JOURNAL OF BACTERIOLOGY, Sept. 1985, p. 882–889
0021-9193/85/090882-08$02.00/0

Vol. 163, No. 3

Plasmid-Mediated Mineralization of 4-Chlorobiphenyl

MALCOLM S. SHIELDS,† SCOTT W. HOOPER, AND GARY S. SAYLER*

Department of Microbiology and The Graduate Program in Ecology, The University of Tennessee, Knoxville, Tennessee 37996

Received 26 November 1984/Accepted 7 May 1985

Strains of *Alcaligenes* and *Acinetobacter* spp. were isolated from a mixed culture already proven to be proficient at complete mineralization of monohalogenated biphenyls. These strains were shown to harbor a 35 × 10^6-dalton plasmid mediating a complete pathway for 4-chlorobiphenyl (4CB) oxidation. Subsequent plasmid curing of these bacteria resulted in the abolishment of the 4CB mineralization phenotype and loss of even early 4CB metabolism by *Acinetobacter* spp. Reestablishment of the *Alcaligenes* plasmid, denoted pSS50, in the cured *Acinetobacter* spp. via filter surface mating resulted in the restoration of 4CB mineralization abilities. 4CB mineralization, however, proved to be an unstable characteristic in some subcultured strains. Such loss was not found to coincide with any detectable alteration in plasmid size. Cultures capable of complete mineralization, as well as those limited to partial metabolism of 4CB, produced 4-chlorobenzoate as a metabolite. Demonstration of mineralization of a purified ^{14}C-labeled chlorobenzoate showed it to be a true intermediate in 4CB mineralization. Unlike the mineralization capability, the ability to produce a metabolite has proven to be stable on subculture. These results indicate the occurrence of a novel plasmid, or evolved catabolic plasmid, that mediates the complete mineralization of 4CB.

Polychlorinated biphenyls (PCBs) represent truly ubiquitous environmental pollutants of strictly anthropogenic origin (16). Their global distribution; potential for toxic, mutagenic, and carcinogenic effects on human populations; and proven bioconcentration and bioaccumulation properties demonstrate them to be of ecological and environmental health concern (12, 21, 22). Because of their unreactive chemical structure and low water solubility, they generally are considered to be nondegradable (16). Despite this, there have been several reports of PCB degradation in mixed (4, 11, 13, 18, 24) and pure (1, 14, 20, 23, 26) bacterial cultures. Plasmid-mediated metabolism of other aromatic and aliphatic pollutants is well documented (3, 6–8). Two plasmids have been reported that are responsible for the conversion of a single PCB congener: 4-chlorobiphenyl (4CB) to 4-chlorobenzoate (4CBZ) (9, 17). The existence of these degradative plasmids encourages the speculation that bacteria in stressed environments have a potential for what might be considered accelerated evolution toward novel catabolic abilities via such plasmid vectors. This certainly seems to be the case in PCB biodegradation since there are no known sources of PCB before their commercial synthesis in 1929 (13). Recently, we have reported the total mineralization of 4CB by a river sediment microbial population (18). From this population, several *Acinetobacter* and *Alcaligenes* isolates competent for the complete mineralization of 4CB were obtained. In this study, the genetic basis for this 4CB mineralization ability was investigated, and the role of a 35 × 10^6-dalton plasmid, designated pSS50, in the oxidation of the chlorophenyl ring of 4CB to CO_2 was demonstrated.

MATERIALS AND METHODS

Bacterial strains. Bacterial strains used in this investigation are listed in Table 1. Two plasmid molecular weight marker strains were used: *Escherichia coli* V517 and containing eight known plasmids (19) and *Pseudomonas putida* (*arvilla*) (ATCC 23793) carrying the TOL plasmid pWWO. From the original mixed culture capable of 4CB mineralization (18), seven wild-type pure cultures were obtained that promoted 4CB mineralization. The cultures include *Alcaligenes* sp. strains A2, A2D, and A5 and *Acinetobacter* sp. strain A8. The wild-type A2 strain carries a 35 × 10^6-dalton plasmid designated pSS50. Strains A2D and A5 both carry two plasmids designated pSS50 and pSS21 (35 × 10^6 and 58 × 10^6 daltons, respectively) and pSS50 and pSS51 (35 × 10^6 and 50 × 10^6 daltons, respectively). A cured derivative of A2D designated *Alcaligenes* sp. strain A2D(pSS50$^-$ and pSS21$^-$) was also used in these studies.

Strain A8 was found to have a 35 × 10^6-dalton plasmid designated pSS50. This strain was cured of pSS8 to yield *Acinetobacter* sp. strain A8(pSS50$^-$). *Acinetobacter* sp. strain AX2(pSS50) resulted from a filter mating between *Acinetobacter* sp. strain A8(pSS50$^-$) and *Alcaligenes* sp. strain A2(pSS50). These strains were maintained on yeast extract polypeptone glucose agar (YEPGA) supplemented with 100 mg of 4BC per liter or stored at −70°C in 15% glycerol-YEPG broth according to previously described methods (5). All strains are currently available as frozen (−80°C) stored cultures.

Media. Purified nutrient agar was obtained from BBL Microbiology Systems, Cockeysville, Md. Yeast extract polypeptone glucose broth (YEPGB; pH 7.0) contained (in grams per liter) glucose (Fisher Scientific Co., Pittsburgh, Pa.) 1.0; polypeptone (BBL), 2.0; yeast extract (BBL), 0.2; NH_4NO_3, 0.2. YEPGA is YEPGB with 18 g or purified agar (Fisher) per liter. The minimal salts medium (pH 7.0) contains (in grams per liter): $NaNO_3$, 4.0; KH_2PO_4, 1.5; $Na_2HPO_4 \cdot 7H_2O$, 0.0011; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2 \cdot 2H_2O$; 0.01 $FeCl_3 \cdot 6H_2O$, 0.0005.

Chemical reagents. 4CB was purchased from Analab Inc., North Haven, Conn. *U*-^{14}C-chlorophenyl ring-labeled 4CB was purchased from Pathfinder Laboratories, Inc., St. Louis, Mo. The radiochemical purity was greater than 99%, and the specific activity was 18.05 Ci mol^{-1}.

