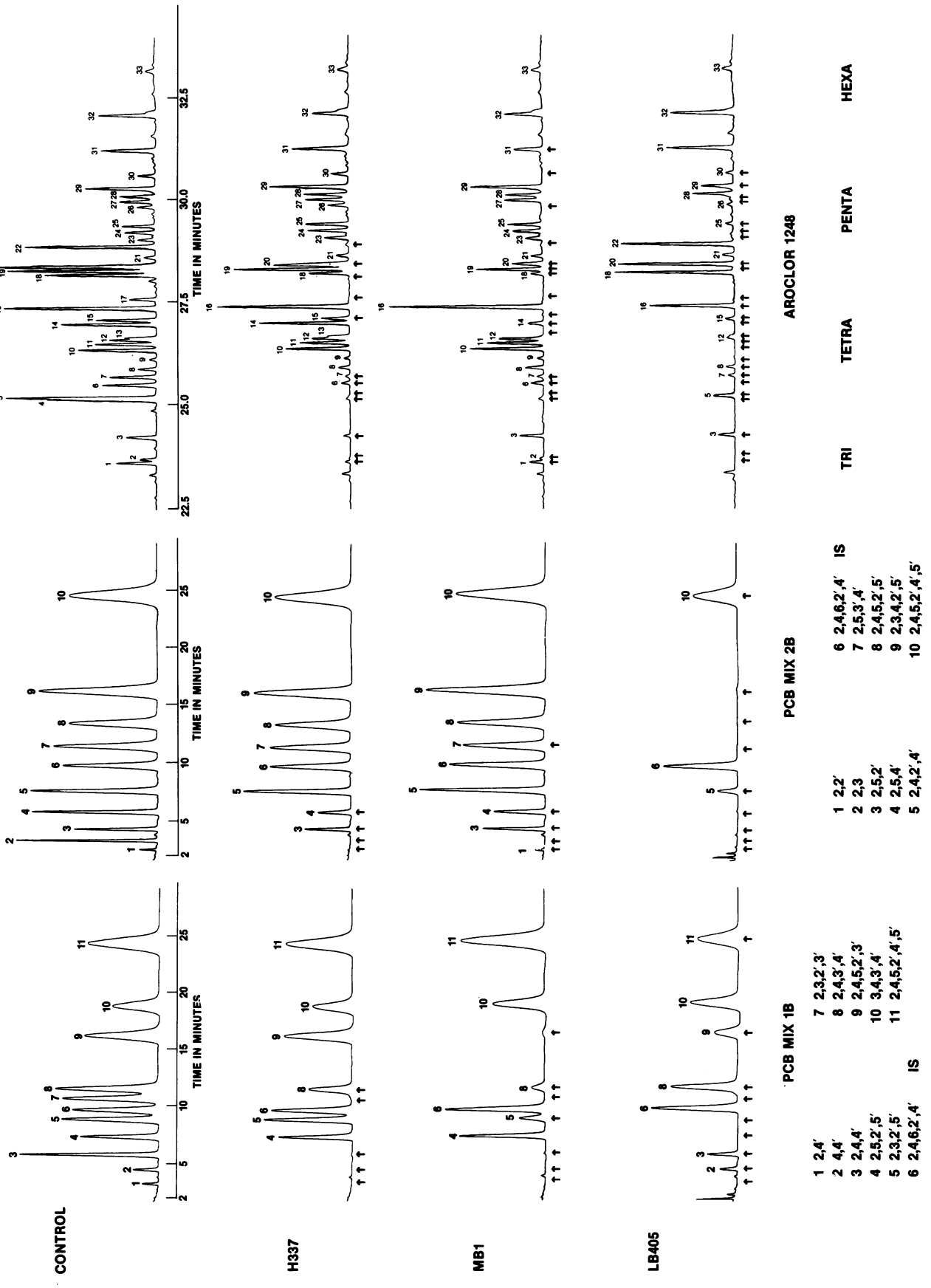
The seven-congener mixture was used to assay 20 mixed bacterial cultures derived by enrichment from PCB-contaminated sites and a strain of *Corynebacterium* sp. Table 2 shows the results obtained for *Corynebacterium* sp. MB1 and one of the mixed cultures, H8. Most of the samples, including our reference culture, MB1, showed little

