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A Novel Transformation of Polychlorinated Biphenyls by *Rhodococcus* sp. Strain RHA1

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We have characterized a biphenyl degrader, *Rhodococcus* sp. strain RHA1. Biphenyl-grown cells of strain RHA1 efficiently transformed 45 components in the 62 major peaks of a polychlorinated biphenyl (PCB) mixture of Kanechlors 200, 300, 400, and 500 within 3 days, which includes mono- to octachlorobiphenyls. Among the intermediate metabolites of PCB transformation, di- and trichlorobenzoic acids were identified. The gradual decrease of these chlorobenzoic acids during incubation indicated that these chlorobenzoic acids would also be degraded by this strain. The effect of the position of chlorine substitution was determined by using PCB mixtures that have chlorine substitutions mainly at either the *ortho* or the *meta* position. This strain transformed both types of congeners, and strong PCB transformation activity of RHA1 was indicated. RHA1 accumulated 4-chlorobenzoic acid temporally during the transformation of 4-chlorobiphenyl. The release of most chloride in the course of 2,2′-dichlorobiphenyl degradation was observed. These results suggested that RHA1 would break down at least some PCB congeners into smaller molecules to a considerable extent.

Polychlorinated biphenyls (PCBs) are synthetic chemicals that were used widely for industrial purposes. Because of their chemical stability, PCBs were spread over many industries. PCBs are also highly recalcitrant to biodegradation, and these chemicals are persistent in the environment for a long time. PCBs in the environment cause serious problems as pollutants.

The first report about the biodegradation of PCBs was published by Ahmed and Focht in 1973 (2). After this investigation, a large number of microorganisms that can degrade PCBs were isolated from soil (4, 5, 8, 10, 14, 17, 21). Microorganisms which are able to grow on biphenyl usually can cometabolize various PCB congeners (3, 7, 9, 15). A common pathway of PCB degradation in aerobic bacteria begins with the attack of biphenyl dioxygenase on an unsubstituted 2,3 position of a biphenyl ring. The dihydroxy metabolites are transformed through *meta*-cleavage products, and chlorobenzoic acid is produced. Interestingly, Bedard et al. described a novel 3,4-dioxygenase attack on PCB congeners by *Alcaligenes eutrophus* H850 (3), and other reports also suggest that some of the metabolites of PCB degradation are produced through an attack at the 3,4 position (17, 21). The aerobic degradation of PCBs is generally limited to the congeners that have five or less chlorines (4, 9, 17), and research on PCB-degrading microorganisms has been rather concentrated on gram-negative bacteria. Recently, novel characteristics of biphenyl/PCB degradation genes in a gram-positive PCB degrader, *Rhodococcus* sp. strain RHA1, were described (16). Thus the detailed study on a PCB degradation activity of RHA1 in combination with genetic characterization would provide important information to address the genetic features that provide strong PCB transformation activity and for the breeding of microbial PCB degradation activity.

In this paper, we carried out congener-specific PCB transformation analysis and characterized the intermediate metabolites of chlorobiphenyls. The results obtained indicated a strong and unique PCB transformation activity of RHA1.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The isolated strain was identified as a gram-positive bacterium, *Rhodococcus* sp., by the National Collections of Industrial and Marine Bacteria Limited (Aberdeen, United Kingdom). Strain RHA1 was a gram-positive, nonsporulating, nonmotile microorganism and showed dual cell morphology of rods and cocci. The cell wall contained *meso*-diaminopimelic acid and mycolic acids. From these criteria and other features, including assimilation characteristics and fatty acid profiles, the strain RHA1 was assigned to the genus *Rhodococcus* (details will be described elsewhere). This strain, *Rhodococcus* sp. strain RHA1, was isolated from a soil sample that was contaminated by an insecticide, γ-hexachlorocyclohexane by repeated transfer to a minimal salt medium (W medium) containing biphenyl as a sole carbon source. The minimal salt medium was composed of KH_2PO_4 (1.7 g/liter), Na_2HPO_4 (9.8 g/liter), $(NH_4)_2SO_4$ (1.0 g/liter), $MgSO_4 \cdot 7H_2O$ (0.1 g/liter), $FeSO_4 \cdot 7H_2O$ (0.95 mg/liter), MgO (10.75 mg/liter), $CaCO_3$ (2.0 mg/liter), $ZnSO_4 \cdot 7H_2O$ (1.44 mg/liter), $CuSO_4 \cdot 5H_2O$ (0.25 mg/liter), $CoSO_4 \cdot 7H_2O$ (0.28 mg/liter), H_3BO_4 (0.06 mg/liter), and HCl (51.3 μl/liter).

