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Evidence for Novel Mechanisms of Polychlorinated Biphenyl Metabolism in *Alcaligenes eutrophus* H850

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Previous studies indicated that *Alcaligenes eutrophus* H850 attacks a different spectrum of polychlorinated biphenyl (PCB) congeners than do most PCB-degrading bacteria and that novel mechanisms of PCB degradation might be involved. To delineate this, we have investigated the differences in congener selectivity and metabolite production between H850 and *Corynebacterium* sp. strain MB1, an organism that apparently degrades PCBs via a 2,3-dioxygenase. H850 exhibited a superior ability to degrade congeners via attack on 2-, 2,4-, 2,5-, or 2,4,5-chlorophenyl rings in PCBs but an inferior ability to degrade congeners via attack on a 4-chlorophenyl ring. Reactivity preferences were also reflected in the products formed from unsymmetrical PCBs; thus MB1 attacked the 2,3-chlorophenyl ring of 2,3,2′,5′-tetrachlorobiphenyl to yield 2,5-dichlorobenzoic acid, while H850 attacked the 2,5-chlorophenyl ring to yield 2,3-dichlorobenzoic acid and a novel metabolite, 2′,3′-dichloroacetophenone. Furthermore, H850 oxidized 2,4,5,2′,4′,5′-hexachlorobiphenyl, a congener with no adjacent unsubstituted carbons, to 2′,4′,5′-trichloroacetophenone. The atypical congener selectivity pattern and novel metabolites produced suggest that *A. eutrophus* H850 may degrade certain PCB congeners by a new route beginning with attack by some enzyme other than the usual 2,3-dioxygenase.

The principal route of polychlorinated biphenyl (PCB) degradation in aerobic bacteria appears to involve 2,3-dioxygenase attack at an unsubstituted 2,3 (or 5,6) position (10, 23). However, in earlier studies, we established that *Alcaligenes eutrophus* H850 degrades a spectrum of PCB congeners (isomers and homologs) that differs substantially from the expected substrates for such an enzyme (4–6). For example, H850 rapidly degrades several congeners, such as 2,5,2′,5′-chlorobiphenyl (2,5,2′,5′-CB) and 2,4,5,2′,5′-CB, that have no unchlorinated 2,3 site. To explain these observations, we proposed that a significant route of PCB degradation in H850 involves a 3,4-dioxygenase (4). The present study was undertaken to gain a clearer understanding of the mechanism of PCB degradation in *A. eutrophus* H850. Because earlier results revealed that *Corynebacterium* sp. strain MB1 degrades a spectrum of PCB congeners consistent with the expected substrates for a 2,3-dioxygenase (4–6), we have also characterized PCB degradation in this organism to provide a basis for comparison.

MATERIALS AND METHODS

Bacterial strains. The isolation, characterization, and maintenance of *A. eutrophus* H850 and *Corynebacterium* sp. strain MB1 are described in the accompanying paper (6).

Chemicals. PCB congeners were obtained from Foxboro Analabs, Inc., North Haven, Conn., and Ultra Scientific, Hope, R.I. Chlorobenzoic acids were purchased from Aldrich Chemical Co., Inc., Milwaukee, Wis., and from Pfaltz and Bauer, Inc., Stamford, Conn. Chloroacetophenones were purchased from Aldrich Chemical Co. and from Eastman Kodak Co., Rochester, N.Y. Two chloroacetophenones that are not commercially available, 2′,3′-dichloroacetophenone and 2′,4′,5′-trichloroacetophenone, were synthesized and purified by our colleagues Stephen Mendelson and Herman Finkbeiner by slow addition of 0.05 mol (3.9 g) of acetyl chloride to a solution of 0.05 mol (6.7 g) of anhydrous aluminum chloride in 25 ml of 1,2-dichlorobenzene or 1,2,4-trichlorobenzene, respectively, followed by heating at 85°C for 1 h. Subsequently, the mixture was added to 100 ml of water containing 10 ml of concentrated HCl. The organic materials were extracted with ether, and the ether was dried and removed. The residue was distilled to separate the acetophenones from the excess chlorobenzene. Both 2′,3′- and 3′,4′-dichloroacetophenones were formed from 1,2-dichlorobenzene and were separated by preparative gas chromatography (GC). The identities of the chloroacetophenones were established by using 300 mHz proton magnetic resonance spectroscopy including comparison with a computer simulation for an $A_1B_1C_1$ system (2′,4′,5′-trichloroacetophenone) or an A_1X_1 system (2′,3′-dichloroacetophenone). Each compound gave the expected mass spectrum.

Resting-cell assays to measure PCB degradation. Biphenyl-grown cells (5) were washed and suspended in 0.05 M sodium phosphate buffer (pH 7.5) at an optical density at 615 nm of 1.0 (approximately 3.8×10^9 cells per ml for H850 and 1.2×10^8 cells per ml for MB1). The concentrations of individual PCB congeners used in assays ranged from 1.25 μM to 5 mM. Actual concentrations are given in the figure legends. In each case, PCB degradation was assessed by direct comparison with a dead-cell control incubated in parallel and subjected to all the same procedures as the experimental sample. A PCB congener that is not degradable by strains MB1 and H850, either 3,5,3′,5′-CB or 2,4,6,2′,4′-CB, was included in each incubation as an internal standard to permit correction for possible physical loss of PCBs during incubation and extraction. The samples (generally 1 ml) were incubated for up to 72 h at 30°C at 250 rpm in a gyratory shaker. Additional experimental details have been published elsewhere (5, 6).

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TABLE 1. Correlation of chlorophenyl ring substituents with differential oxidation of PCB congeners by *A. eutrophus* H850 and *Corynebacterium* sp. strain MB1[a]

Common Structure	PCB Congener	Percent Degradation			
		5 μM		25 μM	
		MB1	H850	MB1	H850
	2,3	100	100	100	100
	2,4′	100	100	100	100
	4,4′	100	61	100	0
	2,4,4′	100	76	98	0
	2,3,2′,3′	100	99	96	53
	2,4,5,2′,3′	98	46	58	0
	2,4,3′,4′	93	22	64	0
	3,4,3′,4′	21	0	0	0
	2,2′	87	100	0	100
	2,5,2′	61	100	0	98
	2,5,4′	86	99	22	86
	2,3,2′,5′	100	98	32	53
	2,5,2′,5′	17	100	0	71
	2,5,3′,4′	56	90	5	54
	2,4,5,2′,5′	9	70	0	30
	2,3,4,2′,5′	0	59	0	0
	2,4,2′,4′	0	54	0	5
	2,4,5,2′,4′,5′	0	16	0	0

[a] PCB congeners in groups of 10 or 11 were incubated with resting cells of H850 or MB1 at 30°C for 24 h (5). Assays were conducted with individual congeners at 5 and 25 μM. Data represent the mean values obtained in several experiments: MB1, 5μM, three experiments; H850, 5 μM, five experiments; MB1 and H850, 25 μM, two experiments each.