TABLE 2. Degradation of PCBs by two cultures assayed by the seven-congener mixture

PCB congener	% Degradation at:[a]			
	50 μM[b]		5 μM[b]	
	MB1	H8	MB1	H8
2,4,4'	44		95	53
3,4,2'		85	98	98
2,5,2',5'		71		100
2,3,5,6				
2,3,2',3'		50	92	96
2,5,3',4'		35		91
3,4,3',4'				

[a] Observed degradation less than 20% is not considered significant and is not reported.

[b] Concentration of individual congeners in the assay mixture.


CONTROL
H337
MB1
LB405
TIME IN MINUTES
PCB MIX 1B
1 2,4'
2 4,4'
3 2,4,4'
4 2,5,2',5'
5 2,3,2',5'
6 2,4,6,2',4' IS
7 2,3,2',3'
8 2,4,3',4'
9 2,4,5,2',3'
10 3,4,3',4'
11 2,4,5,2',4',5'
PCB MIX 2B
1 2,2'
2 2,3
3 2,5,2'
4 2,5,4'
5 2,4,2',4'
6 2,4,6,2',4' IS
7 2,5,3',4'
8 2,4,5,2',5'
9 2,3,4,2',5'
10 2,4,5,2',4',5'
AROCLOR 1248
TRI
TETRA
PENTA
HEXA

ability to degrade the PCBs at 50 μM concentrations. In contrast, H8 degraded several congeners at this level yet had no activity against the one congener that MB1 degraded, 2,4,4′-trichlorobiphenyl. At the lower PCB concentration, MB1 degraded significant fractions of two more congeners but still had no activity against 2,5,2′,5′- or 2,5,3′,4′-tetrachlorobiphenyls. Conversely, H8 readily degraded the latter congeners but exhibited limited ability to degrade 2,4,4′-trichlorobiphenyl.

This assay successfully identified a mixed culture with exceptional and novel PCB-degrading activity. Nevertheless, the assay had limitations; most of the cultures we screened could degrade only two or three of the congeners (generally 2,4,4′-, 3,4,2′-, and 2,3,2′,3′-chlorobiphenyls). Although this information gave an indication of which cultures were exceptional (such as H8), the assay lacked the sensitivity to discriminate among cultures with less extensive degradative competence.

(ii) Improved congener mixtures. Because of the limitations of the seven-congener mixture, we developed two defined congener mixtures encompassing a broader range of congeners (dichloro- to hexachlorobiphenyls). Our intent was to define assay mixtures that would allow us to (i) assess the degradative competence and congener specificity of a large number of isolates, (ii) readily identify strains capable of degrading highly chlorinated biphenyls, and (iii) distinguish among those strains which degrade PCBs by alternative pathways.

A brief discussion of the structure of PCBs and the most commonly observed route of PCB degradation will provide the basis for understanding our choice of congeners. Figure 1 shows examples of several structural classes of PCB. There are four equivalent *ortho* positions (2, 6, 2′, and 6′), four equivalent *meta* positions (3, 5, 3′, and 5′), and two equivalent *para* positions (4 and 4′).