Chemicals. 4-Chlorobiphenyl was purchased from Nacalai Tesque Co., Ltd., Kyoto, Japan. The PCB congeners and Kanechlors 200, 300, 400, and 500 were obtained from GL Sciences Inc., Tokyo, Japan. PCB 48 was purchased from Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan. All other chemicals were obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan.

Analysis of PCB degradation competence. Cells were grown on W-biphenyl medium and transferred to fresh W-biphenyl medium at an optical density at 600 nm of 0.4. Ethylacetate solutions containing PCB 48 (which is an equivalent to Aroclor 1248); mixtures of Kanechlors 200, 300, 400, and 500; or recombined mixtures of PCB congeners were added to the cell suspensions. Total PCB concentration was adjusted to 10 μg/ml. Control cells were inactivated by autoclaving at 121°C for 15 min prior to the addition of PCBs. Cells were incubated in separate tubes for three days at 30°C in a reciprocal shaker. Cultures were acidified by the addition of hydrochloric acid at a final concentration of 2.0%. A quarter volume of ethylacetate was added and mixed for 5 min, and the mixture was centrifuged at 4,000 × *g* for 15 min. Extraction was repeated twice. The solvent layer was concentrated 20-fold by evaporation and subjected to gas chromatography-mass spectrometry (model 5971A, Hewlett Packard Co., Palo Alto, Calif.) by using a Ultra-2 capillary column (50 m by 0.2 mm [inner diameter], an SE-54 equivalent, Hewlett Packard Co.). Analytical procedure of PCB transformation activity was essentially that of Quensen et al. (18). The PCB-congener-specific ions were collected to provide the selected ion chromatograms

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TABLE 1. Analysis of the degradation of Kanechlor 200, 300, 400, 500 mixture and PCB 48 by *Rhodococcus* sp. strain RHA1

