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Aromatic hydrocarbon degradation by *Sphingomonas yanoikuyae* B1

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***Sphingomonas yanoikuyae* B1 is able to grow on a wide variety of aromatic compounds including biphenyl, naphthalene, phenanthrene, toluene, *m*-, and *p*-xylene. In addition, the initial enzymes for degradation of biphenyl have the ability to metabolize a wide variety of different polycyclic aromatic hydrocarbons. The catabolic pathways for the degradation of both the monocyclic and polycyclic aromatic hydrocarbons are intertwined, joining together at the level of (methyl)benzoate and catechol. Both upper branches of the catabolic pathways are induced when *S. yanoikuyae* B1 is grown on either class of compound. An analysis of the genes involved in the degradation of these aromatic compounds reveals that at least six operons are involved. The genes are not arranged in discrete pathway units but are combined in groups with genes for the degradation of both classes of compounds in the same operon. Genes for multiple dioxygenases are present perhaps explaining the ability of *S. yanoikuyae* B1 to grow on a wide variety of aromatic compounds.**

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

Introduction

Biphenyl has long been utilized as a model compound in the analysis of the degradation of polychlorinated biphenyls (PCBs). Extensive studies have been made on the genes and enzymes involved as well as on the types of PCBs that can be transformed by each strain. However, not all biphenyl-degrading organisms are capable of oxidizing PCBs containing several chlorine groups. One class of organisms falling into this latter class (non-PCB oxidizing biphenyl degraders) is comprised of organisms that are also capable of degrading polycyclic aromatic hydrocarbons (PAHs). This is a little recognized class of organism, probably due to the fact that more attention is placed on the biphenyl-degrading organisms as models for PCB degradation and the naphthalene-degrading organisms as models for PAH degradation. This class of organisms (biphenyl and PAH degraders) in general have the ability to utilize a wide variety of PAHs as carbon sources and co-metabolize a number of PAHs (see below). This is not surprising as the biphenyl structure can be found in a variety of different PAHs. One organism capable of biphenyl and PAH degradation is David T Gibson's strain B1. This organism was preliminarily classified as a *Beijerinckia* species [8] but has subsequently been found to be *Sphingomonas yanoikuyae* through the use of several new biochemical and phylogenetic techniques [12]. This paper will focus on what is known about the ability of strain B1 to metabolize aromatic compounds.

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Metabolism of aromatic compounds by *S. yanoikuyae* B1

S. yanoikuyae B1 was originally isolated for the ability to grow on biphenyl for the purpose of elucidating the biphenyl degradation pathway [8]. Gibson and coworkers wereable to identify *cis*-2,3-dihydroxy-1-phenyl-cyclohexa-4,6-diene and 2,3-dihydroxybiphenyl (*cis*-biphenyl dihydrodiol) as intermediates in the catabolic pathway [8]. This indicated that metabolism proceeds through the addition of two atoms of molecular oxygen into the aromatic nucleus at the 2,3 position (Figure 1). A ring cleavage dioxygenase would subsequently cleave this compound at the 1,2 position to form 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate. In order to investigate the inital step in this pathway in more detail, Gibson *et al* constructed a mutant strain, B8/36, which accumulates *cis*-biphenyl dihydrodiol when grown on succinate in the presence of biphenyl. This mutant was instrumental in showing that B1 initiates metabolism of a wide variety of different PAHs through dioxygenase attack on the aromatic nucleus as shown in Figure 1.

