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Functional analysis of genes involved in biphenyl, naphthalene, phenanthrene, and *m*-xylene degradation by *Sphingomonas yanoikuyae* B1

E Kim[1,2] and GJ Zylstra[2]

[1]*Department of Biology, Yonsei University, Seoul, South Korea;* [2]*Biotechnology Center for Agriculture and the Environment, Cook College, Rutgers University, New Brunswick, New Jersey 08901–8520, USA*

Sphingomonas yanoikuyae B1 is able to utilize toluene, _m_-xylene, _p_-xylene, biphenyl, naphthalene, phenanthrene, and anthracene as sole sources of carbon and energy for growth. A forty kilobase region of DNA containing most of the genes for the degradation of these aromatic compounds was previously cloned and sequenced. Insertional inactivation of _bphC_ results in the inability of B1 to grow on both polycyclic and monocyclic compounds. Complementation experiments indicate that the metabolic block is actually due to a polar effect on the expression of _bphA3_, coding for a ferredoxin component of a dioxygenase. Lack of the ferredoxin results in a nonfunctional polycyclic aromatic hydrocarbon dioxygenase and a nonfunctional toluate dioxygenase indicating that the electron transfer components are capable of interacting with multiple oxygenase components. Insertional inactivation of a gene for a dioxygenase oxygenase component downstream of _bphA3_ had no apparent effect on growth besides a polar effect on _nahD_ which is only needed for growth of B1 on naphthalene. Insertional inactivation of either _xylE_ or _xylG_ in the _meta_-cleavage operon results in a polar effect on _bphB_, the last gene in the operon. However, insertional inactivation of _xylX_ at the beginning of this cluster of genes does not result in a polar effect suggesting that the genes for the _meta_-cleavage pathway, although colinear, are organized in at least two operons. These experiments confirm the biological role of several genes involved in metabolism of aromatic compounds by _S. yanoikuyae_ B1 and demonstrate the interdependency of the metabolic pathways for polycyclic and monocyclic aromatic hydrocarbon degradation.

Keywords: phenanthrene; naphthalene; biphenyl; xylene; *Sphingomonas*; biodegradation

Introduction

Sphingomonas yanoikuyae B1 is able to utilize a wide variety of aromatic hydrocarbons as carbon and energy sources. This organism, originally called *Beijerinckia* species strain B1 but reclassified as *Sphingomonas yanoikuyae* [28], was originally isolated for its ability to utilize biphenyl as a carbon source [22]. The organism is able to grow on several additional polycyclic aromatic hydrocarbons, including naphthalene, phenanthrene, and anthracene [1,22,25], as well as the monocyclic aromatic hydrocarbons toluene, *m*-, and *p*-xylene [39]. Metabolism of polycyclic or monocyclic aromatic compounds is coinduced by growth on either class of substrate [31,38,39]. Once the catabolic pathways are induced the organism is capable of oxidizing a wide range of aromatic compounds including dibenzofuran [4], dibenzothiophene [36], acenaphthene [44], acenaphthylene [44], carbazole [42], dibenzo-*p*-dioxin [34], benz(*a*)anthracene [21,26,38], benz(*a*)pyrene [21], 3-methylcholanthrene [29], 6,7-dihydro-5H-benzocycloheptene [41], and 1,2-dihydronaphthalene [12]. Metabolism of biphenyl, naphthalene, phenanthrene, toluene, and the xylenes is thought to proceed as shown in Figure 1. A common set of enzymes is responsible for the initial metabolic steps in degradation of the polycyclic aromatic compounds. An initial dioxygenase attack results in the formation of a *cis*-dihydrodiol. Although the initial dioxygenase from *S. yanoikuyae* B1 has not been purified, analogous enzymes from other microorganisms consist of three components: a reductase, a ferredoxin, and a dioxygenase comprised of a large and small subunit [13,23]. Following the initial dioxygenase attack a dehydrogenase rearomatizes the ring structure to form a dihydroxylated product which is *meta*-cleaved [22]. These first three enzymes have broad substrate ranges and thus *S. yanoikuyae* B1 is capable of metabolizing a wide range of polycyclic compounds through the ring cleavage step. Following ring cleavage metabolism of biphenyl proceeds through a hydrolase to form benzoate while metabolism of naphthalene proceeds through an isomerase, a hydratase-aldolase, a dehydrogenase, and salicylate hydroxylase to form catechol [5,10,11]. Phenanthrene is metabolized by a similar pathway [15]. The metabolism of monocyclic aromatic compounds proceeds via a TOL plasmid-type pathway (Figure 1) involving successive oxidation of the methyl group [39]. The 'upper' catabolic pathways for monocyclic and polycyclic aromatic compounds intersect at a 'lower' *meta*-cleavage pathway, presumably with metabolic reactions similar to those found in the TOL plasmid pathway [2,47,48] and shown in Figure 1.