Plasmid detection. The basic colony plasmid isolation procedure of Kado and Liu (15) was used with the following modifications: surface colonies from YEPGA were sus-

* Corresponding author.
† Present address: Mayo Clinic, Rochester, MN 55905.

TABLE 1. Bacterial strains, phenotypes, and associated plasmids used in demonstrating plasmid-mediated mineralization of 4CB

Strain	Identification	Phenotype[a]	Plasmid(s)	Size ($\times 10^6$ dalton)	Reference or source
V517	*E. coli*	Cit^+, K^r, Ce^r, Ak^r	Eight size reference plasmids		19
ATCC 23793	*P. putida*	TOL^+	pWWO	78	3
A8	*Acinetobacter* sp.	Mtb^+ Min^+ starch$^+$ Str^s Cm^s Ap^s Pen^s Nal^r	pSS50	35	This study
A8 (pSS50$^-$)	*Acinetobacter* sp.	Mtb^- Min^- starch$^+$ Str^s Cm^s Ap^s Pen^s Nal^r	None		This study
A2	*Alcaligenes* sp.	Mtb^+ Min^+ starch$^-$ Str^r Cm^s Ap^r Pen^r Nal^s	pSS50	35	This study
A2D	*Alcaligenes* sp.	Mtb^+ Min^+ starch$^-$ Str^r Cm^s Ap^r Pen^r Nal^s	pSS50, pSS21	35, 38	This study
A2D(pSS50$^-$, pSS21$^-$)	*Alcaligenes* sp.	Mtb^- Min^- starch$^-$ Str^r Cm^s Ap^r Pen^r Nal^s	None		This study
A5	*Alcaligenes* sp.	Mtb^+ Min^+ starch$^-$ Str^r Cm^s Ap^r Pen^r Nal^s	pSS50, pSS51	35, 50	This study
AX2	*Acinetobacter*	Mtb^+ Min^+ starch$^+$ Str^s Cm^s Ap^s Pen^s Nal^r	pSS50	35	This study

[a] Mtb^+ and Mtb^- refer to the ability to produce an HPLC-detectable metabolite (presumably 4CBZ from 4CB). Min^+ and Min^- refer to the ability to produce CO_2 from 4CB. Antibiotic sensitivities were interpreted on the basis of disk assays. No zone of inhibition was read as resistant. Abbreviations: K, kanamycin; Ce, cephalithin; Ak, amikacin; Str, streptomycin (10 μg per disk); Cm, chloramiphenicol (30 μg per disk); Ap, ampicillin (10 μg per disk); Pen, penicillin (10 U per disk); Nal, nalidixic acid (30 μg per disk).

pended in 70 μl of E buffer in a 500-μl microfuge tube (Fisher). A total of 140 μl of lysis buffer (consisting of 3% sodium dodecyl sulfate in 50 mM Tris adjusted to 62 mM NaOH) was added, and the tubes were heated at 60 to 65°C for 20 min. A total of 420 μl of the 50:50 chloroform-phenol mixture was added and blended with a vortex mixer briefly. The tubes were then centrifuged for 7 min at 15,600 × *g* in an Eppendorf model 5415 microfuge. A total of 60 μl of the aqueous phase was combined with 10 μl of a loading dye containing Ficoll (30% [wt/vol]), bromcresol purple (0.25% [wt/vol]), and glycerol (50% [vol/vol]) in E buffer. Electrophoresis in 0.5% ME agarose (Seakem Corp., Rockland, Maine) was carried out at 100 V for 3 h.

Isolation of plasmid DNA for restriction and hybridization analysis was performed by the method of Anderson and McKay (2). Isolated DNA was purified by dye-bouyant density gradient isopycnic centrifugation in CsCl (0.95 g ml^{-1}) and ethidium bromide (0.9 mg ml^{-1}).

Plasmid curing. Two 4CB mineralizing strains, A2D(pSS50, pSS21) and A8(pSS50) were cured of their respective plasmids by growing the cells at 40°C for 24 to 48 h in YEPGB. These cultures were then diluted and plated on YEPGA. Single-colony isolates were picked, restreaked on YEPGA, and examined for loss of detectable plasmid(s) DNA. These isolates were examined for phenotypic similarity to the plasmid-bearing strain and assayed for metabolic activity with the [^{14}C]4CB substrate.

Plasmid restriction and hybridization. Restriction enzymes *Eco*RI, *Bam*HI, and *Hin*dIII and nick-translation enzymes (Bethesda Research Laboratories, Gaithersburg, Md.) were used according to the manufacturer's directions. After digestion, DNA fragments were resolved by 0.8% agarose gel electrophoresis at 100 V for 3 h. Resolved restriction fragments were transferred to GeneScreen-plus (New England Nuclear Corp., Boston, Mass.) hybridization transfer membranes by electroblotting with a Trans-Blot transfer cell (Bio-Rad Laboratories, Richmond, Calif.) by the manufacturer's instructions.

A ^{32}P-labeled DNA probe was prepared by nick-translation of pSS50-purified plasmid DNA. [^{32}P]dCTP (3,000 Ci $mmol^{-1}$; New England Nuclear) was used as the sole labeling nucleotide. Hybridizations were conducted on GeneScreen-plus by the manufacturer's instructions. Hybridization was detected by autoradiography for 24 to 48 h (−70°C) using Kodak X-Omat x-ray film and a single intensifier screen.

Mating. *Acinetobacter* sp. strain A8(pSS50$^-$) (Nal^r) and *Alcaligenes* sp. strain A2(pSS50) (Nal^s) were cultivated separately in 5.0 ml of YEPGB tubes overnight at 24°C. The cultures were mixed, and the resulting 10-ml suspension was filtered onto a sterile polycarbonate filter (0.2 μm pore size; Nucleopore Corp., Pleasanton, Calif.). Filters were transferred to a nutrient agar plate and maintained at 24°C for 48 h. The entire filter was then transferred to 100 ml of minimal salts medium with 100 μg of 4CB (saturated) and 50 μg of nalidixic acid per ml as sole carbon additions in a 500-ml Erlenmeyer flask. The cells were incubated with shaking at 24°C for 72 h, and 0.1-ml fractions of this were plated onto

TABLE 2. History of strains used in filter mating experiments

Strain	Plasmid	Plasmid size ($\times 10^6$ daltons)	Source	Phenotype[a]	
				4CBZ	CO_2
Alcaligenes sp. strain A2	pSS50	35	Wild-type sediment isolate	+	+
Acinetobacter sp. strain A8	pSS50	35	Wild-type sediment isoate	+	−(+)[b]
Acinetobacter sp. strain A8	pSS50$^-$		Cured derivative of pSS8	−	−
Acinetobacter sp. strain AX2	pSS50	35	Exconjugant result from mating A8(pSS50$^-$) × A2(pSS50)	+	+

[a] A minus sign indicates the lack of 4CBZ or CO_2 production; a plus sign indicates the converse.
[b] Variable reaction.

