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Extradiol Cleavage of Two-ring Structures of Biphenyl and Indole Oxidation by Biphenyl Dioxygenase in *Commamonas Acidovorans*

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Abstract *Commamonas acidovorans* SMN4 showed wide growth substrate spectra for various aromatic hydrocarbons. Strain SMN4 was able to grow on biphenyl producing a *meta*-cleavage compound, yellow 2-hydroxy-6-oxophenylhexa-2,4-dienoic acid with a spray of 2,3-dihydroxybiphenyl, while it also grew on catechol, developing yellow 2-hydroxymucoic semialdehyde with a spray of 100 mM catechol. Thus these results indicate that two-ring structures of biphenyl were cleaved by *meta*-mode in upper and lower pathways. Strain SMN4 metabolized various substituted biphenyl compounds and xylene to the corresponding benzoate derivatives through oxidation of the ring structures. It was clearly shown that biphenyl can be a common inducer in the oxidation of biphenyl and 2,3-dihydroxybiphenyl. Various compounds were examined for their suitability to serve as substrates for indole oxidation, indicating that biphenyl, benzoate, and succinate are quite good inducers of indigo production due to the activity of biphenyl dioxygenase. This results suggest that indigo formation is by means of the combined activities of biphenyl dioxygenase and tryptophanase.

Key words: Biphenyl, cleavage of ring structures, indole oxidation, Biphenyl dioxygenase, *Commamonas acidovorans*

Biphenyls have become serious environmental pollutants distributed widely in the world. Several studies have reported the biodegradation and catabolic pathway of biphenyl [2, 3, 5, 6, 9, 11, 12].

Usually biphenyl-utilizing bacteria metabolize biphenyls [4]. Oxygen molecules are introduced at the 2,3-position of the ring of biphenyl to produce a dihydrodiol by the action of biphenyl dioxygenase. The dihydrodiol is then dehydrogenated to a 2,3-dihydroxybiphenyl by dihydrodiol dehydrogenase. The 2,3-dihydroxybiphenyl is then cleaved at the 1,2-position by 2,3-dihydroxybiphenyl dioxygenase. The yellow *meta*-cleavage compound, 2-hydroxy-6-oxo-phenylhexa-2,4-dienoate, is hydrolyzed to the corresponding benzoic acid by a *meta*-cleavage compound hydrolase. Benzoate is further degraded by a *meta*-cleavage pathway [7, 16].

Beijerinckia sp. strain B1 was isolated on minimal medium supplemented biphenyl as the sole source of carbon [8]. The strain B1 degrades biphenyl through an initial dioxygenase attack at the 2 and 3 positions on the aromatic ring to form (+)-*cis*-(1S,2R)-dihydroxy-3-phenylcyclohexa-3,5-diene (*cis*-biphenyl dihydrodiol) [18]. The two upper metabolic pathways for the degradation of biphenyl and *m*-xylene are induced after growth on either biphenyl or *m*-xylene, suggesting a common regulatory element [15].

Pseudomonas paucimobilis strain Q1, capable of utilizing biphenyl as its sole carbon source, grew not only on substituted biphenyl, but also on salicylate and substituted benzoate [6]. Evidence has been presented that the catabolism of biphenyl and salicylate is regulated by a common unit in this strain [6]. The catabolisms of biphenyl, xylene/toluene, and salicylate are interrelated, since benzoate and toluate are common metabolic intermediates of biphenyl and xylene/toluene, and salicylate is produced from 2-hydroxybiphenyl (*o*-phenylphenol). Growth of the *P. paucimobilis* Q1 cells with benzoate as the sole carbon source allowed the induction of only the *ortho*-pathway enzymes, suggesting that biphenyl, xylene/toluene, or salicylate especially did not induce the *meta*-pathway enzymes for the oxidative degradation of these compounds [6].