Substitution no.	Peak no.	Congener identification	% Degradation[a] (SD)			
			RHA1		P6 (Aroclor 1254)[b]	H850 (Aroclor 1254)[c]
			PCB 48	KC mix[d]		
1,2	2	4		100 (0)		
	3	2,2′, 2,6		100 (0)		
	4	2,4, 2,5		100 (0)		
	5	2,3′		100 (0)		
	6	2,4′, 2,3	100 (0)	100 (0)		
3,4	7	2,6,2′	97 (3)	80 (19)		
	9	2,5,2′, 4,4′	100 (0)	100 (0)		
	10	2,4,2′	100 (0)	100 (0)		
	11	2,6,3′, 2,3,6	100 (0)	100 (0)		
	12	2,3,2′, 2,6,4′	100 (0)	100 (0)		
	14	2,4,5		100 (0)		
	15	2,5,3′	100 (0)	100 (0)		
	16	2,4,3′	100 (0)	100 (0)		
	17	2,5,4′	100 (0)	100 (0)		100
	18	2,4,4′, (2,4,6,2′)	100 (0)	98 (3)		45
	19	3,4,2′, 2,3,4, 2,3,3′, 2,5,2′,6′	93 (4)	93 (2)		100
	20	2,3,4′, (2,4,2′,6′)	93 (4)	93 (1)		
4,5	21	2,3,6,2′	69 (9)	—		
	22	2,3,2′,6′	43 (13)	—		
	23	2,5,2′,5′, 2,6,3′,5′	92 (4)	51 (29)	77	100
	24	2,4,2′,5′	97 (1)	67 (23)	49	95
	25, 26	2,4,2′,4′, 2,4,5,2′, 2,4,6,4′	95 (3)	67 (17)		
	28	2,3,2′,5′	100 (0)	100 (0)	100	100
	29	2,3,2′,4′, 2,3,6,3′, 3,4,4′	97 (4)	93 (9)		
	30	2,6,3′,4′, 2,3,4,2′, 2,3,6,4′, 2,5,3′,5′	62 (4)	31 (13)	21	15
	32	2,3,2′,3′	100 (0)	93 (10)	88	
	33	2,3,5,3′, 2,4,5,3′, 2,4,6,2′,4′, 2,4,6,2′,5′	100 (0)	85 (20)		
	34	2,3,3′,5′, 2,3,5,4′	100 (0)	73 (33)		
	35	2,4,5,4′, (2,3,5,2′,6′)	97 (2)	73 (22)	99	—
	36	2,5,3′,4′, 3,4,5,2′	99 (1)	93 (10)	100	95
5,6	37	2,3,6,2′,5′ 2,4,5,2′,6′ 2,4,3′,4′	82 (1)	52 (10)	63	50
	38	2,3,4,3′ 2,3,6,2′,4′	42 (15)	—	12	—
	39	2,3,3′,4′ 2,3,4,4′ (2,3,6,2′,3′, 2,3,5,2′,5′)	98 (0)	81 (14)	80	20
	40	2,4,5,2′,5′, 2,3,5,2′,4′	61 (6)	22 (24)	60	85
	41	2,4,5,2′,4′	90 (4)	56 (27)	—	20
	42	2,3,6,2′,4′,6′, 2,3,5,6,3′, 2,4,6,3′,4′	97 (4)	78 (22)		
	43	2,4,5,2′,3′, 2,3,4,5,2′, (2,3,5,6,2′,6′)	99 (1)	92 (9)	99	25
	44	2,3,4,2′,5′, 2,3,4,6,4′, 2,3,5,3′,5′	74 (4)	41 (29)	—	60
	45,46	2,3,4,2′,4′	80 (6)	22 (24)	—	—
	47	3,4,3′,4′, 2,3,6,3′,4′	26 (11)	—	—	20
6,7	48	2,3,5,6,2′,5′	94 (2)	27 (9)	—	40
	49	2,3,5,2′,3′,6′, 3,4,5,2′,5′, 2,3,4,6,2′,5′	68 (11)	—		15
	50	2,4,5,3′,4′, 2,3,6,2′,4′,5′, 2,3,4,5,3′	36 (29)	—	75	—
	51	2,3,5,6,2′,3′, 2,3,4,5,2′,6′, 2,3,4,5,4′		29 (10)	82	—
	52, 53	3,4,5,2′,3′, 2,3,4,6,2′,3′, 2,3,5,3′,5′, 2,3,5,2′,4′,5′, 2,3,4,6,3′,5′	82 (7)	30 (14)	24	25
	54	2,4,5,2′,4′,5′	34 (34)	—	—	20
	55	2,3,4,2′,3′,6′, 2,3,4,3′,4′	—	—	71	
	56	2,3,4,5,2′,5′	38 (20)	—	—	20
	57	2,3,5,6,2′,3′,6′		24 (14)		
	58	2,3,4,5,2′,4′		25 (16)	—	—
	59	2,3,4,6,2′,3′,6′		—		
	60, 61	2,3,4,2′,4′,5′, 2,3,5,6,3′,4′, 2,3,4,6,3′,4′		—	—	—
7, 8	62	2,3,5,6,2′,3′,5′		62 (13)		
	64	2,3,5,6,2′,4′,5′, 2,3,4,5,2′,4′,6′		—		—
	65	2,3,4,6,2′,4′,5′		30 (7)		
	66	2,4,5,3′,4′,5′		—		—
	68	2,3,4,5,2′,3′,6′, 2,3,4,5,6,2′,4′		—		—
	69	2,3,5,6,2′,3′,4′		21 (17)		—
	70	2,3,4,6,2′,3′,4′, 2,3,4,5,3′,4′, 2,3,5,6,2′,3′,5′,6′		—	13	—
	71, 72	2,3,4,5,6,2′,3′, 2,3,4,6,2′,3′,5′,6′, 2,3,4,5,6,2′,4′,6′ 2,3,4,5,2′,3′,5′, 2,3,4,5,6,3′,5′		21 (16)	—	—
	73	2,3,4,5,2′,4′,5′		—	—	—
	77	2,3,4,5,2′,3′,4′		—		—