In addition to biphenyl, *S. yanoikuyae* B1 is also capable of utilizing the two- and three-ring PAHs naphthalene, anthracene, and phenanthrene as carbon sources for growth [8]. The initial step in the metabolism of these compounds also involves the formation of a *cis*-dihydrodiol (Figure 1; [10]) as determined using the mutant strain B8/36. This not only demonstrated that these compounds are metabolized via a *cis*-dihydrodiol but also showed that a single *cis*-dihydrodiol dehydrogenase, lacking in B8/36, is involved in the metabolism of all four PAHs. Besides these initial two steps the metabolic pathway(s) by which naphthalene, phenanthrene, and anthracene are metabolized have not been analyzed in detail in *S. yanoikuyae* B1. However, based on work with other microorganisms (reviewed by Yen and

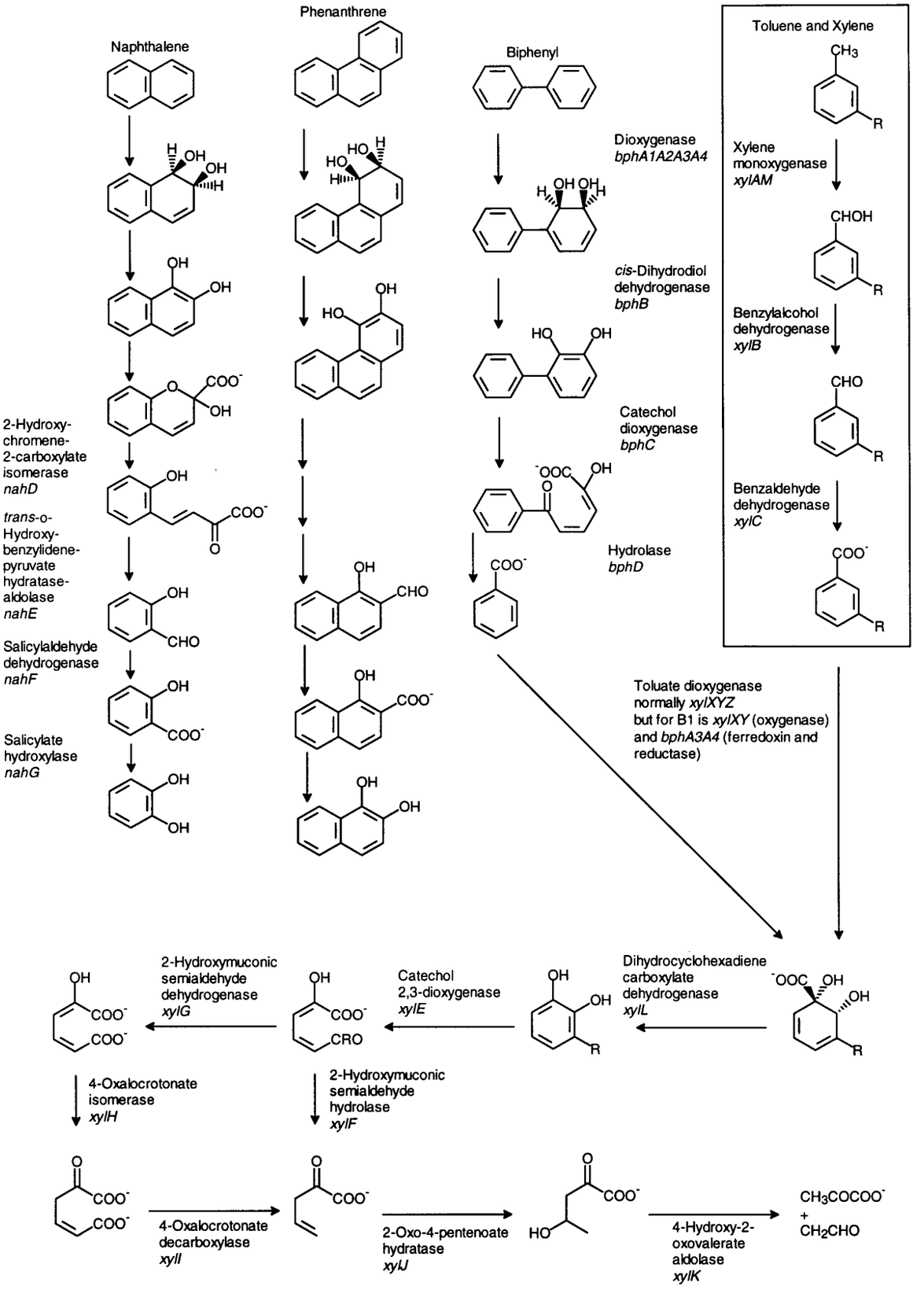

Figure 1 Catabolic pathways for the degradation of naphthalene, phenanthrene, biphenyl, toluene, and *m*-xylene. The enzyme and gene designations for each step in a pathway are shown. R═H for toluene and R═CH_3 for *m*-xylene.

