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NOTE

Association of a Common Reductase with Multiple Aromatic Terminal Dioxygenases in *Sphingomonas yanoikuyae* Strain B1

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The aromatic dioxygenase system in *Sphingomonas yanoikuyae* strain B1 consists of three components, an oxygenase, a ferredoxin, and a reductase. The insertional knockout of the *bphA4* gene encoding a reductase and subsequent complementation experiments showed that the reductase encoded by *bphA4* in *S. yanoikuyae* strain B1 is associated with multiple dioxygenase components including that of toluate dioxygenase (XylXY).

Key words: *Sphingomonas*, aromatic dioxygenase, reductase, ferredoxin, iron sulfur protein

Sphingomonas yanoikuyae strain B1 (formerly known as *Beijerinckia* sp. strain B1) (10) is able to metabolize a wide variety of aromatic compounds including *m*-, *p*-xylenes, toluene, biphenyl, naphthalene, phenanthrene, anthracene, carbazole, dibenzothiophene, acenaphthene, acenaphthylene, dibenzo-*p*-dioxin, and benz[a]anthracene (21). Previous studies showed that the same genes are involved in the degradation of various aromatic hydrocarbons by *S. yanoikuyae* strain B1 (Fig. 1) (12, 13, 14).

Aromatic dioxygenase, the initial enzyme of the aromatic degradative pathway, is normally a three-component enzyme system, which consists of a flavoprotein reductase, a ferredoxin containing a Rieske-type [2Fe-2S] center and an iron sulfur protein. It has been known that reductase and ferredoxin components form a short electron transport chain that supplies electrons to an iron sulfur protein component (terminal dioxygenase), which adds both atoms of molecular oxygen to one of the aromatic rings (5, 6). The iron sulfur protein component also contains a Rieske-type [2Fe-2S] center and comprises a large and a small subunit. It is generally accepted that the large subunit plays a greater role in determining substrate specificity (3, 8, 20). Interestingly, *S. yanoikuyae* strain B1 was found to have at least six different sets of an iron sulfur protein component. The presence of multiple dioxygenases as well as the relaxed specificity of initial enzymes of the upper degradative pathway might be possible explanations for the broad substrate spectrum of *S. yanoikuyae* strain B1 (12). In contrast to the iron sulfur protein, a single gene was identified for ferredoxin and reductase components of aromatic dioxygenase, respectively (21). In particular, the ferredoxin (BphA3) component is involved not only as a component in the initial ring-oxidizing dioxygenase but also as a component in toluate dioxygenase (12). Since the toluate dioxygenase is generally a two component enzyme system, in which a ferredoxin and a reductase are combined into a single component, it is very interesting to observe that a ferredoxin component of the initial aromatic dioxygenase, which is a three component system, is involved even in *m*-toluate degradation. This observation suggests the possibility that the reductase component (BphA4) of aromatic dioxygenase is also associated with the iron sulfur protein (XylXY) of toluate dioxygenase. Thus, this study attempted to verify the putative association of the reductase component (BphA4) with multiple terminal dioxygenase components including XylXY.

The insertional mutant strain *S. yanoikuyae* strain MB1 (*bphA4*::Cm) was constructed by inserting a chloramphenicol resistance cassette into the *Nru*I site, which cleaves the *bphA4* gene (Fig. 2). A 2.0 kb *Sph*I to *Pst*I DNA fragment was cloned from pGJZ1530 (11) into pGEM3Z (Promega, Madison, USA). The resulting plasmid was digested with *Nru*I and subsequently ligated with a 0.9 kb *Sma*I DNA fragment from p34S-cm containing the chloramphenicol resistance gene (1). The hybrid plasmid was digested with *Pst*I and *Hin*dIII (located on the multiple cloning site of pGEM3Z) and transferred to the broad host range vector pRK415 (9). *S. yanoikuyae* strain MB1 was constructed through homologous recombination