Several investigators (1, 6, 8, 12) have proposed that bacterial PCB degradation is initiated by the attack of a dioxygenase at carbon positions 2,3 (or 5,6). Available evidence (6) suggests that a necessary requirement is the availability of a 2,3 (or 5,6) site free of chlorines. Theoretically, a 2,3-dioxygenase should be capable of degrading 4,4′-dichlorobiphenyl (Fig. 1, panel C), which has four free 2,3 sites, but not 2,5,2′,5′-tetrachlorobiphenyl (panel D), which has none. We reasoned that some bacteria might, instead, possess a dioxygenase which preferentially attacks at carbon positions 3,4 (or 4,5). These bacteria should be capable of degrading 2,5,2′,5′-tetrachlorobiphenyl but not 4,4′-dichlorobiphenyl. Both types of bacterium would be expected to degrade congeners chlorinated on a single ring (panel B), and neither would be expected to attack congeners that lack adjacent unchlorinated sites (panel E). Alternatively, some bacteria might have a monooxygenase that would attack such congeners in addition to all the congeners attacked by either type of dioxygenase. PCBs with two or more *ortho* chlorines (panel F) are particularly resistant to degradation by 2,3-dioxygenases (7), possibly because steric interference from the *ortho* chlorines impairs approach to the active site of the enzyme. We included congeners of each of these structural classes (panels B through E) in our two basic PCB congener mixtures.

An additional consideration was the degree of chlorination. We deliberately included congeners that we thought would be readily degradable (2,3- and 2,4′-dichlorobiphenyls) and others that we thought would be accessible to both 2,3- and 3,4-dioxygenase attack but would be increasingly difficult to degrade owing to the degree of chlorination (2,5,4′-, 2,3,2′,5′-, and 2,4,5,2′,3′-chlorobiphenyls.) To allow normalization of results, we included, as an internal standard, a PCB congener that was not degradable by our cultures. Our initial choice of internal standard was 2,4,5,2′,4′,5′-hexachlorobiphenyl, which has no adjacent unchlorinated sites. However, during our studies, we isolated two bacterial strains capable of degrading this congener and modified the mixtures to include 2,4,6,2′,4′-pentachlorobiphenyl as an internal standard.

The final considerations in selecting congeners were GC retention times (so that all congeners in a mixture would be resolved by packed-column GC) and response factors (so that most congeners would yield comparable areas on an electron capture detector). The components of our basic defined congener mixtures, PCB mixtures 1B and 2B, are given in Fig. 2.

Degradation of defined congener mixtures and Aroclor 1248 by three bacterial strains. We chose three bacterial strains which differed in PCB-degradative ability to evaluate the performance of the defined congener mixtures. Since the value of this assay depends on its ability to predict degradative competence on those PCB congeners which are common environmental contaminants, we also studied the degradation of Aroclor 1248. This Aroclor contains nearly all of the PCB congeners present in two of the most common PCB mixtures found in the environment, Aroclors 1242 and 1254.

The defined congener assay clearly demonstrates the differences and similarities in the PCB-degradative competence of these strains (Fig. 2). The chromatograms reveal that MB1 and H337 degraded many of the same congeners, whereas LB405 attacked a different group of congeners. Closer analysis shows that MB1 degraded all of the congeners that were metabolized by H337 (some to a greater extent) but also had activity against three more highly chlorinated congeners. The results with the Aroclor 1248 assay were comparable; MB1 degraded all but one of the peaks attacked by H337 but degraded many to a greater extent, including five tetrachloro- and pentachlorobiphenyl peaks that were not attacked by H337.

The results for LB405 also illustrate the close correlation of the data obtained from the defined congener assay and the Aroclor 1248 assay. It is evident from both assays that LB405 has a greater range of degradative ability and different congener specificity than either of the other strains. However, whereas the defined congener assay permits immediate identification of those congeners which were degraded, this information is difficult to derive from the Aroclor assay for the reasons discussed earlier. An analysis of congener specificity shows that LB405 completely degraded 2,5,2′,5′- and 2,4,5,2′,5′-chlorobiphenyls, which have no available 2,3 (or

FIG. 2. Degradation of defined congener mixtures and Aroclor 1248 by three bacterial strains. The GC profiles of heat-killed controls for congener mixtures 1B and 2B and Aroclor 1248 are shown at the top of the figure, and the profiles of degraded PCBs for each of the strains are shown in the three lower sets of panels. The congener composition of each defined assay mixture is shown. The concentration of 2,4′-dichlorobiphenyl was 1 μM; all other congeners in mixtures 1B and 2B were present at 5 μM. Resting cells were incubated with the defined congener mixtures for 24 h and with Aroclor 1248 for 72 h. PCBs were extracted and chromatographed as described in Materials and Methods. Arrows designate congeners or peaks that were degraded by at least 20%. IS, Internal standard.