Serdar [30]) it may be inferred that degradation of these three compounds proceeds as shown in Figure 1. Essentially, the metabolism of the three compounds initially proceeds by identical biochemical reactions as shown for biphenyl (dioxygenase attack, dihydrodiol dehydrogenation, and *meta*-ring cleavage). However following ring cleavage, metabolism of naphthalene, phenanthrene, and anthracene diverges from that for biphenyl. For example, following cleavage of 1,2-dihydroxynaphthalene, 2-hydroxychromene-2-carboxylate is spontaneously formed [4]. This requires the action of an isomerase to change the *cis* double bond in the side chain to the *trans* configuration. Cleavage of the latter compound by a hydratase-aldolase results in the formation of salicyladehyde and a three-carbon fragment. Salicyladehyde is subsequently converted to salicylate which can be metabolized through catechol and the *meta*-cleavage pathway. Phenanthrene and anthracene are metabolized by a similar pathway for the removal of at least the first aromatic ring. This has been demonstrated by the fact that a *nahD* mutant of B1 (EK504) accumulates analogous compounds from naphthalene, phenanthrene, and anthracene (Kim and Cerniglia, personal communication; [18]). The mechanism of removal of the second aromatic ring and metabolism of the remaining aromatic compound is not yet known for *S. yanoikuyae* B1. Two different metabolic pathways have been proposed in other microorganisms for the continued degradation of phenanthrene following removal of the first aromatic ring [6,19].

In addition to growth on PAHs *S. yanoikuyae* B1 also has the ability to grow on several monocyclic aromatic compounds as well. Growth substrates in this class include *m*-xylene, *p*-xylene, toluene, *p*-ethyltoluene, and 1,2,4-trimethylbenzene [24]. Metabolism of these compounds proceeds via a TOL-plasmid type catabolic pathway with successive oxidation of the methyl group to form an aromatic acid (Figure 1). Benzoate (or toluate) is subsequently metabolized via the well-known *meta*-cleavage pathway to acetaldehyde and pyruvate. This pathway for monocyclic aromatic hydrocarbon degradation is metabolically linked to that for the degradation of PAHs. The initial (or 'upper') pathways for the metabolism of each compound intersect at the *meta*-cleavage (or 'lower') pathway. Biphenyl and the monocyclic compounds are metabolized to (methyl)benzoates and (methyl)catechol while naphthalene is metabolized to salicylate and catechol. Linkage of the pathways for monocyclic and polycyclic aromatic hydrocarbon degradation also occurs at the level of gene regulation. Growth on a monocyclic aromatic compound induces not only the genes for its own degradation but also the genes responsible for the 'upper pathway' of PAH degradation [24]. The alternative case, growth on biphenyl, also results in the induction of the genes for the 'upper pathway' of *m*-xylene degradation. The reasons for this unusual regulation can partially be explained by the gene organization as discussed below.

Co-oxidation of aromatic substrates by *S. yanoikuyae* B1

In addition to the ability to utilize biphenyl, naphthalene, anthracene, and phenathrene as sole sources of carbon for growth, *S. yanoikuyae* B1 is able to oxidize a wide variety of PAH substrates due to the relaxed specificity of the enzymes involved. Most, but not all, of the studies on the substrate range of the B1 PAH catabolic pathway have taken place using the *cis*-dihydrodiol dehydrogenase mutant B8/36. The advantage to using this strain in the metabolic studies is that one can unequivocally determine the position of attack of the initial dioxygenase on the aromatic compound. This is due to the fact that B8/36 will accumulate a *cis*-dihydrodiol(s) from an oxidizable PAH substrate. The compound can be purified relatively easily and its structure determined. The disadvantage is that one does not know how far the starting compound will be metabolized in the catabolic pathway. Based on the position of initial enzymatic attack on the substrate one can often predict how far through the pathway a substrate can be metabolized based on what is known for similar substrates and the properties of the enzymes involved.