PCB extraction and GC analysis. At appropriate times, congener depletion assays were stopped and the PCBs were extracted as described previously (5, 6). Samples to be analyzed for chlorobenzoic acids and hydroxylated metabolites were acidified by the addition of perchloric acid (final concentration, 0.7%) and extracted with 4 volumes of ether, and then 1 ml of each extract was derivatized with 0.3 ml of N,O-*bis*(trimethylsilyl)acetamide (Pierce Chemical Co., Rockford, Ill.).

The extracts were analyzed on either a 5880 GC (Hewlett-Packard Co., Palo Alto, Calif.) or a Vista 6000 GC (Varian, Sunnyvale, Calif.) with an automatic sampler, an electron capture detector, and a glass column (6 ft [1.83 m] by 4 mm) packed with 1.5% SP-2250/1.95% SP-2401 on 100/120 Supelcoport (Supelco, Inc., Bellefonte, Pa.). Defined congener depletion assays were analyzed by isothermal (190°C) GC as described previously (5). All other samples were chromatographed for 32 min with a temperature program which began at 150°C, was raised to 220°C at a rate of 5°/min, and then held at 220°C. The carrier gas was nitrogen (flow rate, 60 ml/min). The injection and detector temperatures were 250 and 300°C, respectively.

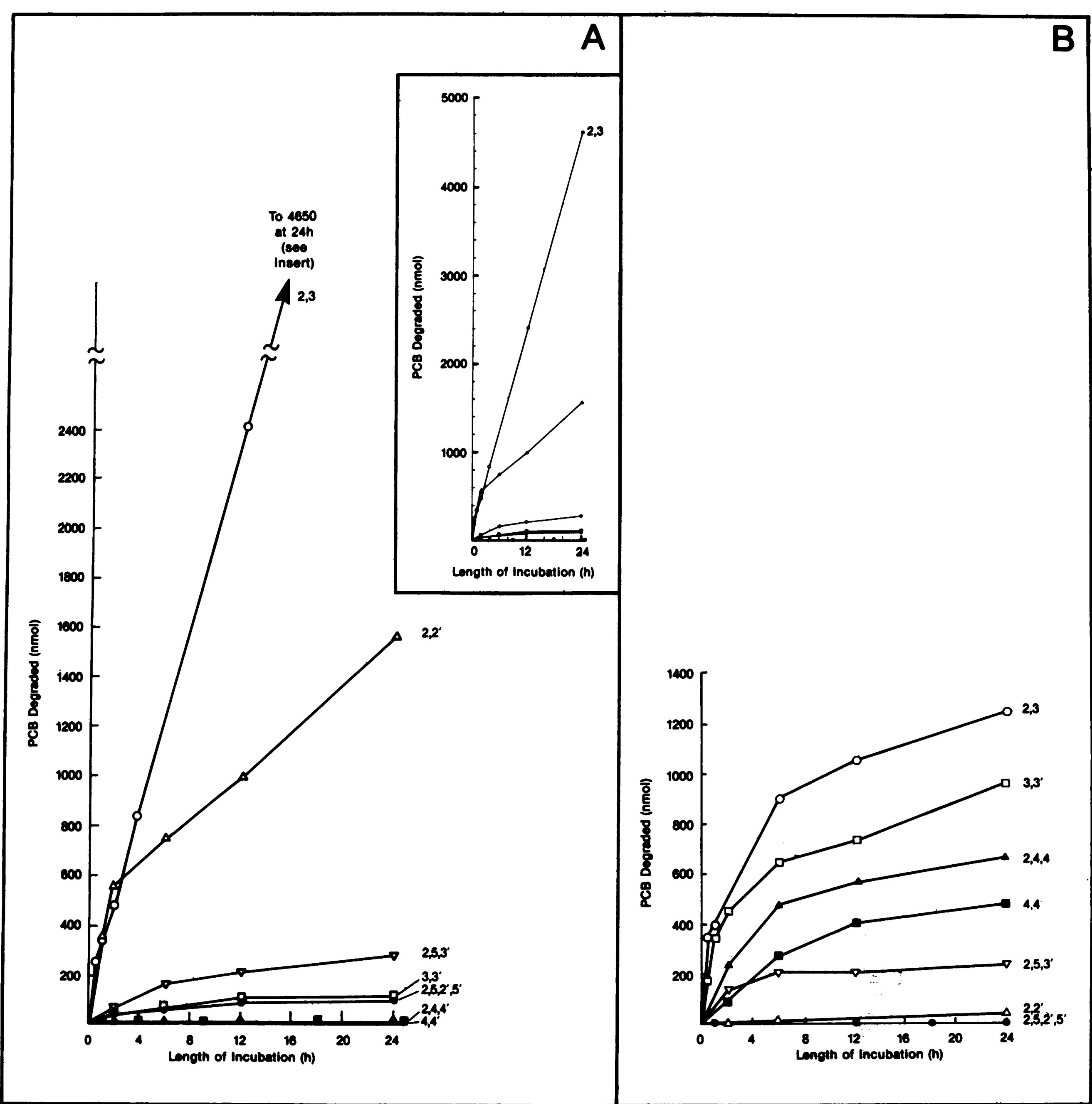

FIG. 1. Time course of oxidation of selected PCB congeners by *A. eutrophus* H850 and *Corynebacterium* sp. strain MB1. PCB congeners were incubated individually with resting cells of H850 (A) and MB1 (B) for the times indicated. Preliminary experiments were conducted with each strain to establish appropriate concentrations for each PCB congener, e.g., the concentration of 2,3-CB was 5 mM for each strain, but 2,2'-CB was assayed at 5 mM for H850 and at 250 μM for MB1. A congener that is not degradable by either organism, 3,5,3',5'-CB, was included as an internal standard to correct for possible physical losses during extraction. Samples were assayed by GC as described.