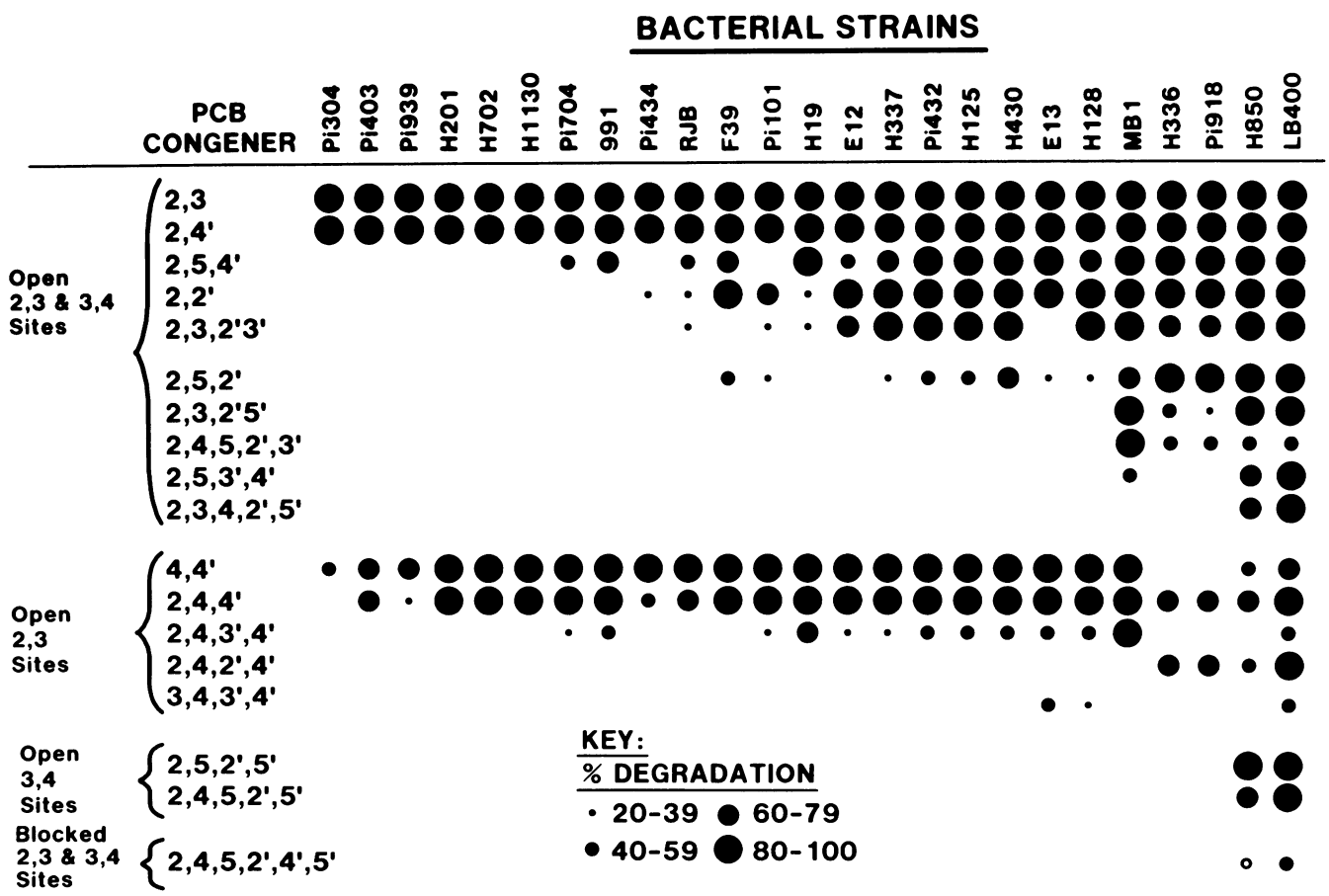


FIG. 3. PCB-degradative competence of environmental isolates. (○) H850 degraded less than 20% of 2,4,5,2',4',5'-hexachlorobiphenyl when assayed in this mixture but was clearly demonstrated to degrade this congener and to generate a metabolite when the congener was assayed separately. As noted elsewhere in this paper, the degradation of some congeners may be adversely affected by other congeners in the assay mixture.

5,6) sites, but exhibited limited ability to degrade congeners chlorinated at positions 4,4'. This is precisely the pattern of activity that would be expected for an organism that utilizes a 3,4-dioxygenase (3; D. L. Bedard and M. J. Brennan, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, Q51, p. 213).

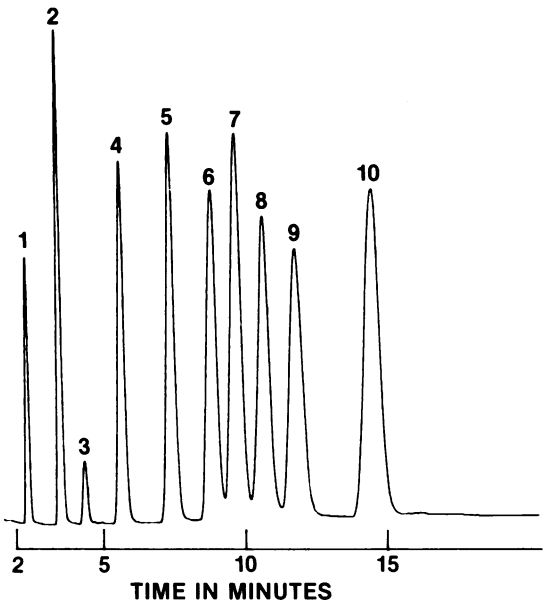
The information that the defined congener assay provides on congener specificity permits us to speculate about the nature of the enzymes responsible for PCB degradation. The similarities in congener specificity between H337 and MB1 suggest that the initial PCB-degradative enzymes in these strains are basically very similar, with only those differences that might be expected for closely related enzymes which differ slightly in binding site geometry or in rate of reaction. This is particularly interesting, since MB1 is a *Corynebacterium* sp. (gram positive) derived from a site in Wisconsin, whereas H337 is an unidentified gram-negative organism isolated from Hudson River sediment in New York. These two strains attack only congeners with open 2,3 sites; hence, they probably use a 2,3-dioxygenase. On the other hand, the third strain, LB405, may utilize a 3,4-dioxygenase.

Our comparison of the degradative profiles of each of these strains on the defined congener mixtures and on Aroclor 1248 permits us to draw several conclusions. The use of PCB mixtures 1B and 2B to assess PCB-degradative competence allows us to (i) determine the range of degradative competence, (ii) predict degradative competence on commercial PCB mixtures (Aroclors), (iii) determine differences in congener specificity, and (iv) identify differences in the extent of degradation of individual congeners.

Screening environmental isolates for PCB-degradative competence. We used our defined congener mixtures to screen the PCB-degradative competence of 25 bacterial strains. The results (Fig. 3) confirm the sensitivity of the assay for discriminating PCB-degradative ability. This permitted rapid identification of two organisms with superior degradative competence, *A. eutrophus* H850 and *P. putida* LB400.