We previously reported on the cloning and nucleotide sequence of two genes coding for *meta*-cleavage dioxygenases from *S. yanoikuyae* B1 [31]. Biochemical characterization of the enzymes encoded by these two genes ident-

Correspondence: GJ Zylstra, Biotechnology Center for Agriculture and the Environment, Foran Hall, 59 Dudley Road, Cook College, Rutgers University, New Brunswick, NJ 08901–8520, USA
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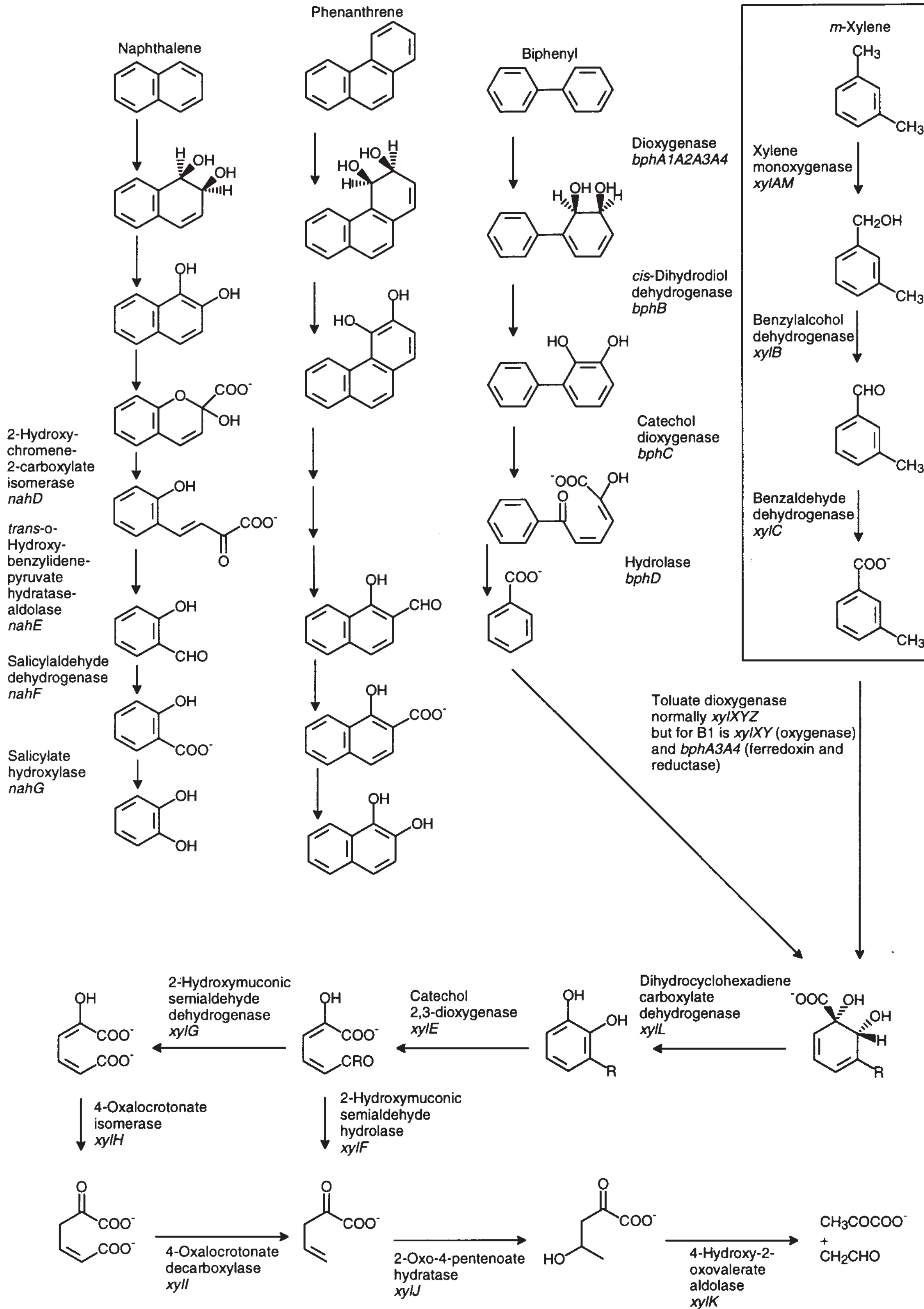

Figure 1 Catabolic pathway for the degradation of biphenyl, naphthalene, phenanthrene, and *m*-xylene by *S. yanoikuyae* B1. Toluene and *p*-xylene are metabolized by a pathway similar to that shown for *m*-xylene. Obtained from Zylstra and Kim [51] with permission.

ified one as 2,3-dihydroxybiphenyl 1,2-dioxygenase (encoded by the *bphC* gene) and the other as catechol 2,3-dioxygenase (encoded by the *xylE* gene). The two genes are contained on the same cosmid clone (pGJZ1510), about seven kilobases apart, and are diametrically transcribed. The nucleotide sequence of a 40-kbp region around *bphC* and *xylE* was determined with the concomitant identification of a number of genes involved in polycyclic and monocyclic aromatic hydrocarbon degradation [51]. The current paper presents biochemical and biological data on the function of genes located in the two putative operons containing *bphC* and *xylE* (Figure 2). Preliminary reports of this work have appeared elsewhere [30,32,33].

