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Characterization of Biphenyl Biodegradation, and Regulation of Biphenyl Catabolism in *Alcaligenes xylosoxydans*.

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***Alcaligenes xylosoxydans* strain SMN3 capable of utilizing biphenyl grew not only on phenol, and benzoate, but also on salicylate. Catabolisms of biphenyl and salicylate appear to be interrelated since benzoate is a common metabolic intermediate of these compounds. Enzyme levels in the extract of SMN3 cells pregrown in media containing benzoate clearly showed that benzoate induced catechol 2,3-dioxygenase which is *meta*-cleavage enzyme of catechol, but did not induce catechol 1, 2-dioxygenase. All the oxidative enzymes of biphenyl and 2,3-dihydroxybiphenyl (23DHBP) were induced when the cells were grown on biphenyl and salicylate, respectively. Biphenyl and salicylate could be a good inducer in the oxidation of biphenyl and 2,3-dihydroxybiphenyl. The two enzymes for the degradation of biphenyl and salicylate were induced after growth on either biphenyl or salicylate, suggesting the presence of a common regulatory element. However, benzoate could not induce the enzymes responsible for the oxidation of these compounds. Biphenyl and salicylate were good inducers for indigo formation due to the activity of biphenyl dioxygenase. These results suggest that indole oxidation is a property of bacterial dioxygenase that form *cis*-dihydrodiols from aromatic hydrocarbon including biphenyl.**

Key words: Biphenyl degradation, dioxygenase, catechol, salicylate, *Alcaligenes xylosoxydans*

Biphenyls have become serious environmental pollutants which are widely distributed in the world. Several studies have reported the biodegradation and catabolic pathway of biphenyl (3, 4, 6, 7, 10, 15, 16).

Catabolism of biphenyl was shown to be specified by plasmids (5, 14, 17), and also by the chromosomal DNA in some bacteria (3, 10, 15, 16). Usually biphenyl-utilizing bacteria co-metabolize certain polychlorinated biphenyls (5). Oxygen molecules are introduced at the 2,3-position of the biophenyl ring to produce a dihydrodiol by the action of a biphenyl dioxygenase (product of gene *bphA*). The dihydrodiol is then dehydrogenated to a 2,3-dihydroxybiphenyl by dihydrodiol dehydrogenase (product of gene *bphB*). The 2,3-dihydroxybiphenyl is then cleaved at the 1,2-position by 2,3-dihydroxybiphenyl dioxygenase (product of gene *bphC*). 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(product of gene *bphD*). Benzoate is further degraded by a *meta*-cleavage pathway (8, 24).

Pseudomonas paucimobilis strain *Q1* is capable of utilizing biphenyl as a sole carbon source. This strain grew not only on substituted biphenyl, but also on salicylate and substituted benzoate (7). Evidence show that the catabolism of biphenyl and salicylate is regulated by a common unit in this strain (7). 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). All the oxidative enzymes of the biphenyl, xylene/toluene, or salicylate in degradative pathways were induced when the cells were grown on either biphenyl, xylene/toluene, or salicylate. The *P. paucimobilis Q1* cells showed induction of *meta*-cleavage enzymes for both 2,3-dihydroxybiphenyl and catechol. Growth of the *P. paucimobilis Q1* cells were with benzoate as a sole carbon source allowed the induction of only the *ortho*-pathway enzymes, suggesting that biphenyl, xylene/toluene, or salicylate did not induce

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the *meta*-pathway enzymes for the oxidative degradation of these compounds (7).

Beijerinckia sp. strain B1 was originally isolated for its ability to grow on biphenyl as the sole source of carbon and energy for growth (9). The strain B1 degrades biphenyl through an initial dioxygenase attack at the 2 and 3 positions of the aromatic ring to form (+)-*cis*-(1S,2R)-dihydroxy-3-phenylcyclohexa-3,5-diene (*cis*-biphenyl dihydrodiol) (27). Thus, degradation of biphenyl and *m*-xylene by *Beijerinckia* sp. strain B1 proceeds by separate "upper" metabolic pathways that intersect at the "lower" pathway for benzoate (or toluate) degradation. 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 (23).