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E. coli pE317 contains genes from *Pseudomonas putida* PpG7 which encoded for enzymes responsible for the conversion of naphthalene to salicylic acid. The plasmid, NAH7, carries two gene clusters that enable the organism to grow on naphthalene as the sole carbon source [1]. Ensley *et al.* [1] reported a detailed genetic and physical analysis of the NAH7 plasmid. They found that the entire pathway for the conversion to salicylic acid is encoded by genes that can be expressed in *E. coli*. The results also led to the unexpected finding that one of these genes is responsible for the microbial production of indigo. In addition, they have showed that indigo formation is a property of the dioxygenase enzyme systems that form *cis*-dihydrodiols from aromatic hydrocarbons.

In our previous paper [13], we described a new bacterial strain, *Alcaligenes xylosoxydans* SMN3, which can grow not only on biphenyl but also on catechol, salicylate, benzoate, and phenol. Biphenyl dioxygenase plays a critical role in biphenyl degradation by catalyzing the first step in the oxidative pathway. All the oxidative enzymes of biphenyl dioxygenase and 2,3-dihydroxybiphenyl dioxygenase were induced when the cells were grown on biphenyl. The two degrative enzymes for the degradation of biphenyl and salicylate are induced after growth on biphenyl, suggesting the presence of a regulatory element.

In this present study, a new strain, *Commamonas acidovorans* was investgated for its degradative characteristics of biphenyl and other aromatics in reference to cleavage of the ring structures of biphenyl and indole oxidation.

MATERIALS AND METHODS

Organisms and Culture Conditions

The biphenyl-utilizing bacterium, *Commamonas acidovorans* SMN4, was used throughout the experiment [13]. Strain SMN4 was isolated from soil by enrichment culture with biphenyl as the sole source of carbon and energy. Cells were grown in basal salt medium (pH 7.0) composed of: 4.3 g K_2HPO_4, 3.4 g KH_2PO_4, 2.0 g $(NH_4)_2SO_4$, 0.16 g $MgCl_2$, 1 μg $MnCl_2 \cdot 4H_2O$. 0.6 μg $FeSO_4 \cdot 7H_2O$, 26 μg $CaCl_2 \cdot 2H_2O$, and 2 μg $Na_2MoO_4 \cdot 2H_2O$ in 1 liter of distilled water. Biphenyl was added at a final concentration of 1.0 mg/ml.

For the test of biodegradability, other aromatic substrates such as catechol, benzoate, salicylate, and phenol were added into basal salt medium at a final concentration of 5 mM. For the investigation of growth characteristics, biphenyl was given as vapor phase on the lid of a basal salt agar plate [6]. The dish was subsequently sealed with polyethylene tape.

Preparation of Cell-free Extracts

Cells were grown in basal salt medium with appropriate substrates such as succinate, salicylate, and benzoate to the late logarithmic phase. After centrifugation, the cells were washed twice with 50 mM phosphate buffer (pH 7.5) and disrupted by a Fisher sonic dismembractor (Model 300). The extract was centrifuged at 28,000×g for 30 min and then at 78,000×g for 90 min. The supernatant fluid was used as a cell-free extract for enzyme assay.

Enzyme Assays

For the determination of biphenyl dioxygenase activities, the reaction mixture contained 50 mM phosphate buffer (pH 7.5), 150 μM NADH, and an appropriate volume of cell extract by a modification of Mondello [16]. The reaction was initiated with the addition of the appropriate substrate and biphenyl at a final concentration of 40 μM. The activity was assayed by measuring the rate of degradation of biphenyl photometrically at 250 nm wavelength. One unit of enzyme activity is defined as the decrease of 1.0 μmole of biphenyl per min at 25°C. Catechol 1,2-oxygenase activity was assayed by measuring the rate of formation of *cis, cis*-muconate at 260 nm as described by Hegeman [10]. The oxidation of 0.1 μmole of catechol to *cis, cis*-muconate causes an increase in absorbancy at 260 nm of 0.56 absorbance unit. Catechol 2,3-oxygenase activity was assayed by determining the rate of accumulation of 2-hydroxymuconic semialdehyde at 375 nm as described by Hegeman [10]. Oxidation of 0.1 μmole of catechol to 2-hydroxymuconic semialdehyde results in an absorbancy increase of 0.98 optical density units at 375 nm. 2,3-Dihydroxybiphenyl oxygenase was assayed by measuring the rate of formation of 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate by following absorption at 434 nm [11]. The molar extinction coefficient (E_{434}) of this compound was determined to be 22,000. One unit of enzyme activity is defined as the increase of 1.0 μmole of 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate per min at 25°C.

