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Beijerinckia sp strain B1: a strain by any other name . . . . .

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***Beijerinckia* sp strain B1 grows with biphenyl as its sole source of carbon and energy. A mutant, strain B8/36, oxidized biphenyl to *cis*-(2*S*,3*R*)-dihydroxy-l-phenylcyclohexa-4,6-diene (*cis*-biphenyl dihydrodiol). Strain B8/36 oxidized anthracene, phenanthrene, benz[*a*]anthracene and benzo[*a*]pyrene to *cis*-dihydrodiols. Other substrates oxidized to *cis*-dihydrodiols were dibenzofuran, dibenzothiophene and dibenzo-*p*-dioxin. Biphenyl dioxygenase activity was observed in cells of *Beijerinckia* B1 and B8/36 after growth in the presence of biphenyl, *m*-, *p*-xylene and salicylate. Recent studies have led to the reclassification of *Beijerinckia* B1 as *Sphingomonas yanoikuyae* strain B1. Subsequent biotransformation studies showed that *S. yanoikuyae* B8/36 oxidized chrysene to a *bis*-*cis*-diol with hydroxyl substituents at the 3,4- and 9,10-positions. Dihydronaphthalene was oxidized to *cis*-1,2-dihydroxy-1,2,3,4-tetrahydronaphthalene, naphthalene, *cis*-1,2-dihydroxy-1,2-dihydronaphthalene and 2-hydroxy-1,2-dihydronaphthalene. Anisole and phenetole were oxidized to phenol. Thus the *S. yanoikuyae* biphenyl dioxygenase catalyzes *cis*-dihydroxylation, benzylic monohydroxylation, desaturation and dealkylation reactions. To date, the genes encoding biphenyl dioxygenase have not been cloned. However, the nucleotide sequence of a *S. yanoikuyae* B1 DNA fragment contains five different α subunits as determined by conserved amino acids coordinating iron in a Rieske [2Fe-2S] center and mononuclear iron at the catalytic site. The specific role of the different putative oxygenases in biotransformation reactions catalyzed by *S. yanoikuyae* is not known and presents an exciting challenge for future studies.**

Keywords: biphenyl; naphthalene; polycyclic aromatic hydrocarbons; dioxygenase; *cis*-dihydrodiol

Introduction

In the late 1960s and early 1970s, polychlorinated biphenyls (PCBs) were identified as worldwide environmental pollutants [45]. At that time little was known about the microbial degradation of biphenyl [8,36] or any of the chlorinated biphenyls found in commercial PCB mixtures (Aroclors).

In 1973 we isolated a bacterium that could utilize biphenyl as its sole source of carbon and energy for growth. The organism was a Gram-negative, motile rod that formed a copious opaque elastic slime when grown aerobically with glucose on nitrogen-free solid medium. Growth was observed at acid pH and cysts were not observed under any growth conditions. All of the preceding features are characteristic of the genus *Beijerinckia* as described in Bergey's Manual of Determinative Bacteriology and the isolate was tentatively identified as a *Beijerinckia* species which was subsequently given the strain designation B1 [17].

Beijerinckia B1 utilized biphenyl, naphthalene, phenanthrene and anthracene as sole sources of carbon and energy for growth. Benzene, toluene and ethylbenzene did not serve as growth substrates.

The following text is a chronology of the metabolic activities in the life of strain B1 since its isolation in 1973.

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Early childhood (1973–1984)

(a) Oxidation of biphenyl and chlorinated biphenyls

The growth of *Beijerinckia* B1 with biphenyl resulted in the excretion of a bright yellow product into the culture medium. This metabolite was tentatively identified as a ring-fission compound formed from a catechol intermediate. Treatment of the parent B1 strain with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine led to the isolation of a mutant, designated *Beijerinckia* sp strain B8/36. The mutant, when grown with succinate in the presence of biphenyl, accumulated a neutral product in the culture medium that was isolated and identified as *cis*-2,3-dihydroxy-l-phenylcyclohexa-4,6-diene (*cis*-biphenyl dihydrodiol) [17]. Subsequent experiments showed that the dihydrodiol was a single enantiomer in which the hydroxyl groups had a (2*R*,3*S*)-absolute configuration [51]. Cell extracts prepared from biphenyl-grown cells of strain B1 catalyzed an NAD^+-dependent oxidation of *cis*-biphenyl dihydrodiol to 2,3-dihydroxybiphenyl which was oxidized further to a yellow ring-fission product in the presence of oxygen. The initial reactions in the catabolism of biphenyl by *Beijerinckia* B1 are shown in Figure 1. *cis*-Biphenyl dihydrodiol has been implicated, but rarely isolated, as the initial product formed in the metabolism of biphenyl by almost all organisms isolated since 1973. The 'dihydrodiol pathway' is now the paradigm for the metabolism of many PCB congeners and unchlorinated aromatic hydrocarbons.

Biphenyl-induced cells of *Beijerinckia* B8/36 oxidized 3-chloro- and 4-chlorobiphenyl on the unchlorinated ring to *cis*-2,3-dihydroxy-1[3-chlorophenyl]-cyclohexa-4,6-diene and *cis*-2,3-dihydroxy-1[4-chlorophenyl]-cyclohexa-4,6-diene, respectively. In contrast, the major product formed

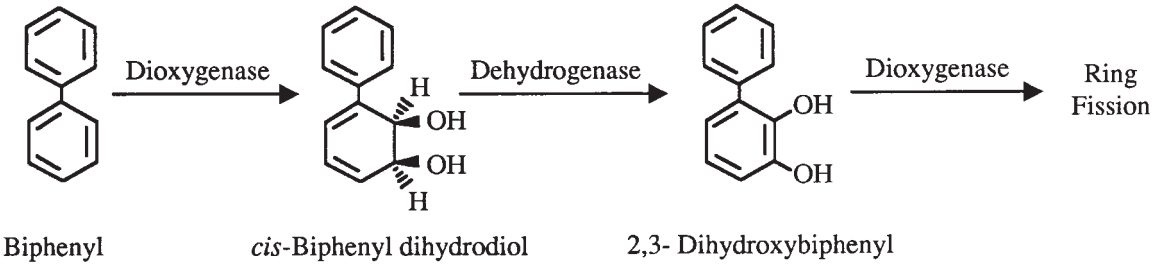


Figure 1 Initial reactions in the degradation of biphenyl by *Beijerinckia* strain B1.