A list of PAH substrates known to be oxidized by B1 is given in Figure 2. One notable theme in the oxidation of these compounds is that there seem to be two preferred modes of attack: a naphthalene dioxygenase and a biphenyl dioxygenase attack. A naphthalene dioxygenase type of enzymatic attack is one such that a *cis*-dihydrodiol is formed at the 1,2 position of a naphthalene moiety found in the PAH compound. A biphenyl dioxygenase attack is one such that a *cis*-dihydrodiol is formed at the 2,3 position of a biphenyl moiety found in the PAH compound. Examples of a naphthalene dioxygenase type of attack on a PAH compound include the formation of *cis*-dihydrodiols from naphthalene, anthracene, and benz[*a*]pyrene. Examples of a biphenyl dioxygenase type of attack on a PAH compound include the formation of *cis*-dihydrodiols from biphenyl, phenanthrene, 3-methylcholanthrene, benz[*a*]pyrene, carbazole, and dibenzothiophene. *S. yanoikuyae* B1 attacks benz[*a*]anthracene at three different positions, illustrating both modes of attack. These two different types of enzymatic attacks do not necessarily suggest that two different enzymes are involved as it is known that naphthalene dioxygenase from *P. putida* NCIB 9816-4 has an extremely broad substrate range. However, this cannot be ruled out as a possibility, especially in light of the genetic data discussed below.

S. yanoikuyae B1 is one of the few microorganisms which have been conclusively shown to oxidize PAHs of four or more aromatic rings. B1 is able to oxidize the four-ring compounds benz[*a*]anthracene [11,23] and 3-methylcholanthrene [13] and the five-ring compound benz[*a*]pyrene [7]. Interestingly, *S. yanoikuyae* B1 attacks several of these compounds at more than one position. These multiple sites of attack may again represent either the action of one dioxygenase with a relaxed substrate range or the action of multiple dioxygenases each with their own preferred substrate and position of attack. Whichever may be the case only one *cis*-dihydrodiol dehydrogenase is involved in the metabolism of each of these compounds since the mutant strain B8/36 accumulates a *cis*-dihydrodiol from each of the PAHs. It is interesting to note that although B1 has not been reported to grow on benz[*a*]anthracene it is capable of removing at least one of the four rings. Mahaffey *et al* [23] showed that 1-hydroxy-2-anthranoic acid, 2-hydroxy-