Indigo Assay

Indigo production was measured with 1.0 ml of the reaction mixture at various time intervals. The indigo was extracted twice with equal volumes of ethyl acetate. The organic phases were combined and the absorbance of the ethyl acetate solution at 600 nm was determined [1]. The concentrations of indigo were calculated from a standard curve for synthetic indigo (Kodak) dissolved in ethyl acetate.

Protein Assay

Protein concentration was determined by the method of Lowry *et al.* [14] with bovine serum albumin as a standard.

Table 1. Utilization of aromatic hydrocarbons and their intermediates by *Commamonas acidovorans* SMN4.

Corbon source (5 mM)	Growth
Biphenyl	++
4-Chlorobiphenyl	+++
Phenol	+++
Benzoate	++
2,3-Dihydroxybiphenyl	+
Catechol	+++
m-Xylene	+
Benzylalcohol	+++
Salicylate	-
Benzylaldehyde	++

+++; very good growth, ++; good growth, +; poor growth, –; no growth.

RESULTS AND DISCUSSION

Catabolic Pathway of Biphenyl

In order to examine the degradative pathway of biphenyl, various hydrocarbons and their intermediates were tested for the utilization by the SMN4. As shown in Table 1, *Commamonas acidovorans* SMN4 showed wide growth substrate spectra for various aromatic compounds. Especially strain SMN4 was able to grow on biphenyl, benzoate and 4-chlorobiphenyl, but did not grow on salicylate. The SMN4 grew on biphenyl quite well, producing a yellow *meta*-cleavage compound on the basal salt medium containing various aromatics. Strain SMN4 also grew on medium containing catechol and benzoate, as well as methylbenzylalcohol as the intermediates of degradation of *m*-xylene. Phenol was also utilized as the sole carbon and energy source by strain SMN4 for growth.

P. paucimobilis Q1 had broad growth substrate spectra for various armatics [6]. The strain converted various substituted biphenyls to the corresponding benzoate derivatives, so that benzoate was produced from chlorobiphenyl [6]. Furukawa *et al.* [6] previously reported that a number of chlorinated biphenyls were converted to the corresponding chlorobenzoates in *Acinetobacter* sp. strain P6 through an oxidative route that included *meta*-cleavage of one of the biphenyl rings [7].

From the result of Table 1, strain SMN4 had also broad growth substrate spectra for a variety of aromatic hydrocarbons and their metabolic intermediates. It was assumed that the strain SMN4 converted substituted biphenyls to the corresponding benzoate. In addition, strain SMN4 was able to metabolize *m*-xylene to benzylalcohol and benzaldehyde, followed by an oxidation pathway of the ring to produce benzoate which is a common metabolic intermediate of biphenyls and xylene.

Biphenyl Degradation

We could measure biphenyl degradation at the time intervals during the culture periods using a spectrophotometer.

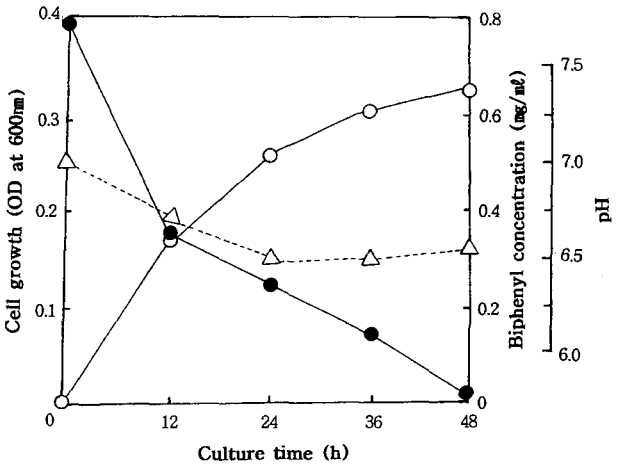

Fig. 1. Changes of cell growth of *Commamonas acidovorans* SMN4, biphenyl biodegradation, and pH in the basal salt medium containing biphenyl during cultivation.
Symbols: (○) Cell growth, (●) Biphenyl, (△) pH.