Analysis by GC-MS. Samples were analyzed by GC-mass spectrometry (MS) with a Varian 1400 GC interfaced directly to a VG Analytical ZAB 2f mass spectrometer (VG Instruments, Inc., Stamford, Conn.). Polar metabolites were derivatized with N,O-*bis*(trimethylsilyl)trifluoroacetamide (Pierce Chemical Co.) prior to analysis. Neutral metabolites were not derivatized. The separations were done on a fused silica DB-1 capillary column (30 m by 0.25 mm [inner diameter]; J & W Scientific, Inc., Folsom, Calif.) connected through a heated transfer line directly to the mass spectrometer ion source. Injections were made in the splitless mode with the detector flushed 30 s after injection. Injector and transfer line temperatures were both 270°C. The carrier gas was helium (flow rate, 25 cm/s). The initial temperature (60°C) was held for 2 min, raised to 280°C at a rate of 8°C/min, and held at 280°C. The mass spectrometer was

TABLE 2. Estimated initial rate of oxidation of selected PCB congeners by *A. eutrophus* H850 and *Corynebacterium* sp. strain MB1

PCB congener identification	Degradation rate[a] (nmol/ml per h) by:	
	H850	MB1
2,3	325	400
2,2′	350	1
3,3′	15	350
4,4′	0.5	50
2,4,4′	1	115
2,5,3′	35	80
2,5,2′,5′	15	0.4

[a] Both cultures were incubated at an optical density at 615 nm of 1.0, which corresponds to a cell concentration of approximately 3.8×10^9 cells per ml for H850 and 1.2×10^8 cells per ml for MB1.

operated in the electron ionization mode (70 eV) at a resolution of 1,000 ($m/\Delta m$, 10% valley). The source temperature was 230°C. The mass range of 30 to 600 atomic mass units was repetitively scanned at a rate of 2 s per scan.

RESULTS

Congener specificity of PCB degradation in *A. eutrophus* H850 and *Corynebacterium* sp. strain MB1. To gain a clearer understanding of the specificity of the PCB-degradative enzymes of *A. eutrophus* H850 and *Corynebacterium* sp. strain MB1, we compared their abilities to oxidize defined mixtures of PCBs at two different concentrations. The results (Table 1) indicate differences in the relative susceptibilities of the various constituent chlorophenyl rings to attack by these two organisms. Chlorophenyl rings substituted at positions 4-, 2,3-, or 3,4- were apparently oxidized more easily by MB1, while those substituted at positions 2-, 2,4-, 2,5-, or 2,4,5- were more susceptible to attack by H850.

To further distinguish the degradative capabilities of H850 and MB1, we compared the time course for the degradation of seven PCB congeners in these strains (Fig. 1) and, from this, estimated the initial rates of oxidation of these congeners (Table 2). For MB1, the shapes of the curves are all similar; a period of rapid initial oxidation was followed by a period of slower oxidation, possibly reflecting depletion of a necessary cofactor, inhibition by metabolite accumulation, or a combination of both. For H850, at least three different degradation patterns were observed: (i) 2,3-CB was degraded linearly until the substrate was exhausted; (ii) 2,2′-CB was degraded at a constant rate for 2 h, and then an abrupt decrease in the rate of degradation occurred; (iii) the remaining congeners were degraded much more slowly and with kinetics similar to those observed for MB1. There were substantial differences in the relative rates of degradation of the congeners within each strain. MB1 degraded 3,3′-CB much more rapidly than 2,5,3′-CB, but the converse was true for H850, even though both strains attack the 3-chlorophenyl ring of each of these congeners. (See Table 3 for degradation products observed in H850. Data are not shown for MB1.) The structures of 3,3′-CB and 2,5,3′-CB differ by a single *ortho* chlorine. Apparently, the *ortho* chlorine on the unattacked ring of 2,5,3′-CB makes this congener more degradable by H850 than 3,3′-CB but has the opposite effect for MB1. Indeed, MB1 barely metabolized 2,2′-CB, which has a single *ortho* chlorine on each ring, while H850 initially oxidized this congener as rapidly as 2,3-CB. H850 metabolized 2,5,2′,5′-CB at the same rate as 3,3′-CB, thus clearly demonstrating that the availability of an unchlorinated 2,3 site is not a major factor in determining whether a congener is a substrate for the PCB-degradative enzyme(s) in H850. On the other hand, the low oxidation rates of 4,4′-CB and 2,4,4′-CB by H850 indicate that 4-chlorophenyl rings are poor substrates for H850, whereas they are good substrates for MB1.

Oxidation products of selected congeners produced by H850 and MB1. In the major pathway proposed for bacterial oxidation of PCBs, a dioxygenase introduces molecular oxygen at an unchlorinated 2,3 site (or a 5,6 site), to produce a cyclic peroxide intermediate and subsequently a dihydrodiol (1, 10, 11). Subsequent dehydrogenation of the dihydrodiol and *meta*-cleavage of the ensuing dihydroxybiphenyl between carbons 1 and 2 ultimately yield chlorobenzoic acid (1, 10, 11). If MB1 metabolizes congeners via a 2,3-dioxygenase, it should attack and oxidize only chlorophenyl rings with open 2,3 sites, thereby yielding a chlorobenzoic acid product with a chlorination pattern corresponding to that of the unattacked ring. Furthermore, if H850 metabolizes PCBs by an alternate route, there should be significant differences in the metabolic products generated by these two organisms from the same PCB congener. Therefore, we examined the metabolites produced from the degradation of several PCB congeners by both H850 and MB1.

Figure 2 compares the GC profiles of the oxidation products of 4,4′-CB, 2,3,2′,5′-CB, and 2,5,2′-CB in H850 and MB1. MB1 oxidized 4,4′-CB to a single product which coeluted with the trimethylsilyl (TMS) ester of 4-chlorobenzoic acid (Fig. 2B). This chlorobenzoic acid is also the end product for the metabolism of 4,4′-CB by other PCB-degrading bacteria (1, 11, 28). However, we were unable to detect any metabolites of this congener following incubation with H850 (Fig. 2C).

Both H850 and MB1 partially metabolized 2,3,2′,5′-CB, but the oxidation products differed substantially in the two organisms (Fig. 2E and F). The metabolite peaks of MB1 included two unidentified compounds (Fig. 2E, peaks e and g) and a compound that coelutes with the TMS ester of 2,5-dichlorobenzoic acid (Fig. 2E, peak b). This suggests that MB1 attacks the 2,3-chlorophenyl ring of this congener and subsequently oxidizes and cleaves that ring to produce 2,5-dichlorobenzoic acid. Two of the metabolite peaks produced by H850 (Fig. 2F) coeluted with peaks e and g; hence some of this congener might be metabolized via the same route as in MB1. However, no 2,5-dichlorobenzoic acid was produced by H850.

More significantly, H850 produced five metabolite peaks (Fig. 2F, peaks a, c, d, f, and h) from 2,3,2′,5′-CB that were not seen for MB1. Peak c coelutes with the TMS ester of 2,3-dichlorobenzoic acid, demonstrating that H850 attacks the 2,5-chlorophenyl ring of 2,3,2′,5′-CB. Metabolite peaks d, f, and h have not yet been identified.

Metabolite a (Fig. 2F) was analyzed by GC-MS. The mass spectrum (Fig. 3A) revealed structurally significant fragments characteristic of a dichloroacetophenone. The molecular ion (m/z 188) lost a methyl group (m/z 173) and a $COCH_3$ group (m/z 145). Furthermore, the isotope distribution for the molecular ion matches the pattern for a dichlorinated compound. Subsequent comparison of metabolite a with 2′,3′- and 2′,5′-dichloroacetophenone standards by GC and GC-MS showed that the GC retention time and fragmentation pattern match those of 2′,3′-dichloroacetophenone.