The biphenyl degradative pathway of the strain B1 has been implicated in the oxidation of polycyclic aromatic hydrocarbons such as phenanthrene (12, 19), anthracene (12), dibenzothiophene (20), acenaphthene (26), acenaphthylene (26), carbazole (25), dibenzo-*p*-dioxin (18), and benz[a]anthracene (13, 22). The wide range of substrates oxidized by the strain B1 may be attributed to the relaxed specificity of the initial enzymes involved in biphenyl degradation. The metabolic pathways for biphenyl and *m*-xylene degradation by the strain B 1 are coordinately induced. And constitutive mutant strains express the enzymes at high levels for both *m*-xylene and biphenyl degradation (23). This suggests that a single regulatory gene may be involved and/or that the genes for the degradation of *m*-xylene and biphenyl are in the same operon(s).

E. coli pE317 contains genes from *Pseudomonas putida* PpG7 which encodes for enzymes responsible for the conversion of naphthalene to salicylic acid. The oxidation of naphthalene by *Pseudomonas putida* PpG7 is catalyzed by enzymes that are encoded by a plasmid. The plasmid, NAH7, carries two gene clusters that enable the organism to grow on naphthalene as a sole carbon source (2). Several compounds produced during naphthalene oxidation, including naphthoquinone and salicylic acid, are widely used in chemical and pharmaceutical industries. To determine the feasibility of utilizing microorganisms to produce these compounds, Ensley *et al.* (1983) reported a detailed genetic and physical analysis of the NAH7 plasmid (2). 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 shown that indigo formation is a property of the dioxygenase enzyme systems that form *cis*-dihydrodiols from aromatic hydrocarbons.

It has been demonstrated that the differences in congener specificity among the bacteria reflect differences in the genes encoding their biphenyl-degrading enzymes(the products *bph* genes). They have described the cloning and characterization of the *bph* genes from *Pseudomonas* strain LB400 (24). Southern hybridization experiments were used to test a variety of biphenyl-degrading bacteria for the presence of DNA sequences similar to any of the LB400 *bph* genes. This result suggests the existence of at least two analogous 2,3-dioxygenase pathways encoded by significantly different gene sets. A bacterium *Alcaligenes eurtrophus* H850, with biphenyl-degrading ability very similar to that of LB400 contained *bph* genes which hybridized strongly to those of LB400 (24).

In isolating biphenyl-utilizing microorganisms, we obtained 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. The activity of the biphenyl dioxygenase of strain SMN3 has led us to study the structure and function of the genes encoding this enzyme. In order to investigate the ability of the strain to degrade biphenyl and other aromatics in more detail, the present work was initiated.

Materials and Methods

Organisms and culture conditions

Biphenyl-utilizing bacteria *Alcaligenes xylosoxydans* SMN3 and *Commanonas acidovorans* SMN4 were used throughout the experiment. Strain SMN3 was isolated from sewage of Ulsan chemical companies in Korea by enrichment culture with biphenyl as the sole source of carbon and energy. Cells were grown in basal salt medium(pH 7.0) containing (in grams per liter): K_2HPO_4, 4.3; KH_2PO_4, 3.4; $(NH_4)_2SO_4$ 2.0; $MgCl_2$, 0.16; $MnCl_2 \cdot 4H_2O$, 0.001; $FeSO_4 \cdot 7H_2O$, 0.0006; $CaCl_2 \cdot 2H_2O$, 0.026; and $Na_2MoO_4 \cdot 2H_2O$, 0.002. Substrate was added at a final concentration of 1.0 mg/ml.

For the test of biodegradability, other aromatic substrates such as catechol, salicylate, *m*-xylene, benzoate, and phenol were added to basal broth medium at the final concentration of 5 mM. For the investigation of growth characteristics, biphenyl was given as vapor on the lid of a basal agar plate

(7). The dish was subsequently sealed with polyethylene tape.

Preparation of cell-free extracts

Cells were grown in basal salt broth 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 assaying the following enzymes: biphenyl dioxygenase, catechol 1,2-dioxygenase (C12O), catechol 2,3-dioxygenase (C23O), and 23DHBP dioxygenase (*meta*-cleavage enzyme of 2,3-dihydroxybiphenyl).