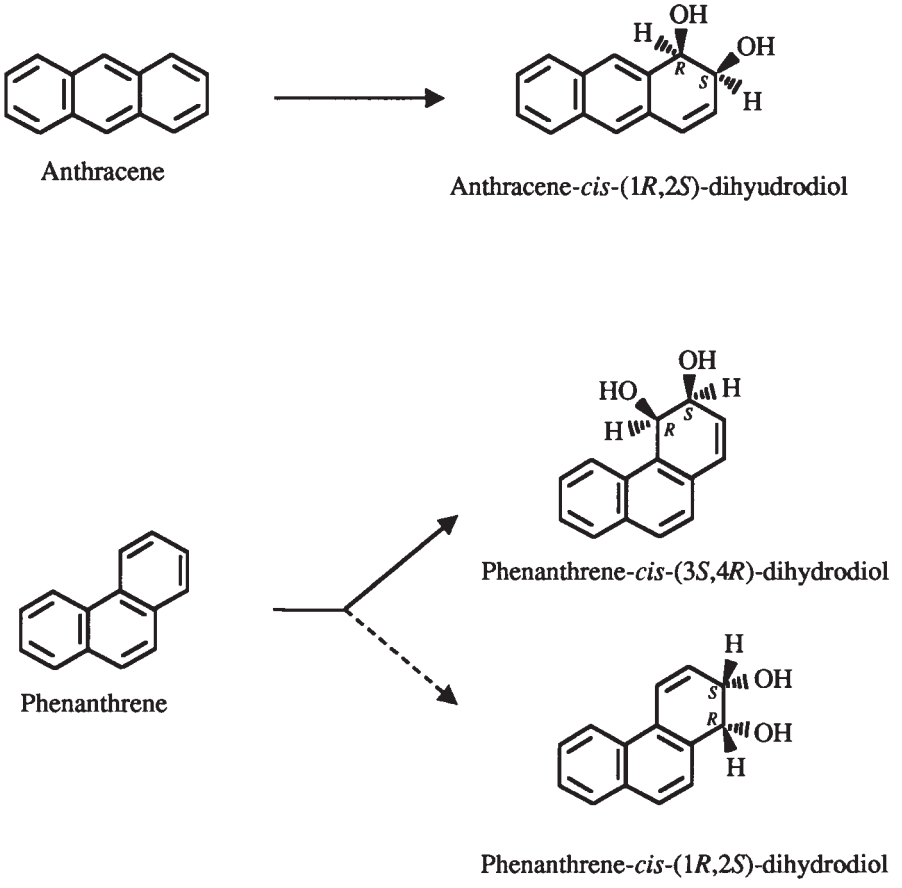


Figure 2 *cis*-Dihydrodiols formed from anthracene and phenanthrene by *Beijerinckia* strain B8/36. Dashed line indicates minor phenanthrene dihydrodiol.

from 2-chlorobiphenyl was identified as 2-chloro-*cis*-5,6-dihydroxy-1-phenylcyclohexa-1,3-diene. Strain B8/36 oxidized 2,4-, 2,5- and 3,4-dichlorobiphenyls to dihydrobiols as evidenced by the detection of individual parent ions at m/e 256 and phenolic dehydration products at m/e 238. PCB congeners containing more than three chlorine substituents were not oxidized [39].

(b) Oxidation of anthracene and phenanthrene

The major products formed by mammals from anthracene and phenanthrene are *trans*-1,2-dihydroxy-1,2-dihydroanthracene and *trans*-9,10-dihydroxy-9,10-dihydrophenanthrene, respectively. In contrast, *Beijerinckia* B8/36 oxidizes anthracene and phenanthrene to *cis*-dihydrodiols at the 1,2- and 3,4-positions, respectively. A minor product formed from phenanthrene was identified as *cis*-1,2-dihydroxy-1,2-dihydrophenanthrene [23]. Subsequent detailed analytical studies showed that the configuration of the hydroxyl groups in the phenanthrene 3,4- and 1,2-dihydrodiols were (3*S*,4*R*) and (1*R*,2*S*), respectively [33]. These configurations are reflected in the structures of the dihydrodiols shown in Figure 2.

(c) Oxidation of polycyclic aromatic hydrocarbons

Beijerinckia B1 does not grow with aromatic hydrocarbons that contain more than three fused rings. However, cells of strain B8/36 grown with succinate in the presence of biphenyl, oxidized benzo[*a*]pyrene to two products that were separated by preparative high pressure liquid chromatography. The major product was identical to a synthetic sample of

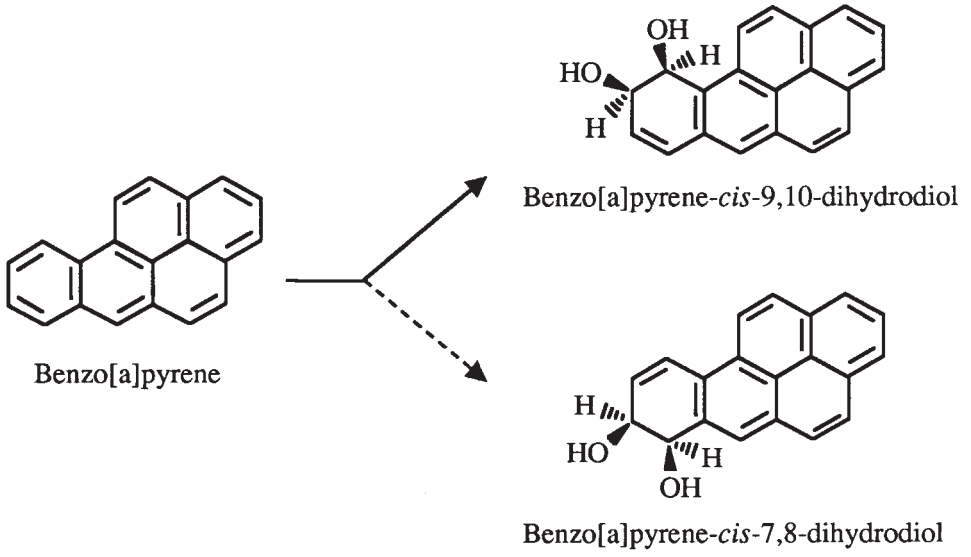


Figure 3 *cis*-Dihydrodiols formed from benzo[*a*]pyrene by *Beijerinckia* strain B8/36. Dashed line indicates minor dihydrodiol. Absolute chemistry not intended.

