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Roles of the *meta*- and the *ortho*-Cleavage Pathways for the Efficient Utilization of Aromatic Hydrocarbons by *Sphingomonas yanoikuyae* B1

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Catabolic pathways for the degradation of various aromatics by *Sphingomonas yanoikuyae* B1 are intertwined, joining at the level of substituted benzoates, which are further degraded via ring cleavage reactions. The mutant strain EK497, which was constructed by deleting a large DNA region containing most of the genes for biphenyl, naphthalene, *m*-xylene, and *m*-toluate degradation, was unable to grow on all of the aromatics tested except for benzoate as the sole source of carbon and energy. *S. yanoikuyae* EK497 was found to possess only catechol *ortho*-ring cleavage activity due to deletion of the genes for the *meta*-cleavage pathway. Wild-type *S. yanoikuyae* B1 grown on benzoate has both catechol *ortho*- and *meta*-cleavage activity. However, *m*-xylene and *m*-toluate, which are metabolized through methylbenzoate, and biphenyl, which is metabolized through benzoate, induce only the *meta*-cleavage pathway, suggesting the presence of a substrate-dependent induction mechanism.

Key words : ring-cleavage dioxygenase, *Sphingomonas*, aromatic hydrocarbons

Sphingomonas yanoikuyae B1 (formerly known as *Beijerinckia* sp. strain B1) (8, 12) is able to metabolize a wide variety of aromatic compounds including *m*- and *p*-xylenes, toluene, biphenyl, naphthalene, phenanthrene, anthracene, carbazole, dibenzothiophene, acenaphthene, acenaphthylene, dibenzo-*p*-dioxin, and benz[a]anthracene (7). This versatile catabolic ability is apparently due to the relaxed specificity of the initial degradative enzymes of separate upper metabolic pathways, which channel many structurally different aromatics into central metabolites such as benzoate and *m*-toluate (14, 24). One unique trait of *S. yanoikuyae* B1 is that the genes for aromatic hydrocarbon degradation in this strain are not arranged in discrete pathway units but are combined in groups for the degradation of both monocyclic and polycyclic aromatic compounds in the same operon. For example, *bphB*, which encodes *cis*-2,3-biphenyl dihydrodiol dehydrogenase, is present at the end of a *meta*-cleavage operon for the catabolism of aromatic acids (14). This novel organization might reflect the evolutionary process of recruiting, modifying, and reorganizing the appropriate genes to obtain the catabolic versatility of *S. yanoikuyae* B1 (14, 15, 17). The coexistence of the genes for degrading both monocyclic and polycyclic aromatic hydrocarbons in the same operon, which could allow for the simultaneous induction of the two catabolic pathways, might be beneficial when different classes of compounds are available at the same time. However, it would be extravagant for this strain to co-induce the degradative genes of the upper metabolic pathway in a situation where catabolic intermediates including benzoate are the only usable substrates.

Benzoate and *m*-toluate, which are central intermediates of biphenyl and *m*-xylene degradation by *S. yanoikuyae* B1, respectively, are metabolized via the *meta*-cleavage pathway when the strain is grown on the latter aromatic compounds (8, 13). However, recent studies demonstrated the presence of an *ortho*-cleavage pathway for benzoate degradation in *S. yanoikuyae* B1 (2, 14). The present work was initiated to investigate the roles of the *meta*-cleavage and *ortho*-cleavage pathways in the degradation of different aromatic compounds and to characterize the *ortho* pathway in more detail at the biochemical level.

Materials and Methods

Bacterial strains, media, and growth conditions

S. yanoikuyae strains B1 and EK497 were grown in mineral salts basal (MSB) medium (18). MSB was supple-

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mented with 20 mM sodium succinate when needed. Biphenyl was provided as crystals in the petri dish lids or 100 ppm in dimethylformamide for liquid medium. *m*-Xylene was provided in the vapor phase in cotton stoppered glass vials for solid media or in a glass bulb for liquid culture. Benzoate and *m*-toluate were added to MSB media at a final concentration of 5 mM. Luria-Bertani (LB) broth was used as complete medium. Solid media contained 1.5% agar. Ampicillin, kanamycin, and tetracycline were added to the medium when needed at 100 μg ml^{-1}, 50 μg ml^{-1}, and 15 μg ml^{-1}, respectively. *Sphingomonas* strains were grown at 30°C and *E. coli* strains were grown at 37°C.