Enzyme assays

Biphenyl dioxygenase activity was determined using, the reaction mixture containing 50 mM phosphate buffer(pH 7.5), 150 μM NADH, and an appropriate volume of cell extract by a modified method of Mondello (24). The reaction was initited 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 the 250 nm wavelength. One unit of enzyme activity is defined as the decrease of 1.0 mole of biphenyl per min at 25°C. Catechol 1,2-dioxygenase activity was assayed by measuring the rate of formation of *cis, cis*-muconate at 260 nm as described by Hegeman (11). The oxidation of 0.1 mole of catechol to *cis, cis*-muconate caused an increase in absorbancy at 260 nm of 0.56 absorbance unit. Catechol 2,3-dioxygenase activity was assayed by determining the rate of accumulation of 2-hydroxymuconic semialdehyde at 375 nm as described by Hegeman (11). Oxidation of 0.1 μmoles of catechol to 2-hydroxymuconic semialdehyde resulted in an absorbancy increase of 0.98 optical density unit at 375 nm. The 2,3-dihydroxybiphenyl dioxygenase was assayed by measuring the rate of formation of 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate by absorption at 434 nm (15). 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

Amount of indigo formation was measured by removing 1.0 ml of the reaction mixture at various time intervals and extracting it twice with the equal volume of ethyl acetate. The organic phases were combined and the absorbance of the ethyl acetate solution at 600 nm was determined (2). The concentrations of indigo were taken 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* (21) with bovine serum albumin as the standard.

Results

Identification and properties of the strain SMN3 and SMN4

Strain SMN3 is a gram-negative, motile, rod , 0.5~1.0 μm in diameter and 0.5~2.6 μm in length. The optimal growth temperature was between 20 to 37°C. The strain was oxidase positive , catalase positive, and urease negative. The strain was capable of anaerobic respiration in the presence of nitrate or nitrite. Cellulose, gelatin, and ensulin were not hydrolyzed. Acid formation was observed from *D*-glucose and *D*-xylose. The guanine and cytosine content of the DNA was 56~70 mole% by melting temperature measurements. By these criteria, strain SMN3 was shown to belong to the *Alcaligenes xylosoxydans* group of bacteria.

Strain SMN4 is a gram-negative, motile by several polar flagella, curved rod, 0.8~1.1 μm in diameter and 2.5~4.1 μm in length. The optical growth temperature was 30°C. The strain was oxidase positive. Gelatin and starch were not hydrolyzed, but lipase was hydrolyzed. The guanine and cytosine content of the DNA was 67 mole% by melting temperature measurement. By these criteria, strain SMN4 was shown to belong to the *Commamonas acidovorans* group of bacteria.

Table 1. Growth of *Alcaligenes xylosoxydans* SMN3 and *Comamonas acidovorans* SMN4. utilizing aromatic hydrocarbons and their intermediates as sole carbon source

Aromatic carbon (5 mM)	SMN3	SMN4
Biphenyl	+++	++
Phenol	+++	+++
Benzoate	++	++
Salicylate	+++	–
Catechol	+++	++
Methyl benzyl alcohol	+++	+++
Benzyl alcohol	+++	+++
m-Xylene	–	+

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

Growth characteristics

Strain SMN3 showed wide growth substrate spectra for various aromatic compounds (Table 1). The biphenyl-degrading *Comamonas acidovorans*, strain SMN4 grew only on biphenyl but not grow on salicylate. *Alcaligenes xylosoxydans* strain SMN3 grew on biphenyl quite well, producing a yellow *meta*-cleavage compound on the agar medium and on medium containing various aromatics. Strain SMN3 also grew on medium containing methylbenzyl alcohol, and benzyl alcohol. Benzoate and salicylate, also supported growth of strain SMN3. In addition to these aromatic substrates, catechol and phenol were also utilized as a sole carbon and energy source by strain SMN3 and strain SMN4 for growth.

Biphenyl degradation

As shown in Fig. 1, it was observed that the maximum absorption spectrum of biphenyl was 250 nm. We measured biphenyl degradation at different the time intervals during the culture periods using spectrophotometer (Fig. 1). Fig. 2 shows growth of cells on biphenyl minimum broth. Biodegradation of biphenyl increased continuously to 630 μg/ml during the 48 hour cultivation.