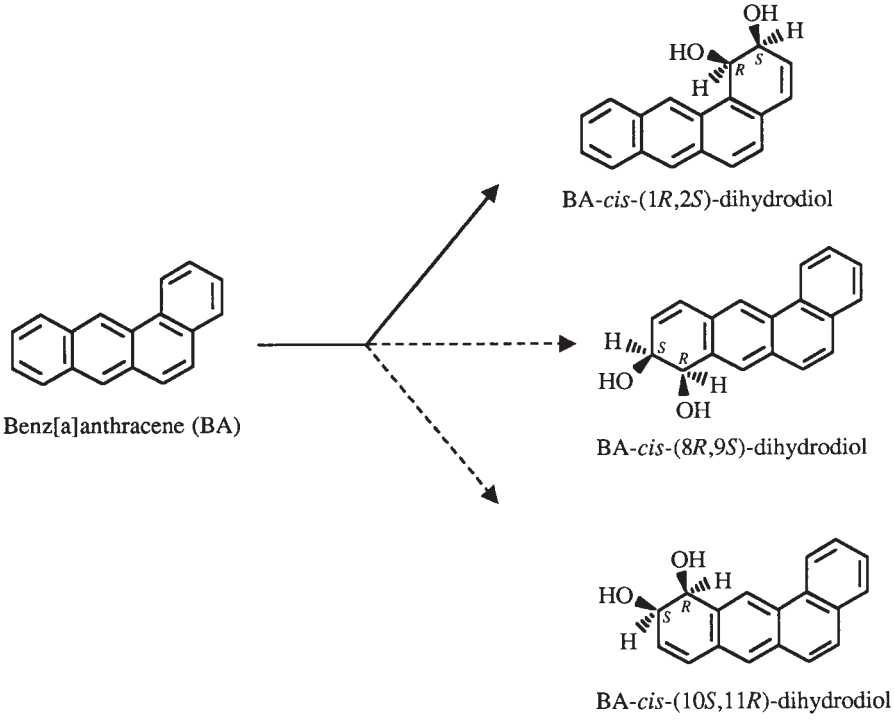


Figure 4 *cis*-Dihydrodiols formed from benz[*a*]anthracene by *Beijerinckia* strain B8/36. Dashed lines indicate minor dihydrodiols.

cis-9,10-dihydroxy-9,10-dihydrobenzo[*a*]pyrene. When the metabolite and the synthetic dihydrodiol were treated with acid both compounds were dehydrated to 9-hydroxybenzo[*a*]pyrene. The minor product formed by strain B8/36 was identified as *cis*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene by conventional analytical techniques [18] (Figure 3). Although *Beijerinckia* strain B1 oxidizes benzo[*a*]pyrene to acidic products, these have yet to be identified.

The oxidation of benz[*a*]anthracene by biphenyl-induced cells of *Beijerinckia* B8/36 led to the formation of three *cis*-dihydrodiols which were separated by high pressure liquid chromatography. The major product gave an identical absorption spectrum to that given by synthetic 1,2-dihydrobenz[*a*]anthracene. Acid treatment of the product gave a single phenol which was identical to synthetic 2-hydroxybenz[*a*]anthracene. These observations together with the data provided by the proton magnetic resonance (PMR) spectrum, identified the product as *cis*-1,2-dihydroxy-1,2-dihydrobenz[*a*]anthracene. The minor products formed from benz[*a*]anthracene were identified from their PMR spectra as *cis*-dihydrodiols at the 8,9- and 10,11-positions [18]. Subsequent studies led to the determination of the

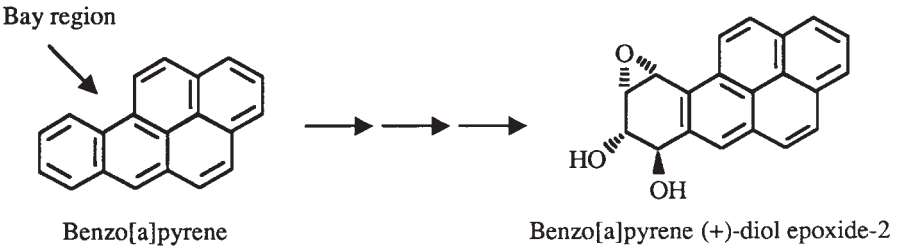


Figure 5 Oxidation of benzo[*a*]pyrene to (+)-diol epoxide-2 by mammalian microsomes (cytochrome P450).

absolute configuration of the three benz[*a*]anthracene *cis*-dihydrodiols shown in Figure 4 [24].