[a] Data values are averages of quadruple experiments. —, degradation less than 20% (not considered significant).
[b] The result on *R. globerulus* P6 (formally designated *Acinetobacter* sp. P6) was reported by Kohler et al. (15). Cells were incubated for 6 days in minimal salt medium containing 10 μg of Aroclor 1254 per ml and biphenyl.
[c] The result on *A. eutrophus* H850 was reported by Bedard et al. (5). Cells grown on biphenyl were incubated for 3 days in the phosphate buffer containing 10 μg of Aroclor 1254 per ml.
[d] Kanechlor 200, 300, 400, 500 mixture (1:1:1:1).

TABLE 2. Mass spectra of intermediate metabolites[a]

Metabolite[b]	GC retention time (min)	Mass spectrum *m/z* (relative intensity)
I (2,4-dichlorobenzoate/2,5-dichlorobenzoate)	17.33	73 (16), 75 (14), 109 (17), 145 (34), 147 (29), 173 (75), 175 (51), 247 (100), 249 (71), 262 (9), 264 (6)
II (3,4-dichlorobenzoate)	17.98	73 (8), 75 (12), 109 (17), 145 (37), 147 (26), 173 (58), 175 (37), 247 (100), 249 (68), 262 (7), 264 (5)
III (2,3-dichlorobenzoate)	18.35	73 (15), 75 (13), 109 (15), 145 (31), 147 (23), 173 (71), 175 (47), 247 (100), 249 (69), 262 (7), 264 (5)
IV (trichlorobenzoate)	22.06	73 (43), 75 (10), 109 (9), 144 (10), 179 (29), 181 (27), 207 (61), 209 (58), 281 (100), 283 (95), 296 (10), 298 (8)
V (trichlorobenzoate)	23.61	73 (24), 75 (8), 109 (9), 144 (10), 179 (31), 181 (30), 207 (68), 209 (67), 281 (96), 283 (100), 296 (6), 298 (7)

[a] Products were identified from the *Rhodococcus* sp. strain RHA1 metabolism of PCB48. Cultures were incubated and extracted, and products were analyzed as described in the text.

[b] Metabolites I, II and III were assigned according to the GC retention times and the mass spectra of the respective authentic compounds. Metabolites IV and V were specified on the basis of the mass spectrum of authentic 2,4,6-trichlorobenzoate.

of PCBs. A library search was performed by the G1030A chemistation program (Hewlett Packard Co.) with the Wiley mass spectrum library (John Wiley & Sons, Inc., New York, N.Y.). Transformation percentages of PCB congeners were calculated by comparing each peak area with a control that was obtained by heat-inactivated cells. The whole PCB transformation experiment, including culturing the cells in the medium containing PCB, extraction, and gas chromatography-mass spectrometry, was repeated several times as indicated in the table footnotes.

Analysis of the metabolites. Cells grown on W-biphenyl medium were washed and resuspended in fresh W medium. After the substrate addition, the cell suspension was incubated at 30°C. The samples drawn periodically were extracted as described above. To generate trimethylsilyl (TMS) derivatives, the samples were treated with TMSI-H (hexamethyldisilazane:trimethylchlorosilane: pyridine [2:1:10]). The resultant samples were subjected to gas chromatography-mass spectrometry analysis. The optical density of the cell suspension at 600 nm was adjusted to 0.1 for PCB 48 and 4.0 for 4-chlorobiphenyl prior to the incubation. PCB 48 or 4-chlorobiphenyl was added at a final concentration of 10 μg/ml or 2.65 mM, respectively. The extracted samples were concentrated 1,000-fold for PCB 48 or 3-fold for 4-chlorobiphenyl. In the case of 4-chlorobiphenyl, the TMSI-H treatment was omitted.

Release of chloride. Cells grown on W-biphenyl medium were washed and resuspended in a fresh chloride-free medium based on W medium containing 33 mM biphenyl. After the addition of 2,2′-chlorobiphenyl at a final concentration of 90 μM, incubation was started at 30°C. Samples were drawn periodically, and each sample was divided to carry out both the analysis of PCB transformation as mentioned above and the determination of the amount of chloride released by the mercuric thiocyanate method described by Iwasaki et al. (13). We repeated twice the whole experiment, including cell culture in the medium containing 2,2′-chlorobiphenyl, extraction, gas chromatography-mass spectrometry analysis, and the determination of the amount of chloride released.