The oxidation products of 2,5,2′-CB (Fig. 2H and I) also revealed differences between the two strains. Both H850 and

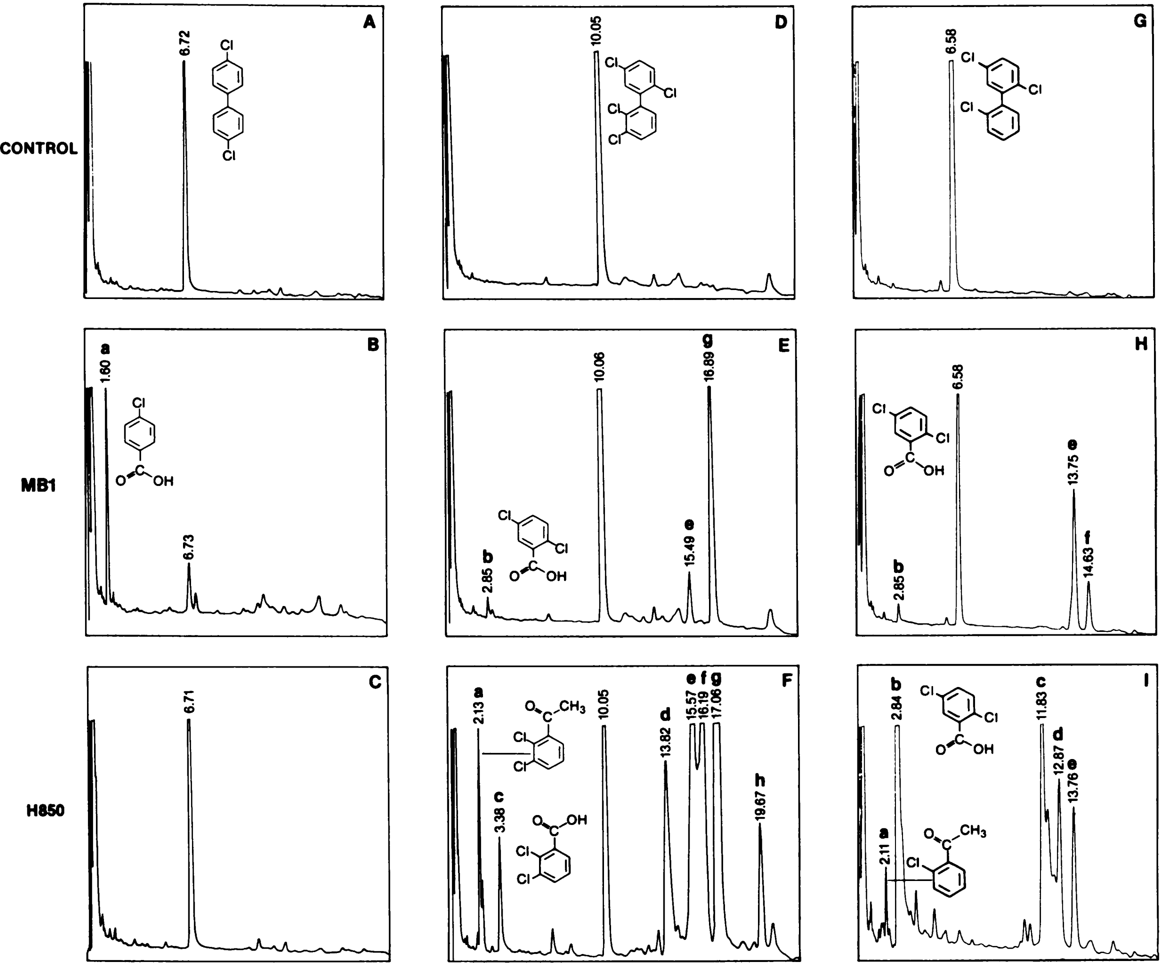

FIG. 2. GC profiles of TMS-derivatized metabolites produced from the oxidation of 4,4'-CB, 2,3,2',5'-CB, and 2,5,2'-CB by *A. eutrophus* H850 and *Corynebacterium* sp. strain MB1. Each congener (200 μM) was incubated at 30°C for 21 h with resting cells of H850 or MB1. Panels A, D, and G show the GC profile (plot of detector response versus time) of the three congeners extracted from acid-killed control cells. Panels B, E, and H show the metabolite profile after the incubation of each congener with *Corynebacterium* sp. strain MB1. Panels C, F, and I show the metabolite profiles after incubation of each congener with *A. eutrophus* H850.

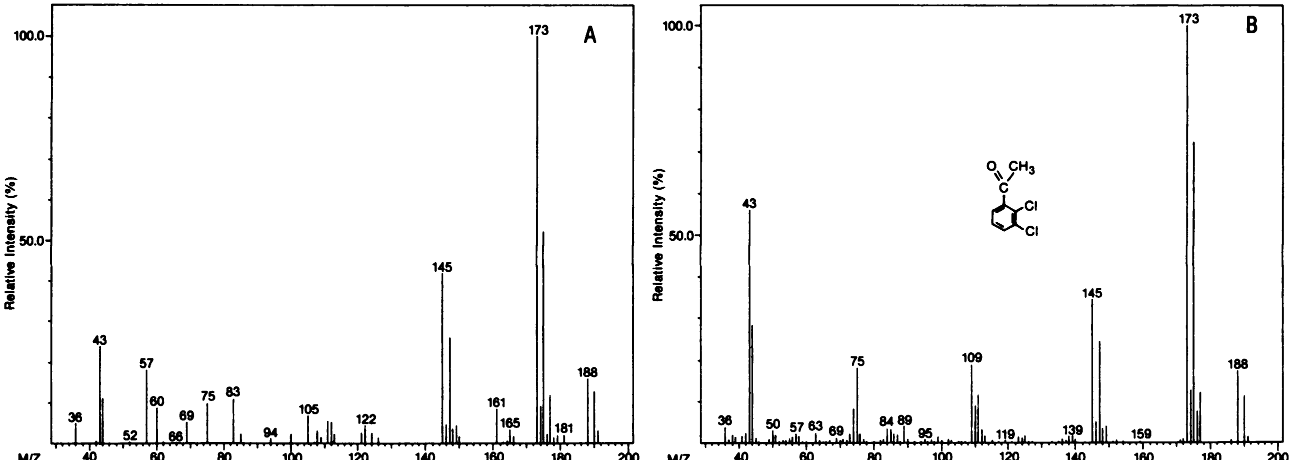

FIG. 3. Mass spectrum of the 2',3'-dichloroacetophenone metabolite produced from the oxidation of 2,3,2',5'-CB by *A. eutrophus* H850. (A) Mass spectrum of metabolite a (Fig. 2F). (B) Mass spectrum of 2',3'-dichloroacetophenone standard.