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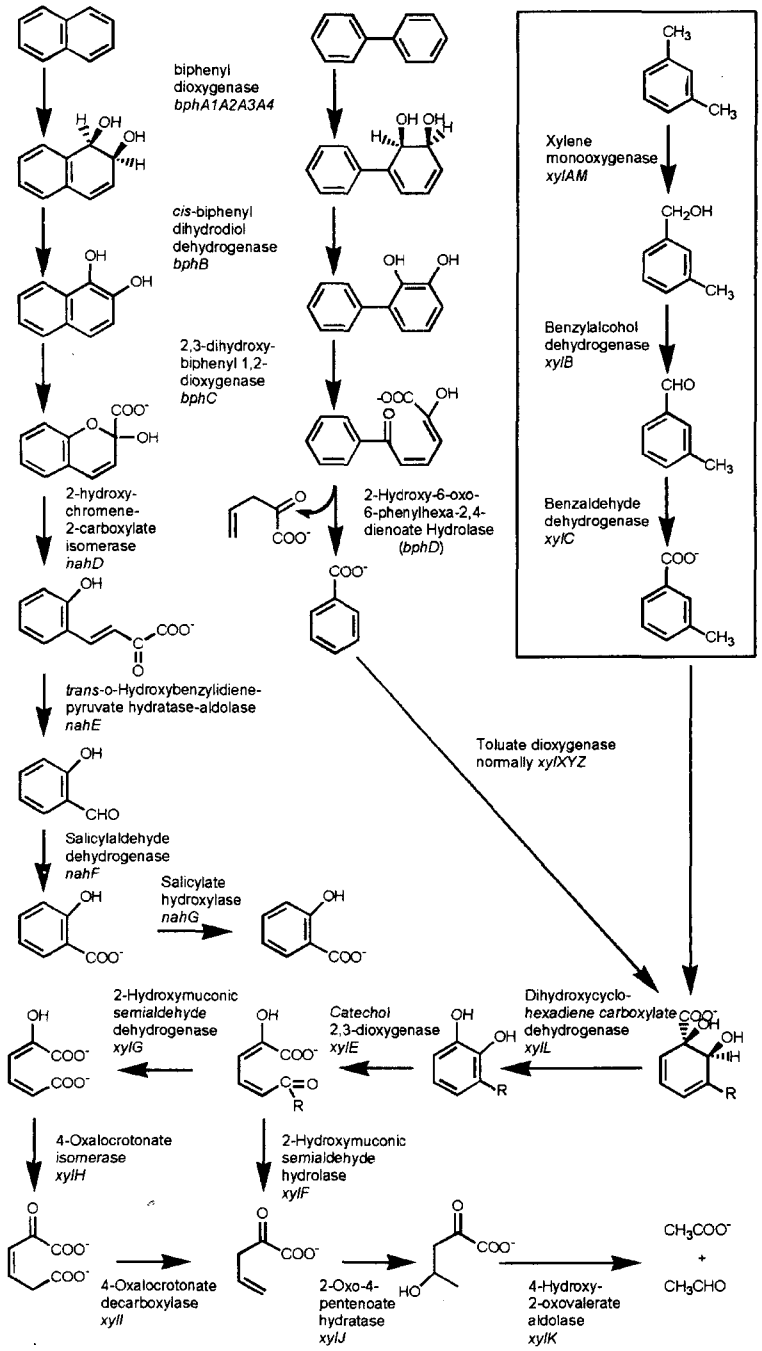

Fig. 1. Catabolic pathway for the degradation of biphenyl, naphthalene, phenanthrene, and *m*-xylene by *S. yanoikuaye* strain B1. Toluene and *p*-xylene are metabolized by a pathway similar to that shown for *m*-xylene. Obtained from Zylstra and Kim (21).

between the genomic DNA of *S. yanoikuyae* strain B1 and the hybrid plasmid after conjugal transfer of the construct. Exconjugants were isolated on tetracycline and chloramphenicol resistance. Subsequently, the cells were inoculated into 5 ml of MSB (mineral salts basal) medium (19) containing 20 mM succinate without the antibiotics and incubated overnight at 30°C with shaking. One ml of the overnight culture was transferred to 5 ml of fresh medium for further incubation. The same overnight culture with proper dilution was also plated out on MSB-succinate plates containing 15 g/ml of tetracycline. The number of colonies on these plates was compared with that of plates without the antibiotics. This procedure was repeated and the insertion mutant was selected for acquisition of chloramphenicol resistance and loss of tetracycline resistance.