TABLE 3. 4CB mineralization by wild-type strains

Strain	$^{14}CO_2$ (dpm ± SD)	% [^{14}C]4CB ± SD[a]
A8(pSS50) (Mtb$^+$ Min$^+$)	17,530 ± 540	14.92 ± 0.46
A2(pSS50) (Mtb$^+$ Min$^+$)	16,490 ± 7,360	14.03 ± 6.26
A5(pSS50, pSS51) (Mtb$^+$ Min$^+$)	22,320 ± 870	19.00 ± 0.74
A2D(pSS50, pSS21) (Mtb$^+$ Min$^+$)	39,260 ± 4,000	14.93 ± 1.52
E. coli	60 ± 10	0.05 ± 0.01

[a] Percentage of [^{14}C]4CB loaded into the reaction on day 0 that is accounted for by the $^{14}CO_2$ dpm.

nutrient agar plates containing 50 μg of nalidixic acid per ml. Colonies were selected and screened for plasmid presence, A8(pS550$^-$) phenotypic markers (Table 1), and the ability to mineralize or metabolize 4CB.

Selection against the plasmid donor *Alcaligenes* sp. strain A2(pSS50) (Nal^s) was achieved by nalidixic acid incorporation into the medium. Selection for the exconjugant was achieved by cell survival and growth for 72 h on minimal salts-4CB broth cultivation. Eight isolates were obtained by plating 0.5 ml of the enrichment broth on nutrient agar. A parental history of donor, recipient, and exconjugant strains is given in Table 2.

Quantification of 4CB mineralization. Liberation of $^{14}CO_2$ from the *U*-^{14}C-chlorophenyl ring-labeled 4CB was used to measure total mineralization as described previously (18). Bacterial growth conditions, assay methods, and collection of $^{14}CO_2$ were as described previously (18) with the following exceptions. Inocula consisted of a single loopful of freshly grown cells from YEPGA; after a 20-day incubation, the $^{14}CO_2$ was trapped in 1 ml of 0.2 N NaOH and quantitated by liquid scintillation spectrometry with a Tracor 6892 liquid scintillation counter; confirmation of ^{14}C was made by infrared spectrometry for confirmation of CO_2 and $Ba^{14}CO_3$ precipitation as has been previously demonstrated (18).

Quantification of ^{14}C-labeled metabolite production. The 1.2 ml of reaction media remaining after $^{14}CO_2$ was collected (1.0 ml of reaction media plus 0.2 ml of 2.0 N H_2SO_4) received 0.1 ml of methanol to further solubilize [^{14}C]4CB bound to the glass vial and Teflon-lined rubber cap. A total of 200 μl of this solution was subjected to high-pressure liquid chromatography (HPLC; series 2 high-pressure liquid chromatograph; Perkin Elmer) equipped with a C18/10 column (Perkin Elmer). Elution was in 80% methanol–20% water at a total flow rate of 2.5 ml min^{-1}.

Eluant fractions were collected directly into scintillation vials over 1-min intervals from the time of injection. The amount of ^{14}C in each fraction was quantitated by liquid scintillation spectrometry.

For nonradioactive samples, HPLC column effluent, containing 4CB and metabolites, was monitored by measuring the absorption at 254 nm (Perkin Elmer LC75 spectrophotometric detector and LC autocontrol).

Isolation of ^{14}C-labeled metabolite. A strain of A2, incapable of producing CO_2 from the chlorinated ring of 4CB but capable of producing large amounts of extracellular metabolite in the growth medium, was used to generate ^{14}C-labeled metabolite from [^{14}C]4CB. Minimal salts medium (2 ml) with 2.1×10^6 dpm of [^{14}C]4CB and approximately 10^{10} A2 cells were incubated at 24°C for 4 days. The spent medium was filtered through a sterile 0.4-μm-pore-size Nucleopore membrane filter. The sterile filtrate, containing the ^{14}C-labeled metabolite, was combined with 8 ml of sterile minimal salts medium. One-milliliter fractions of this mixture were divided into sterile reaction vessels. Because of a combination of the high precentage of turnover of [^{14}C]4CB to the ^{14}C-labeled metabolite by this A2 strain and the extremely high affinity of 4CB for any surface (e.g., Nucleopore membranes), the filtered reaction medium was free of any detectable [^{14}C]4CB (as determined by HPLC).

RESULTS

Strain identification. Mixed cultures, found by Kong and Sayler (18) to be proficient for the total mineralization of 4CB, yielded two genera of bacterial isolates that were competent for the same trait. Isolates A2, A5, and A2D were found to be gram-negative, motile rods capable of reducing NO_3^- to NO_2^-. They could not liquify gelatin or produce acid, either oxidatively or fermentatively, from lactose, sucrose, or glucose. The organisms could not hydrolyze starch, but they gave positive oxidase reactions. As a result, these strains were identified as *Alcaligenes* spp. Another isolate, A8, was also found to be a gram-negative rod that