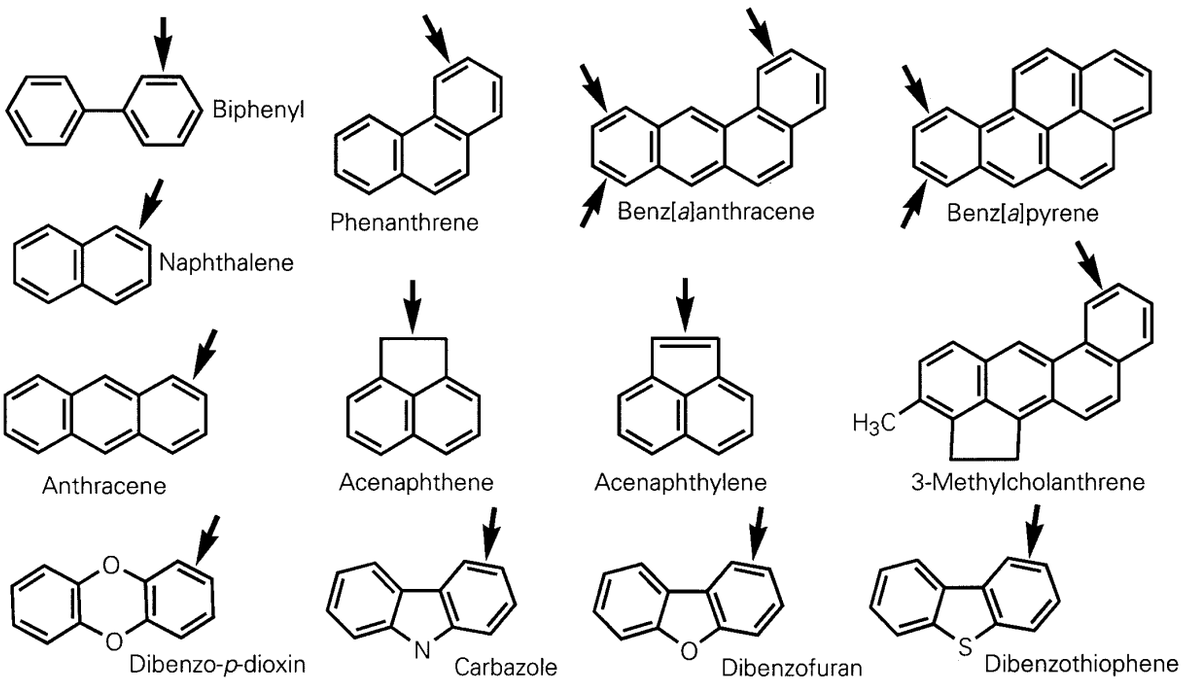

Figure 2 Polycyclic aromatic hydrocarbons which are oxidized by *S. yanoikuyae* B1 to *cis*-dihydrodiols. The arrow indicates the position on the compound where the oxygens are added by the initial dioxygenase enzyme to biphenyl [8,31], naphthalene [8], anthracene [1,10], phenanthrene [10,21], benz[*a*]anthracene [7,11], benz[*a*]pyrene [7], 3-methylcholanthrene [13], dibenzofuran [2], dibenzothiophene [22], carbazole [26], acenaphthene [29], acenaphthylene [29], and dibenzo-*p*-dioxin [20].

3-phenanthroic acid, and 3-hydroxy-2-phenanthroic acid could be detected in culture supernatants following incubation of induced B1 cells with benz[*a*]anthracene. These three compounds result from the initial enzymatic attack at the 1,2; 8,9; and 10,11 positions of benz[*a*]anthracene presumably followed by the actions of the following enzymes in the catabolic pathway. Whether metabolism proceeds to remove the remaining three rings of this compound is not known.

Genetic analysis of *S. yanoikuyae* B1

The wide variety of PAHs that *S. yanoikuyae* B1 can metabolize, along with the fact that it can also metabolize monocyclic compounds, makes the strain a prime candidate for in-depth analysis of the genes and enzymes involved. Investigation at the molecular level of the ability of *S. yanoikuyae* B1 to degrade aromatic compounds has lagged behind those for many other strains. However, as will be seen below, the structure and organization of the genes tells us much about how this strain has evolved, how the genes for catabolic pathways can be recruited, and why the strain is able to degrade such a wide variety of aromatic compounds.