It is of interest to note that the major *cis*-dihydrodiols formed from polycyclic aromatic hydrocarbons by *Beijerinckia* B8/36 are always 'bay-region' dihydrodiols. The term 'bay-region' describes the angular junction of three aromatic rings by analogy to a bay as shown in Figure 5 [22]. Jerina and his associates have conducted extensive studies on the mammalian metabolism of polycyclic aromatic hydrocarbons. This work has led to the suggestion that the key structural feature of a carcinogenic metabolite is an epoxide on a saturated, angular benzo-ring which forms part of the 'bay-region.' Thus the most potent benzo[*a*]pyrene metabolite in terms of carcinogenicity is (+)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene [(+)-diol epoxide-2] (Figure 5) [25]. The filamentous fungus, *Cunninghamella elegans* also oxidizes benzo[*a*]pyrene to (+)-diol epoxide-2 [9,10]. However, *Beijerinckia* strains B1 and B8/36 do not oxidize polycyclic aromatic hydrocarbons to epoxides or *trans*-dihydrodiols. Succinate-grown cells of the parent B1 strain did not oxidize benz[*a*]anthracene. However, cells grown with succinate in the presence of biphenyl were able to oxidize [12-^{14}C]benz[*a*]anthracene to $^{14}CO_2$. These results showed that strain B1 can degrade at least two rings of the parent hydrocarbon and this conclusion was substantiated by the isolation and identification of 1-hydroxy-2-anthroic acid as the major metabolite formed from benz[*a*]anthracene. Two minor products were isolated and identified as 2-hydroxy-3-phenanthroic acid and 3-hydroxy-2-phenanthroic acid [37]. The formation of 1-hydroxy-2-anthroic acid as the major product clearly indicates that it is formed from *cis*-1,2-dihydroxy-1,2-dihydrobenz[*a*]anthracene. Thus the major pathway for the degradation of benz[*a*]anthracene by *Beijerinckia* B1, shown in Figure 6, involves the stereospecific oxidation of the substrate to a *cis*-dihydrodiol at the 1,2-position followed by dehydrogenation to 1,2-dihydroxybenz[*a*]anthracene. Ring cleavage followed by the reactions proposed by Evans *et al* [15] for the oxidation of phenanthrene to 1-hydroxy-2-naphthoic acid accounts for the formation of 1-hydroxy-2-anthroic acid from benz[*a*] anthracene. In addition, the formation of 2-hydroxy-3-phenanthroic acid and 3-hydroxy-2-phenanthroic acid can be explained by dehydrogenation of *cis*-dihydrodiols at the 8,9- and 10,11-positions to the corresponding catechols followed by ring fission and an analogous sequence of reactions proposed for the oxidation of anthracene to 2-hydroxy-3-naphthoic acid [15]. The recent identification of 2-hydroxybenzochromene-2-carboxylate as

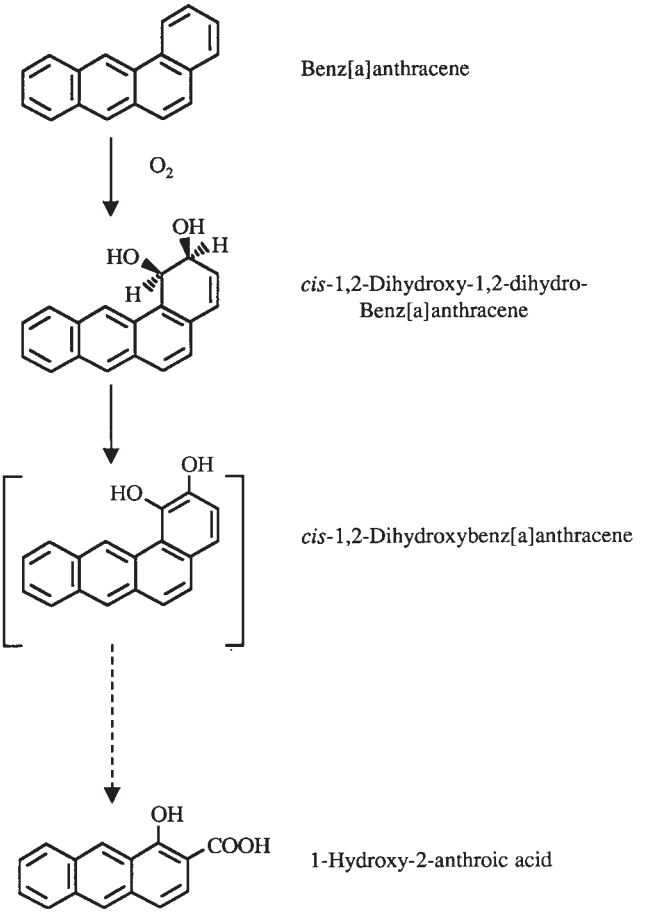


Figure 6 Degradation of benz[*a*]anthracene to 1-hydroxy-2-naphthoic acid by *Beijerinckia* strain B1.

an intermediate in the degradation of anthracene by *Beijerinckia* B1 [30] suggests that analogous benzochromene carboxylates are also intermediates in the degradation of benz[*a*]anthracene.

The differences between fungal and bacterial metabolism of polycyclic aromatic hydrocarbons probably reflect the functions of the different enzyme systems that are involved. Fungi appear to have evolved an efficient detoxification system for the elimination of lipophilic aromatic hydrocarbons from the cell. In contrast, bacteria have evolved suites of enzymes for degradation of unsubstituted aromatic hydrocarbons to intermediates that can support growth. *Beijerinckia* B1 does not grow on aromatic hydrocarbons

larger than phenanthrene. Thus, it seems probable that the enzymes involved in the degradation of biphenyl to molecules that can enter the pathways of central metabolism are the same enzymes that initiate the oxidation of benzo[*a*]pyrene and benzo[*a*]anthracene.

(d) Metabolism of heterocyclic substrates

Preliminary studies showed that *Beijerinckia* B8/36, when grown with succinate in the presence of dibenzothiophene, accumulated two products in the culture medium. The major product was purified by silica gel chromatography and identified as (+)-*cis*-1,2-dihydroxy-1,2-dihydrobenzothiophene (dibenzothiophene dihydrodiol). The dihydrodiol was oxidized to 1,2-dihydroxydibenzothiophene by a purified preparation of (+)-*cis*-naphthalene dihydrodiol dehydrogenase. 1,2-Dihydroxydibenzothiophene was also formed when cell extracts prepared from dibenzothiophene-induced cells of *Beijerinckia* B1 were incubated under anaerobic conditions with dibenzothiophene dihydrodiol and NAD^+. The same cell extracts in the presence of air oxidized 1,2-dihydroxybiphenyl through a yellow ring-fission compound to 3-hydroxy-2-formylthionaphthene which was resistant to further degradation. The minor product formed from dibenzothiophene by strain B8/36 was isolated in pure form and shown to be identical to a synthetic sample of dibenzothiophene-5-oxide [34].

When crude preparations of *cis*-1,2-dihydroxy-1,2-dihydrodibenzothiophene were analyzed by high pressure liquid chromatography, a minor product was detected and identified as a dihydrodiol by mass spectrometry. Subsequent PMR analysis identified this product as *cis*-3,4-dihydroxy-3,4-dihydrodibenzothiophene. Partially purified preparations of *cis*-biphenyl dihydrodiol dehydrogenase oxidized *cis*-1,2-dibenzothiophene dihydrodiol and *cis*-3,4-dibenzothiophene dihydrodiol to 1,2-dihydroxydibenzothiophene and 3,4-dihydroxydibenzothiophene, respectively. In separate experiments 1,2- and 3,4-dihydroxydibenzothiophene were oxidized by a partially purified preparation of 2,3-dihydroxybiphenyl 1,2-dioxygenase to yellow ring-fission products. The product formed from 1,2-dihydroxydibenzothiophene was slowly converted to 3-hydroxy-2-formylthionaphthene and pyruvate. In contrast, the ring-fission product formed from 3,4-dihydroxydibenzothiophene was not metabolized further [38].