Moreover, Fig. 1 shows that growth of cells on biphenyl minimum broth increased to 0.31 optical density at 600 nm in the 48 h culture period. Biodegradation of biphenyl decreased continually to 180 μg/ml during the 36 h cultivation.

Extradiol cleavage of Biphenyl Degradation

Incubating strain SMN4 cells on the plate with biphenyl or 4 chlorobiphenyl led to the production of a yellow *meta*-cleavage compound, 2-hydroxy-6-oxophenylhexa-2, 4-dienoic acid with a spraying of 100 mM 2,3-dihydroxybiphenyl as shown in Table 2. By means of utilization of the intermediate of aromatic compounds (Table 1), biphenyl, 2,3-dihydroxybiphenyl, or 4-chlorobiphenyl was degraded to a *meta*-cleavage compound, and to benzoic acid. Moreover, by spraying with 100 mM catechol solution the strain SMN4 produced yellow 2-hydroxymuconic semialdehyde [17]. Another interesting feature of strain SMN4 is that it was able to grow on *m*-

Table 2. Detection of 2-hydroxy-6-oxophenylhexa-2,4-dienoic acid and 2-hydroxy muconic semialdehyde on colonies grown on Luria-Bertani plates.

Strain	2-Hydroxy-6-oxo-phenylhexa-2,4-dienoic acid	2-Hydroxymuconic semialdehyde
Pseudomonas putida		
mt-2	+	+
PRS2000	-	-
Commamonas acidovorans		
SMN4	+	+

2-Hydroxy-6-oxo-phenylhexa-2,4-dienoic acid was detected with yellow color production (+) on the plate by spraying of 100 mM 2,3-dihydroxybiphenyl solution, while 2-hydroxymuconic semialdehyde was detected with spraying of 100 mM catechol solution.

xylene, and its alcohol, aldehyde, and carboxylic acid. These results suggested that the catabolism of biphenyl, xylene, and benzoate was related to each other. Biphenyls and *m*-xylene can be further metabolized to catechol, followed by the *meta*-cleavage of the ring to produce yellow 2-hydroxymuconic semialdehyde. Thus these results indicate that two-ring structures of biphenyl were cleaved by extradiol-mode in upper and lower pathways. Moreover, these results coincided with finding that colonies of the strain SMN4 showed a yellow color production on the minimal agar plate supplemented with biphenyl as described in Table 2.

Induction of Oxidizing Activities

Since the catabolism of biphenyl and xylene appeared to be interrelated, strain SMN4 cells were grown on various substrates to compare the oxidation of biphenyl and 2,3-dihydroxylbiphenyl, as shown in Table 1.

It was found that enzyme levels in cell-free extracts of strain SMN4 cells clearly showed that biphenyl induced biphenyl dioxygenase, and 2,3-dihydroxybiphenyl dioxygenase which means *meta*-cleavage enzyme as shown in Table 3.

On the other hand, the cell-free extract from benzoate-grown cells had very low biphenyl dioxygenase and 2,3-dihydroxybiphenyl dioxygenase activity, similar to the low activities in the catechol-grown cell-free extracts. Salicylate, however, could not induce the enzymes responsible for the oxidation of these substrates and intermediates. These results clearly show that biphenyl can be a common inducer in the oxidation of biphenyl and 2,3-dihydroxybiphenyl.

However, many of *Pseudomonas* strains capable of decomposing catechol by the *meta*-cleavage pathway also possess the *ortho*-cleavage pathway. In naphthalene-degrading fluorescent pseudomonads, the enzymes of the *meta*-cleavage pathway are induced after growth with benzoate [13]. In the present work, the strain SMN4 is similar to a previous result in that benzoate serves as an inducer at the lower level for the enzymes of biphenyl and catechol degradation, which are all metabolized through the *meta*-pathway. In our previous study [13], it was compared with the strain *P. putida* mt-2, which are catabolized biphenyl through the *meta*-pathway shown by a high activity of catechol 2,3-dioxygenase, whereas *P. putida* PRS2000 through the *ortho*-pathway.