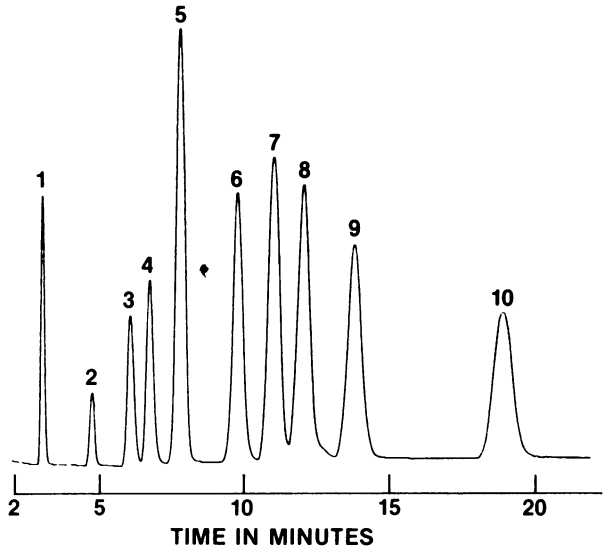
Furthermore, the data obtained from the assay afford some insight regarding the nature of the PCB-degradative enzymes operative in these organisms. The PCB congeners are arranged into four groups according to whether they have free 2,3 sites, free 3,4 sites, both, or neither. Within each group, the congeners are arranged by relative ease of degradation by these strains. Although the data suggest that most of our isolates utilize a 2,3-dioxygenase, it is quite clear that open 2,3 or 3,4 sites or both are not the only criteria for degradation. Several examples will illustrate the kinds of information that these assays can yield about the PCB-degradative enzymes responsible for the initial attack.

Since all of these bacteria degraded 2,4'-dichlorobiphenyl, they are all capable of attacking a chlorinated ring, yet they differed considerably in their abilities to degrade 2,2'- and 4,4'-dichlorobiphenyls. Strains H336, Pi918, H850, and



PCB MIX 3B

1	2,6	6	2,3,2′,5′
2	2,4,6	7	2,4,6,2′,4′ IS
3	4,4′	8	2,4,6,3′,5′
4	2,4,4′	9	2,4,6,2′,4′,6′
5	2,4,6,4′	10	2,4,6,3′,4′



PCB MIX 4B

1	2,5	6	2,4,6,2′,5′
2	4,4′	7	3,5,3′,5′ IS
3	2,6,2′6′	8	2,4,3′,4′
4	2,5,2′,6′	9	2,4,5,2′,5′
5	2,4,6,4′	10	2,3,6,2′,3′,6′

FIG. 4. GC profiles and compositions of additional PCB congener mixtures designed to address finer details of congener specificity. IS, Internal standard.

LB400 were partially distinguished from the majority by their limited ability to degrade 4,4′-dichlorobiphenyl (Fig. 3), yet these same strains readily degraded 2,2′-dichlorobiphenyl. Curiously, they degraded 2,4,4′-trichlorobiphenyl more efficiently than the simpler 4,4′-dichlorobiphenyl. These data suggest that the *ortho* chlorine may facilitate enzymatic attack by these four organisms and that they may preferentially attack the dichlorinated ring of 2,4,4′-trichlorobiphenyl. This was substantiated in H850 by the observation that this strain stoichiometrically degraded 2,4,4′-trichlorobiphenyl to 4-chlorobenzoic acid (data not shown), hence it is the dichlorinated ring which is attacked and cleaved. It is further supported by the observation that these four strains all degraded 2,4,2′,4′-tetrachlorobiphenyl.

The assay identified two strains, *A. eutrophus* H850 and *P. putida* LB400, which were able to degrade congeners blocked at all 2,3 (and 5,6) positions. Both of these strains exhibited exceptional PCB-degradative competence, and both had the ability to degrade 2,4,5,2′,4′,5′-hexachlorobiphenyl, a congener with no adjacent unchlorinated carbons. These unique abilities suggest the presence of novel PCB-degradative enzymes, such as a 3,4-dioxygenase, monooxygenase, or dehalogenase, or a dioxygenase capable of attacking at a chlorinated site, in these two strains (3; L. H. Bopp, J. Ind. Microbiol., in press).