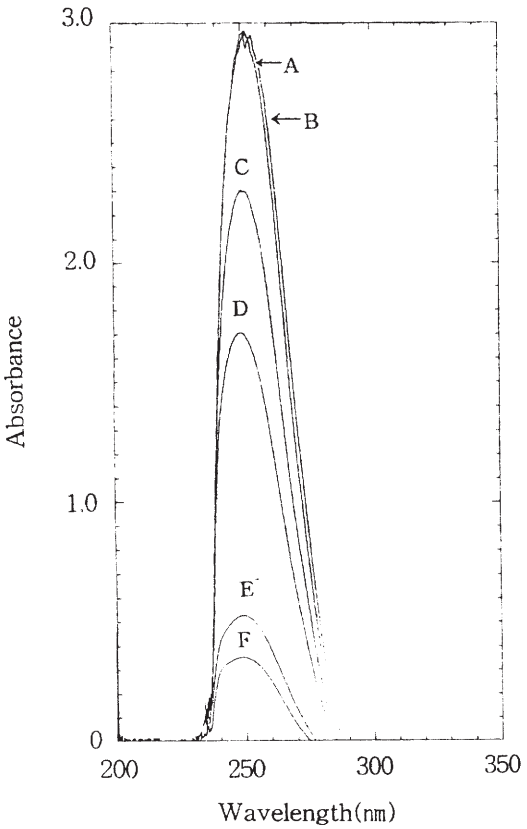

Fig. 1. UV-scanning spectra of biphenyl and its biodegradation remaining in liquid medium supporting growth of the strain SMN3 at time intervals. Induction was carried out with minimal medium containg biphenyl (1 mg/ml) as shown in Materials and Methods. A, B, C, D, E, and F represent 0, 6, 12, 24, 36, and 48 hour incubation, respectively.

Indigo production

Several bacteria that oxidize aromatic hydrocarbons to *cis*-dihydrodiols also oxidize indole to indigo. The indigo formation is thought to result from combined activities of tryptophanase and dioxygenase. Indigo formation occurred when the cells were grown in the culture medium supplemented with 1 mM indole. Indigo formation was enhanced when indole was added to the culture of *Alcaligenes xylosoxydans*, induced with as shown in Fig. 3.

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, salicylate or biphenyl induced catabolic enzymes of biphenyl in the broth culture had higher indigo formation. Combination of biphenyl and salicylate, salicylate and succinate, biphenyl and succinate showed higher production of indigo as shown in Table 2. These results indicate that biphenyl or salicylate are quite good inducer for indigo formation.

Catabolic pathway

Biodegradation of biphenyl by *P. putida* mt-2 cells was carried out by the *meta*-pathway shown by a high activity of catechol-2,3-dioxygenase(C230); whereas, *P. putida* PRS2000 was carried out by *ortho*-pathway, indicated by a high activity of

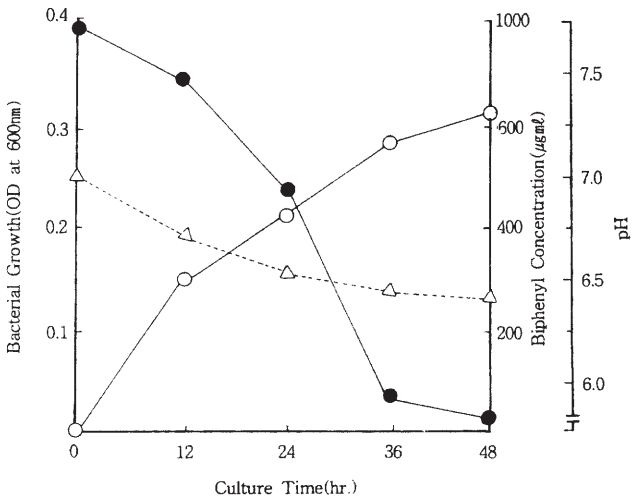

Fig. 2. Changes in bacterial growth, biphenyl biodegradation, and pH change of culture by *Alcaligenes xylosoxydans* SMN3 during culture period on minimal broth medium containing 1 mg/ml of biphenyl. ○-○, bacterial growth; ●-●, biphenyl remaining in the broth; △-△, pH.