Table 3. Enzyme levels in the crude extracts of strain SMN4 grown on various inducers.

Inducer	Biphenyl dioxygenase (unit/mg protein)	2,3-Dihydroxybiphenyl dioxygenase (unit/mg protein)
Biphenyl	0.68	4.20
Benzoate	0.04	0.22
Salicylate	ND[a]	ND
Catechol	0.04	0.41

Cells were grown in 20 ml of Luria-Bertani broth overnight, harvested, resuspended in 100 ml of minimal media containing 5 mM of inducers, and incubated for 36 h for enzyme induction.
[a]ND; not detected.

Table 4. Substrate profile of 2,3-dihydroxybiphenyl dioxygenase and catechol 2,3-dioxygenase from the crude extract of SMN4 cells.

Substrate	2,3-Dihydroxybiphenyl dioxygenase (unit/mg protein)	Catechol 2,3-dioxygenase (unit/mg protein)
Biphenyl	0.07	6.68
2,3-Dihydroxybiphenyl	0.06	4.68
Catechol	0.88	0.41
3-Methylcatechol	1.09	0.51
Methylbenzoate	0.83	0.21

Substrate Profile of 2,3-dihydroxybiphenyl and Catechol 2,3-dioxygenase

In a previous study [13], we examined the benzoate induction of catechol 2,3-oxygenase, which is a *meta*-cleavage enzyme of catechol, but not catechol 1,2-dioxygenase, which is an *ortho*-cleavage enzyme in the low pathway of aromatic compounds. Since the catabolism of biphenyl was carried out through *meta*-cleavage of the ring structure in an upper pathway, the substrate profile was investigated and we compared the oxidation of biphenyl and 2,3-dihydroxybiphenyl, as shown in Table 4. It was found that substrate specificities of biphenyl and 2,3-dihydrohydroxybiphenyl appeared to be lower in 2,3-dihydroxybiphenyl dioxygenase in the upper pathway, but those of 3-methylcatechol, catechol, and methylbenzoate were at a higher level in catechol 2,3-dioxygenase in a lower pathway of biphenyl biodegradation.

Indigo Production

Several bacteria oxidize aromatic hydrocarbons to *cis*-dihydrodiols and also oxidize indole to indigo [1]. Indigo production was investigated in culture medium from indole, supplemented with 5 mM succinate. Indigo formation was increased when the cells were grown in the culture medium supplemented with 1 mM indole for 5 h (Fig. 2).

We examined various compounds to serve as substrates for the production of indigo in a culture medium. Indigo formation was not observed in cultures containing glucose. However, biphenyl or benzoate induced catabolic enzymes of biphenyl in the broth culture to produce higher indigo formation. Combinations of biphenyl and benzoate, benzoate and succinate, biphenyl and succinate, showed higher production of indigo as shown in Table 5. These results indicate that biphenyl, benzoate, and succinate were quite good inducers for indigo production.

Biphenyl and 2,3-dihydroxybiphenyl dioxygenase activities were induced when strain SMN4 was grown on biphenyl

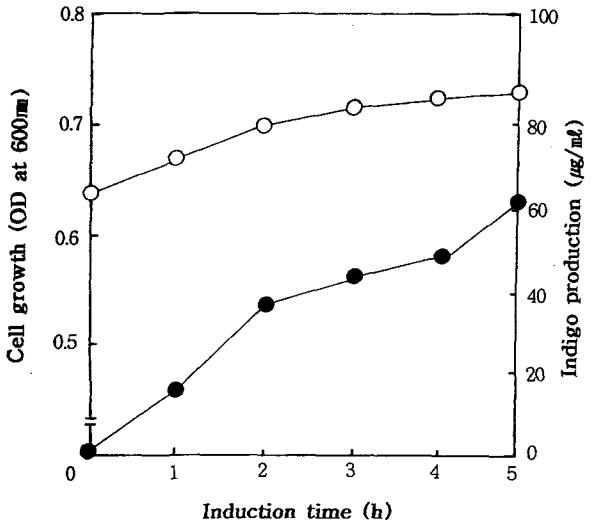

Fig. 2. Indigo production during the cell growth of *Commamonas acidovorans* SMN4.