Materials and methods

Bacterial strains, plasmids, and media

S. yanoikuyae B1 is the wild-type strain which is capable of growth on biphenyl, naphthalene, phenanthrene, toluene, *m*-, and *p*-xylene [22]. *S. yanoikuyae* B8/36 was derived from B1 by chemical mutagenesis and lacks *cis*-dihydrodiol dehydrogenase activity [22]. *P. putida* PPO200 is *P. putida* mt-2 [47] cured of the TOL plasmid [52]. *E. coli* DH5α (F^- *ϕ80dlacZΔM15 Δ(lacZY-argF)U169 deoR recA1 endA1 hsdR17(rK$^-$,mK$^+$) supE44 thi-1 gyrA96 relA1*; Gibco-BRL, Gaithersburg, MD, USA) was used as the recipient strain in all of the cloning experiments. The pGEM series of cloning vectors (Promega, Madison, WI, USA) were utilized to construct subclones for DNA sequencing. The cloning vector pRK415 [27] was used to construct the plasmids for mutant complementation experiments. The helper plasmid pRK2013 was used in triparental mating experiments to mobilize the pRK415-based clones into the *S. yanoikuyae* B1 mutants as described previously [9]. The cosmid clone pGJZ1510 [31] is the source of DNA for the subclones. Minimal medium (MSB) [45] used for the metabolite accumulation experiments or transconjugant selection was supplemented with 20 mM sodium succinate when needed. Screening for growth on plates was accomplished by adding biphenyl and naphthalene as crystals to the plate lids, spraying the surface of the agar with an ethereal solution of phenanthrene, or by adding toluene, *m*-, or *p*-xylene to cotton-stoppered small glass vials in the plate lid. Polycyclic aromatic hydrocarbon substrates were added to broth cultures as crystals while monocyclic aromatic hydrocarbons were added to the culture medium in a glass bulb suspended above the medium. L-broth [37] was used as complete medium. Solid MSB or L medium contained 2% agar. Ampicillin, kanamycin, and tetracycline were added to the culture medium when needed at 100, 50, and 15 $\mu g\ ml^{-1}$, respectively. *S. yanoikuyae* B1 was grown at 30°C while *E. coli* were routinely cultured at 37°C. Complementation tests of the *pdxA* mutation in *E. coli* CGSC 5630 [6] were performed with MSB medium with and without pyridoxine HCl (1 $\mu g\ ml^{-1}$). The medium used for this purpose was MSB agar containing glucose (50 $mg\ ml^{-1}$), L-glutamine (100 $\mu g\ ml^{-1}$), L-isoleucine (20 $\mu g\ ml^{-1}$), L-leucine (20 $\mu g\ ml^{-1}$), L-proline (30 μg ml), L-threonine (80 $\mu g\ ml^{-1}$), L-valine (40 $\mu g\ ml^{-1}$), and thiamine HCl (1 $\mu g\ ml^{-1}$). *E. coli* CGSC 5630 (pGEM3Z) was used as a negative control.

Molecular techniques

Plasmid DNA was isolated by the alkaline-sodium dodecyl sulfate technique [3] or by the QIAprep spin column procedure (Qiagen, Chatsworth, CA, USA). Restriction digests, ligations, and transformation into *E. coli* DH5α competent cells were performed as recommended by the supplier (Bethesda Research Laboratories, Gaithersburg, MD, USA). Agarose gel electrophoresis was performed in 40 mM Tris, 20 mM acetate, 2 mM EDTA buffer. Southern hybridizations were performed as recommended by the nylon membrane supplier (Bio-Rad Laboratories, Rockville Center, NY, USA). DNA restriction fragments were isolated from agarose gels using the procedure of Vogelstein and Gillespie [46]. DNA fragments were labeled by the random priming method of Feinberg and Vogelstein [16].

Generation and characterization of insertional knockout mutants

Insertional knockout mutants were constructed by replacing the wild-type DNA with DNA that has a kanamycin resistance gene insertion. This was accomplished by taking advantage of the inherent instability of pRK415. A kanamycin resistance gene cassette was inserted into a convenient restriction site in a DNA fragment. The DNA fragment was

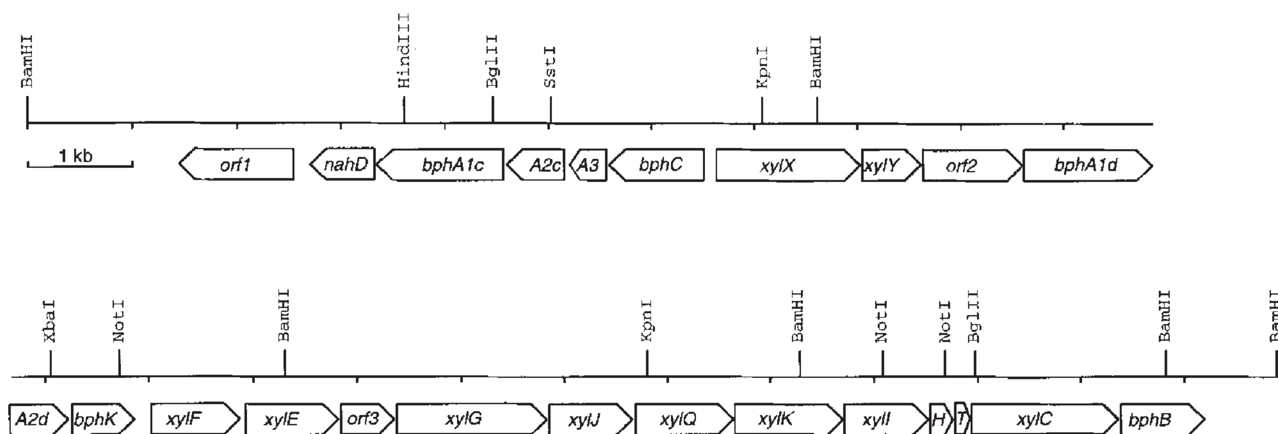

Figure 2 Nucleotide sequence cartoon of the 23-kb gene cluster showing the locations of the genes for biphenyl, naphthalene, phenanthrene, and *m*-xylene degradation described in this paper [51]. Each tick mark represents one kilobase pair.

then cloned into pRK415 and mobilized into *S. yanoikuyae* B1 by triparental mating. Transconjugants were selected for kanamycin and tetracycline resistance on MSB succinate plates. The cells were then cultured overnight in MSB succinate broth without antibiotics. An aliquot of the culture was plated on MSB succinate plates containing kanamycin and 1.0 ml of the broth culture was subcultured into 5 ml of fresh medium without antibiotics. This procedure was repeated until colonies were obtained which were kanamycin-resistant but tetracycline-sensitive. This process generally took 3–5 days. The insertional mutants were analyzed by Southern blotting to verify that a double reciprocal recombination event actually did occur and that the original chromosomal DNA had been replaced with the kanamycin resistance gene. HPLC analysis of culture supernatants for accumulating metabolites was performed with a Beckman System Gold HPLC (Fullerton, CA, USA) fitted with a reverse phase 5-μm C18 column (4.6 mm × 25 cm) with diode array spectral detection using a gradient of 50–100% methanol in water (1 ml min^{-1}). Acetic acid (1%) was added to the mobile phase for the detection of *p*-toluate. The identity of all accumulating metabolites was confirmed by comparing retention time and UV/Vis spectrum with those of authentic standards.

