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ORIGINAL PAPER

Identification, cloning, and characterization of a multicomponent biphenyl dioxygenase from *Sphingobium yanoikuyae* B1

Sinéad M. Ní Chadhain · Elizabeth M. Moritz · Eungbin Kim · Gerben J. Zylstra

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Abstract *Sphingobium yanoikuyae* B1 utilizes both polycyclic aromatic hydrocarbons (biphenyl, naphthalene, and phenanthrene) and monocyclic aromatic hydrocarbons (toluene, *m*- and *p*-xylene) as its sole source of carbon and energy for growth. The majority of the genes for these intertwined monocyclic and polycyclic aromatic pathways are grouped together on a 39 kb fragment of chromosomal DNA. However, this gene cluster is missing several genes encoding essential enzymatic steps in the aromatic degradation pathway, most notably the genes encoding the oxygenase component of the initial polycyclic aromatic hydrocarbon (PAH) dioxygenase. Transposon mutagenesis of strain B1 yielded a mutant blocked in the initial oxidation of PAHs. The transposon insertion point was sequenced and a partial gene sequence encoding an oxygenase component of a putative PAH dioxygenase identified. A cosmid clone from a genomic library of *S. yanoikuyae* B1 was identified which contains the complete putative PAH oxygenase gene sequence. Separate clones expressing the genes encoding the electron transport components (ferredoxin and reductase) and the PAH dioxygenase were constructed. Incubation of cells expressing the dioxygenase enzyme system with biphenyl or naphthalene resulted in production of the corresponding *cis*-dihydrodiol confirming PAH dioxygenase activity. This demonstrates that a single multicomponent dioxygenase enzyme is involved in the initial oxidation of both biphenyl and naphthalene in *S. yanoikuyae* B1.

Keywords *Sphingobium* · Polycyclic · Biodegradation · Dioxygenase · Biphenyl · Naphthalene · Phenanthrene

S. M. Ní Chadhain · E. M. Moritz · G. J. Zylstra
Biotechnology Center for Agriculture and the Environment, Rutgers, The State University of New Jersey, 59 Dudley Road, New Brunswick, NJ 08901-8520, USA

E. M. Moritz
Department of Microbiology, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

E. Kim
Yonsei University, Seoul, Republic of Korea

S. M. Ní Chadhain (✉)
Biotechnology Center for Agriculture and the Environment, Rutgers University, 59 Dudley Road, New Brunswick, NJ 08901-8520, USA
e-mail: sinead@aesop.rutgers.edu

Introduction

Sphingobium yanoikuyae B1 (formerly *Sphingomonas yanoikuyae* B1and *Beijerinckia* sp. strain B1) was originally isolated for the ability to grow on biphenyl as its sole source of carbon and energy [14]. *S. yanoikuyae* B1 and a dihydrodiol accumulating mutant of B1, *S. yanoikuyae* B8/36, oxidize a wide range of aromatic compounds including the polycyclic aromatic hydrocarbons naphthalene, anthracene, phenanthrene, chrysene, benz[a]anthracene, and benzo[a]pyrene [6, 13, 26, 27, 35, 43]. While many genes implicated in the degradation of biphenyl, naphthalene, xylene and toluate, as well as enzymes involved in the downstream metabolism of biphenyl have been identified [4, 7, 23–25, 36, 43], the genes encoding the biphenyl dioxygenase which catalyzes the initial step in polycyclic aromatic hydrocarbon degradation by *S. yanoikuyae* B1 have yet to be identified (Fig. 1). Previous work identified a large 39 kb segment of chromosomal DNA encoding multiple genes involved in aromatic hydrocarbon degradation, including single ferredoxin and ferredoxin reductase genes

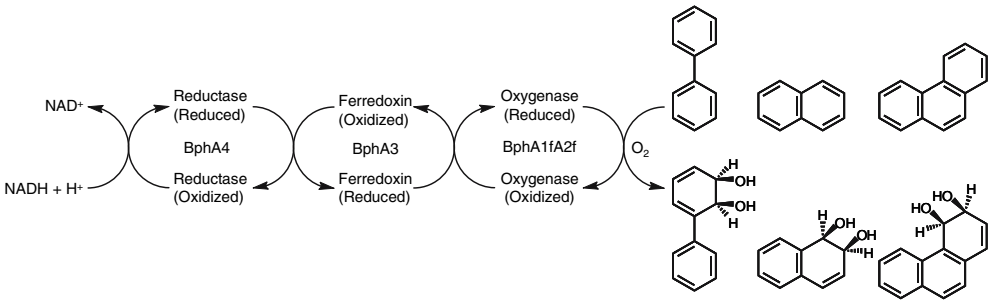

Fig. 1 Initial step in the aerobic degradation pathway of biphenyl, naphthalene and phenanthrene. BphA4, ferredoxin reductase; BphA3, ferredoxin; BphA1f, biphenyl dioxygenase alpha subunit; BphA2f, biphenyl dioxygenase beta subunit

and multiple alpha and beta subunit dioxygenase gene pairs. However, a biphenyl dioxygenase gene was not identified in this region [7, 23–25, 36, 43].