Deletion mutagenesis

Deletion mutagenesis was performed by gene replacement as described previously (14). A hybrid plasmid was constructed in the vector pRK415 (11) where a 1.7 kb *Pst*I to *Sst*I fragment from mini-Tn*5Km1* containing the kanamycin resistance gene was inserted in place of a deleted 33,188 kb region of the cloned fragment (Fig. 1). Strain EK497 was then generated through homologous recombination of the regions flanking the kanamycin resistance cassette and the corresponding genomic region of *S. yanoikuyae* B1.

Enzyme assays

Following growth on aromatic substrates, *S. yanoikuyae* B1 and EK497 were harvested, washed, resuspended in 50 mM MOPS buffer (pH 7.8) containing 10% acetone, 10% glycerol, 1 mM ascorbic acid, and 100 μM $FeSO_4$, and disrupted by sonication. Unbroken cells and cell debris were removed by centrifugation at 10,000 × *g* for 30 min. The resulting supernatant was used as the enzyme solution. Catechol 2,3-dioxygenase (C23O) activity was assayed spectrophotometrically by measuring the increase in the absorbance at the corresponding wavelength of the *meta*-cleavage product of catechol: λ_{max}=375 nm and ε =33,400 $cm^{-1}M^{-1}$ (3). The activity of catechol 1,2-dioxygenase (C12O) was assayed spectrophotometrically by monitoring the increase in the absorbance at 260 nm for *cis,cis*-muconate: ε=16,800 $cm^{-1}M^{-1}$ (4). The activity of *cis,cis*-muconate cycloisomerase (CCMC) was assayed spectrophotometrically by measuring the rate of decrease in the absorbance at 260 nm as described by Ornston (16). *cis,cis*-Muconate was provided by Professor, Sang-Ho Choi at Chonnam National University, Korea. One unit of enzyme activity is defined as the formation of 1 μmol of product per minute at 25°C.

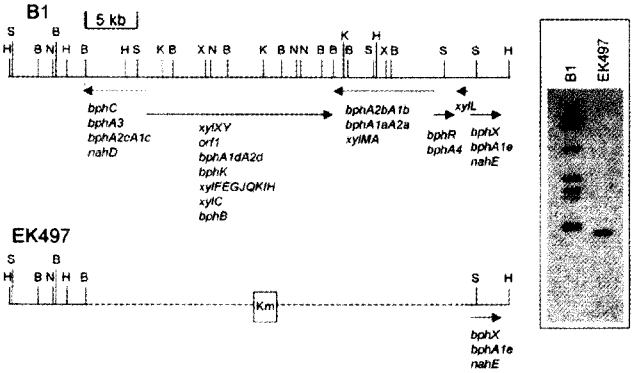

Fig. 1. Construction of the deletion mutant EK497 from *S. yanoikuyae* B1. A kanamycin resistance cassette was inserted in place of the indicated genes. EK497 cannot grow on biphenyl, naphthalene, phenanthrene, toluene, *m*-xylene, and *m*-toluate due to loss of genes encoding the oxygenases that initially metabolize these aromatic compounds. Abbreviations: B, *Bam*HI; H, *Hin*dIII, K, *Kpn*I; N, *Not*I; S, *Sst*I; X, *Xba*I; Km, kanamycin resistance gene. The inset shows Southern blot using a *S. yanoikuyae* B1 *bph/xyl* gene probe and *Bam*HI digested total genomic DNA prepared from B1 and EK497.

Slot blot analysis

Total RNA was prepared by the method of Summers (19). Ten micrograms of total RNA was denaturated in 50% formamide-6% formaldehyde and transferred to nylon membranes (Sigma, USA) using a slot blot apparatus. DNA restriction fragments to be used as probes in slot blot experiments were separated by gel electrophoresis and eluted from gel fragments by the procedure of Vogelstein and Gillespie (20). DNA fragments were labeled with [α-^{32}P]-dATP by the random priming method of Feinberg and Vogelstein (5). Hybridization was performed overnight at 60 °C in a solution containing (2 SSPE, 7% SDS, 10% PEG8000, 100 g ml^{-1} denatured sonicated herring sperm DNA). Washes were done at 63°C sequentially in solutions containing 2 × SSC plus 0.1% SDS (15 min), 1 × SSC plus 0.1% SDS (15 min), and 0.1 × SSC plus 0.1% SDS (10 min).