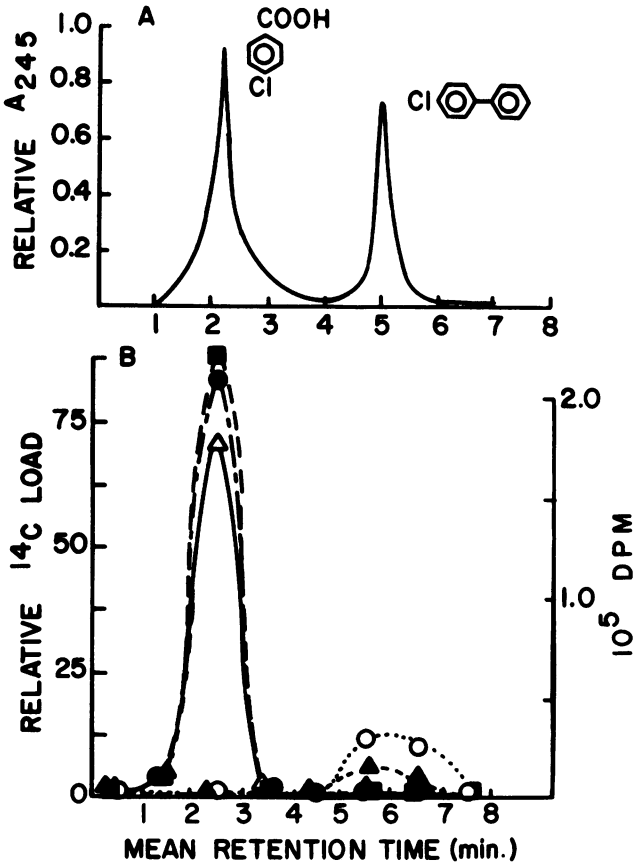

FIG. 1. HPLC fractions from growth medium containing [^{14}C]4CB as the sole source of carbon and energy. One-minute fractions of HPLC column eluant were collected and quantitated by liquid scintillation spectroscopy. (A) Observed retention times of 4CBZ (25) and 4CB standards under the same HPLC conditions. (B) Strains A8 (△) (containing pSS50) and A2 (■) (containing pSS50) are the wild *Acinetobacter* and *Alcaligenes* isolates, respectively. A8 (▲) (pSS50$^-$) is the cured derivative of A8. AX2 (●) (containing pSS50) is the exconjugant from the mating of A2(pSS50) × A8(pSS50$^-$). *P. putida* (○) with the TOL plasmid was included as a negative control. A_{245}, Absorbance at 245 nm.

could not liquify gelatin or produce acid oxidatively or fermentatively from lactose, sucrose, or glucose. This group, however, was nonmotile, oxidase negative, and could not reduce NO_3^- and was proficient in the hydrolysis of starch. This organism was tentatively identified as an *Acinetobacter* sp.

Routine confirmative identification of the two groups during the course of study was by means of their respective antibiotic resistances (Table 1).

Mineralization and metabolism of 4CB by pure culture isolates. *Alcaligenes* sp. strains A2, A5, and A2D and *Acinetobacter* sp. strain A8 were found to be competent for the total mineralization (Min^+) of 4CB (Table 3), with concomitant production of metabolite (Mtb^+) as demonstrated by strains A2 and A8 (Fig. 1). The metabolite cochromatographed with authentic 4CBZ, indicating that it was 4CBZ (Fig. 1A). Previous work showed a 4CBZ metabolite from the mixed cultures from which these isolates were taken (18). Similar evidence exists that other 4CB-metabolizing *Alcaligenes* and *Acinetobacter* spp. accumulate 4CBZ as the major metabolite (11). A typical yellow metabolite, speculated by Furukawa and Matsumura (10) to be 2-hydroxy-6-oxo-6-(4-chlorophenyl)-hexa-2,4 dienoic acid, was also produced by mineralizing strains.

Role of plasmids in 4CB mineralization. Plasmid profiles of the isolates most closely examined in this investigation are given in Fig. 2. A2(pSS50) and A8(pSS50), each with a 35×10^6-dalton plasmid, were completely stable with respect to their ability to metabolize 4CB to 4CBZ but unstable in their ability to mineralize 4CB. A2D (Mtb^+ Min^+) carrying pSS21 (58×10^6 daltons) and pSS50 (35×10^6 daltons) is an *Alcaligenes* sp. isolated from the same sediment (with an identical antibiotic resistance profile to A2[pSS50]) and is stable in relation to its 4CB mineralization abilities. *Alcaligenes* sp. strain A5 (Mtb^+ Min^+) also exhibited an identical antibiotic resistance profile to that of A2(pSS50), but when originally isolated it was found to contain a 35×10^6-dalton plasmid (pSS50) and what may be a 50×10^6-dalton plasmid (pSS51) or an open circular form of pSS50. The putative pSS51 was transient in nature. Like A2D(pSS50, pSS21), A5 retained stable 4CB mineralization ability. DNA from a representative pSS50 (A5 strain) was examined by electron microscopy, and contour length measurements confirmed the size as approximately 53 kilobases or 35×10^6 daltons (Fig. 3).

Curing of pSS50 from strain A8(pSS8) (Mtb^+ Min^+) by growth at restrictive temperatures yielded the plasmidless deriviative A8(pSS50$^-$) (Mtb^- Min^-; Fig. 2). Abilities for both $^{14}CO_2$ production (Table 4) as well a metabolite production (Fig. 1) from [^{14}C]4CB were absent from A8(pSS50$^-$) (Mtb^- Min^-) while they were readily detectable in the parent A8(pSS50). To further establish the role of pSS in the degradation of 4CB, a mating between a cured *Acinetobacter* sp. strain A8(pSS8$^-$) (Mtb^- Min^-) and *Alcaligenes* sp. strain A2(pSS50) (Mtb^+ Min^+) was done. The resulting exconjugant, AX2(pSS50) (Mtb^+ Min^+) retained the antibiotic resistance and starch hydrolysis phenotype of A8(pSS50$^-$) (Table 1); however, it now contained the 35×10^6-dalton plasmid pSS50 (Fig. 2) and demonstrated the ability to produce 4CBZ (Fig. 1) and $^{14}CO_2$ from 4CB (Table 4). This provides additional evidence that the same 35×10^6-dalton plasmid functionally mediates 4CB mineralization in two different bacterial isolates.