Beijerinckia B8/36, when grown with glucose in the presence of dibenzofuran, produced two unstable dihydrodiols which were identified by isolation and characterization of their phenolic dehydration products. The major and minor diols were tentatively identified as *cis*-2,3-dihydroxy-2,3-dihydrodibenzofuran and *cis*-1,2-dihydroxy-1,2-dihydrodibenzofuran, respectively. These results are in contrast to those obtained in concurrent experiments with *Cun-*

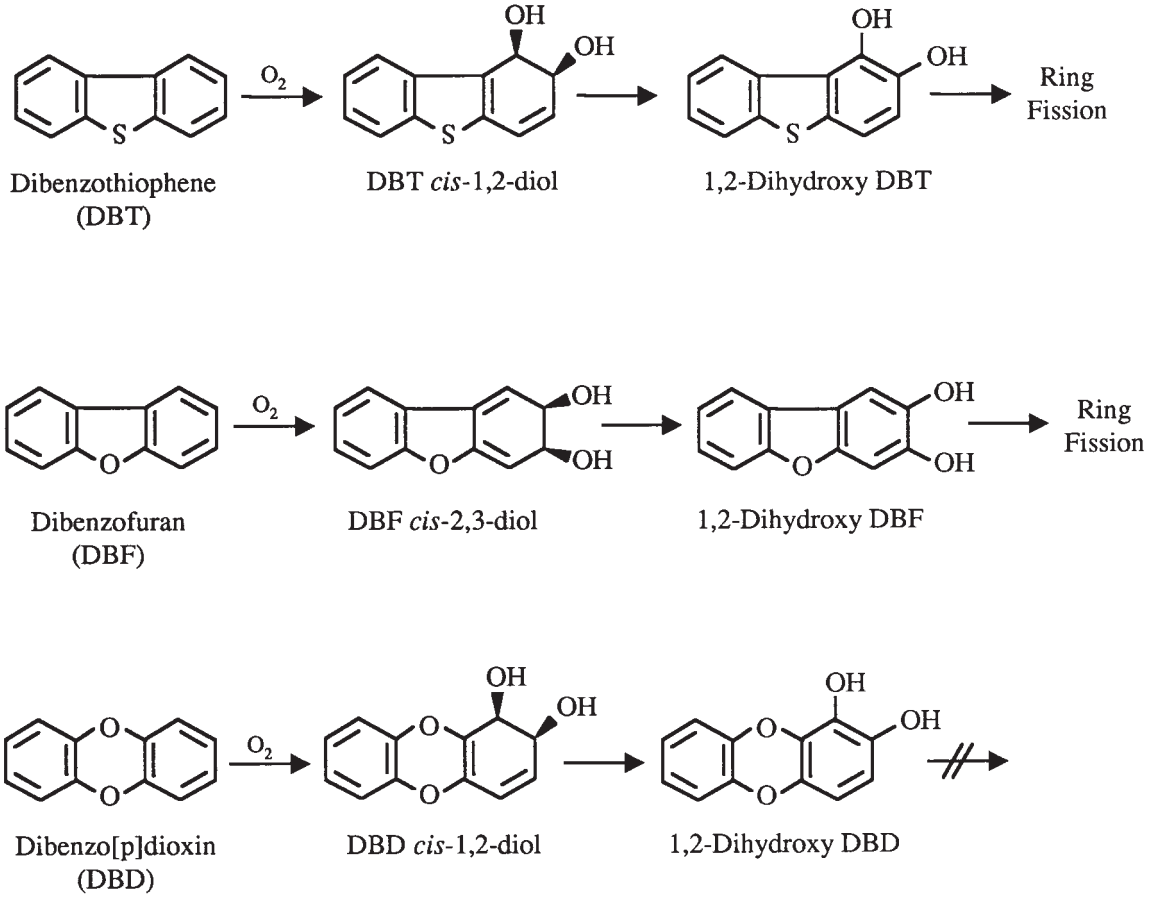


Figure 7 Initial reactions in the oxidation of dibenzothiophene, dibenzofuran, and dibenzo-*p*-dioxin by *Beijerinckia* strain B1.

Table 1 Aromatic compounds that support the growth of *Beijerinckia* strains B1 and B8/36[a]

Substrate	Strain B1	Strain B8/36
Biphenyl	+++	–
Naphthalene	+	–
Phenanthrene	+	–
Anthracene	+	–
m-Xylene	+++	+++
m-Methyl benzyl alcohol	+++	nt
m-Toluic acid	+++	nt
p-Xylene	+++	+++
p-Toluic acid	+++	nt
1,2,4-Trimethylbenzene	+++	nt
m-Ethyltoluene	+++	nt
Salicylate	+++	+++

[a] Good growth, +++; poor growth, +; no growth, –; not tested, nt.

ninghamella elegans which led to the isolation and identification of *trans*-2,3-dihydroxy-2,3-dihydrodibenzofuran as the major product. The latter was shown to be much more stable to acid-catalyzed dehydration than the bacterial *cis*-isomer [11].

Biphenyl-induced cells of *Beijerinckia* B1 oxidized dibenzo-*p*-dioxin and several chlorinated derivatives. The rates of oxidation decreased with increasing chlorine substitution. The mutant B8/36 strain oxidized dibenzo-*p*-dioxin to *cis*-1,2-dihydroxy-1,2-dihydrodibenzo-*p*-dioxin which was further oxidized to 1,2-dihydroxybenzo-*p*-dioxin by the wild-type B1 strain [31]. 1-Chloro- and 2-chlorodibenzo-*p*-dioxin were oxidized by strain B8/36 to chlorinated *cis*-dihydrodiols. The yields of both dihydrodiols were low and the positions of the hydroxyl substituents were not established [32].