The pRK415-based clones for construction of the insertional knockout mutants were made as follows. Strain EK121 was constructed by inserting a kanamycin resistance gene from pUC4-KIXX (Pharmacia, Uppsala, Sweden) as a *Sma*I fragment into the *Eco*RV site in *xylE* on a 4.4-kb *Xba*I-*Sst*I fragment. Strain EK385 was constructed by inserting a kanamycin resistance gene from mini-Tn*5Km1* [7] into the *Eco*RI site in *bphC* on a 2.3-kb *Sst*I-*Kpn*I fragment. Strain EK504 was constructed by inserting a kanamycin resistance gene from pUC4-KIXX into the *Bgl*II site in *bphA1c* on a 7.5-kb *Bam*HI fragment. Strain EK533 was constructed by inserting a kanamycin resistance gene from mini-Tn*5Km1* into the *Bam*HI site in *xylX* on a 6.8-kb *Bgl*II-*Xba*I fragment. The transcriptional direction of the kanamycin resistance gene in all constructs is opposite that of the gene into which it was inserted.

Chemicals

All organics were purchased from Aldrich Chemical Company, Milwaukee, WI, USA, and were of the highest purity available. *cis*-2,3-biphenyl dihydrodiol was synthesized from biphenyl using the mutant B8/36 following standard procedures [22].

Results

Role of operon one in polycyclic and monocyclic aromatic hydrocarbon degradation

The previously identified *bphC* [31] is the first gene in a putative operon of at least five genes (Figure 2). Analysis of the nucleotide sequence [51] suggests that the genes code for a dioxygenase ferredoxin component (*bphA3*), a dioxygenase terminal oxygenase large and small subunit (*bphA1cA1d*), and 2-hydroxychromene-2-carboxylate isomerase (*nahD*). An insertional knockout mutation was made in order to determine whether these genes are indeed operonic and to verify the role of the genes in aromatic metabolism by *S. yanoikuyae* B1. A kanamycin resistance cassette was cloned into the *Eco*RI site in *bphC* and inserted by homologous recombination into the genome of *S. yanoikuyae* B1 as described in Materials and Methods. The resulting strain, EK385, not only lost the ability to grow on the polycyclic aromatic hydrocarbons biphenyl, naphthalene, and phenanthrene but also lost the ability to grow on the monocyclic compounds *m*-xylene and *m*-toluate as well. No accumulating metabolic pathway intermediates were detected in the culture supernatants when EK385 was grown on succinate in the presence of biphenyl, naphthalene, or phenanthrene. Since a dihydroxylated compound would be expected to accumulate with a *bphC* mutation, this result suggests that the kanamycin gene insertion in EK385 resulted in a polar phenotype and that a gene for one or more of the initial dioxygenase components is present downstream of *bphC*. This result was confirmed by the fact that EK385 was no longer able to oxidize indole to indigo, a trait known to be associated with ring hydroxylating dioxygenase enzymes [14]. EK385 grown on succinate in the presence of *m*-xylene accumulates *m*-toluate (HPLC retention time 9.5 min) in the culture medium. This result, along with the fact that EK385 does not grow on *m*-toluate, indicates that one or more of the genes responsible for toluate dioxygenase are located downstream of *bphC*.

A clone, designated pGJZ1553, containing a 7.5-kb *Bam*HI fragment in the vector pRK415 is able to restore the ability of EK385 to grow on biphenyl, naphthalene, phenanthrene, *m*-xylene, and *m*-toluate (Figure 3). Smaller clones containing either *bphC* and *bphA3* (pGJZ1554) or only *bphA3* (pGJZ1555) are able to restore the ability of EK385 to grow on biphenyl, phenanthrene (slower than normal growth), *m*-xylene, and *m*-toluate but did not restore the ability to grow on naphthalene. These data indicate that *bphA3*, coding for a ferredoxin component of an aromatic dioxygenase, is required for growth on both polycyclic and monocyclic aromatic compounds. The data also indicate that XylE can take the place of BphC. Since the EK385 mutant is polar, the data indicate that *nahD* and *bphA1cA2c* are not needed for growth on biphenyl, phenanthrene, *m*-xylene, and *m*-toluate but one or both are required for growth on naphthalene.

A new insertional knockout mutation was constructed in order to determine the roles of *bphA1cA2c* and *nahD* in degradation of aromatic compounds by *S. yanoikuyae* B1. A kanamycin resistance cassette was cloned into the *Bgl*II site in *bphA1c* and reinserted into the genome of *S. yanoikuyae* B1 as described in Materials and Methods. The resultant mutant strain, designated EK504, is not able to grow on naphthalene but is able to grow on biphenyl, phenanthrene, *m*-xylene, and *m*-toluate. Analysis of the culture supernatant of EK504 following growth on succinate in the presence of naphthalene revealed the accumulation of a compound with an absorption spectrum identical to that previously published [11] for *trans*-*o*-hydroxybenzylidenepyruvic acid (Figure 4). Although a *nahD* mutation would be expected to accumulate 2-hydroxy-chromene-2-carboxylate (see Figure 1), it has previously been shown by Eaton and Chapman [11] that this compound is slowly spontaneously converted to *trans*-*o*-hydroxybenzylidenepyruvic acid and would be the expected detected compound