Cloning of the genes for aromatic hydrocarbon degradation usually begins by constructing a genomic library in *E. coli* and screening for activity of key enzymes in the catabolic pathway, such as the initial dioxygenase or the *meta*-ring cleavage dioxygenase. This often works if the cloned genes are near a promoter active in *E. coli* (either native or in the vector) and, in the case of the initial oxygenase, if the multiple genes encoding the individual components are clustered. These experiments are aided by the fact that the *meta*-cleavage of catechol results in an easily observable bright yellow compound and that the initial dioxygenase oxidizes indole to indigo which accumulates as a blue precipitate inside the cells [5]. Construction and screening of a cosmid library consisting of 40-kb fragments did not yield any clones which perform these reactions [16]. In order to clone the genes, a different approach was then taken. Several transposon mutant strains deficient in aromatic metabolism were constructed [16] using the mini-transposon mini-Tn*5Km1* [3]. A disproportionate number of these strains lack *cis*-biphenyl dihydrodiol dehydrogenase activity (Kim and Zylstra, unpublished observations). This suggested that there was either a hot spot for insertion of the transposon or that the gene for *cis*-biphenyl dihydrodiol dehydrogenase is located in a large operon where transposon insertions upstream would have polar effects (see below). One of these transposon mutants, EK3, was chosen for further study due to the fact that it is unable to grow on either *m*-xylene or biphenyl and accumulates *cis*-biphenyl dihydrodiol when grown on succinate in the presence of biphenyl. A 6-kb *Not*I fragment of genomic DNA was cloned from EK3 taking advantage of the unique *Not*I site in the mini-Tn*5Km1* transposon. A gene for a *meta*-cleavage dioxygenase was located next to the transposon insertion and this fragment used to locate a cosmid clone containing the analogous region of *S. yanoikuyae* B1 genomic DNA [16]. Subclones of this approximately 40-kb cosmid clone indicated that it contained the genes for two *meta*-cleavage dioxygenase enzymes, approximately 7 kb apart from one another and transcribed in opposite directions. The fact that there are genes for two *meta*-cleavage dioxygenases makes sense due

to the fact that there are two steps in the catabolic pathway where this type of enzyme is required: ring cleavage of a dihydroxylated polycyclic compound such as 1,2-dihydroxynaphthalene or 2,3-dihydroxybiphenyl and ring cleavage of a dihydroxylated compound such as catechol or 3-methylcatechol (Figure 1). Indeed, an analysis of the deduced amino acid sequences of these two enzymes reveals that one falls into the class of enzymes which are found in upper pathway operons for biphenyl and naphthalene catabolism and the other falls into the class of enzymes which are found in lower pathway operons for (methyl)catechol metabolism [16]. This finding is supported by the substrate range of each enzyme: one has a preference for 2,3-dihydroxybiphenyl and the other has a preference for catechol. The gene encoding the former enzyme was thus designated *bphC* for 2,3-dihydroxybiphenyl 1,2-dioxygenase and the gene encoding the latter designated *xylE* for catechol 2,3-dioxygenase.

The location of the genes for two-ring cleavage dioxygenases 7 kb apart from one another and transcribed in opposite directions suggested that there are perhaps at least two operons in this organism, one containing genes for an upper pathway for the conversion of biphenyl to benzoate and another containing genes for a lower pathway for the metabolism of (methyl)benzoate. A third operon might be present for the conversion of toluene and the xylenes to (methyl)benzoates. This hypothesis is based on what is known about the well-studied toluene, naphthalene, and biphenyl pathways. However, *S. yanoikuyae* B1 does not fit this general rule, as already evidenced by the fact that the gene (*bphB*) for *cis*-biphenyl dihydrodiol dehydrogenase must be present downstream of *xylE* based on the phenotype of the transposon mutant EK3. Additionally, a knockout mutant of *bphC*, designated EK385, resulted in the loss of the ability to grow on biphenyl, naphthalene, *m*-xylene, and *m*-toluate [15]. This suggests that genes are present downstream of *bphC* which are involved in both the upper and lower pathways of aromatic metabolism. Analysis of the nucleotide sequence downstream of *bphC* reveals the presence of several genes which are involved in aromatic metabolism (Figure 3). These include genes for a ferredoxin component of a dioxygenase (*bphA3*), a large subunit of an oxygenase component of a dioxygenase (*bphA1c*), and 2-hydroxychromene-2-carboxylate isomerase (*nahD*). Immediately following these genes is one for a transposase (designated orf1 in Figure 3). The ferredoxin component is involved not only in the initial dioxygenase that is involved in attacking the initial substrate (biphenyl, naphthalene, etc) but also as a component in the dioxygenase that attacks (methyl)benzoate. Normally benzoate and toluate dioxygenases consist of a reductase and a two-subunit oxygenase [9,25]. However, in the case of *S. yanoikuyae* B1 the reductase component is split into two components, a reductase and a ferredoxin. This shows conservation of electron transfer components as the same reductase and ferredoxin can be used with multiple oxygenase components. This is not unprecedented as *ortho*-halobenzoate 1,2-dioxygenase has also been shown to be a three-component enzyme [27].