The major reactions used by *Beijerinckia* B1 to oxidize dibenzothiophene, dibenzofuran and dibenzo-*p*-dioxin are shown in Figure 7.

Adolescence (1985–1989)

(a) Growth and induction studies

It has been known for many years that salicylate is an inducer of naphthalene dioxygenase and other enzymes of the naphthalene upper pathway [1,42]. In addition, Furukawa *et al* showed that the oxidation of biphenyl, toluene, *m*-xylene and salicylate is regulated by a common unit in *Pseudomonas paucimobilis* [16]. These results prompted us to investigate the role of these and related monocyclic aromatic compounds as growth substrates and inducers for *Beijerinckia* strains B1 and B8/36. The results of these studies are shown in Table 1. The parent B1 strain grew with all of the substrates tested, whereas the mutant B8/36 strain did not grow with biphenyl, naphthalene, phenanthrene or anthracene. Polarographic studies showed that cells of strain B1, grown with the alkylbenzenes and the alcohol and acid derivatives shown in Table 1, oxidized biphenyl, naphthalene, phenanthrene, anthracene, dibenzothiophene and dibenzofuran to yellow ring-fission products which were slowly metabolized further. The reverse situation was also observed. That is, cells of strain B1, grown with biphenyl, naphthalene, phenanthrene and anthracene oxidized *m*-xylene, *p*-xylene and their respective alcohol and acid derivatives. *m*-Xylene- and *p*-xylene-grown cells of the mutant B8/36 oxidized biphenyl, phenanthrene, anthracene, dibenzofuran and dibenzothiophene to their respective *cis*-dihydrodiols. At the time (1984–1986), the ability of *Beijerinckia* to oxidize a wide range of monocyclic and polycyclic aromatic compounds was attributed to the expression of suites of catabolic enzymes that have relaxed substrate specificities and/or common regulatory mechanisms (F Mondello and WR Mahaffey, unpublished results). Preliminary transposon mutagenesis studies with the *Tn5* vector pRKTV14 led to the isolation of seven mutants that were unable to grow with biphenyl and all other compounds listed in Table 1. Reversion experiments with one of these mutants (strain B702) indicated that a single mutation in a regulatory gene product may be responsible for loss of the phenotypic properties exhibited by *Beijerinckia* B1 [41].

At this stage of our studies we decided to clone the structural and regulatory genes for biphenyl degradation. Over an 18-month period, all attempts to locate these genes in a cosmid library of 40-kb fragments of *Beijerinckia* B1 DNA were unsuccessful.

Separation (1989–1996)

In 1989 we suspended studies on the catabolism of aromatic hydrocarbons by *Beijerinckia* B1. At the same time an in-

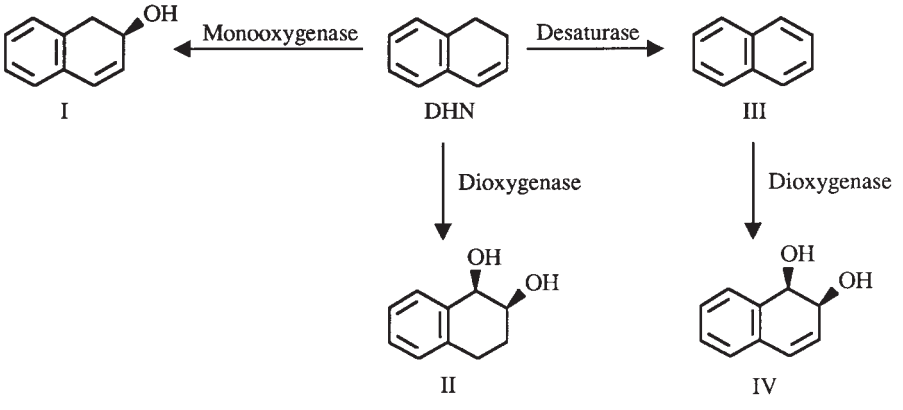


Figure 8 Oxidation of dihydronaphthalene (DHN) by *Beijerinckia* strain B8/36. Structural designations: I, (+)-(2*R*)-hydroxy-1,2-DHN; II, (–)-*cis*-(1*R*,2*S*)-dihydroxy-1,2,3,4-THN; III, naphthalene; IV, (+)-(1*R*,2*S*)-*cis*-1,2-dihydroxy-1,2-dihydronaphthalene.

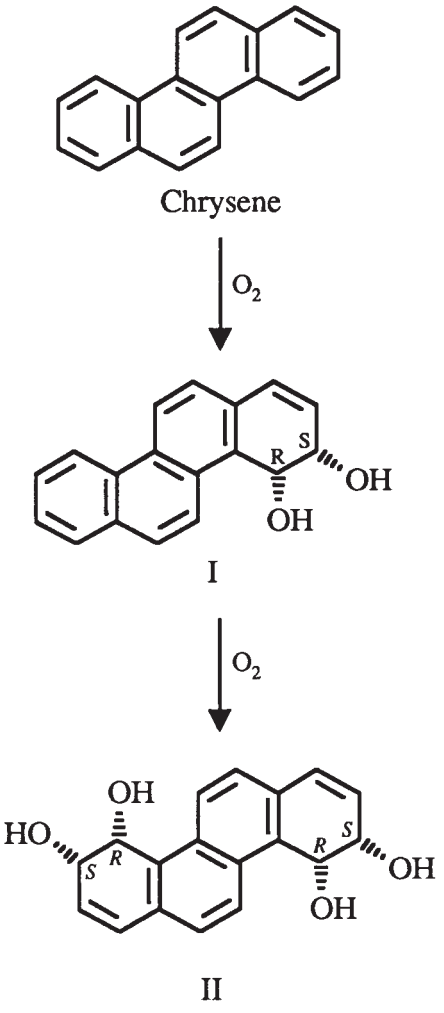


Figure 9 Oxidation of chrysene by *Beijerinckia* strain B8/36. Structural designations: I, (+)-*cis*-(3*S*,4*R*)-dihydroxy-3,4-dihydrochrysene; II, (+)-(3*S*,4*R*,9*S*,10*R*)-3,4,9,10-tetrahydroxy-3,4,9,10-tetrahydrochrysene.

depth genetic analysis of this organism was initiated in the laboratory of Gerben Zylstra at Rutgers University. Our own studies focused on the characterization of the naphthalene and biphenyl dioxygenase systems expressed by *Pseudomonas* sp NCIB 9816-4 and *Burkholderia* sp LB400, respectively.