RESULTS

Transformation of PCBs. Biphenyl-grown cells of strain RHA1 transformed most of the 10-μg/ml PCB 48, which is equivalent to Aroclor 1248. This strain transformed all the detectable mono-, di-, tri-, tetra-, penta-, and hexachlorobiphenyls, except two congeners at peak 55, at least to some extent in 3 days (Table 1). Almost all the tri- and tetrachlorobiphenyl congeners of PCB 48 were extensively transformed (80 to 100% transformation).

Within 3 days, *Rhodococcus* sp. strain RHA1 transformed 45 components including heptachlorobiphenyls in the 62 major peaks of the 10-μg/ml Kanechlor mixture, which consisted of Kanechlor 200, 300, 400, and 500. The transformation profile for PCB congeners by RHA1 was compared with those reported for the strong PCB degraders, *A. eutrophus* H850 (3), and *Rhodococcus globerulus* P6 (15) (Table 1). The results demonstrated the remarkably wide range of PCB-degradative activity in *Rhodococcus* sp. strain RHA1. Some of the congeners, represented by the peak numbers 35, 39, and 43, were hardly transformed by *A. eutrophus* H850 (3), but strain RHA1 and *R. globerulus* P6 transformed these congeners (15) (Table 1). The strain RHA1 also transformed hexa- and heptachlorinated PCB congeners of the peaks numbered 48, 56, 62, and 65 (Table 1).

Intermediate metabolites of PCBs. The cells were grown on biphenyl and 10 μg of PCB per ml, and the metabolites were extracted and concentrated. The TMS derivatives were generated and analyzed by gas chromatography-mass spectrometry. The mass spectrum of each metabolite corresponding to each total ion peak was analyzed (Table 2). Metabolite I showed the same retention time as and a fragmentation pattern identical to that of authentic 2,4- and 2,5-dichlorobenzoic acids. Metabolites II and III were identified as 3,4- and 2,3-dichlorobenzoic acid, respectively, on the basis of the mass spectra and retention times. Both of the metabolites IV and V were identified as trichlorobenzoic acid from the mass spectrum patterns. They showed mass spectra identical to that of 2,4,6-trichlorobenzoic acid, although their retention times were different. The base ion peaks of these metabolites are presented in Fig. 1. The amounts of these metabolites seem to be very small. Because of the difficulty in distinguishing them from other existing compounds in total ion peaks, the accurate estimation of the metabolite concentration would be impossible. However, the peak heights of metabolites II and V from the data in 6 days were apparently lower than those in 2 days. This would indicate that the chlorobenzoic acids were also degraded during incubation.

Effects of position of chlorine substitution on PCB transformation. We examined the effects of the position of chlorine substitution on PCB transformation in *Rhodococcus* sp. strain RHA1. Ten pure PCB congeners were mixed and used for this experiment. Solution A was a mixture of five *ortho*-substituted PCB congeners that consisted of 2,2′-, 2,5,2′-, 2,3-, 2,5,2′,5′-, and 2,4,5,2′,5′-chlorobiphenyls. Solution B was a mixture of five *para*-substituted PCB congeners that consisted of 4,4′-, 2,4,2′,4′-, 2,4,3′,4′-, 3,4,3′,4′-, and 2,4,5,2′,4′,5′-chlorobiphenyls. The result was compared with those reported for *Pseudomonas pseudoalcaligenes* KF707 and *Pseudomonas* sp. LB400 (11) (Table 3). Strain KF707 exhibited low transformation activity on the *ortho*-substituted PCB congeners such as 2,2′-, 2,5,2′-, and 2,5,2′,5′-chlorobiphenyls. Strain LB400 showed low transformation activity on *para*-substituted congeners such as 4,4′- and 2,4,3′,4′-chlorobiphenyls but showed higher activity when these *para*-substitutions were combined with chlorine substitutions at the *ortho* positions such as 2,4,2′,4′-chlorobiphenyl. Strain RHA1 exhibited high transformation activity on both types of congeners regardless of the position of substitution except 3,4,3′,4′- and 2,4,5,2′,4′,5′-chlorobiphenyls (Table 3).