Results

Identification of an ortho-cleavage pathway in S. yanoikuyae B1

S. yanoikuyae EK497 was constructed by replacing a 33,188 bp region of DNA, from the *Pst*I site in *nahD* to the *Sst*I site upstream of *bphX* with a kanamycin resistance gene cassette (Fig. 1). This mutation is missing all of the genes for the *meta*-cleavage pathway and many of the genes for the upper biphenyl and *m*-xylene pathways. *S. yanoikuyae* EK497 is still able to grow on benzoate as a sole carbon and energy source although it lost the ability to grow on *m*-toluate, *m*-xylene, biphenyl, naphthalene, and phenanthrene. This observation clearly demonstrates that *S. yanoikuyae* B1 possesses an additional pathway for benzoate degradation. In order to characterize this pathway in more detail activities of C23O, C12O, and CCMC were examined in the cells of *S. yanoikuyae* EK497 grown on benzoate and succinate. As summarized in

Table 1, two key enzymes of the *ortho*-pathway (C12O and CCMC) are induced in EK497 grown on benzoate while no C23O activity was detected. This means that benzoate is metabolized solely via an *ortho*-cleavage pathway in *S. yanoikuyae* EK497.

Induction of meta- and ortho-cleavage enzymes in S. yanoikuyae B1

Kim and Zylstra (13) showed that the *meta*-cleavage pathway is induced by either *m*-xylene or biphenyl, which are degraded via *m*-toluate and benzoate, respectively. *m*-Toluate, which is converted to 3-methylcatechol, was expected to be metabolized more efficiently by the *meta*-cleavage pathway than by the *ortho*-cleavage pathway because C23O is able to tolerate alkyl substituents on the catechol (9). However, benzoate, which is formed as an intermediate during biphenyl degradation, can be channeled into either the *meta*- or *ortho*-cleavage pathway. One question that remains is which pathway predominates upon growth on either biphenyl or benzoate. In order to address this question the activities of C12O, CCMC, and C23O were examined in cells of *S. yanoikuyae* B1 grown on benzoate, *m*-toluate, and biphenyl. As summarized in Table 1, although a large amount of C23O activity was detected in *m*-toluate-grown cells, there is no detectable C12O or CCMC activity in the same crude extract. In contrast, when *S. yanoikuyae* B1 was grown on benzoate, C12O and CCMC activities were high while C23O activity was near the basal level. Furthermore, despite the fact that biphenyl is converted to benzoate during its degradation, neither C12O nor CCMC activity was detected in B1 grown on biphenyl. The different induction patterns of the two ring-cleavage dioxygenases and CCMC following growth on different aromatic substrates indicate that *S. yanoikuyae* B1 is able not only to distinguish two structurally homologous aromatic acids, but also to modulate the expression of both *meta*- and *ortho*-pathways depending on available substrates.

Induction of the xylX gene for toluate dioxygenase

Table 1. Induction of key enzymes in the *meta*- and *ortho*-cleavage pathways in *S. yanoikuyae* B1 and EK497. Enzyme activities are the average of at least three independent experiments and are expressed as units per mg protein

Strain	Growth substrate	C23O	C12O	CCMC
EK497	Succinate	ND[1]	ND	ND
	Benzoate	ND	1,519.0 ± 136.7	509.9 ± 40.7
B1	Succinate	66.7 ± 2.7	ND	ND
	Benzoate	76.4 ± 3.5	679.7 ± 25.9	218.7 ± 6.6
	m-Toluate	7,475.5 ± 526.2	ND	ND
	Biphenyl	2,923.4 ± 116.9	ND	ND

[1]Not detected.

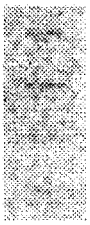

Fig. 2. Slot blot analysis of total RNA isolated from *S. yanoikuyae* B1 using the *xylX* gene as a probe. The growth substrate is listed next to each slot.