Here we report the identification and preliminary characterization of the initial biphenyl dioxygenase gene, *bphA1fbphA2f* from *S. yanoikuyae* B1. The gene was identified in an indigo production deficient mini-transposon mutant of *S. yanoikuyae* B1, which is unable to grow on biphenyl. Expression of *bphA1fA2f* with the previously described electron transfer components *bphA3bphA4* in *E. coli* resulted in the transformation of biphenyl and naphthalene to the corresponding *cis*-dihydrodiols. Complementation of the indigo production minus mutant with *bphA1fA2f* restored the ability to convert indole to indigo and to grow on biphenyl as the sole carbon and energy source, an indication that the cloned gene is indeed responsible for B1's ability to grow on biphenyl.

Materials and methods

Bacterial strains, plasmids and media

S. yanoikuyae B1 is the wild type strain and is capable of growth on biphenyl, naphthalene, and *m*-xylene [13]. Strain B8/36 is derived from *S. yanoikuyae* B1 and is capable of growth on *m*-xylene and accumulates *cis*-biphenyl dihydrodiol when grown on succinate in the presence of biphenyl [14]. Mineral salts broth (MSB) was used for carbon source and metabolite accumulation studies [37]. MSB was supplemented with 10 mM glucose when needed. Biphenyl and naphthalene were provided as crystals in the petri dish lids or dissolved in dimethylformamide and added to liquid cultures. *m*-Xylene was supplied in the vapor phase in cotton stoppered glass vials for solid media or in glass bulbs for liquid cultures. *S. yanoikuyae* strains were grown at 30°C and *E. coli* strains were grown at 37°C. Expression experiments were conducted at 30°C (Table 1).

Mutant and genomic library construction

The construction of the genomic library was described previously [24]. Colonies were screened on L agar for the production of indigo. A mutant library of *S. yanoikuyae* B1 was made using mini-Tn5Km1 [8, 24] following procedures described previously [24]. Colonies containing a mutation in the initial dioxygenase were identified by their inability to grow on biphenyl and to produce indigo from indole. Genomic DNA was isolated from one biphenyl minus strain, digested with NotI and cloned into pGEM5Zf(−). Transformants were plated on kanamycin to select for clones containing the transposon integration site. The DNA flanking the transposon integration site was sequenced to identify the gene that had been disrupted in the mutant.

Locating the genes for the large and small subunit of the oxygenase component of biphenyl dioxygenase

PCR primers were designed to amplify the region adjacent to the transposon integration site in the biphenyl minus mutant EK110. The primers were used to screen a genomic library of *S. yanoikuyae* B1 by PCR. One positive clone from a genomic library of *S. yanoikuyae* B1 was identified. The cosmid DNA was isolated and sheared to fragments of approximately 3 kb using a DNA hydroshear machine (Genomic Solutions, Ann Arbor, MI) The sheared cosmid DNA was then end repaired using the EndIt repair kit (Epicentre, Madison, WI) and cloned into pCR Blunt (Invitrogen). Ninety-six clones were picked and arrayed using a QPix II (Genetix USA, Boston, MA). Plasmid DNA was isolated using the PureLink 96 kit (Invitrogen) and sequenced on an ABI Prism 3100 Genetic Analyzer using M13f and M13r primers and Big Dye v3.1 dye terminator chemistry (Applied Biosystems, Foster City, CA). The sequences were trimmed and assembled using SeqMan (DNAStar, Madison, WI). Sequencing primers were designed to walk across any remaining gaps in the cosmid

Table 1 Bacterial strains and plasmids used in this study

Bacterial strains and plasmids	Genotype or phenotype	Reference/source
S. yanoikuyae B1	PAH degrading isolate	[13]
S. yanoikuyae B8/36	Diol accumulating mutant of B1	[14]
E. coli TOP10	F$^-$ *mcr*A Δ (*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*Δ139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G	Invitrogen
E. coli BL21(DE3)pLysS	F$^-$ *omp*T *hsdS*$_B$(r$_B^-$ m$_B^-$) *gal dcm* (DE3) pLysS (Cam^R)	Invitrogen
E. coli BL21 Star (DE3)	F$^-$ *omp*T *hsd*S$_B$ (*r*$_B^-$ *m*$_B^-$) *gal dcm rne*131 (DE3)	Invitrogen
E. coli JM109	F′ *traD36 proA*$^+$*B*$^+$ *lacI*^{q} *Δ(lacZ)M15/ Δ(lac-proAB) glnV44 e14*$^-$ *gyrA96 recA1 relA1 endA1 thi hsdR17*	
pET101D	colE1 *ori*, Ap^r	Invitrogen
pALTER-Ex2	Tc^r, p15a *ori*	Promega
pRK415	Tc^r, broad host range vector	[21]
pB1Af	*bphA1fbphA2f* in pET101D	This study
pB1Fd	*bphA3* in pALTER-Ex2	This study
pB1Rd	*bphA4* in pALTER-Ex2	This study
pB1FdRd	*bphA3bphA4* in pALTER-Ex2	This study
pRKAf	*bphA1fbphA2f* in pRK415	This study

assembly. The assembled sequence was analyzed using Blast and GeneMark software [2, 3, 5].