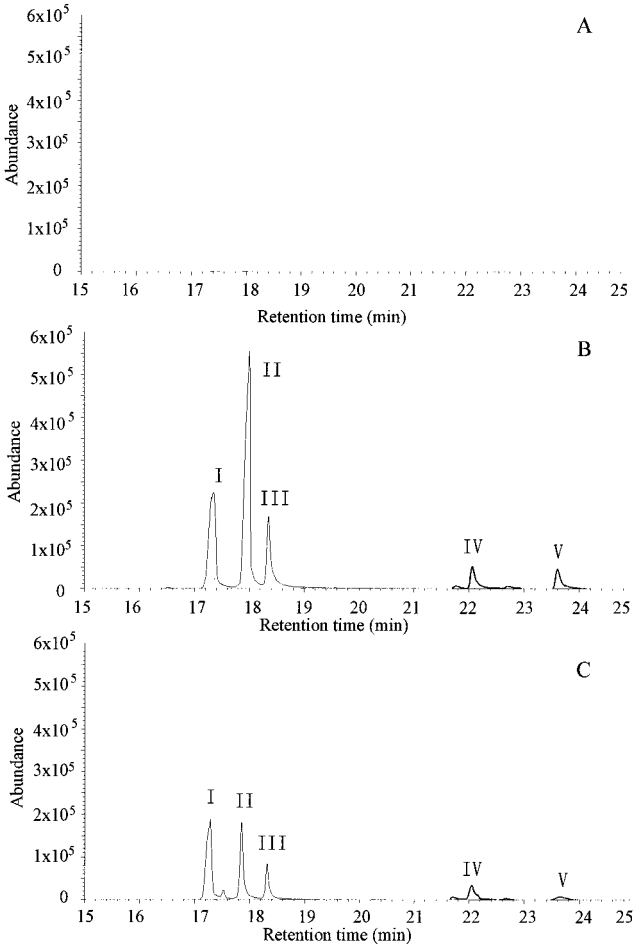

FIG. 1. Accumulation of intermediate metabolites during the transformation of PCB 48. The RHA1 cells were incubated in W medium containing 10 μg of PCB 48 per ml plus 33 mM biphenyl. The ethylacetate extracts were prepared after 0 (A), 2 (B), and 6 (C) days from one-tenth aliquots of the culture. The TMS derivatives of the concentrated extract were subjected to gas chromatography-mass spectrometry analysis. The selected ion chromatograms at *m/z* of 247 (peaks I, II, and III) and 283 (peaks IV and V) are presented to demonstrate the transient accumulation of dichlorobenzoates and trichlorobenzoates, respectively. Assignment of each metabolite is shown in Table 2.

Release of chloride accompanied by 2,2′-dichlorobiphenyl degradation. To determine whether the dechlorination occurred during the transformation of PCBs, the release of chloride and the amount of remaining 2,2′-dichlorobiphenyl were measured in the growth medium and shown in Fig. 2. The 2,2′-dichlorobiphenyl completely disappeared in 2 days after incubation. The chloride release started to increase after this period. The chloride release continued until 4 days. The molar ratio of chloride against biphenyl was about 2. These data showed that almost all the chloride should be removed and released from 2,2′-dichlorobiphenyl.

The metabolism of 4-chlorobiphenyl. Intermediate metabolites of 4-chlorobiphenyl by strain RHA1 were determined to provide an insight into the metabolic pathway of PCB. Biphenyl-grown RHA1 cells rapidly transformed 4-chlorobiphenyl, and the 4-chlorophenol and 4-chlorobenzoic acid accumulated. The identity of each metabolites was confirmed by gas chromatography-mass spectrometry comparison with authentic standards. The 4-chlorophenol accumulated gradually (Fig. 3). The transformation of 4-chlorobenzoic acid seemed to be rather slow, on the basis of the slow disappearance of 4-chlorobenzoic acid (Fig. 3).