The role of pSS50 and pSS21 is not clear, since the curing of these plasmids should result in the loss of 4CB metabolism as well as 4CB mineralization abilities. The generation of A2D(pSS50$^-$, pSS21$^-$) from A2D(pSS50, pSS21) results in a strain that no longer has the ability to produce $^{14}CO_2$ from

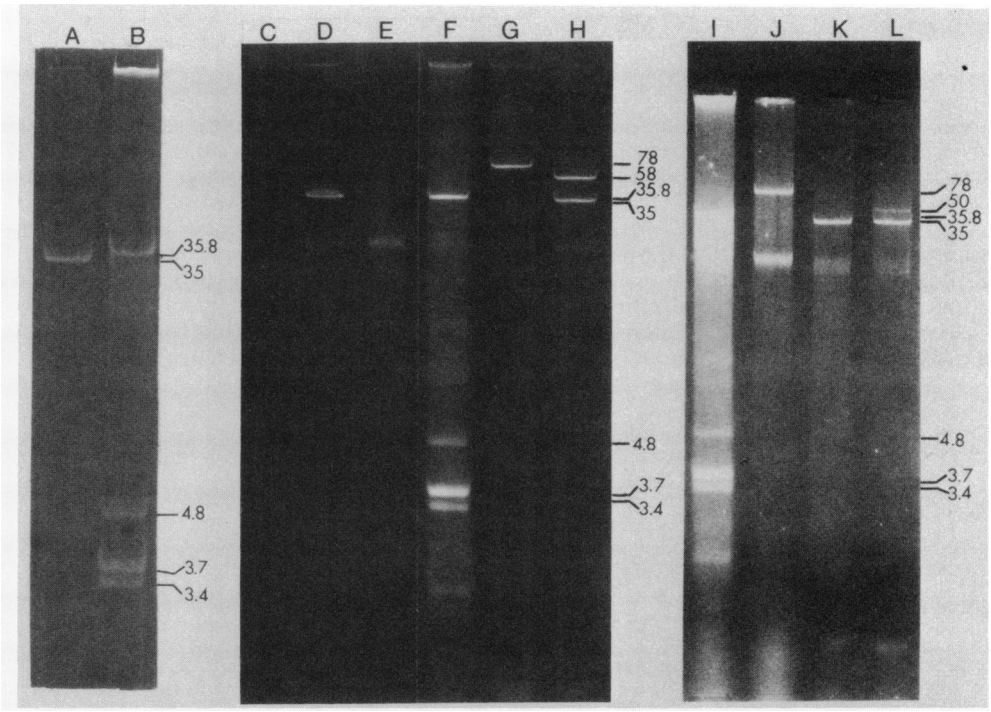

FIG. 2. Plasmid profile of 4CB-mineralizing strains. Lane A, A8 with 35×10^6-dalton pSS50; lanes B, F, and I, *E. coli* V517 reference; lane C, A8(pSS50$^-$); lane D, A2(pSS50); lane E, A2D(pSS50$^-$, pSS21$^-$); lanes G and J, *P. putida arvilla* (pWWO); lane H, A2D(pSS50, pSS21); lane K, AX2(pSS50); lane L, A5(pSS50, pSS51). Numbers are in megadaltons.

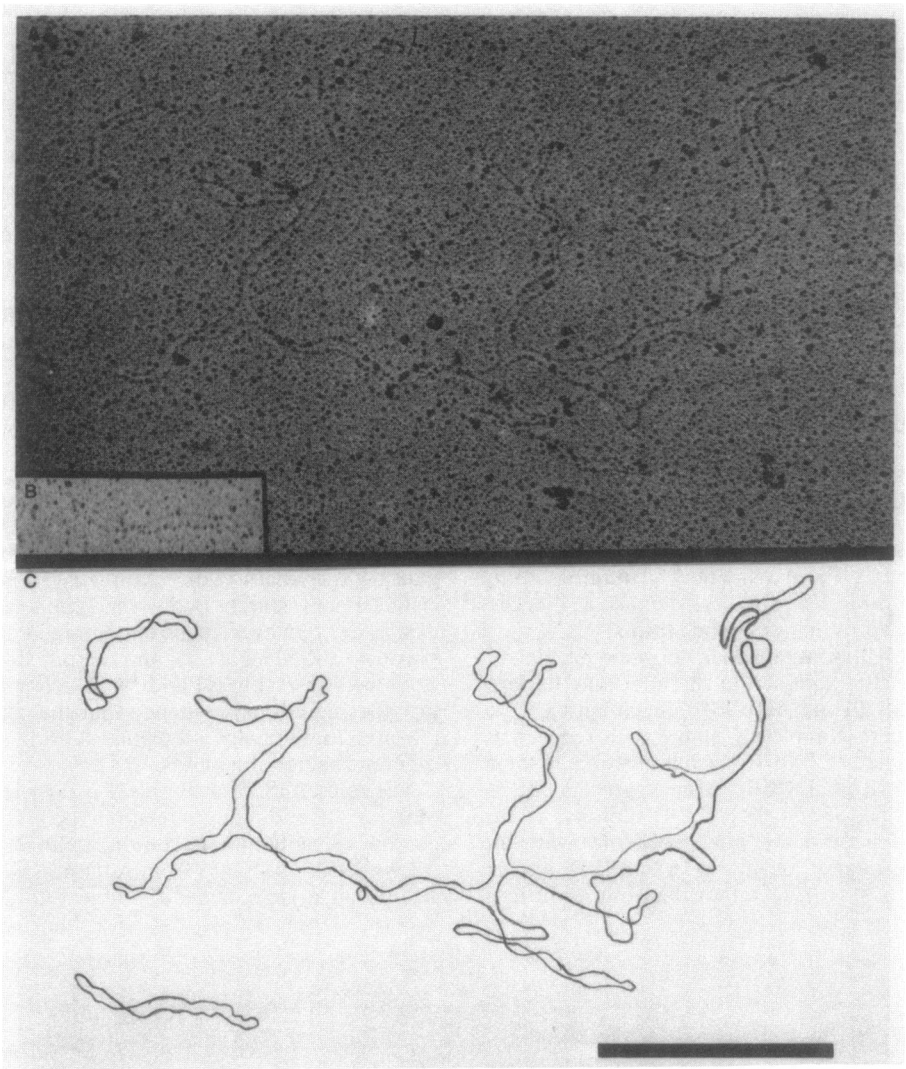

FIG. 3. Electron micrograph and contour length tracing of plasmid pSS50. Samples were uranyl acetate stained, rotary shadowed with Pt/Pd, and examined on a Zeiss EM9S electron microscope. (A) Center, pSS50; upper left (A) and (B), ϕX174 DNA RFII (5,378 base pairs) size reference markers. (C) contour tracing of pSS50 with a calculated contour length of 53.2 kilobases. Bar, 1 μm.

4CB. The cured strain, A2D(pSS50$^-$, pSS21$^-$), however, is still capable of 4CBZ production from 4CB.