Expression clone construction

The putative biphenyl/naphthalene dioxygenase gene was identified and PCR primers designed to the 5′ end of the large subunit and the 3′ end of small subunit. The primer sequences were as follows: bphA1f_f: ATGAGCAGCG ACGCCACAC, bphA2f_r: TTAGCAAAAGAAATACA GGTTC. The two subunits were amplified by PCR and cloned into pCR2.1 TOPO (Invitrogen). The dioxygenase was then amplified from this construct with the primers bphA2f_r and bphA1f_f2: CACCATGAGCAGCGAC GCCACAC using *Pfu* polymerase and cloned into the expression vector pET101D (Invitrogen). The ferredoxin and ferredoxin reductase genes from *S. yanoikuyae* B1 were amplified and cloned into pCR2.1 TOPO (Invitrogen) using the following restriction enzyme tagged primers: B1Fdf_Eco, GAATTCAGGAGATATACCCATGTCGA ACAAATTGCGCC, B1Fdr_Nsi, ATGCATTCAGGCG CTCTCTTTCGG, B1Rdf_Nsi, ATGCATTCGATTGCG ATTGTTGGTGCG, B1Rdr-Hin, AAGCTTTCAGCCCG CCTGCTTGAGC. The DNA sequence was confirmed and the ferredoxin and reductase were then excised from pCR2.1_FdRd by digestion with *Eco*RI and *Hin*dIII and ligated into pALTER-Ex2 (Promega). Both constructs were then transformed together into the expression hosts *E. coli* BL21 (DE3) pLysS or *E. coli* BL21 Star (Invitrogen).

Dioxygenase assays

Clones containing the genes encoding the terminal dioxygenase genes and the electron transfer components were grown up overnight in LB. Two percent was inoculated into 50 ml MSB containing 10 mM glucose and grown to an OD_{600} of 0.5. The cultures were then induced by the addition of IPTG to 0.5 mM and grown to an OD_{600} of 1.0. The cells were then harvested by centrifugation and resuspended in 25 ml 50 mM phosphate buffer (pH 7.25) and biphenyl or naphthalene added to 0.25 mg/ml. Control incubations with *S. yanoikuyae* B8/36, a *cis*-dihydrodiol accumulating mutant of *S. yanoikuyae* B1, were also performed [14]. The cultures were incubated for 4 h to overnight at 30°C and 200 rpm. The culture supernatants were then extracted three times with an equal volume of ethyl acetate, dried, and resuspended in methanol before analysis by gas chromatography-mass spectrometry on an HP 5890 Series II gas chromatograph coupled to an HP 5971 mass selective detector. One microliter was injected in split mode with a 5 min solvent delay. The initial oven temperature was 70°C rising 10°C per minute to a final temperature of 280°C. The samples were run on an HP-5MS column (HP part number 19091S-433, 30 × 0.25 mm, 0.25 μM film thickness) with helium as the carrier gas.

Complementation studies

In order to confirm that the cloned genes are indeed responsible for catalyzing the initial step in the degradation of

biphenyl by *S. yanoikuyae* B1, the *bphA1fA2f* genes were cloned into the *Eco*RI site of the broad host range vector pRK415 [21]. The dioxygenase genes were amplified from pCR2.1-Af using the following primers which had EcoRI fragments at the 5′ end to facilitate cloning, bphA1f_f_Eco: GAATTC ATGAGCAGCGACGCCACAC, bphA2f_r_Eco: GAATTCTTAGCAAAAGAAATACAGGTTC. The amplicon was cloned into pCR2.1 TOPO and the DNA sequenced confirmed. The *bphA1fA2f* genes were excised from pCR2.1_AfEco with *Eco*RI and cloned into pRK415. The resulting construct was then mobilized into the biphenyl minus mutant EK110 by triparental mating and assayed for the ability to produce indigo on L agar and to grow on biphenyl.

Nucleotide sequence accession numbers

The nucleotide sequences reported in this study have been deposited in the National Center for Biotechnology Information (NCBI) under the following accession numbers EF152282 and EF151283.