TABLE 3. Degradation of the mixed PCB congeners by *Rhodococcus* sp. strain RHA1

Solution and congener (2 ppm each)	% Degradation[a] (SD)		
	RHA1	LB400[b]	KF707[b]
A			
2,**2**′-CB	100 (0)	100	18
2,5,**2**′-CB	98 (2)	100	10
2,3-CB	100 (0)	100	100
2,5,**2**′,5′-CB	76 (13)	100	9
2,4,5,**2**′,5′-CB	29 (11)	100	0
B			
4,**4**′-CB	95 (2)	25	100
2,**4**,2′**4**′-CB	83 (6)	81	0
2,**4**,3′,**4**′-CB	99 (1)	43	31
3,**4**,3′,**4**′-CB	0	6	0
2,**4**,5,2′,**4**′,5′-CB	0	41	0

[a] Data values are averages of triplicate experiments. Degradation less than 20% is not considered significant and is not reported.
[b] The results on *Pseudomonas* sp. LB400 and *P. pseudoalcaligenes* KF707 were reported by Gibson et al. (11). Cells grown on biphenyl were incubated for one day in phosphate buffer containing PCBs.

DISCUSSION

A gram-positive PCB degrader, *Rhodococcus* sp. strain RHA1, that was originally isolated by growth on biphenyl showed the ability to degrade an extremely wide range of congeners in commercial PCB mixtures. It transformed even the highly chlorinated PCB congeners, including heptachlorobiphenyls. On some PCB congeners, it showed transformation activities superior or equivalent to those of the previously reported strong PCB degraders, including *R. globerulus* P6 (15) and *A. eutrophus* H850 (5). Although the experimental conditions are not the same, the comparison mentioned above seems to be meaningful, because the composition of Aroclor 1254 is essentially equivalent to that of the KC mix we used. The incubation period of our experiment is the same as or shorter than those used in the other experiments. The incubation temperature we used is the same as that used in the other experiments. In our similar experiments with other isolates, the weak PCB degraders hardly transformed PCB congeners with more than five chlorine substitutions (unpublished data). Such dependence of the result on PCB transformation activity of each strain would support the significance of the comparison we made. We also compared the effect of the position of chlorine substitutions on the transformation of PCBs from strain RHA1 with the substrate specificities of biphenyl-degrading bacteria described in the literature (11). The experimental conditions are also different in this case, but the relative comparison between congeners and between strains would be meaningful. In comparison to *Pseudomonas* sp. LB400 and *P. pseudoalcaligenes* KF707 (Table 2), strain RHA1 has good transformation activity on both *ortho*- and *para*-substituted PCB congeners and the ability was not so affected by the position of substitutions. Thus, strain RHA1 seems to have PCB transformation activity as strong as those of the PCB degraders mentioned above.

In the case of 2,2′-dichlorobiphenyl degradation, most of the substituted chlorines were released. So, RHA1 seems to break down at least some PCB congeners into smaller molecules to a considerable extent. It would metabolize them probably through chlorobenzoic acids, because chlorobenzoic acids were

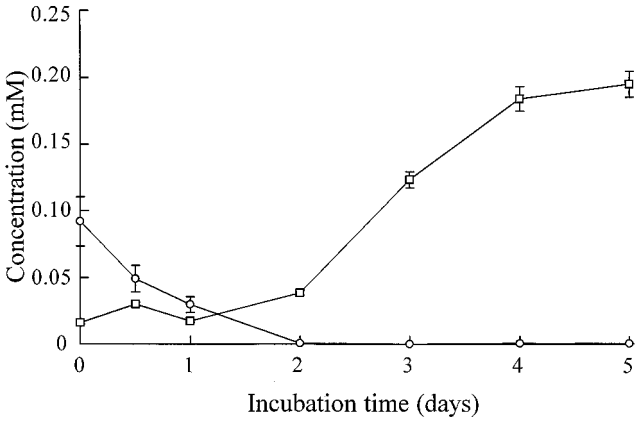

FIG. 2. The release of chloride associated with the degradation of 2,2′-dichlorobiphenyl by *Rhodococcus* sp. strain RHA1. The result was presented as the mean values of duplicate experiments. The RHA1 cells grown on biphenyl were incubated for 5 days in W medium containing 90 mM of 2,2′-dichlorobiphenyl plus 33 mM of biphenyl. Aliquots of the cultures were withdrawn and used to determine the residual amount of 2,2′-dichlorobiphenyl (○) and the release of chloride (□).

detected in the metabolites of PCBs and the transient accumulation of a good amount of 4-chlorobenzoic acid was observed during the degradation of 4-chlorobiphenyl. On the basis of the delay of the start of chlorine release in Fig. 2, chlorines seems to be released in the course of degradation following the accumulation of chlorobenzoic acid.