Defined PCB mixtures tailored to address finer details of congener specificity. Additional congener mixtures can be designed to gain more information about the congener specificity of bacterial strains of particular interest. For example, a mixture can be designed to screen for the ability to degrade particularly recalcitrant PCB congeners or to learn how the chlorine substitution pattern on each ring influences biodegradability.

Several rules apply in formulating convenient assay mixtures. (i) One or more degradable PCB congeners should be included in each mixture to act as positive controls for 2,3- and 3,4-dioxygenase activity. (ii) A PCB congener which is not degradable by the microorganisms being tested should be included as an internal standard. (This may involve some trial and error. One can make some guesses as to which congeners will not be degraded, but the final test is the assay. If, as in the case of LB400, the internal standard is degraded, another must be chosen.) (iii) The congeners should be easily separable by packed-column GC. (iv) The concentrations of congeners should be such that detector response is in the linear range.

Figure 4 shows two congener mixtures specifically designed to assess how the chlorination pattern of the second ring affects the degradation of a set of congeners which are chlorinated at positions 2,4,6 on the first ring and how the

TABLE 3. Degradation of PCB mixtures 3B and 4B by *A. eutrophus* H850

PCB congener	% Degradation at:[a]	
	24 h	72 h
Mixture 3B		
2,6	31	50
4,4'	40	50
2,4,4'	36	49
2,4,6	18	30
2,3,2',5'	99	100
2,4,6,4'		
2,4,6,2',4' IS[b]	0	0
2,4,6,3',4'		
2,4,6,3',5'		
2,4,6,2',4',6'		
Mixture 4B		
2,5	95	100
4,4'	55	85
2,4,3',4'	22	45
2,4,6,4'		
2,5,2',6'	91	100
2,6,2',6'		
3,5,3',5' IS	0	0
2,4,5,2',5'	85	100
2,4,6,2',5'	41	61
2,3,6,2',3',6'	18	33

[a] Degradation which was less than 20% at 72 h is not considered significant and is not reported.
[b] IS, Internal standard.

degree of *ortho* substitution affects degradation. PCB congeners 4,4'; 2,4,4'; 2,4,3',4'; 2,3,2',5'; and 2,4,5,2',5' were included as positive controls for degradative activity, and 2,4,6,2',4'- and 3,5,3',5'-chlorobiphenyls were included as internal standards.

Table 3 gives the results obtained from these assays with *A. eutrophus* H850. Of the seven congeners chlorinated at positions 2,4,6 on one ring, 2,4,6- and 2,4,6,2',5'-chlorobiphenyls were partially degraded, whereas the others were not. These results suggest that the 2,4,6 ring is not readily attacked by H850 and that the chlorination pattern on the second ring will determine whether a congener substituted at positions 2,4,6 can be degraded by this strain. Furthermore, 2,6-dichlorobiphenyl and 2,4,6-trichlorobiphenyl were poorly degraded, even though each of these congeners has an unsubstituted ring. Remarkably, however, congeners with these chlorination patterns were more readily degraded by H850 when the second ring was chlorinated at carbon positions 2' and 5'; i.e., 2,5,2',6'- and 2,4,6,2',5'-chlorobiphenyls were more rapidly degraded than 2,6- and 2,4,6-chlorobiphenyls, respectively. Moreover, 2,3,6,2',3',6'-hexachlorobiphenyl (which is equivalent to 2,5,6,2',5',6'-hexachlorobiphenyl) was more readily degraded by H850 than was 2,6,2',6'-tetrachlorobiphenyl. Thus, these assays suggested that, in H850, degradation of congeners chlorinated at both *ortho* positions on a single ring (i.e., 2,6) is enhanced by chlorination of the second ring at carbon positions 2',5'. These conclusions have been confirmed (D. L. Bedard, R. J. May, and M. L. Haberl, manuscript in preparation).

The examples given above further illustrate the kind of information that can be obtained from defined congener assays. However, we caution that absolute rates of degradation should be determined with individual congeners in the presence of an appropriate internal standard, since we now have evidence that the degradation of any one congener in a mixture may be influenced by the other congeners present (compare the degradation of 4,4' in mixtures 3B and 4B [Table 3]).