The nucleotide sequence immediately to the right of *bphC* shows the presence of a second operon involved in aromatic metabolism (Figure 3). This operon is highly reminiscent of the TOL plasmid *meta*-cleavage or lower pathway operon with several exceptions. The *xylZ* gene, coding for the reductase component of toluate dioxygenase, is missing. This is in agreement with the data discussed in the previous paragraph where a reductase and a ferredoxin take the place of a single bifunctional reductase in this multicomponent enzyme. In addition, several rearrangements of this operon have taken place in comparison with the TOL plasmid. The *xylL* gene has been removed and is present elsewhere, *xylT* has been moved to a position just after *xylH*, *xylF* has been moved to a new location, and at least three new genes have been added to the middle of the operon: orf2, *bphA1d* coding for a large subunit of a dioxygenase, and *bphK*, encoding a glutathione *S*-transferase. In addition, *xylC* and *bphB* have been placed at the end of the operon. Experiments with insertional mutagenesis of this group of genes suggest that there are at least two promoters, one in front of *xylX* and the other between *xylX* and *xylE* [15]. Thus, insertions (either by insertional mutagenesis or by random transposon insertion) in *xylE* result in polar effects on *bphB*. This explains the large proportion of *bphB* mutations obtained in transposon mutagenesis experiments described above: there is a large target area (nine kilobases) for the transposon to exert a polar effect on *bphB*.

Several genes required for aromatic metabolism in *S. yanoikuyae* B1 are still missing from the nucleotide sequence discussed above. This being the case, nucleotide sequencing was continued to the left of *bphB* in the hope of finding the remaining genes in the pathway. At least four additional operons were discovered in this region of DNA (Figure 3). The first contains the genes for two different oxygenase components of a dioxygenase (*bphA1a*, *bphA2a*, *bphA1b*, *bphA2b*) and the reductase and oxygenase components of xylene monooxygenase (*xylAM*). Genes to the right of this operon are arranged in small operonic units. These include genes encoding a regulatory protein (*bphR*), a reductase component of a dioxygenase (*bphA4*), a *cis*-(methyl)benzoate dihydrodiol dehydrogenase (*xylL*), a 2-hydroxybenzalpyruvate aldolase (*nahE*), an oxygenase component of a dioxygenase (*bphA1e*), and a protein of unknown function (*bphX*).

Although over 30 genes have been identified in *S. yanoikuyae* B1 and shown to be involved in metabolism of polycyclic and monocyclic aromatic compounds [15,17] there still remain several to be found. For instance, the genes for benzylalcohol dehydrogenase (*xylB*), salicylate hydroxylase (*nahG*), and 2-hydroxy-6-oxo-6-phenylhexa-2,4-dieonate hydrolase (*bphD*) have not yet been identified. In addition, genes for at least six oxygenase components have been identified in the nucleotide sequence. One of these, *xylXY*, codes for the oxygenase component of toluate dioxygenase. Analysis of the remaining five sets of genes (labeled *bphA1A2*) indicates that they encode oxygenases of unknown function. Deletion of an oxygenase gene or insertional mutagenesis of an oxygenase gene results in no detectable phenotype as measured by the ability to oxidize naphthalene or biphenyl. Thus, the genes for the true initial oxygenase component have not yet been sequenced. The