According to the intermediate metabolites observed in the course of 4-chlorobiphenyl degradation, chlorobenzoic acids produced from chlorobiphenyls seem to be metabolized through chlorophenols. In this case, the extraction of metabolites was performed after acidifying the sample. The high-performance liquid chromatography analysis showed no 4-chlorophenol before the acidification (data not shown). The same kind of result was observed by Hernandez et al. (12). So, 4-chlorophenol detected would not be a true intermediate metabolite of 4-chlorobiphenyl. The 4-chlorophenol was suggested to be generated from the intermediate metabolite of the transformation of 4-chlorobenzoic acid to 4-chlorocatechol and 1-carboxy-1,2-dihydroxy-4-chlorocyclohexadiene by acid hydrolysis during the acidification step (12, 20). It is possible to figure out the putative degradation pathway of 4-chlorobiphenyl via 4-chlorobenzoic acid to 4-chlorocatechol as proposed in the literature mentioned above, where 1-carboxy-1,2-dihydroxy-4-chlorocyclohexadiene is generated from 4-chlorobenzoic acid by dihydroxylation and converted to 4-chlorocatechol by dehydrogenation.

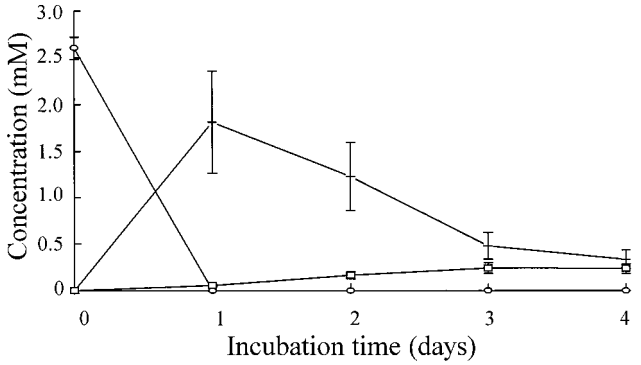

FIG. 3. The accumulation of metabolites associated with the degradation of 4-chlorobiphenyl by *Rhodococcus* sp. strain RHA1. The concentrations of 4-chlorobiphenyl (○), 4-chlorobenzoic acid (+), and 4-chlorophenol (□) at different times are presented as the mean values of duplicate experiments. The experimental conditions were described in Materials and Methods.

Strain RHA1 hardly transformed the congeners of PCB 48 at concentrations as high as 100 μg/ml (data not shown). The growth of RHA1 on biphenyl was repressed in the presence of 100 μg of PCB 48 per ml. Both the transformation of PCB 48 and the growth on biphenyl plus PCB 48 were completely inhibited at the concentration of 100 μg/ml. Sondossi et al. indicated the inhibition of chlorobiphenyl degradation by intermediates including chlorobenzoic acids and chlorophenols (20). This may be the case in strain RHA1. In the preliminary experiment, however, a derivative of RHA1 deficient in the initial step of biphenyl-PCB transformation did not grow in one-third-diluted Luria broth in the presence of 100 μg of PCB 48 per ml. The complete inhibition of PCB transformation would have originated from the toxicity of PCB itself. It is also possible to figure out the production of the inhibitory intermediates generated unexpectedly in the alternative pathway. Recently, we detected another PCB transformation pathway in RHA1 (19). Because this alternative pathway was not induced by biphenyl, sensitivity of RHA1 to PCB seems be a main problem. If so, we plan to introduce the PCB transformation genes of RHA1 into the other PCB-tolerant microorganisms to create a stronger PCB-transforming microorganism.

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