The above enzyme assay data demonstrated that C23O was induced specifically by growth on either *m*-toluate or biphenyl, but not by growth on benzoate. One question that remains is whether other enzymes in the *meta*-cleavage pathway are also induced in this manner. The *xylX* gene encodes the large subunit of toluate dioxygenase and is the first gene in the *meta*-cleavage operon of B1, although it is suspected that there may be a second promoter between *xylX* and *xylE* (14). In order to analyze the induction patterns of *xylX* following growth on *m*-toluate, biphenyl, benzoate, and succinate, slot blots were performed with total RNA prepared from *S. yanoikuyae* B1 grown on each substrate and the *xylX* gene as a probe. As shown in Fig. 2, expression of the *xylX* gene increased significantly in the presence of *m*-toluate or biphenyl but benzoate and succinate failed to induce expression of *xylX*. These data suggest that all the genes in the *meta*-cleavage pathway are induced by *m*-toluate or biphenyl, but not by benzoate, which can be metabolized more efficiently by the *ortho*-cleavage pathway.

Discussion

In general the genes for aromatic hydrocarbon degradation in most known bacteria are organized into operons based on catabolic segments (1, 23). One operon might contain those genes required for conversion of polycyclic aromatic hydrocarbons to simple aromatic acids (i.e. the upper *bph* or *nah* operons), a second operon might contain those genes required for conversion of monocyclic aromatic compounds to aromatic acids (i.e. the upper *xyl* operon), and a third operon might contain those genes required for conversion of aromatic acids to TCA cycle intermediates (i.e. the *meta*-operon of the TOL plasmid). However, this is not the case for *S. yanoikuyae* B1. Rather, the aromatic degradative genes in *S. yanoikuyae* B1 are combined not in discrete pathway units but in groups with genes for the degradation of monocyclic and polycyclic aromatic hydrocarbons (14). This novel genetic organization of *S. yanoikuyae* B1 leads to simultaneous induction of the *meta*-cleavage pathway along with the upper catabolic pathways (14) and thus B1 needs a mechanism for

the selective activation of the appropriate genes to avoid the uneconomical expression of unnecessary genes.

One significant difference between *meta-* and *ortho-*pathways is that the former can serve as a route for growth substrates which are converted to alkylcatechol such as 3-methylcatechol and 4-methylcatechol mainly because C23O is able to tolerate alkyl substituents on the catechol (9). This might explain why only C23O activity is detected in *S. yanoikuyae* B1 grown on *m*-toluate and why *S. yanoikuyae* EK497 is unable to grow on *m*-toluate, which is metabolized via 3-methylcatechol. Also, it is generally accepted that the *meta*-cleavage pathway is induced by substituted benzoates *per se* while *cis,cis*-muconate, the *ortho*-cleavage product of catechol, is the inducer for the *ortho*-cleavage route (10, 21). Thus, when both pathways coexist in the same cell the former might be induced earlier, which results in channeling the majority of substrate molecules into the *meta*-cleavage pathway. As a result, the concentration of *cis,cis*-muconate might not reach high enough to induce the *ortho*-pathway at full scale. In this context, exclusive induction of the *meta*-pathway by *m*-toluate or biphenyl can be expected. In fact, the presence of the *bphB* gene at the end of the *meta*-operon also contributes to the earlier induction of the *meta*-pathway. However, although the benzoate formed during biphenyl degradation is degraded exclusively by the *meta*-pathway, directly fed benzoate is channeled into the *ortho*-pathway as evidenced by enzyme assay and slot blot results. These results are in agreement with the previous report that growth of *S. paucimobilis* Q1 (formerly known as *Pseudomonas paucimobilis* Q1) with benzoate as a sole carbon source allowed the induction of only the *ortho*-pathway enzymes although biphenyl-grown Q1 cells showed induction of the *meta*-cleavage enzymes (6). It should be noted that C23O has a significant basal activity during growth on either succinate or benzoate. Therefore, some benzoate catabolism is directed through the *meta*-cleavage pathway which decreases induction of the *ortho* pathway in B1 during growth on benzoate. Indeed, this is one reason why induction levels of C12O and CCMC in B1 grown on benzoate are 55% less than those in EK497 (Table 1). The ability to utilize only the *ortho*-pathway for growth on benzoate is a significant evolutionary advantage for B1. *P. putida* mt-2, where both the *ortho* and the *meta* pathway are induced by growth on benzoate, readily loses the *meta*-cleavage pathway genes following growth on benzoate because there is a growth advantage to using the *ortho*-cleavage pathway (22). In mt-2 loss of the *meta* pathway genes results in the loss of the ability to grow on *m*- and *p*-xylene. In contrast, separate regulation of the *meta* and *ortho* pathways in B1 prevents competition between the two pathways and prevents loss of the *meta* pathway which would result in the loss of the ability to grow on the xylenes, biphenyl, naphthalene, and phenanthrene.

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