During this period, Cerniglia and his colleagues reevaluated the identity of *Beijerinckia* B1. The results provided by 16S rRNA sequencing, biochemical tests, fatty acid methyl ester analysis, polyacrylamide gel electrophoresis of protein and DNA-DNA hybridization led to the reclassification of *Beijerinckia* B1 as *Sphingomonas yanoikuyae* strain B1 [27]. The genus *Sphingomonas* was not recognized prior to 1990 and in addition to *Beijerinckia* B1, several bacterial strains identified as pseudomonads have now been reclassified as sphingomonads [summarized in 49]. One taxonomic property retained by *Sphingomonas yanoikuyae* B1 is its ability to grow on nitrogen-free media. The ability of other sphingomonads to grow under these conditions has not been reported.

Reconciliation (1996–present)

(a) Oxidation of 1,2-dihydronaphthalene by Sphingomonas yanoikuyae strains

The increasing demand for single enantiomers of chiral drugs has been accompanied by an increased interest in enzyme-catalyzed enantiospecific reactions [48]. Toluene (TDO) and naphthalene (NDO) dioxygenases oxidize a wide range of aromatic substrates to homochiral *cis*-diols [4,44], and some of these diols have been used in the enantioselective synthesis of a diverse array of biologically active products [6,7,21,35,47,50]. In addition, NDO catalyzes monohydroxylation, sulfoxidation, desaturation, *O*- and *N*-dealkylation reactions with selected substrates [44] and oxidizes indole to indigo [13]. These results led us to investigate the ability of *S. yanoikuyae* to oxidize dihydronaphthalene (DHN). The results obtained (Figure 8) show that the biphenyl dioxygenase induced in *S. yanoikuyae* B8/36 can catalyze dioxygenation, monooxygenation and desaturation reactions [12]. In addition, previous studies [43] showed that *m*-xylene-induced cells of strain B8/36 oxidize anisole to phenol (33%) and phenetole to phenol (5%) and ethenyloxybenzene (5%). The substrate specificity of BPDO has not been investigated in detail. Nevertheless, results obtained to date show that BPDO catalyzes diverse reactions analogous to those reported for NDO [44].

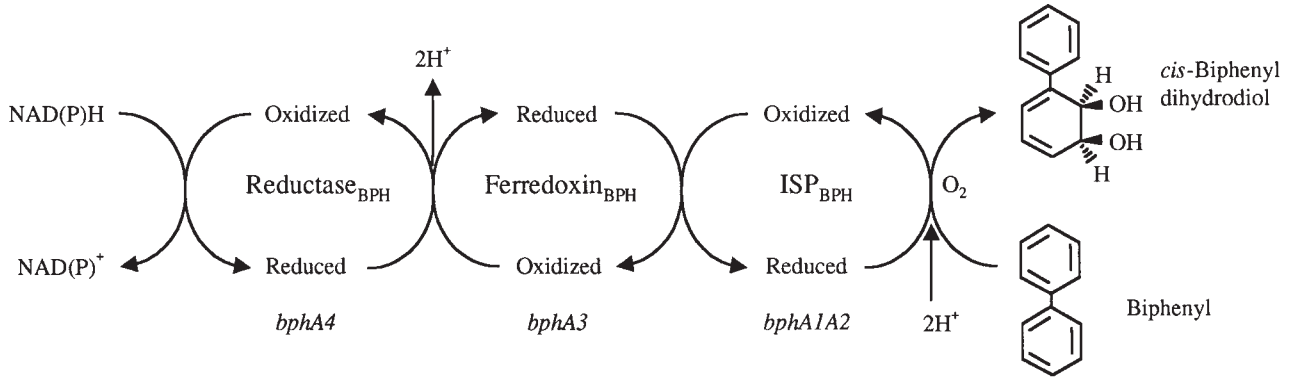
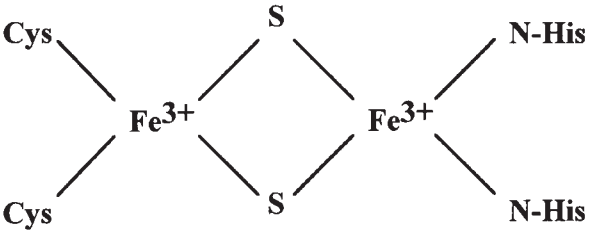


Figure 10 Electron flow and gene designations in the biphenyl dioxygenase system from *Burkholderia* strain LB400.

a

	77												109
9816-4 NahAc	FLNV	C	R	H	RGKTLVSVEAGNA..KGFV	C	SY	H	GWGFG
LB400 BphA	FLNQ	C	R	H	RGMRICRSDAGNA..KAFT	C	SY	H	GWAYD
B1 BphA1c	VVNR	C	A	H	RGALVCRELRGNRPN..LE	C	VY	H	QWAYD
B1 BphA1e	WVNR	C	A	H	KGATVCRSLRGNQADGAFV	C	VY	H	QWAYD
B1 BphA1a	FLNT	C	T	H	RGTASCRAIRGNA..RSFT	C	NY	H	GWSFA
B1 BphA1b	YLNS	C	P	H	RGNKVCFADAGNT..RRFV	C	NY	H	GWAFN
B1 XylX	LVNA	C	A	H	RGAKVCREKAGNK..KNFM	C	PF	H	GWTYS

Consensus sequence CxH-x15-17-CxxH



b

	200					220	350					370
9816-4 NahAc	ENFVGDAY	H	VGWT.	H	ASSLRS.		RTFGPAGFWESD	D	NDNMETAS
LB400 BphA	EQFCSDMY	H	AGTTT	H	LSGILAG		RNFSAGGVFEQD	D	GENWVEIQ
B1 BphA1c	EN.TRDPY	H	ASLL.	H	LFHNTFG		NLIGPAGLISME	D	GEAVEIVQ
B1 BphA1e	EN.SRDSY	H	GALL.	H	LFYPTFG		NLVGPSGLISME	D	GEAVELCQ
B1 BphA1a	ENVCGDGY	H	LGWA.	H	.TGAMMA		QTFGPGGTFEMD	D	GENWENCT
B1 BphA1b	ENFIGDAY	H	AGWT.	H	DSGCRSM		QTFGPGGTGEMD	D	GENWENCT
B1 XylX	EN.GLDGY	H	VGTV.	H	ANYFMTV		DFFNASGMATPD	D	LTEFNNCQ