All of the organisms capable of 4CB mineralization examined appeared to harbor the same 35 × 10^6-dalton plasmid. Restriction enzyme digestion of pSS50 (from A2), pSS50 (from AX2), pSS50 (from an A2D isolate found to harbor only pSS50), and pSS50 (from an A5 strain found to harbor only pSS50) exhibited identical patterns of cleavage for *Eco*RI, *Bam*HI, and *Hin*dIII (based on single and double digests) when compared with the 35 × 10^6-dalton pSS50

TABLE 4. Metabolization and mineralization of [^{14}C]4CB by donor, recipient, and exconjugant strains[a]

Strain	$^{14}CO_2$[b]			^{14}C-labeled metabolite[c]		
	dpm ± SD	% donor activity (A2)	% [^{14}C]4CB load	dpm ± SD[d]	% donor activity (A2)	% [^{14}C]4CB load
A2(pSS50) (Mtb$^+$ Min$^+$)	3,570 ± 750	100	1.9	678,620 ± 72,470	100	63.2
A8(pSS50−) (Mtb$^-$ Min$^-$)	80 ± 160	2.3	<0.1	9,260 ± 870	1.4	0.9
AX2(Mtb$^+$ Min$^+$)	2,640	74.0	1.4	621,660	91.6	57.9
E. coli	60 ± 10.0	0.4	<0.1	11,210 ± 620	1.7	1.0

[a] All data are means of triplicate observations. All data were recorded after a 6-day incubation period. Donor, A2(pSS50); recipient A8(pSS50$^-$); exconjugant, AX2(pSS50).

[b] Day 0 load was 184,800 ± 10,300 dpm.

[c] Day 0 load was 1,073,450 ± 6,970 dpm.

[d] Metabolite counts were derived from pooled HPLC fractions of the [^{14}C]4CBZ peak (Fig. 1).

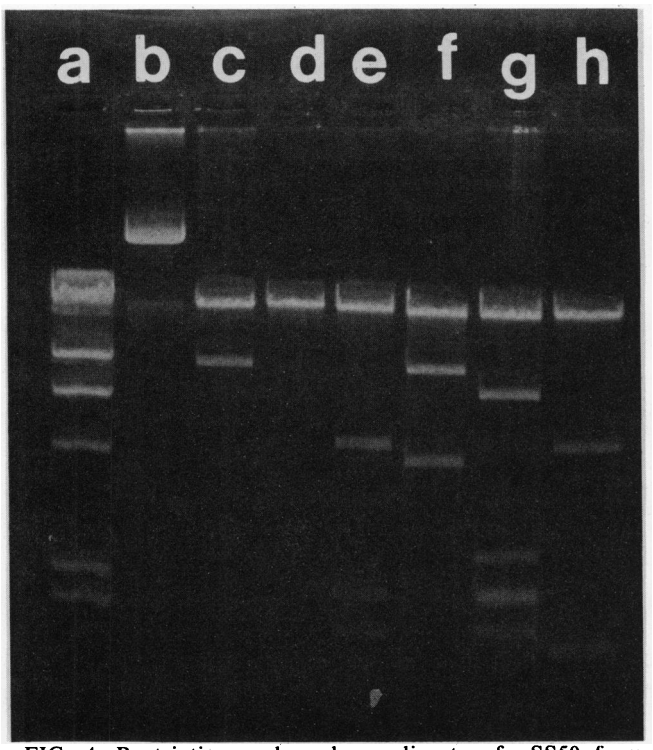

FIG. 4. Restriction endonuclease digests of pSS50 from *Alcaligenes* sp. strain A5. Lane a, λ *Hin*dIII marker; lane b, whole pSS50; lane c, *Eco*RI digest; fragments, 29.3 × 10^6 and 5.7 × 10^6 daltons; lane d, *Bam*HI; fragments 34.5 × 10^6 and 0.5 × 10^6 (not visible) daltons; lane e, *Hin*dIII; fragments, 29.5 × 10^6, 3.0 × 10^6, 1.4 × 10^6, 1.1 × 10^6 daltons; lane f, *Eco*RI × *Bam*HI double digest; fragments, 26.8 × 10^6, 5.7 × 10^6, 2.5 × 10^6, 0.5 × 10^6 (not visible) daltons; lane g, *Eco*RI × *Hin*dIII double digest; fragments, 25.2 × 10^6, 4.1 × 10^6, 1.5 × 10^6, 1.4 × 10^6, 1.1 × 10^6 daltons; lane h, *Bam*HI × *Hin*dIII double digest; fragments, 29.5 × 10^6, 3.0 × 10^6, 1.0 × 10^6, 1.0 × 10^6, 0.5 × 10^6 (not visible) daltons. (size based on comparison to λ *Hin*dIII digest in 0.5% agarose). (Molecular size estimates for high-molecular-weight fragments were calculated after resolution in 0.3% agarose gel, due to stacking in 0.5% agarose gel as indicated.)

plasmid of A5 shown in Fig. 4. In addition, probing of these digests with ^{32}P-labeled pSS50 DNA indicated that all fragments of the 35 × 10^6-dalton plasmid shared homology with pSS50. There is no hybridization between the 58 × 10^6-dalton pSS21 plasmid of A2D and pSS50 (data not shown).

Instability of the 4CB mineralization phenotype. The ability to produce 4CBZ is a stable characteristic in all pSS-bearing strains. The ability to produce CO_2 from the chlorinated ring, however, is frequently lost on subculture. Attempts to identify an agent that would select against this loss of mineralizing ability have had with little success. Incorporation of 4CB into YEPG media at 200 μg/ml has failed to provide noticeably larger retention of the mineralization phenotype (data not shown). Sustained growth of any strain on 4CB as a sole carbon source has been found for only one strain A5(pSS50) (Mtb^+ Min^+). Despite more than 20 passages in YEPG medium, strains A5(pSS50) (Mtb^+ Min^+) and A2D(pSS50, pSS21) (Mtb^+ Min^+) remain stable competent mineralizers.