TABLE 3. Metabolism of selected PCB congeners by *A. eutrophus* H850

PCB congener identification	PCB Concn				Unidentified metabolite peaks[a]	Chlorobenzoic acid detected[b]	Chloroacetophenone detected[c]	Chlorination pattern on attacked ring
	25 μM		250 μM					
	% Degraded	Time (h)	% Degraded	Time (h)				
2,3	ND[d]		100	5	None	2,3-	None	None
2,2′	ND		100[e]	24	None	2-	None	2
2,4′	100	0.2	100	4	None	4-	None	2
3,3′	ND		85	6	None	None[f]	3′-	3
2,5,2′	ND		100	21	3	2,5-	2′-	2 and 2,5
2,5,3′	ND		70	6	4	2,5-	2′,5′-*	3
2,5,4′	100	4	75	24	3	4-	4′-	2,5
2,4,4′	100	24	<5	72	None	4-	None	2,4
2,3,2′,3′	ND		<10	48	2	None	None	2,3
2,3,2′,5′	ND		40	24	5	2,3-	2′,3′-*	2,5
2,4,5,2′,5′	95	24	25	24	2	None	2′,5′- and 2′,4′,5′-*	2,5 and 2,4,5

[a] These are generally TMS compounds. Individual peaks do not necessarily correspond to different metabolites; rather, in some cases, they may represent different states of silylation. However, the presence of one or more peaks is a clear indication of metabolism.

[b] Chlorobenzoic acids were tentatively identified by matching GC retention times with those for appropriate chlorobenzoic acid standards. The 2,5-dichlorobenzoic acid produced from 2,5,2′-trichlorobiphenyl was shown by GC-MS to be a dichlorobenzoic acid.

[c] Chloroacetophenones were identified by matching GC retention times with those of the corresponding standards. In several cases, denoted by an asterisk (*), identification was confirmed by GC-MS.

[d] ND, Not determined.

[e] This congener was assayed at a concentration of 1 mM.

[f] We have determined that 3-chlorobenzoic acid is rapidly metabolized by *A. eutrophus* H850. This may explain why no 3-chlorobenzoic acid was detected in this case.

MB1 oxidized this congener to 2,5-dichlorobenzoic acid (metabolite b), indicating that both organisms attack the 2-chlorophenyl ring of this congener. Metabolite peaks e and f (Fig. 2H) probably represent hydroxylated intermediates resulting from attack on the 2-chlorophenyl ring. H850 produced a metabolite that comigrated with peak e, but none corresponding to peak f. The metabolite profile of H850 (Fig. 2I) also revealed three additional peaks: a, c, and d. Metabolite a has been tentatively identified as 2′-chloroacetophenone on the basis of comparison of retention time with that of a standard. Thus H850 apparently degrades both rings of this congener. Metabolite peaks c and d may therefore represent intermediate metabolites resulting from attack either on the 2,5-chlorophenyl ring or at a novel position on the 2-chlorophenyl ring.

Table 3 summarizes the results obtained from the metabolism of several additional congeners by H850. The data further illustrate the unusual specificity of the PCB-degradative system in H850. Notably, 2,4,4′-CB and 2,5,4′-CB were both attacked on the dichlorophenyl ring rather than the monochlorophenyl ring, and a pentachlorobiphenyl, 2,4,5,2′,5′-CB, was attacked on both rings and was degraded more extensively than 2,4,4′-CB or 2,3,2′,3′-CB. These data indicate that H850 attacks 2,5-chlorophenyl rings in preference to rings chlorinated at the 4-, 2,3-, or 2,4- positions.

Significantly, ring-chlorinated acetophenones were produced from the oxidation of 6 of the 11 congeners examined but only from attack on rings chlorinated at position(s) 3-, 2,5-, or 2,4,5-.

Metabolism of 2,4,5,2′,4′,5′-CB to 2′,4′,5′-trichloroacetophenone. The congener 2,4,5,2′,4′,5′-CB is of interest not only because it is a highly chlorinated biphenyl, but particularly because it lacks vicinal unchlorinated carbon atoms. We expected that the lack of adjacent unchlorinated positions would preclude attack by bacterial dioxygenase enzymes, which attach one atom of oxygen to each of two adjacent unchlorinated atoms. However, our defined congener assays often showed evidence that a small amount of this congener was being degraded by H850 (Table 1). To determine whether this was the case, we incubated H850 cells with 2,4,5,2′,4′,5′-CB (5 μM) for 72 h. During the incubation, 93% of the 2,4,5,2′,4′,5′-CB was oxidized and a single metabolite was seen (Fig. 4A and B). Its mass spectrum (Fig. 4D) revealed the fragmentation pattern predicted for a trichloroacetophenone: the isotope distribution for the molecular ion (*m/z* 222) was consistent with that of a molecule containing three chlorines, and the structurally significant acetophenone peaks corresponding to the loss of a methyl group (*m/z* 207) and a $COCH_3$ group (*m/z* 179) were both present. Subsequent comparison of the metabolite with a 2′,4′,5′-trichloroacetophenone standard by GC retention time and GC-MS (Fig. 4C and E) confirmed that the metabolite was indeed 2′,4′,5′-trichloroacetophenone. Approximately 0.28 nmol of trichloroacetophenone was detected from the oxidation of 4.6 nmol of 2,4,5,2′,4′,5′-CB, indicating that at least 6% of this congener was metabolized to 2′,4′,5′-trichloroacetophenone. However, since the chloroacetophenone was subsequently metabolized, this represents a minimal estimate.

Effect of a 2,5-chlorophenyl ring on the degradation of PCBs. Preliminary defined congener assays had indicated that 2,6,2′,5′-CB and 2,4,6,2′,5′-CB were oxidized more rapidly than 2,6-CB and 2,4,6-CB, respectively (5). This finding was particularly remarkable for the following two reasons: (i) the biodegradability of PCB congeners is generally inversely related to the number of chlorine substituents (2, 11, 12); and (ii) PCB congeners with chlorines at positions 2,6 are unusually recalcitrant to biodegradation (11). Therefore, we have pursued this finding by comparing nine pairs of PCB congeners. Within each pair, the chlorination pattern on the first ring was the same, while the second ring was either unchlorinated or chlorinated at carbons 2′ and 5′. Congeners with an unchlorinated ring were metabolized