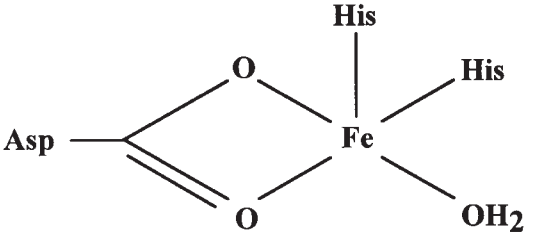


Figure 11 Conserved amino acids coordinating iron in the α subunits of NDO and BPDO dioxygenases. (a) Rieske [2Fe-2S] center. (b) 2-His-1-carboxylate facial triad at the active site. NahAc, *Pseudomonas* sp 9816-4; BphA, *Burkholderia* sp LB400; BphA1a,A1b,A1c,A1e, *Sphingomonas yanoikuyae* B1.

(b) Metabolism of polycyclic arenes

Our previous results on the oxidation of benzo[*a*]pyrene and benz[*a*]anthracene by *Sphingomonas yanoikuyae* B1 showed that BPDO preferentially oxidizes anthracene, phenanthrene, benz[*a*]anthracene and benzo[*a*]pyrene to bay-region regioisomers of chiral *cis*-dihydrodiols [18,23,24]. These properties suggested that chrysene, a symmetrical polycyclic aromatic hydrocarbon with two bay-regions, would be a good substrate for studies on regiospecificity of BPDO. This work, carried out in collaboration with Professor Derek R Boyd and his associates in the Department of Chemistry, the Queen's University of Belfast, UK, showed that *S. yanoikuyae* B8/36 oxidizes chrysene to (+)-*cis*-(3*S*,4*R*)-dihydroxy-3,4-dihydrochrysene [2]. The absolute configuration of the hydroxyl groups in the diol was determined by stereochemical correlation with

(−)-(3*S*,4*R*)-dihydroxy-1,2,3,4-tetrahydrochrysene. Further studies on chrysene oxidation by B8/36 led to the isolation and identification of (+)-(3*S*,4*R*,9*S*,10*R*)-3,4,9,10-tetrahydroxy-3,4,9,10-tetrahydrochrysene, the first *bis*-*cis*-diol to be isolated and identified [3]. These reactions are shown in Figure 9.

Our current biotransformation studies with *S. yanoikuyae* are directed towards the oxidation of azaarenes such as acridine and phenazine. Preliminary studies indicate that both substrates are oxidized to mono-*cis*- and *bis*-*cis*-dihydrodiols (DT Gibson, DR Boyd, unpublished observations). Thus, *S. yanoikuyae* continues to provide new enzyme-catalyzed routes to novel chiral diols.

(c) Aromatic ring-hydroxylating dioxygenases

The elegant genetic analysis of *S. yanoikuyae* B1 by Zylstra and Kim [28,29,52], and the determination of the complete nucleotide sequence of a 184-kb plasmid from *S. aromaticus* F199 by Romine *et al* [46], provide an explanation for the difficulties encountered in our initial efforts to clone the genes for BPDO from strain B1. To date, all of the enzymes responsible for the oxidation of aromatic hydrocarbons to *cis*-dihydrodiols are multicomponent systems that form short electron transport systems with flavins and iron-sulfur clusters as redox components [40]. For example, the BPDO system purified from *Burkholderia* sp strain LB400 (formerly *Pseudomonas* sp strain LB400) consists of a flavoprotein ($Reductase_{BPH}$) [5] which transfers electrons from NAD(P)H through a small Rieske [2Fe-2S] iron sulfur protein ($Ferredoxin_{BPH}$) [20] to the oxygenase component (ISP_{BPH}) [19]. The latter has an $\alpha_3\beta_3$ subunit composition and each α subunit contains a Rieske [2Fe-2S] iron sulfur center which transfers electrons to mononuclear iron at the catalytic site of the enzyme. The organization of the BPDO system and the gene designations for each component are shown in Figure 10. The genes for each dioxygenase component form part of the *bph* operon and are expressed coordinately [14]. In contrast, the nucleotide sequence of a *S. yanoikuyae* B1 DNA fragment, thought to contain the genes for the BPDO system, contains genes for no less than five different α subunits (*bphA1aA1bA1cA1dA1e*) [52]. Each *bphA1* gene contains a consensus sequence for ligands that coordinate iron in the Rieske [2Fe-2S] center and a second consensus sequence for the 2-His-1-carboxylate motif that coordinates mononuclear iron at the active site. Proof for the existence of these coordination sites, which are shown in Figure 11, is derived from the crystal structure of the dioxygenase component of the NDO system [26]. It is of interest to note that only *bphA1a* and *bphA1b* have adjacent genes (*bphA2a* and *bphA2b*) for the β subunit in the strain B1 nucleotide sequence. Other unusual features of this sequence include the presence of single genes for ferredoxin (*bphA3*), ferredoxin reductase (*bphA4*) and *cis*-dihydrodiol dehydrogenase (*bphB*). The presence of a single gene for *cis*-dihydrodiol dehydrogenase would account for the phenotype of *S. yanoikuyae* B8/36. However, deletion or inactivation by insertional mutagenesis of any single oxygenase gene in the B1 nucleotide sequence does not impair the ability of B1 to oxidize biphenyl or naphthalene [52]. These results suggest that the genes encoding BPDO are located on a different region of the B1 chromosome. It is also possible that the *bph(A1cA1dA1e)* genes encoding the α subunits are inactive in the absence of their β subunits. The answers to questions relating to the substrate specificity of the oxygenases in strain B1 may be facilitated by a study of the six $(\alpha\beta)_n$ oxygenase homologs located on the plasmid PNL1 harbored by *S. aromaticivorans* [46]. Clearly, there is much work still to be done.

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