As expected, *S. yanoikuyae* strain MB1 was unable to grow on biphenyl, naphthalene, phenanthrene, *m*-xylene

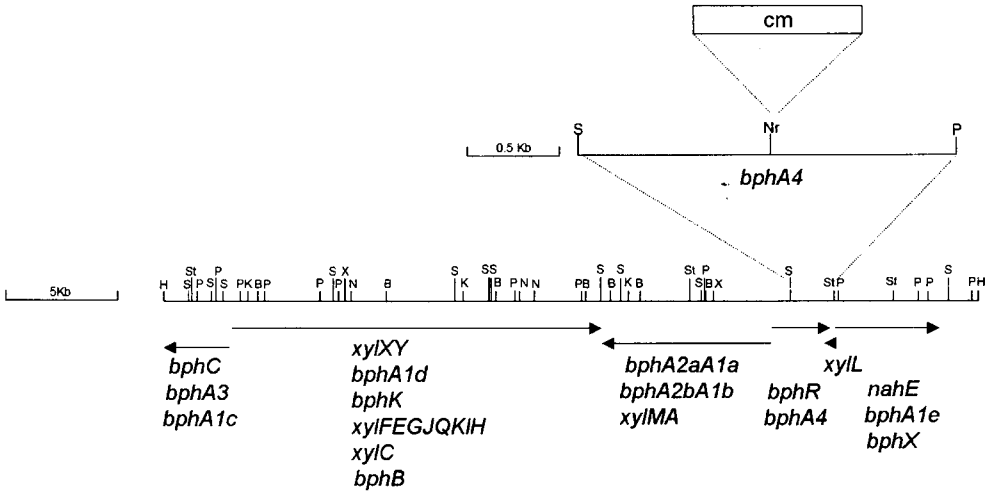

Fig. 2. The location of chloramphenicol resistance cassette in the insertional mutant, *S. yanoikuyae* strain MB1. Abbreviations: B, *Bam*HI; H, *Hin*dIII; K, *Kpn*I; N, *Not*I; Nr, *Nru*I; P, *Pst*I; S, *Sph*I; St, *Sst*I; X, *Xba*I.

Table 1. Characteristics and complementation of *S. yanoikuyae* strain MB1

	Biphenyl	Naphthalene	Phenanthrene	*m*-Xylene	*m*-Toluate	Benzoate
MB1	-	-	-	-	-	+
MB1 (pKEB1105)	+	+	+	+	+	+

[a]The ability of bacterial strains to use the volatile carbon sources naphthalene, biphenyl, and *m*-xylene was determined by providing the source in the vapor phase for cells that were inoculated on MSB agar. Plates were placed in screw-capped jars and were incubated at 30°C. Naphthalene vapor was provided by placing crystals in an uninoculated plate; biphenyl crystals were placed directly in the lids of inoculated plates. A cotton-stoppered glass tube containing *m*-xylene was placed in the lids of uninoculated plates. Growth on phenanthrene was determined by a spray-plate method (15). Plates were inoculated and then lightly sprayed with ether containing 0.5% of phenanthrene. Benzoate and *m*-toluate were provided at the concentration of 5 mM.

and *m*-toluate (Table 1). However, it should be noted that *S. yanoikuyae* strain MB1 is still able to grow on benzoate as a sole carbon and energy source suggesting that *S. yanoikuyae* strain B1 additionally possesses other oxygenase(s) for benzoate degradation. Also, the mutant strain lost the ability to produce indigo (blue colonies) from indole, when cells are exposed to indole after growth on either biphenyl or *m*-xylene, which is indicative of aromatic dioxygenase activity (2). These data indicate that the same reductase component is associated with multiple dioxygenase components (Table 1). In order to confirm this observation, complementation experiments were performed with *S. yanoikuyae* strain MB1.