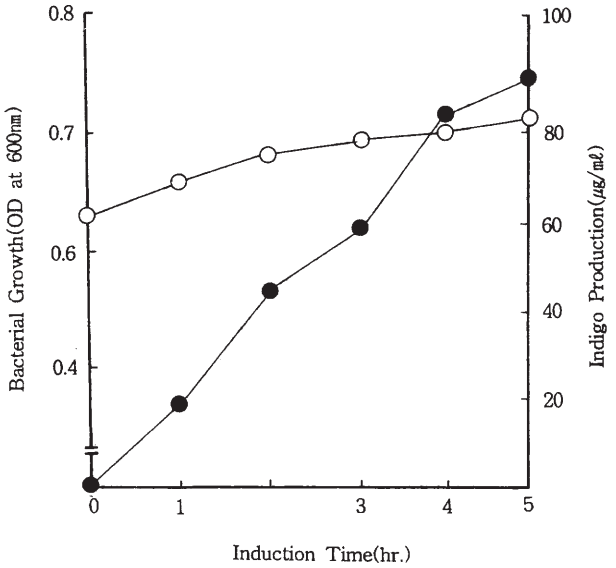

Fig. 3. Indigo production during the cell growth of *Alcaligenes xylosoxydans* SMN3. Bacterial cells were grown in 20 ml of Luria Bertani broth overnight, washed, resuspended in 100 ml of minimal broth containing 5 mM succinate, and followed by incubation with shaking at 180 rpm for 4 hours until A_{600} reached to 0.62. After 1 mM indole was added to the succinate minimal medium,the culture was further incubated 5 hours at time intervals for the production of indigo. ○-○, Bacterial growth; ●-●, Indigo concentration in the broth.

catechol-1,2-dioxygenase(C120). Enzyme levels in the extracts of benzoate-grown strain SMN3 and SMN4 cells showed that benzoate induced catechol 2, 3-dioxygenase, which is *meta*-cleavage enzyme of catechol, but not catechol 1,2-dioxygenase, which is *ortho*-cleavage enzyme of catechol. (Shown in Table 3). Moreover, these results coincided with finding that the colonies of the strain SMN3 and SMN4 showed yellow color production on the minimal agar plate supplemented with biphenyl as demonstrated by their growth characteristics.

Table 2. Indigo production induced by biphenyl and its metabolites in *Alcaligenes xylosoxydans* SMN3

Inducer(5 mM) \ Culture time	Indigo production (μg/ml)		
	1 hr	2 hr	3 hr
Glucose	0	0	0
Biphenyl	7.2	21.0	29.0
Benzoate	3.6	7.2	13.6
Succinate	2.8	4.5	7.5
Salicylate	12.0	24.1	28.5
Succinate + salicylate	12.5	24.0	30.5
Salicylate + biphenyl	19.0	22.0	35.5
Succinate + benzoate	3.0	5.0	9.5
Benzoate + biphenyl	10.5	12.8	15.0
Succinate + biphenyl	10.0	18.0	25.3

Bacterial cells were grown in 2 ml of Luria-Bertani broth overnight, washed, and resuspended in 50 ml of minimal medium supplemented with various inducers (5 mM). The cell suspension was incubated 4 hours. After indole (1 mM) was added to the culture, cells were induced indigo production at time intervals.

Table 3. Enzyme activities of catechol 1,2-dioxygenase and catechol 2,3-dioxygenase from the cell-free extracts of benzoate grown cells

Strain	Catechol 1,2-dioxygenase (unit/mg protein)	Catechol 2,3-dioxygenase (unit/mg protein)
Pseudomionas putida		
*mt-2**	0.15	1.90
PRS2000**	0.40	0.03
Alcaligenes xylosoxydans		
SMN3	0.05	1.30
Comamonas acidovorans		
SMN4	0.03	1.04

* *meta*-pathway with C230 activity.
** *ortho*-pathway with C120 activity.