Bacterial cells were grown in 20 ml of Luria-Bertani broth overnight, washed, resuspended in 100 ml of the basal salt medium containing 5 mM succinate, and followed by incubation with shaking at 180 rpm for 4 h until A_{600} reached 0.62. After 1 mM indole was added into the succinate basal medium, the culture was further incubated for 5 hours for the production of indigo. Symbols: (○) Cell growth, (●) Indigo concentration.

(Table 3). Similarly, Furukawa *et al.* [6] indicated that both the *meta*-cleavage enzymes 2,3-dihydroxybiphenyl oxygenase and catechol 2,3-oxygenase were observed in all biphenyl, *m*-xylene, or *m*-toluate, and salicylate-grown cells. However, in our experiment we did not observe induction on salicylate-grown cells.

In this connection, it was clearly shown that biphenyl and benzoate were good inducers for indigo formation as well as biphenyl dioxygenase, which is metabolized to produce indigo from indole in the strain SMN4.

However, even if biphenyl is a good inducer for indigo production, the combination of biphenyl and salicylate was not appeared induction of indigo formation. This combined effect assumed that salicylate as a carbon source is not a good inducer for indigo production as reported in previous study [13].

The results presented in Table 5 and Fig. 2 suggest that indole oxidation is a property of bacterial dioxygenase that forms *cis*-dihydrodiols from the aromatic hydrocarbon, biphenyl. However, the combination of biphenyl and benzoate produced higher indigo formation than biphenyl and succinate, as shown in Table 5. These results indicate that biphenyl and benzoate are better inducers than salicylate for biphenyl dioxygenase production.

It was reported that the oxidation of naphthalene by *P. putida* PpG7 is catalyzed by enzymes that are encoded by a plasmid [1]. It was found that the entire pathway for the conversion of naphthalene to salicylate is encoded by

Table 5. Indigo production from indole with various inducers in *Commamonas acidovorans* SMN4 cells.

Inducer (5 mM)	Indigo production (μg/ml)		
	1 hr	2 hr	3 hr
Glucose	0	0	0
Biphenyl	6.2	8.4	10.4
Benzoate	3.4	5.8	9.1
Succinate	2.7	4.8	6.4
Salicylate	ND[a]	ND	ND
Succinate + salicylate	ND	ND	ND
Salicylate + biphenyl	ND	ND	ND
Succinate + benzoate	7.5	9.6	13.7
Benzoate + biphenyl	9.0	15.4	20.8
Succinate + biphenyl	6.2	8.7	11.6

Bacterial cells were grown in 2 ml of Luria-Bertain broth overnight, washed, and resuspended in 50 ml of minimal supplemented with various inducers (5 mM). The cell suspension was incubated 4 h. After indole (1 mM) was added to the culture, cells were induced indigo production at time intervals.
ND[a]; not detected.

genes that can be expressed in *E. coli*. The results also led to the unexpected finding that one of these genes is responsible for the microbial production of indigo. In addition, it has also shown that indigo formation is a property of the dioxygenase enzyme systems that form *cis*-dihydrodiol from aromatic hydrocarbons [1].

However, indigo formation was not observed in cultures containing 5 mM glucose as shown in Table 5. It is known that high glucose concentrations can cause catabolite repression of tryptophanase synthesis. Indigo formation occurred when indole was added to cultures of *P. putida strain* PpG7 [1]. All of these observations suggest that indigo synthesis in recombinant *E. coli* is catalyzed by naphthalene dioxygenase. Further support for this hypothesis was provided by the observation that biphenyl-utilizing *Alcaligenes xylosoxydans* strain SMN3 oxidizes indole to indigo like other naphthalene-utilizing pseudomonads [13].

Our results suggest that indigo formation is due to the combined activities of biphenyl dioxygenase and tryptophanase according to a study of Ensley *et al.* [1]. The reaction sequence accounts for the formation of indigo by the strain SMN4 used in this investigation. It also provides a possible explanation in other studies on indigo formation [1] even though we have not been able to detect the formation of *cis*-2,3-dihydroxy-2,3-dihydrodiol.

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