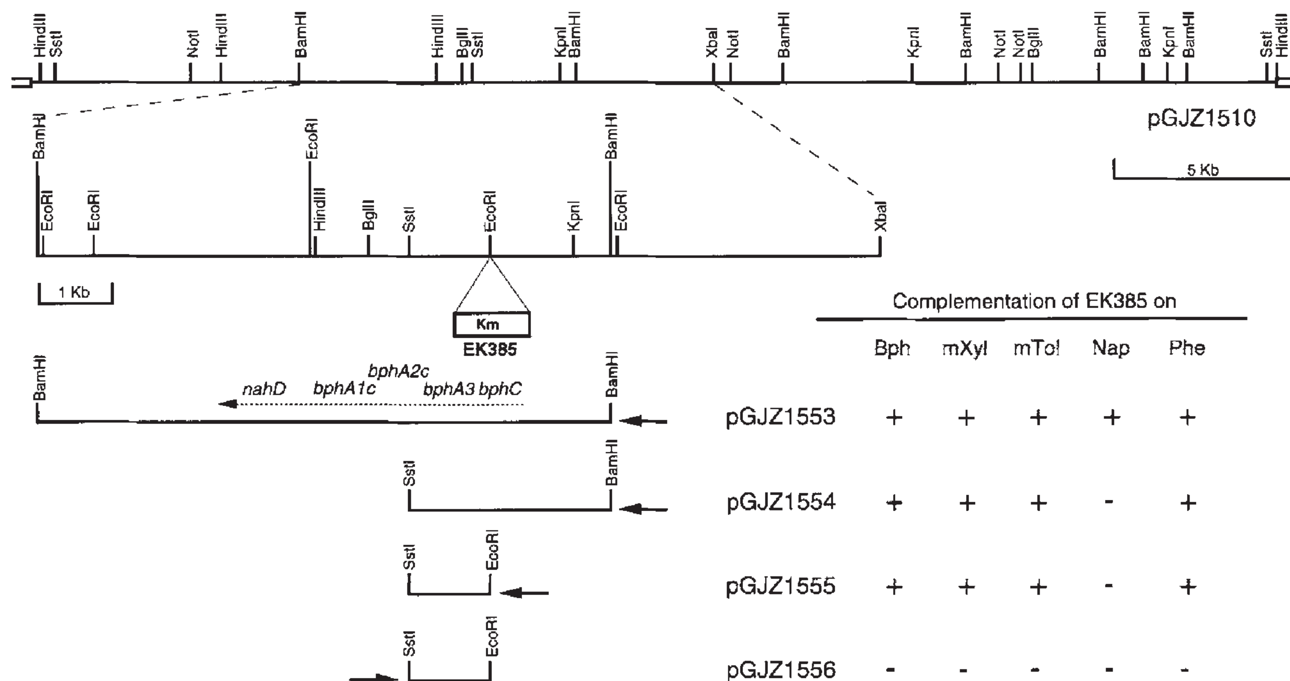

Figure 3 Complementation of the polar insertion mutation EK385. The arrows indicate the direction of transcription from the *lac* promoter on the vector (pRK415). The double line in the pGJZ1510 restriction map is the vector (pHC79). The insertion point for the kanamycin resistance gene in the B1 mutant EK385 is shown for reference. Abbreviations: Bph, biphenyl; mXyl, *m*-xylene; mTol, *m*-toluate; Nap, naphthalene; Phe, phenanthrene.

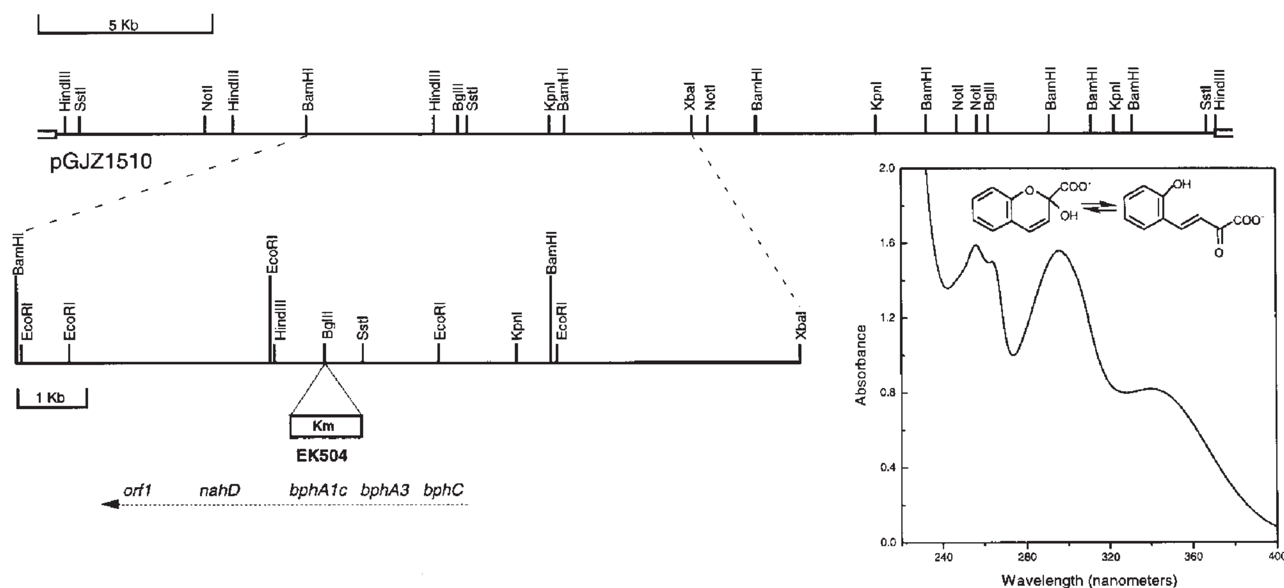

Figure 4 Accumulation of 2-hydroxy-chromene-2-carboxylate (chemically converted to *trans-o*-hydroxybenzylidenepyruvic acid) by the polar insertion mutant EK504. The double line in the pGJZ1510 restriction map is the vector (pHC79). The insertion point for the kanamycin resistance gene in the B1 mutant EK504 is shown for reference.