A 2.0 kb *Sph*I to *Pst*I DNA fragment, containing the intact *bphA4* gene and parts of the *bphR* and *xylL* genes for a putative transcriptional regulator and dihydrocyclohexadiene carboxylate dehydrogenase, respectively, was cloned into the broad host range vector pBBR1MCS-3 (16) for complementation experiments with *S. yanoikuyae* strain MB1. The ability of the recombinant plasmid (pKEB1105) to complement the mutation was determined by transferring the plasmid into the strain by triparental mating using the helper plasmid pRK2013 (4). *S. yanoikuyae* strain MB1 harboring pKEB1105 recovers the ability not only to grow on all of the substrates described above but also to catalyze the indole-indigo reaction (Table 1). These results suggest that reductase encoded by *bphA4* in *S. yanoikuyae* strain B1 is indeed associated with multiple dioxygenase components including that of toluate dioxygenase (XylXY).

As mentioned previously above, toluate dioxygenase and the related enzyme benzoate dioxygenase, consist of two components: a bifunctional reductase encoded by the *xylZ* gene and an oxygenase consisting of a large and small subunit encoded by the *xylXY* genes (7, 17). It was previously suggested that toluate dioxygenase in *S. yanoikuyae* strain B1 consists of three components (an oxygenase, a ferredoxin, and a reductase) by showing that the ferredoxin encoded by *bphA3* is required for toluate dioxygenase activity (12). This study clearly confirms this possibility by showing that the insertional inactivation of the *bphA4* gene simultaneously results in the loss of toluate dioxygenase activity in *S. yanoikuyae* strain B1. Kim and Zylstra (1999) also mentioned the involvement of a three-component dioxygenase in aromatic acid metabolism is not without precedence as *ortho*-halobenzoate 1,2-dioxygenase isolated from *Pseudomonas aeruginosa* strain 142 is known to be a three-component enzyme (18). However, the aromatic oxygenase system in *S. yanoikuyae* strain B1 is peculiar in that both the ferredoxin and reductase play a dual role as an electron transfer component of both toluate dioxygenase and a broad substrate range PAH (polycyclic aromatic hydrocarbon) dioxygenase (Fig. 3). The previous observation that *S. yanoikuyae* strain B1 does not contain the *xylZ* gene for a

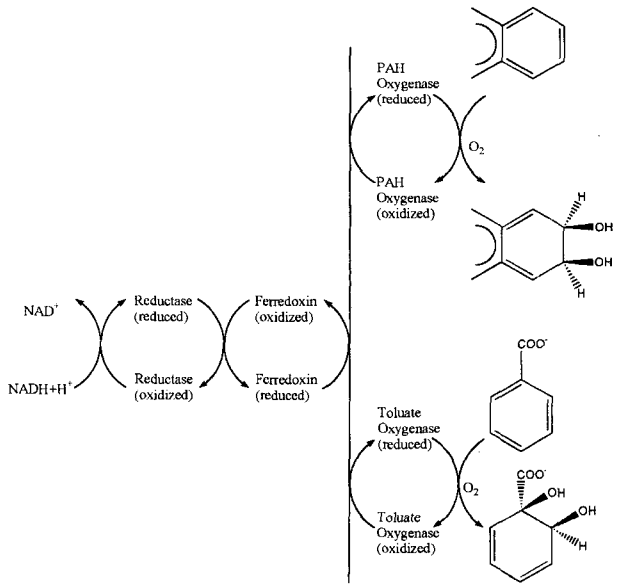

Fig. 3. Schematic description of the electron transport chain for the aromatic dioxygenase components in *S. yanoikuyae* strain B1.

reductase of two-component dioxygenase (21) is confirmed by the data presented here which indicate that the reductase of toluate dioxygenase normally encoded by *xylZ* in the TOL plasmid is not needed by *S. yanoikuyae* strain B1.

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