^{14}C-labeled material balance. ^{14}C-labeled material balances were calculated for recovery of ^{14}C associated with $^{14}CO_2$, [^{14}C]4CBZ in solution and were adsorbed to reaction vessel components. Average ^{14}C recovery for nonmetabolizing strains such as A8(pSS50$^-$) was approximately 85%, with 78% of the [^{14}C]4CB being associated with the reaction vessel serum cap. Average ^{14}C recovery for strains capable of production of 4CBZ but not chlorinated ring CO_2 from 4CB, such as cured A2D(pSS50$^-$, pSS21$^-$) (Mtb^+ Min^-), were approximately 80%, with approximately 75% of the initial [^{14}C]4CB being recovered as [^{14}C]4CBZ. For mineralizing strains, total ^{14}C recovery ranged from 40 to 50% with approximately 25% of the input $^{14}CO_2$ after 7 to 12 days of incubation. The major loss of ^{14}C for mineralizing strains was associated with inefficient $^{14}CO_2$ capture and loss from the reaction vessel, as confirmed by the loss of [^{14}C]HCO_3 in control bicarbonate recovery experiments.

Metabolite oxidation. The question arises as to whether 4CBZ detected by HPLC fractionation is a true intermediate or a terminal by-product in the oxidative pathway of 4CB by these strains. To address this problem, the mineralizing strain A2D(pSS50, pSS21) (Mtb^+ Min^+), the nonmineralizing strain A8(pSS50) (Mtb^+ Min^-) (after loss of mineralization function in this strain), and *E. coli* were tested for their abilities to oxidize ^{14}C-labeled metabolite, which was generated and collected from an A2(pSS50) (Mtb^+ Min^-) (after loss of this strain's mineralization capability) culture filtrate. According to HPLC and liquid scintillation spectrometry analysis, the only detectable ^{14}C source in the ^{14}C-labeled metabolite preparation was 4CBZ. After a 7-day incubation of A8(pSS50) (Mtb^+ Min^-), A2D(pSS50, pSS21) (Mtb^+ Min^+), and *E. coli* (Mtb^- Min^-) with [^{14}C]4CBZ as the sole carbon source, the $^{14}CO_2$ produced was quantified along with the radioactivity remaining in the reaction vials (Table 5). A2D(pSS50, pSS21) (Mtb^+ Min^+) was found to be capable of 10% mineralization of the ^{14}C-labeled metabolite preparation in 7 days under assay conditions. This indicates that the 4CBZ metabolite as isolated by HPLC is not a terminal product in the oxidative degradation of 4CB. Interestingly (as was the case for 4CB mineralizers grown with [^{14}C]4CB) the total recovery of ^{14}C label from mineralizers grown with ^{14}C-labeled metabolite is only about half that of the nonmineralizers. An efficiency check of our CO_2 trapping system using [^{14}C]bicarbonate indicates a trapping efficiency of 91.6%. Therefore, the loss of the ^{14}C label is not due to an inefficient trapping of $^{14}CO_2$, but is probably caused by CO_2 permeability of the reactor vessel.

DISCUSSION

Both *Alcaligenes* and *Acinetobacter* isolates described in this study are capable of 4CB mineralization. Both were found to contain a 35 × 10^6-dalton plasmid (pSS50) that was shown to mediate 4CB metabolism and mineralization. This

TABLE 5. Mineralization of extracellular metabolite (4CBZ) produced from 4CB metabolism

Recovered	Phenotype		dpm from ^{14}C-labeled metabolite				
	Min	Mtb	$^{14}CO_2$	Cap	Vial	% Mineralized[a]	% [^{14}C]4CB loaded
A8(pSS50)[b]	−	+	100	100	151,600	0.06	110
A2D(pSS50, pSS21)[c]	+	+	13,900	400	61,600	10.04	55
E. coli	−	−	200	100	131,500	0.13	95

[a] Based on a day 0 ^{14}C load of 138,000 dpm.
[b] Means of duplicate observations after a 7-day incubation.
[c] Means of six observations.

is not the first plasmid described to mediate 4CB metabolism by bacteria. Kamp and Chakrabarty (17) have described the *Klebsiella* plasmid pAC21 (65×10^6 daltons) that encodes the biodegradation of 4CB to 4CBZ. More recently, Furukawa and Chakrabarty (9) have reported the ability of an *Acinetobacter* spp. and *P. putida* mixed culture to completely degrade 4CB. This was reportedly due to three separate clusters of degradative genes. One cluster, on the pKF1 plasmid (53.7×10^6 daltons) of an *Acinetobacter* spp., encoded the oxidation of 4CB to 4CBZ (as does pAC21). The other two genetic elements involved the TOL transposon- and the pAC27 plasmid- (*P. putida*) encoded enzymes for the subsequent mineralization of 4CBZ.

Unlike pAC21, pKF1, and pAC27, which encode only partial degradation of 4CB or only degradation of 4CB metabolites, pSS50 was found to be responsible for the total mineralization of 4CB by our *Acinetobacter* strain. This was concluded from two observations. (i) Curing pSS50 from strain A8(pSS50) coincided with the loss of 4CBZ production and CO_2 formation. (ii) Subsequent introduction of pSS50 into the ($pSS50^-$) (cured) strain resulted in a reestablishment of both 4CB metabolite formation and mineralization abilities.

The pKF1 plasmid of Furukawa and Chakrabarty (9) undergoes a spontaneous deletion of 1.6×10^6 daltons, resulting in the loss of the 4CB degradation phenotype. Initially the plasmid DNA of the freshly isolated strains reported here was observed to be 50×10^6 daltons (pSS51) and appeared to be reduced in size to 35×10^6 daltons (pSS50) during successive subcultures. This observation suggests a similar spontaneous deletion of DNA that was not under selection during laboratory maintenance. However, simultaneous maintenance of pSS50 and pSS51 (presumably sharing a common replicon) in strains such as A5 is unlikely, except in the event that a mixed population of cells of the same strain may harbor the different-sized plasmids. The plasmid pSS21 (58×10^6 daltons) is exempt from this reasoning since it demonstrates no homology with pSS50. A more likely hypothesis is that pSS51 is an open circular form of pSS50 on agarose gel electrophoresis.

The results presented in this study indicate that 4CB mineralization observed in a freshwater sediment population can be carried out independently by two of its members. These two members, *Acinetobacter* and *Alcaligenes* spp., in turn, owe this mineralization potential to a plasmid denoted pSS50 (35×10^6 daltons). The possibility does exist that the plasmid (pSS50) may activate other chromosomal genes partly involved in mineralization. Since the bacterial isolates and pSS50 plasmid were isolated from PCB-contaminated sediment, they are ideal candidates for genetic manipulation aimed at a broader substrate degradative range and eventual reapplication to contaminated freshwater environments.