in culture supernatants of a *nahD* mutation. Since the only detectable effect of the insertion mutation in EK504 was to produce a polar mutational effect on *nahD* this means that loss of *bphA1c* (the point of insertion of the kanamycin resistance gene) does not have any effect on the metabolism of the tested aromatic compounds.

Role of operon two in polycyclic and monocyclic aromatic hydrocarbon degradation

A second putative operon containing genes involved in aromatic metabolism is present adjacent to the one discussed above. Analysis of the nucleotide sequence (Figure 2) suggests that the genes code for the oxygenase component of

toluate dioxygenase (*xylXY*), a protein with similarity to PdxA of *E. coli* (orf2), an oxygenase component of another dioxygenase (*bphA1dA2d*), a glutathione S-transferase (*bphK*), a meta-cleavage pathway (*xylFEGJQKIHT*), benzaldehyde dehydrogenase (*xylC*), and a *cis*-2,3-biphenyl dihydrodiol dehydrogenase (*bphB*). The *xylE* gene was previously located through a transposon insertion immediately adjacent to and downstream of this gene [31]. This transposon mutant strain, EK3, is unable to grow on *m*-xylene, biphenyl, or naphthalene. Additionally, EK3 accumulates *cis*-2,3-biphenyl dihydrodiol (HPLC retention time 27.2 min) when grown on succinate in the presence of biphenyl. This is the expected phenotype of a *bphB* mutation and thus suggests that the transposon insertion in EK3 exerts a polar effect and that *bphB* is downstream of *xylE*. In order to confirm this suspected gene organization, an insertional knockout mutation of *xylE* was constructed. A kanamycin resistance gene cassette was cloned into the *Eco*RV site in the *xylE* gene and reinserted into the genome as described in Materials and Methods. The resulting mutant strain, EK121, has the same phenotype as the transposon mutant strain EK3: it is not able to grow on *m*-xylene, biphenyl, or naphthalene and accumulates *cis*-2,3-biphenyl dihydrodiol from biphenyl. A 22.5-kb *Hin*dIII fragment cloned into pRK415 is able to restore the ability of EK3 and EK121 to grow on *m*-xylene and biphenyl and the ability of B8/36 (a *bphB* mutant [22]) to grow on biphenyl (Figure 5). In addition, a 4.8-kb *Pst*I-*Sst*I and a 1.1-kb *Pst*I-*Eco*RI fragment can restore the ability of B8/36 to grow on biphenyl (Figure 5). This verifies the role of the sequenced *bphB* in the second step of polycyclic aromatic hydrocarbon degradation and demonstrates that it is in the same operon as *xylE*.

In order to verify the function of the *meta*-cleavage pathway, pGJZ1550 containing the 22.5-kb *Hin*dIII fragment (Figure 5) was moved into *P. putida* PPO200. The resulting strain is able to grow slowly on *m*-toluate, indicating that this DNA encodes the *meta*-cleavage pathway. In addition, an insertional knockout mutant was constructed to confirm the function of *xylX*. A kanamycin resistance cassette was cloned into the *Bam*HI site of *xylX* and reinserted into the genome of *S. yanoikuyae* B1 as described in Materials and Methods. The resulting mutant strain, EK533, accumulates *m*-toluate (HPLC retention time 9.5 min) from *m*-xylene indicating that *xylX* does actually encode a subunit of the oxygenase component of toluate dioxygenase. Unexpectedly, EK533 (unlike EK3 and EK121) grows on biphenyl and naphthalene suggesting that the kanamycin gene insertion does not exert a polar effect on the putative operon or that there is a second promoter downstream. EK533 is able to grow on toluene, but this is expected due to the fact that toluene can be metabolized via the *ortho*-cleavage pathway also present in this strain.

Experiments to assign functions to *bphA1dA2d* and *orf2* were inconclusive. Since *orf2* shows similarity to *E. coli pdxA* (involved in pyridoxine synthesis), complementation experiments were performed to determine if the encoded protein performed a similar enzymatic reaction. A 2.4-kb *Pst*I restriction fragment containing the putative *pdxA* gene was subcloned from pGJZ1510 into pGEM3Z in both orientations. The two clones were transformed into the *pdxA E. coli* CGSC 5630 and examined for their ability to grow with and without pyridoxine. The *Pst*I fragment containing the *pdxA* gene was not able to complement the inability of CGSC 5630 to grow without pyridoxine added to the culture medium. This suggests that *orf2* does not encode a protein with PdxA activity. The putative oxygenase genes, *bphA1dA2d*, immediately following *orf2* also have no apparent function, assuming that the kanamycin gene insertion in *xylX* in EK533 has polar effects on *bphA1dA2d*. Additionally, deletion of *bphA1dA2d* has no effect on the ability to oxidize biphenyl or naphthalene (data not shown).