Induction of biphenyl dioxygenase and 2,3-dihydroxybiphenyl dioxygenase

Since the catabolism of biphenyl and salicylate appeared to be interrelated, strain SMN3 cells were grown on various substrate to compare the oxidation of biphenyl. (Table 4)

Cells grown on biphenyl and its related intermediates such as salicylate, benzoate, and catechol were investigated to find out how biphenyl dioxygenase and 2,3-dihydroxybiphenyl dioxygenase could be induced. Biphenyl or salicylate was a good inducer for the oxidation of biphenyl and 2,3-dihydroxybiphenyl. However, benzoate, in contrast to biphenyl or salicylate, was as poor an inducer as catechol for the catabolism of biphenyl. The benzoate-grown cells extensively did not oxidize biphenyl

Table 4. Induction of biphenyl dioxygenase and 2,3-dihydroxybiphenyl dioxygenase in *Alcaligenes xylosoxydans* SMN3

Inducer	Protein (mg/ml)	Biphenyl dioxygenase		2,3-Dihydroxybiphenyl	
		Activity (unit)	Specific Activity (unit/mg protein)	Activity (unit)	Specific Activity (unit/mg protein)
Biphenyl	0.765	0.24	0.314	5.818	7.605
Benzoate	0.720	0.01	0.007	0.045	0.063
Salicylate	0.725	0.20	0.275	5.773	7.363
Catechol	0.680	0.03	0.047	0.045	0.066

Cells were grown in 20 ml of Luria-Bertani broth for overnight, harvested, resuspended in 100 ml of minimal media containing 5 mM of inducer, and incubated for 36 hours for the induction of enzyme activities.

(Table 2).

These results clearly show that biphenyl could be a common inducer in the oxidation of biphenyl and 2, 3-dihydroxybiphenyl. Salicylate was also shown to be a good inducer for the oxidation of biphenyl or 2, 3-dihydroxybiphenyl. However, benzoate was not able to induce the enzyme responsible for the oxidation of these compounds. Thus, it can be concluded that biphenyl and salicylate are good inducers for indigo formation as well as biphenyl dioxygenase which is metabolized to produce indigo.

Discussion

A. xylosoxydans SMN3 had a broad growth substrate spectra for a variety of aromatic compounds and their metabolic intermediates. (Shown in Table 1) The strain converted aromatics such as biphenyl.

Furukawa *et al.*, (1983) previously reported that a number of chlorinated biphenyls were converted to corresponding chlorobenzoates in *Acinetobacter* sp. strain P6 through an oxidative route that included *meta*-cleavage of one of the biphenyl rings (8). The dissimilatory pathways of various compounds in strain *Q1* was thus basically identical to that of the polychlorinated biphenyl-degrading *Acinetobacter* sp. strain P6.

Accordingly, the metabolic pathway of biphenyl compounds, salicylate, and their dissimilatory relationships have been investigated in our study. The common metabolites of biphenyls and salicylate can be further metabolized to catechol, followed by the *meta*-cleavage of the ring to produce 2-hydroxymuconic semialdehyde. With the strain SMN3, it was assumed that the hydrocarbons (biphenyl and phenol), alcohol (*m*-methylbenzyl alcohol), and carboxylic acids (salicylate) interacted with the regulatory gene product to induce synthesis of catabolic enzymes for both biphenyl and salicylate. Furthermore, catabolic enzymes for biphenyl and salicylate were also shown to be induced by the same substrate in this strain, shown in Table 3 and 4. However, even though biphenyl was shown to be a good inducer for indigo production, combination of biphenyl and benzoate resulted in a low induction of indigo. This combined effect cause us to assume that benzoate as a carbon source is not a good inducer for indigo production.

The results presented in Table 2 and Fig. 3 suggest which indole oxidation is a property of bacterial dioxygenase that form *cis*-dihydrodiols from aromatic hydrocarbon, such as biphenyl. However, combination of biphenyl and benzoate produce lower indigo formation than biphenyl or salicylate as shown Table 2. These results indicate that biphenyl or salicylate is a better inducer than benzoate for biphenyl dioxygenase.

Ensley *et al.*, (1983) reported that the oxidation of naphthalene by *Pseudomonas putida* PpG7 is catalyzed by enzymes that are encoded by a plasmid. They found that the entire pathway for cohverting naphthalene to salicylate 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 also shown that indigo formation is a property of the dioxygenase enzyme systems that form *cis*-dihydrodiol from aromatic hydrocarbons (2).