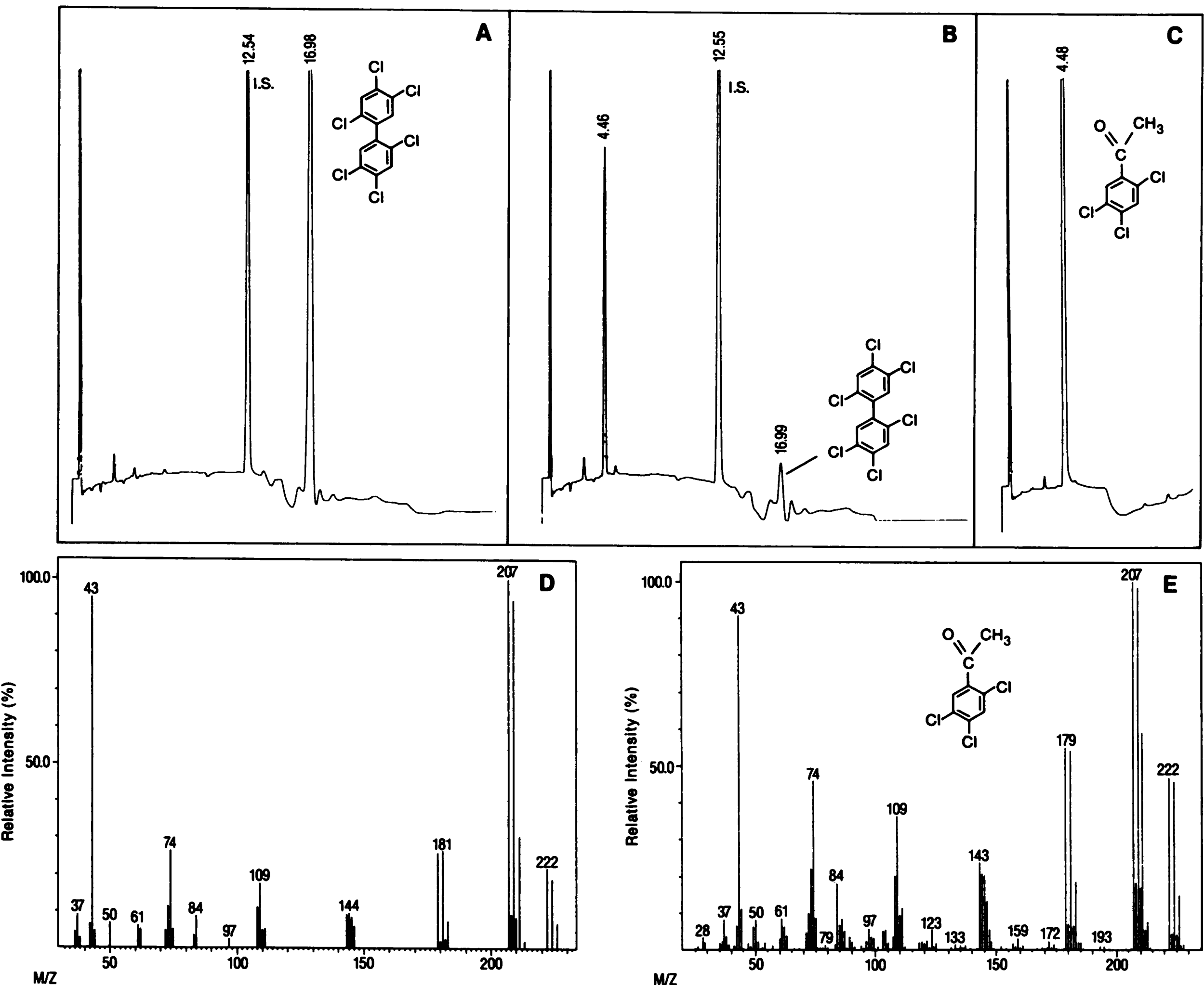

FIG. 4. Metabolism of 2,4,5,2′,4′,5′-hexachlorobiphenyl to 2′,4′,5′-trichloroacetophenone. (A) GC profile (plot of detector versus time) of extract from heat-killed cells incubated with 2,4,5,2′,4′,5′-CB and an internal standard (3,5,3′,5′-CB). (B) GC profile of extract from resting cells of *A. eutrophus* H850 incubated at 30°C for 72 h with 2,4,5,2′,4′,5′-CB (5 μM) and an internal standard (3,5,3′,5′-CB). (C) GC profile of 2′,4′,5′-trichloroacetophenone standard. (D) Mass spectrum of the metabolite eluting at 4.46 min in panel B. (E) Mass spectrum of 2′,4′,5′-trichloroacetophenone standard.

more rapidly as long as the first ring was not chlorinated in both *ortho* positions, i.e., positions 2,6. An example is shown in Fig. 5A. However, when the first ring was chlorinated in both *ortho* positions the congener chlorinated at positions 2′,5′ was oxidized more rapidly, even in the case of a heptachlorobiphenyl (Fig. 5B to E). Subsequent experiments established that several di-*ortho*-substituted congeners chlorinated at position 3′ (which is equivalent to 5′) were also degraded more rapidly than the corresponding congeners in which the second ring was unsubstituted (e.g., 2,6,3′-CB versus 2,6-CB, 2,4,6,3′-CB versus 2,4,6-CB, and 2,3,5,6,3′-CB versus 2,3,5,6-CB). Furthermore, although 2,6,2′,6′-CB was not significantly degraded, a congener with an additional *meta* chlorine on each ring, 2,3,6,2′,3′,6′-CB (which is equivalent to 2,5,6,2′,5′,6′-CB) was oxidized by H850 (Fig. 5F).

DISCUSSION

The principal route of PCB degradation in most procaryotes appears to involve 2,3-dioxygenase attack at an unsubstituted ring or at chlorophenyl rings that have at least one pair of adjacent unchlorinated carbons at positions 2,3 (or 5,6). Furthermore, Furukawa and co-workers have established five additional correlations between PCB structure and biodegradability on the basis of their studies of two strains of bacteria that oxidize PCBs via a 2,3-dioxygenase, *Acinetobacter* sp. strain P6, and *Alcaligenes* sp. strain Y42 (10, 12). (i) Biodegradability decreases as chlorine substitution increases. (ii) Congeners with two *ortho* chlorines are extremely resistant to degradation. (iii) PCBs containing an unsubstituted ring are generally degraded more rapidly than isomers that are chlorinated on both rings. (iv) Tetra- and pentachlorobiphenyls containing a 2,3-chlorophenyl ring are more susceptible to biodegradation than other tetra- and pentachlorobiphenyls. (v) Ring fission generally occurs on the unchlorinated or less chlorinated ring of a congener.