Discussion

S. yanoikuyae B1 has the ability to utilize both monocyclic and polycyclic aromatic hydrocarbons as carbon and energy sources for growth. This catabolic ability seems to be a common and unique trait of the aromatic hydrocarbon-degrading *Sphingomonas* genus [17,18,20,28]. Based on what is known about the genetics of aromatic hydrocarbon

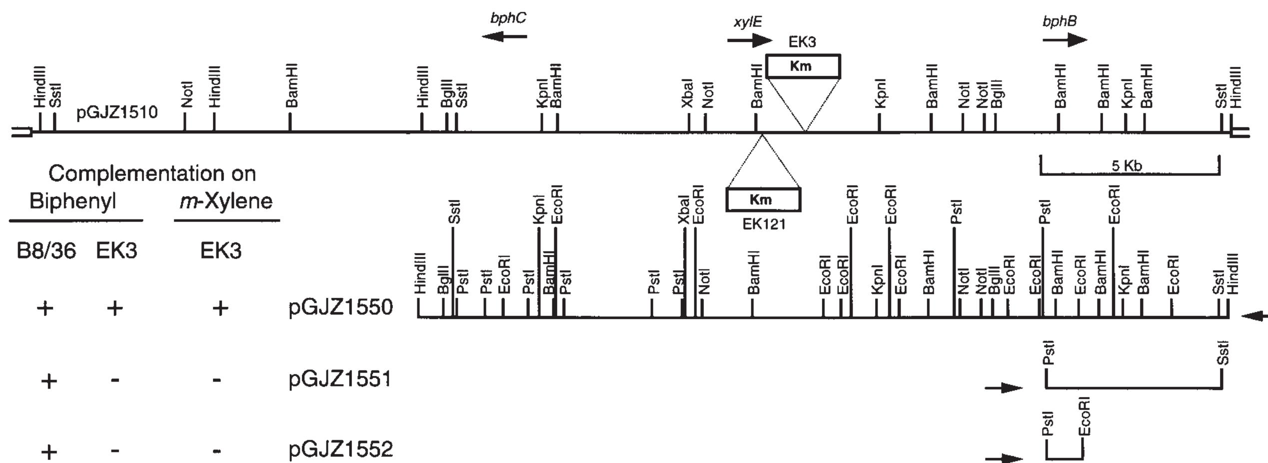

Figure 5 Location of *bphB* downstream of *xylE*. EK121 has the same complementation phenotype as EK3. The double line in the pGJZ1510 restriction map is the vector (pHC79). The arrows next to the subclones indicate the direction of transcription from the *lac* promoter on the vector (pRK415). The insertion point for the kanamycin resistance gene in the B1 mutants EK3 and EK121 is shown for reference.

degradation in related organisms (ie *Pseudomonas* species [2,49,50]), one might expect the genes for aromatic hydrocarbon degradation in *S. yanoikuyae* B1 to be organized into operons based on catabolic segments. For instance, one operon might contain those genes required for conversion of polycyclic aromatic hydrocarbons to simple aromatic acids (similar to the *bph* operon [19] or the upper *nah* operon [8,49]), a second operon might contain those genes required for conversion of monocyclic aromatic compounds to aromatic acids (similar to the upper *xyl* operon [2]), and a third operon might contain those genes required for conversion of aromatic acids to tricarboxilic acid cycle intermediates (similar to the lower *xyl* operon [2]). However, this does not seem to be the case for *S. yanoikuyae* B1. In the process of evolving the capability to degrade a wide variety of aromatic hydrocarbons this organism has recruited, modified, and reorganized the appropriate genes and operons needed to construct the required catabolic pathways. The first operon reported here is a prime example of this reorganization process. This operon (Figure 2) contains the genes that encode 2,3-dihydroxybiphenyl 1,2-dioxygenase (*bphC*), a ferredoxin (*bphA3*), an oxygenase large subunit of unknown function (*bphA1cA2c*), and 2-hydroxychromene-2-carboxylate isomerase (*nahD*). This operon thus contains some but not all of the genes needed for an 'upper' pathway for the conversion of polycyclic aromatic hydrocarbons to aromatic acids. The recruitment of *nahD* into this operon represents a significant event in the evolution of this catabolic pathway. The first three enzymes in the polycyclic aromatic hydrocarbon pathway (the initial aromatic oxygenase, the *cis*-dihydrodiol dehydrogenase, and the 2,3-dihydroxybiphenyl 1,2-dioxygenase [22,35,38]) have broad substrate ranges and can metabolize biphenyl, naphthalene, and phenanthrene to the appropriate ring-cleavage products. However, at this point the catabolic pathways for the three polycyclic aromatic hydrocarbon substrates diverge (Figure 1). Continued degradation of naphthalene past the ring-cleavage step requires 2-hydroxychromene-2-carboxylate isomerase and *trans-o*-hydroxybenzylidene-pyruvate hydratase-aldolase. The gene (*nahD*) for the former enzyme is present in the first operon while the gene for the latter enzyme (*nahE*) has been located elsewhere [51]. A polar insertion mutation (EK504) that no longer produces 2-hydroxychromene-2-carboxylate isomerase can not grow on naphthalene but can still utilize biphenyl and phenanthrene as carbon sources for growth (Figure 4). However, growth on phenanthrene is slower than normal indicating that *nahD* is necessary but not required for growth. EK504 accumulates *trans-o*-hydroxybenzylidenepyruvic acid when grown on succinate in the presence of naphthalene (Figure 4), verifying the identity of *nahD* at both the enzymatic function and DNA sequence homology level. The kanamycin resistance gene insertion in EK504 is in *bphA1c*, immediately upstream from *nahD* (Figure 4). The fact that this insertion mutant has no apparent phenotype besides the polar effect on *nahD* gene expression leads to three possible conclusions: *bphA1c* may be a pseudogene with no biological function, other oxygenases may be present with overlapping specificity, or, more likely, *bphA1c* may encode an enzyme whose function has not yet been discovered.