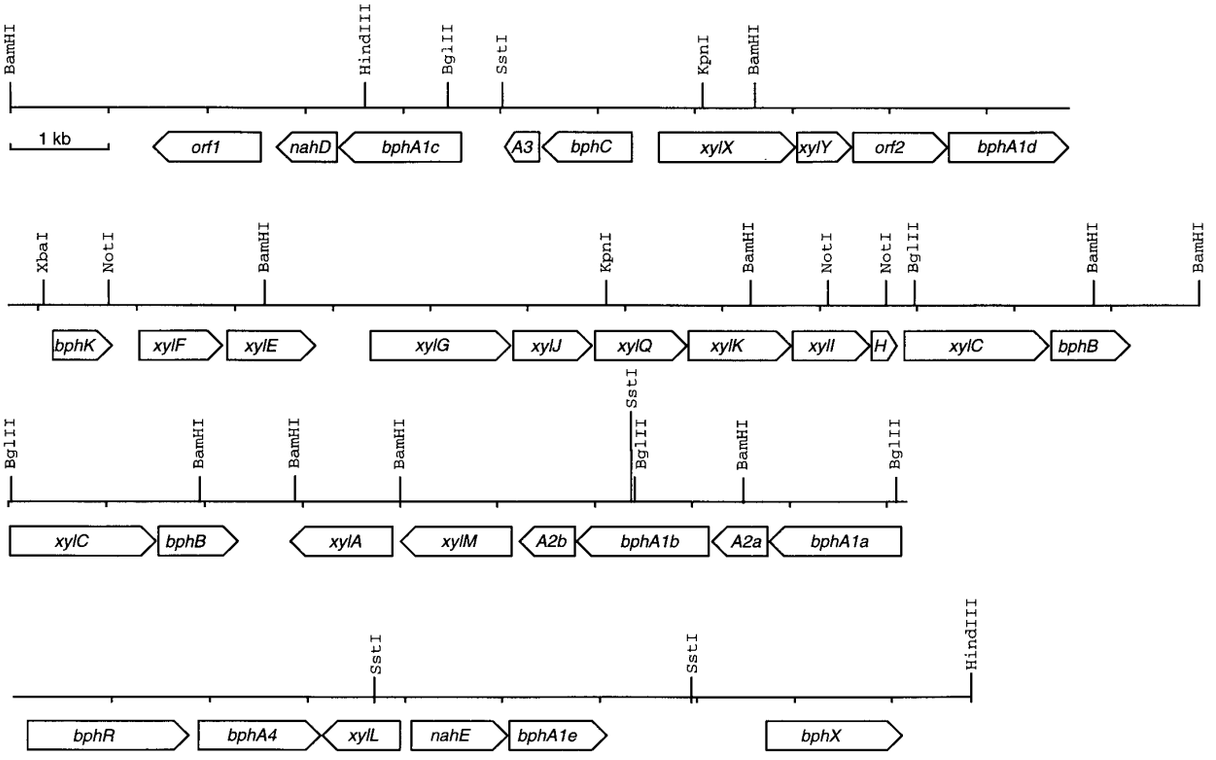

Figure 3 Cartoon of the nucleotide sequence encoding genes for aromatic hydrocarbon degradation from *S. yanoikuyae* B1.

role of these five other oxygenases in aromatic metabolism has yet to be determined.

The organization of genes for polycyclic and monocyclic aromatic metabolism in *S. yanoikuyae* B1 is quite unusual compared with that known for the *xyl* genes of the TOL plasmid or the *nah* genes of the NAH plasmid. However, this may be considered unusual only by the fact that aromatic metabolism encoded by the TOL and NAH plasmids was among the first to be analyzed at the genetic level. *S. yanoikuyae* B1 may not have a unique gene organization, it may just represent a new class of organization that has not yet been seen. In fact, investigations of related *Sphingomonas* strains have shown this to be the case. Southern hybridization of restriction digested genomic DNA from several different *Sphingomonas* strains shows that they all possess cross-hybridizable genes [14]. In fact, *Sphingomonas paucimobilis* Q1 shows an identical RFLP pattern as that found in *S. yanoikuyae* B1. In addition, aromatic hydrocarbon-degrading *Sphingomonas* species strains from the deep subsurface possess cross-hybridizing genes. Since these organisms are thought to have been separated from their surface counterparts for 66–100 million years [28] this means that the gene recruitment and reorganization must have occurred in the far distant past. One interesting observation is that although the genes for polycyclic and monocyclic aromatic metabolism are present in the chromosome of *S. yanoikuyae* B1 they are present on large plasmids in the deep subsurface *Sphingomonas* strains [14].

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