ACKNOWLEDGMENTS

This investigation was supported by grant R808 457020 from the Office of Exploratory Research, U.S. Environmental Protection Agency. Partial support for this investigation was provided by Public Health Service grant T-32-A107123 from the National Institutes of Health. G.S.S. is supported by a research career development award from the National Institute of Environmental Health Sciences.

We gratefully acknowledge the helpful advice and council of Karl Sirotkin and able technical assistance of John Wickman.

LITERATURE CITED

1. **Ahmed, M., and D. D. Focht.** 1973. Degradation of polychlorinated biphenyls by two species of *Achromobacter*. Can. J. Microbiol. **19**:45–52.
2. **Anderson, D. G., and L. L. McKay.** 1983. Simple and rapid method for isolating large plasmid DNA from lactic streptocci. Appl. Environ. Microbiol. **46**:549–552.
3. **Chakrabarty, A. M.** 1980. Plasmids and dissimilation of synthetic environmental pollutants, p. 21–30. *In* Plasmids and transposons. Academic Press, Inc., New York.
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. **Davis, R. W., D. Botstein, and J. R. Roth (ed.).** 1980. Advanced bacterial genetics. A manual for genetic engineering. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
6. **Dunn, N. W., and I. C. Gunsalus.** 1973. Transmissible plasmid coding early enzymes of naphthalene oxidation in *Pseudomonas putida*. J. Bacteriol. **114**:974–979.
7. **Farrell, R., and A. M. Chakrabarty.** 1979. Degradative plasmids: molecular nature and mode of evolution. *In* K. N. Timmis and A. Puhler (ed.), Plasmids of medical, environmental and commercial importance. Elsevier/North Holland Biomedical Press, Amsterdam.
8. **Fisher, P. R., J. Appelton, and J. M. Pemberton.** 1978. Isolation and characterization of the pesticide-degrading plasmid pJP1 from *Alcaligenes paradoxus*. J. Bacteriol. **135**:798–804.
9. **Furukawa, K., and A. M. Chakrabarty.** 1982. Involvement of plasmids in total degradation of chlorinated biphenyl. Appl. Environ. Microbiol. **44**:619–626.
10. **Furukawa, K., and F. Matsumara.** 1976. Microbial metabolism of polychlorinated biphenyls. Studies on the relative degradability of polychlorinated biphenyl components by *Alcaligenes* sp. J. Agric. Food Chem. **24**:251–256.
11. **Furukawa, K., K. Tonomura, and A. Kamibayashi.** 1978. Effect of chlorine substitution on the biodegradability of polychlorinated biphenyls. Appl. Environ. Microbiol. **35**:223–227.
12. **Higuchi, K. (ed.).** 1976. PCB poisoning and pollution. Academic Press, Inc. New York.
13. **Huntzinger O., S. Safe, and V. Zitko (ed.).** 1974. *In* The chemistry of PCBs, p. 1–16. CRC Press, Inc., Boca Raton, Fla.
14. **Jeenes, D. J., W. Reineke, H.-J. Knackmuss, and P. A. Williams.** 1982. TOL plasmid pWWO in constructed halobenzoate-degrading *Pseudomonas* strains: enzyme regulation and DNA structure. J. Bacteriol. **150**:180–187.
15. **Kado, C. I., and S.-T. Liu.** 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. **145**:1365–1373.
16. **Kalmaz, E. V., and G. D. Kalmaz.** 1979. Transport, distribution, and toxic effects of polychlorinated biphenyls in ecosystems. Review. Ecol. Model. **6**:223–251.
17. **Kamp, P. F., and A. M. Chakrabarty.** 1979. Plasmids specifying p-chlorobiphenyl degradation in enteric bacteria. *In* K. N. Timmis and A. Puhler (ed.), Plasmids of medical, environmental and commercial importance. Elsevier/North-Holland Biomedical Press, Amsterdam.
18. **Kong, H.-L., and G. S. Sayler.** 1983. Degradation and total mineralization of monohalogenated biphenyls in natural sediment and mixed bacterial culture. Appl. Environ. Microbiol. **46**:666–672.
19. **Macrina, F. L., D. J. Kopecko, K. R. Jones, D. J. Ayers, and S. M. McCowen.** 1978. A multiple plasmid-containing *Escherichia coli* strain: convenient source of size reference plasmid molecules. Plasmid **1**:417–420.
20. **Mass, R., F. Messier, L. Ploquin, C. Ayotte, and M. Sylvestre.** 1984. Microbial degradation of 4-chlorobiphenyl, a model compound of chlorinated biphenyls. Appl. Environ. Microbiol. **47**:947–951.
21. **Safe, S.** 1980. Metabolism, uptake, storage and bioaccumulation. *In* J. Kimbrough (ed.), Halogenated biphenyls, terphenyls, naphthalenes, dibenzodioxins and related products. Elsevier/North-Holland Biomedical Press, Amsterdam.
22. **Sayler, G. S., M. C. Reid, B. K. Perkins, R. M. Pangi, R. L. Smith, T. K. Rao, J. L. Epler, W. D. Morrison, and R. DuFrain.** 1982. Evaluation of the mutagenic potential of bacterial polychlorinated biphenyl biodegradation products. Arch.

Environ. Contam. Toxicol. **11**:577–581.
23. **Sayler, G. S., M. Shon, and R. R. Colwell.** 1977. Growth of an estuarine *Pseudomonas* sp. on polychlorinated biphenyls. Microbial Ecology **3**:241–255.
24. **Shiaris, M. P., and G. S. Sayler.** 1982. Biotransformation of PCB by natural assemblages of freshwater microorganisms. Environ. Sci. Technol. **16**:367–369.
25. **Stecher, P. G. (ed.)** 1968. Merck index, eighth Ed., p. 241. Merck and Co., Inc. Rathway, N.J.
26. **Yagi, O., and R. Sudo.** 1980. Degradation of polychlorinated biphenyls by microorganisms. J. Water Pollut. Control Fed. **52**:1035–1043.