The nucleotide sequence and the functional biological data presented here have implications for the evolution of enzymes as well. An insertional knockout mutant of *bphC* (EK385) has an initially unexpected phenotype: the inability to grow on *m*-xylene, *m*-toluate, biphenyl, naphthalene, and phenanthrene. The inability to grow on the polycyclic compounds was an expected phenotype due to the fact that the insertion would have a polar effect on *bphA3*, encoding a ferredoxin needed for the initial dioxygenase. Indeed, this mutant does not initiate the degradation of polycyclic compounds and no longer produces indigo from indole, indicative of dioxygenase activity [14]. The inability of EK385 to grow on *m*-xylene and *m*-toluate was unexpected following simple inspection of the nucleotide sequence of this operon (Figure 2). Growth of EK385 on succinate in the presence of *m*-xylene results in the accumulation of *m*-toluate. Introduction of a plasmid containing only *bphA3* into EK385 (Figure 3) results in the restoration of the ability of this mutant strain to grow on both *m*-xylene and *m*-toluate. These data indicate that toluate dioxygenase requires the ferredoxin encoded by *bphA3* for activity. Toluate dioxygenase [24], and the related enzyme benzoate dioxygenase [40], are enzymes that consist of two components: a reductase (product of the *xylZ* gene) and an oxygenase consisting of a large and small subunit (products of the *xylXY* genes). The reductase functions as a simple electron transport protein, transferring electrons from NADH to the oxygenase. The reduced oxygenase component then performs the actual enzymatic transformation of the aromatic acid to the corresponding *cis*-dihydrodiol. The fact that a ferredoxin encoded by *bphA3* is required for toluate dioxygenase activity indicates that toluate dioxygenase in *S. yanoikuyae* B1 consists of three components: a reductase, a ferredoxin, and an oxygenase. The reductase and ferredoxin in *S. yanoikuyae* B1 take the place of the single bifunctional reductase found in other organisms. Data discussed below also suggest that *S. yanoikuyae* B1 has lost *xylZ* which normally would encode such a reductase. The observation that a three-component oxygenase is involved in aromatic acid metabolism in *S. yanoikuyae* B1 is not without precedence as Romanov and Hausinger showed that *ortho*-halobenzoate 1,2-dioxygenase from *P. aeruginosa* 142 is a three-component enzyme [43]. However, in the present work the data indicate that the ferredoxin encoded by *bphA3* plays a dual role: as an electron transfer component of both toluate dioxygenase and a broad substrate range biphenyl/naphthalene/phenanthrene dioxygenase.

The second operon reported in the present paper also has implications for the recruitment, modification, and reorganization of genes and operons for aromatic hydrocarbon degradation. This operon is highly reminiscent of the 'lower' TOL plasmid operon for the catabolism of aromatic acids to pyruvate and acetaldehyde that is found in *P. putida*. However, there are significant differences between these two operons. The 'lower' or *meta*-cleavage TOL plasmid *xyl* operon is organized with the genes in the order *xylXYZLTEGFJQKIH* (see Figure 1 for an explanation of which enzymes are encoded by each of these genes). The enzymes encoded by the analogous genes in *S. yanoikuyae* B1 are related at the level of 60–80% similarity.

However, three major differences are immediately apparent in the gene organization. *S. yanoikuyae* B1 does not contain the *xylZ* or *xylL* genes in this operon. These genes are adjacent to each other in the TOL plasmid operon and thus their lack in *S. yanoikuyae* B1 may be due to a single deletion event. The fact that *xylZ* is missing is not surprising since evidence presented above indicates that the toluate oxygenase reductase normally encoded by *xylZ* in the TOL plasmid is not needed by *S. yanoikuyae* B1. However, the loss of *xylL* is significant since the product of this gene, toluate *cis*-dihydrodiol dehydrogenase, is absolutely required for the catabolism of aromatic acids. This gene has been located elsewhere in the genome [51] but the rationale for why it has been removed from this operon remains unexplained. Four new genes (*orf2*, *bphA1d*, *bphA2d*, and *bphK*) of unknown biological function have been substituted for those which have been deleted. In addition, there is an obvious rearrangement of genes compared to the TOL plasmid lower operon: *xylF* has been moved in front of *xylE* from its position between *xylG* and *xylJ*, and *xylT* has been moved from in front of *xylE* to after *xylH*.

A third rearrangement of the TOL plasmid 'lower' operon occurred through the addition of two genes to its 3′ end: *xylC* and *bphB*. The first of these two genes, *xylC*, encodes benzaldehyde dehydrogenase, involved in the conversion of benzaldehyde, *m*-, and *p*-tolualdehyde to the corresponding acids. This gene is normally present in a TOL plasmid 'upper' operon containing the genes for conversion of toluene, *m*-, and *p*-xylene to the corresponding acids [2]. The second of these genes, *bphB*, encodes *cis*-2,3-biphenyl dihydrodiol dehydrogenase. This enzyme is involved in the dehydrogenation of a wide variety of aromatic *cis*-dihydrodiols as a mutant lacking this enzyme, *S. yanoikuyae* B8/36, accumulates *cis*-dihydrodiols from a wide variety of polycyclic aromatic compounds (see Introduction). The fact that EK3 [31] and EK121 (this work), representing insertions in *xylG* and *xylE* respectively, have polar effects on *bphB* proves that *bphB* (and *xylC* by implication) are operonic with the upstream *xyl* genes. However, an insertional mutation of *xylX* (EK533) did not result in polar effects on *bphB*. This suggests that either the kanamycin gene insertion did not result in a polar effect or that there is a second promoter present after *xylX* but before *xylE*.

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