These generalizations are also valid for *Corynebacterium* sp. strain MB1 and for many other PCB-degrading bacteria (5, 10, 23). However, the congener selectivity pattern of H850 differs substantially from that of most other bacteria

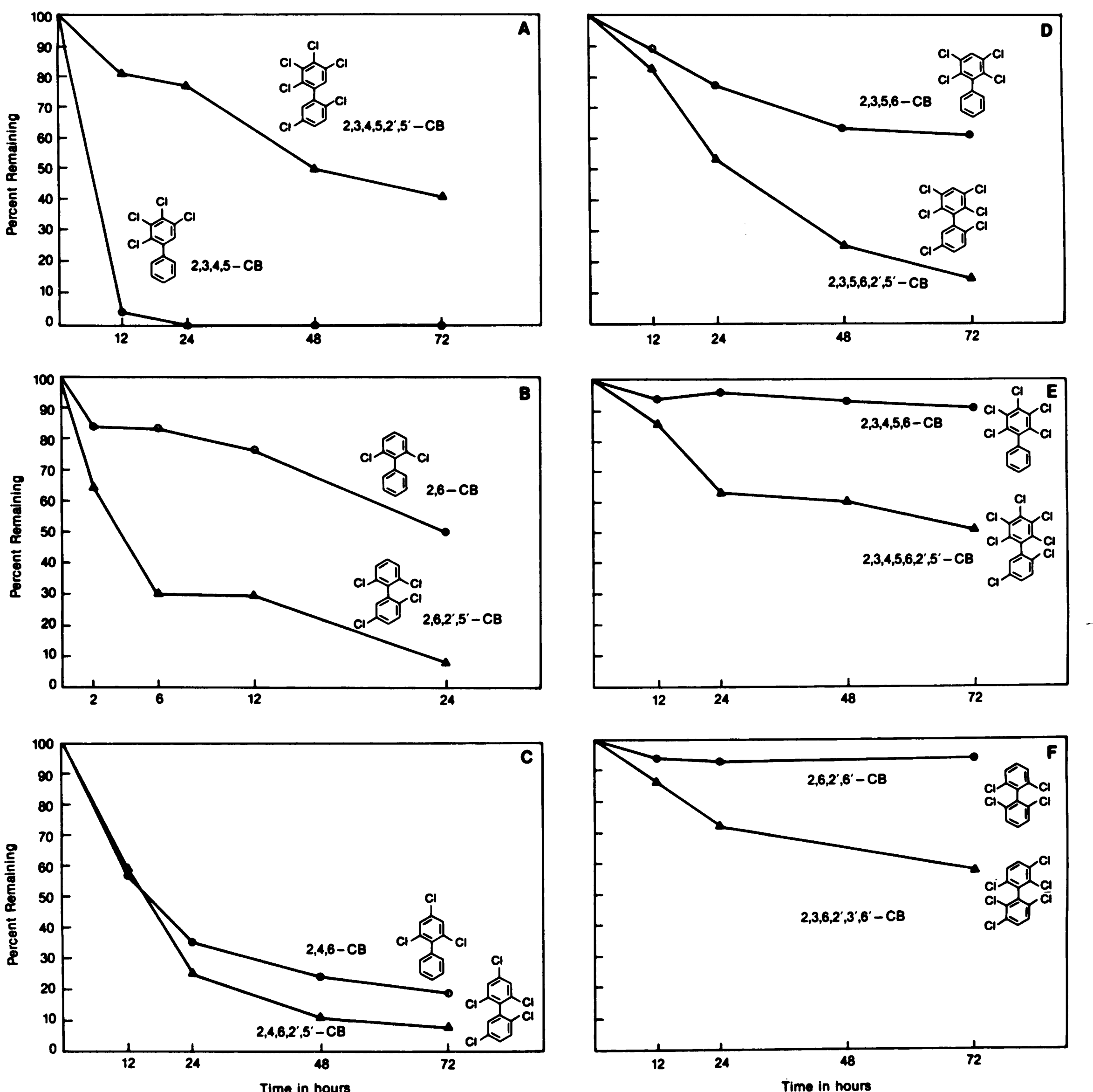

FIG. 5. Effect of a 2,5-chlorophenyl substituent on the degradation of PCBs. Ten pairs of congeners were incubated with resting cells of *A. eutrophus* H850 in the presence of an internal standard (3,5,3',5'-CB). All congeners were assayed at 5 μM except 2,6,2',6'-CB and 2,3,6,2',3',6'-CB, which were assayed at 2.5 μM, and 2,3,4,5,6-CB and 2,3,4,5,6,2',5'-CB, which were assayed at 1.25 μM. In addition to the six pairs of congeners shown, we compared the degradation of four pairs of congeners which contained a 2,3-, 2,4-, 2,3,4-, or 2,4,5-chlorophenyl substituent joined to either a phenyl ring or a 2,5-chlorophenyl ring. In each of these cases, the congener containing an unchlorinated ring was degraded more rapidly than the one containing a 2,5-chlorophenyl ring, as was seen for 2,3,4,5-CB and 2,3,4,5,2',5'-CB (panel A).

(Fig. 1; Tables 1 to 3). These results are consistent with the interpretation that H850 uses a novel mechanism of PCB degradation, possibly a 3,4-dioxygenase. This hypothesis is further supported by the fact that only one of Furukawa's correlations of PCB structure and biodegradability [correlation (iii)] is valid for *A. eutrophus* H850, while the remaining four do not apply for the following reasons. (i) Tetra- and pentachlorobiphenyls containing a 2,5-chlorophenyl ring were degraded more extensively by H850 than lower congeners chlorinated at both *para* positions, i.e., positions 4,4'. (ii) H850 rapidly degraded many di-*ortho*-substituted congeners, including 2,2'-CB, 2,3,6-CB (5), 2,5,2'-CB, 2,5,2',5'-CB, and 2,6,2',5'-CB, and several tri- and tetra-*ortho*-substituted congeners such as 2,4,6,2',5'-CB, 2,3,5,6,2',5'-CB, and 2,3,6,2',3',6'-CB. (iv) Highly chlorinated biphenyls containing a 2,3-chlorophenyl group were less susceptible to degradation by H850 than were those containing a 2,5-chlorophenyl ring (Table 1) (6). (v) H850 degraded both 2,4,4'-CB and 2,5,4'-CB via attack and ring fission of the dichlorinated ring to yield 4-chlorobenzoic acid.

A 2,3-biphenyl dioxygenase pathway has recently been demonstrated for *A. eutrophus* H850 (M. Schocken, L. Nadim, and D. T. Gibson, personal communication; F. J. Mondello, personal communication) and is probably responsible for the oxidation of some PCB congeners in this organism. However, both the unusual congener selectivity

of H850 and the production of novel metabolites suggest that additional enzymes are involved in the degradation of many PCB congeners by this organism.

On the basis of congener selectivity, we proposed previously that a significant route of PCB metabolism in H850 involves a 3,4-dioxygenase (4; D. L. Bedard and M. J. Brennan, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, Q51, p. 213). The first detectable product of such an enzyme would be a 3,4-*cis*-dihydrodiol. Thus, the recent identification of *cis*-3,4-dihydro-*cis*-3,4-dihydroxy-2,5,2′,5′-tetrachlorobiphenyl as a major metabolite of 2,5,2′,5′-CB in H850 (Schocken et al., personal communication) supports our hypothesis.