In addition, because of the volatile and insoluble nature of PCBs, it is critical to substantiate the results of depletion assays such as the one described by demonstrating products of PCB degradation. The results of our metabolite assays have been described elsewhere (3, 11; Bopp, in press).

The use of these congener mixtures to rapidly identify microorganisms with exceptional PCB-degradative competence will further our understanding of the environmental fate of PCBs and the biochemistry of PCB biodegradation and will have an impact on the development of effective biodegradative processes for cleanup of PCBs.

ACKNOWLEDGMENTS

We thank Tah-Mun Su and John F. Brown, Jr., for helpful discussions, Robert E. Wagner for expert capillary analysis of PCBs, and Carolyn Meyer and Marsha Berry for typing the manuscript.

LITERATURE CITED

1. **Ahmed, M., and D. D. Focht.** 1973. Degradation of polychlorinated biphenyls by two species of *Achromobacter*. Can. J. Microbiol. **19**:47–52.
2. **Baxter, R. A., P. E. Gilbert, R. A. Lidgett, J. H. Mainprize, and H. A. Vodden.** 1975. The degradation of polychlorinated biphenyls by microorganisms. Sci. Total Environ. **4**:53–61.
3. **Bedard, D. L., M. J. Brennan, and R. Unterman.** 1984. Bacterial degradation of PCBs: evidence of distinct pathways in *Corynebacterium* sp. MB1 and *Alcaligenes eutrophus* H850, p. 4-101–4-118. *In* G. Addis and R. Komai (ed.), Proceedings of the 1983 PCB Seminar, Electrical Power Research Institute, Palo Alto, Calif.
4. **Clark, R. R., E. S. K. Chian, and R. A. Griffin.** 1979. Degradation of polychlorinated biphenyls by mixed microbial cultures. Appl. Environ. Microbiol. **37**:680–685.
5. **Furukawa, K.** 1982. Microbial degradation of polychlorinated biphenyls, p. 33–57. *In* A. M. Chakrabarty (ed.), Biodegradation and detoxification of environmental pollutants. CRC Press, Inc., Boca Raton, Fla.
6. **Furukawa, K., N. Tomizuka, and A. Kamibayashi.** 1979. Effect of chlorine substitution on the bacterial metabolism of various polychlorinated biphenyls. Appl. Environ. Microbiol. **38**:301–310.
7. **Furukawa, K., K. Tonomura, and A. Kamibayashi.** 1978. Effect of chlorine substitution on the biodegradability of polychlorinated biphenyls. Appl. Environ. Microbiol. **35**:223–227.
8. **Massé, R., F. Messier, L. Péloquin, C. Ayotte, and M. Sylvestre.** 1984. Microbial biodegradation of 4-chlorobiphenyl, a model compound of chlorinated biphenyls. Appl. Environ. Microbiol. **47**:947–951.
9. **Mullin, M. D., C. M. Pochini, S. McCrindle, M. Romkes, S. H. Safe, and L. M. Safe.** 1984. High-resolution PCB analysis: synthesis and chromatographic properties of all 209 PCB congeners. Environ. Sci. Technol. **18**:468–476.
10. **Sayler, G. S., M. Shon, and R. R. Colwell.** 1977. Growth of an estuarine *Pseudomonas* sp. on polychlorinated biphenyl. Microb. Ecol. **3**:241–255.
11. **Unterman, R., D. L. Bedard, L. H. Bopp, M. J. Brennan, C. Johnson, and M. L. Haberl.** 1985. Microbial degradation of polychlorinated biphenyls, p. 481–488. *In* Proceedings: international conference on new frontiers for hazardous waste management. U.S. Environmental Protection Agency, Cincinnati, Ohio.
12. **Yagi, O., and R. Sudo.** 1980. Degradation of polychlorinated biphenyls by microorganisms. J. Water Pollut. Control Fed. **52**:1035–1043.