However, indigo formation was not observed in cultures containing more than 1% glucose. High glucose concentrations caused catabolite repression of tryptophanase synthesis in *E. coli*. Indigo formation occurred when indole was added to cultures of *P. putida* strain PpG7 (2). Unlike *E. coli*, this organism does not produce its own indole. All of these observations suggest that indigo synthesis in the 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 oxidize indole to indigo like other naphthalene-utilizing pseudomonads.

Beijerinckia strain B836 is a mutant that oxidizes biphenyl and various polycyclic aromatic hydrocarbons to *cis*-dihydrodiols, whereas *P. putida* strain 39/D oxidize benzene, toluene, and several moncyclic aromatic hydrocarbons to *cis*-dihydrodiols(16). Both of these mutant strains and their wild type parents oxidize indole to indigo. The ability of both B836 and wild-type strains to oxidize indole to indigo is induced by *m*-xylene.

Our results suggest that indigo formation is due to the combined activities of tryptophanase and biphenyl dioxygenase previously reported by Ensley *et al.*, (1983). The reaction sequence shown in Fig. 4

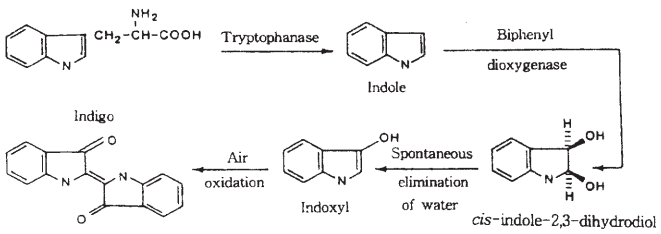

Fig. 4. Proposed pathway for indigo formation in *Alcaligenes xylosoxydans* SMN3. Indole in formed from tryptophan by tryptophanase, followed by indigo formation by biphenyl dioxygenase in the cells.

accounts for the formation of indigo by the strain SMN3 used in this investigation. It also provides a possible explanation for other reports on the indigo formation (2) although we have not been able to detect the formation of *cis*-2,3-dihydroxy-2,3-dihydrodiol.

The cell extracts also had induced levels of the enzymes involved in benzoate catabolism, such as *m*-methylbenzylalcohol dehydrogenase and 3-methylcatechol-2,3-dioxygenase (6). On the other hand, cell extracts from benzoate-grown cells had very low catechol 2,3-dioxygenase, and *m*-methylbenzylalcohol dehydrogenase activity, similar to the low activity in the sodium acetate-grown cell extract. However, high levels of catechol 1,2-dioxygenase activity were observed in the extracts of benzoate-grown cells, suggesting that benzoate does not induce any enzymes for substrates which are catabolized through the *meta* pathway such as biphenyl, xylene/toluene, or salicylate.

However, many of the *Pseudomonas* strain capable of decomposing catechol by the *meta*-cleavage pathway also are capable of the *ortho*-cleavage pathway. In naphthalene-degrading fluorescent pseudomonads, the enzymes of the *meta*-cleavage pathway were induced after growth with benzoate (1). The strain SMN3 is thought to be similar to these *Pseudmonads* in that benzoate induces the enzymes for biphenyl and catechol degradation, which are all metabolized through the *meta* pathway. The strain *P. putida* mt-2 and PRS 2000 which catabolize though the *meta*-pathway and *ortho*-pathway, respectively, could be compared (Table 3).

Biphenyl-and 2,3-dihydroxybiphenyl-oxidizing activity were induced when strain SMN3 was grown on biphenyl or salicylate; wereas, low activity was induced with benzoate (Table 4). Similarly, Furukawa *et al.*(7) reported that salicylate oxidation was observed in biphenyl, *m*-xylene-, or *m*-toluate-grown cells. Both the *meta*-cleavage enzymes 2,3-dihydroxybiphenyl dioxygenase and catechol 2,3-dioxygenase were observed in all biphenyl, *m*-xylene, or *m*-toluate, and salicylate-grown cells.

These observation led us to conclude that biphenyl and salicylate are good inducers for indigo formation as well as biphenyl dioxygenase which are metabolized to produce indigo in the strain SMN3.

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