Additional support for 3,4-dioxygenase activity comes from our observations (Fig. 5) concerning the effect of a 2,5-chlorophenyl ring on PCB oxidation. One explanation for these results is illustrated in Fig. 6. According to this model, congeners containing an unchlorinated ring are degraded by 2,3-dioxygenase attack on that ring. However, when both *ortho* positions on the opposite ring are chlorinated, attack by the 2,3-dioxygenase is sterically hindered by the *ortho* chlorines. The more rapid degradation of di-*ortho*-substituted congeners containing a 2,5- or a 3-chlorophenyl ring suggests that these congeners may be degraded by an enzyme capable of oxidation at carbon positions 3,4 (or 4,5) and that this enzyme may have greater affinity for a 2,5- or a 3-chlorophenyl ring than for an unsubstituted ring.

PCB metabolites that may result from dioxygenase attack at the 3,4 position have been reported for several bacteria (11, 19, 29). However, in each of these cases, the major route of PCB metabolism appeared to be via a 2,3-dioxygenase pathway. The relative contributions of the 2,3- and 3,4-dioxygenase activities to PCB degradation in *A. eutrophus* H850 have not yet been clearly defined. Furthermore, we do not know whether these activities represent separate enzymes or a single enzyme with broad substrate specificity. Experiments with cell extracts and with mutants blocked at various steps in the 2,3-dioxygenase pathway are now in progress and should aid us in determining the nature of the enzyme(s) involved.

X = Cl or H

FIG. 6. Model proposed to explain why 2,6-substituted congeners containing a 2,5-chlorophenyl ring are degraded more rapidly by H850 than those containing an unchlorinated ring. Top: The relative susceptibilities of an unchlorinated ring and a 2,5-chlorophenyl ring to H850 attack at positions 2,3 (or 5,6) and 3,4 (or 4,5), explaining the more rapid degradation of congeners containing an unchlorinated ring. Bottom: The relative susceptibilities of an unchlorinated ring and a 2,5-chlorophenyl ring in a 2′,6′-substituted PCB to H850 attack at positions 2,3 and 3,4. The chlorines at positions 2′,6′ on the unattacked ring sterically block attack at position 2,3 (or 5,6) but permit attack at position 3,4 (or 4,5). Chlorines at position 2,5 enhance oxidation at position 3,4.

The oxidation of 2,4,5,2′,4′,5′-hexachlorobiphenyl also indicates novel enzymatic activity. This highly recalcitrant congener can also be degraded by *Pseudomonas putida* LB400, a recently described strain with a range of PCB congener selectivity that closely resembles that of H850 (5, 7). No early metabolites of this congener have been observed in H850, but in LB400, hydroxylated hexachlorobiphenyls were identified by GC-MS (7). There are at least four possible mechanisms for the oxidation of this congener. The first, dioxygenase attack at a chlorinated site of an aromatic ring, has never been demonstrated. However, since there is evidence for 2,3- and 3,4-dioxygenase activities in both H850 and LB400, it is possible that one of these enzyme activities is capable of attacking a chlorinated carbon. A second possibility involves monooxygenase metabolism via an epoxide. This is a common route of metabolism for a variety of aromatic compounds in eucaryotes (15), including 4,4′-CB (22) and 2,5,2′,5′-CB (9), and is believed to be responsible for the oxidation of 2,4,5,2′,4′,5′-CB in rabbits (26). This mechanism of oxidation also occurs in procaryotes (8), albeit rarely. A third possible mechanism of oxidation of 2,4,5,2′,4′,5′-CB involves non-arene oxide hydroxylation by a monooxygenase, most probably by direct insertion of an oxygen atom into a carbon-hydrogen bond (27). This mechanism has been proposed for the *meta*-hydroxylation of 2,5,2′,5′-CB (20) and of chlorobenzene (24, 25) in the rat. The fourth possibility, the direct replacement of a chlorine by a hydroxyl group, has been demonstrated for 3-chlorobenzoic acid in a *Pseudomonas* sp. (16) and for 4-chlorobenzoic acid in *Arthrobacter* (18, 21, 30) and *Nocardia* (17) spp. In at least one case, the hydroxyl group was derived from water, not molecular oxygen, and the displacement was catalyzed by a novel aromatic hydroxylase, not a mixed-function oxidase (18).

It will be necessary to isolate and characterize early metabolites of 2,4,5,2′,4′,5′-CB to determine whether any of the above mechanisms is responsible for the oxidation of this congener in H850. In any case, the identification of 2′,4′,5′-trichloroacetophenone as a metabolite of 2,4,5,2′,4′,5′-CB clearly demonstrates that H850 harbors all of the enzymes necessary to oxidize and cleave a 2,4,5-chlorophenyl ring.

Our discovery of ring-chlorinated acetophenones among the metabolites of PCBs having 3-, 2,5-, or 2,4,5-chlorophenyl groups was unexpected. Baxter and Sutherland reported that chloroacetophenones can be formed by photolysis of the yellow ring-fission products [hydroxyoxo(chlorophenyl)chlorohexadienoic acids] that accumulate following oxidation of some PCB congeners (3). However, we consistently identified chloroacetophenones, but no yellow metabolites, from the oxidation of 3,3′-CB, 2,5,2′-CB, 2,3,2′,5′-CB, 2,4,5,2′,5′-CB, and 2,4,5,2′,4′,5′-CB. Furthermore, comparable amounts of 2′,4′,5′-chloroacetophenone were produced in parallel oxidations of 2,4,5,2′,4′,5′-CB conducted under normal laboratory light or in the absence of light. Thus, the ring-chlorinated acetophenones produced by *A. eutrophus* H850 do represent products of microbial metabolism.

It is unclear how much PCB is oxidized via chloroacetophenones, because these metabolites are further metabo-

lized. Both chloroacetophenones and chlorobenzoic acids are produced from the oxidation of several PCB congeners (Table 3). One goal of our research is to determine whether they arise by independent pathways or whether the chlorobenzoic acid might arise from metabolism of the chloroacetophenone (13, 14). Experiments to elucidate the biochemical mechanisms by which chloroacetophenones are formed and metabolized in H850 and to determine how much PCB is oxidized via this route are currently under way.

In summary, PCB degradation in *A. eutrophus* H850 differs substantially from that seen in most other bacterial systems that have been studied, but bears a striking resemblance to that observed in the recently described *P. putida* LB400 (5, 7). *A. eutrophus* H850 and *P. putida* LB400 have a much broader range of PCB-degradative ability than does any other PCB-degrading organism reported to date. It is apparent from available information on PCB congener selectivity and routes of metabolism in these and other PCB-degrading organisms (5–7, 11, 12, 19, 29) that bacteria have developed various mechanisms for metabolizing PCBs and have much greater abilities to degrade PCBs than was previously expected. This information should make it possible to develop effective processes for the biodegradation of PCBs.

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