Results

In previous work we identified and sequenced a 39 kb region of *S. yanoikuyae* chromosomal DNA that encodes a number of genes for aromatic compound degradation. While this region contains genes encoding the large and small subunits of five different oxygenases [23, 24] none of these genes encode the oxygenase component of biphenyl dioxygenase. In order to locate the genes encoding the biphenyl dioxygenase a transposon mutant library was constructed with mini-Tn5*Km1*. Kanamycin resistant colonies were screened for the inability to grow on biphenyl while retaining the ability to grow on *m*-xylene and *m*-toluate. Mutants falling into this category must be blocked in one of the first four steps in the biphenyl degradation pathway (between biphenyl and benzoate). It is well known that certain aromatic dioxygenases have the ability to convert indole to indigo, including biphenyl dioxygenase from *S. yanoikuyae* B1 [1, 11, 19, 28, 30, 34]. Mutants lacking biphenyl dioxygenase were thus located by screening the mutants unable to grow on biphenyl for the inability to produce indole from indigo. One mutant, designated EK110, falls into this category and thus most likely has the mini-Tn5*Km1* insertion in the genes encoding for the biphenyl dioxygenase component. The region adjacent to the mini-Tn5*Km1* insertion was cloned and partially sequenced in order to positively identify the gene that had been disrupted in EK110. A 10 kb NotI fragment of EK110 genomic DNA hybridizes to the kanamycin resistance gene of mini-Tn5*Km1*. This NotI fragment was cloned by excising the band from a gel and cloning into the NotI site of pGEM5Zf(−). A partial gene sequence encoding an oxygenase component of a putative PAH dioxygenase was identified. This sequence was used to design PCR primers to screen a cosmid library of *S. yanoikuyae* B1 in order to obtain the DNA sequence of genes flanking the biphenyl dioxygenase gene.

Identification and sequence analysis of the initial dioxygenase containing cosmid clone

Screening of a genomic library of *S. yanoikuyae* B1 by PCR with primers to the transposon site in *S. yanoikuyae* EK110, the indigo deficient mutant, identified one positive cosmid clone. The region flanking the biphenyl dioxygenase genes was sequenced. The sequence was assembled using SeqMan and 11 putative open reading frames (ORF) were identified (Table 2). The function of each ORF was

Table 2 Best BlastP matches in the GenBank database to the sequence reported in this study

Start	End	Accession number	Identity (%)	Name	Organism
1	581	NP_106596	84	Transposase	*Mesorhizobium loti* MAFF303099
1983	873	ZP_01445243.1	58	Transposase	*Roseovarius* sp. strain HTCC 2601
4133	2201	YP_497892.1	73	AMP-dependent synthetase and ligase	[1]*N. aromaticivorans* DSM 12444
5085	4133	NP_049063.1	46	MFS DMT transporter	[a]*N. aromaticivorans* F199
5218	6582	CAG17576.1	90	Ring-hydroxylating dioxygenase alpha subunit	*Sphingomonas* sp. strain CHY-1
6623	7151	CAG17577.1	93	Ring-hydroxylating dioxygenase beta subunit	*Sphingomonas* sp. strain CHY-1
7187	8285	CAG17578.1	93	Aryl-alcohol dehydrogenase	*Sphingomonas* sp. strain CHY-1
8390	11055	NP_049059.1	73	Pyruvate phosphate dikinase	[a]*N. aromaticivorans* F199
11107	12070	NP_049058.1	74	Hyp 010	[a]*N. aromaticivorans* F199
12094	13098	NP_049057.1	84	Hyp 009 (ketoadipate reductase)	[a]*N. aromaticivorans* F199
13125	13552	NP_049056.1	82	3-Isopropyl malate dehydrogenase	[a]*N. aromaticivorans* F199

[a] The nomenclature/strain designation for *N. aromaticovorans* is inconsistent. The genome sequence is reported as *N. aromaticovorans* DSM 12444, the sequence of the 184 kDa catabolic plasmid is reported for *N. aromaticovorans* F199, they refer to the same strain [12]

deduced from sequence homology to genes in the GenBank database identified by Blast searches (Table 2). Two homologues (*bphA1f, bphA2f*) to the large and small subunits of a ring hydroxylating dioxygenase were identified. *bphA1f* is 1,365 nt long and encodes a protein with a predicted MW of 50,978 Da. BlastP analysis revealed that the large subunit (BphA1f) is most closely related to a ring hydroxylating dioxygenase from *Sphingomonas* sp. CHY-1 (90% identity), a strain isolated for it's ability to grow on chrysene and which can also grow on naphthalene, phenanthrene, and anthracene [9, 41]. The next closest match is a putative naphthalene/biphenyl dioxygenase from *Novosphingium aromaticovorans* F199 (BlastP = 79%) [33]. Phylogenetic analysis showed that BphA1f forms a distinct cluster with the large subunits of bph/nap dioxygenase enzymes from various Sphingomonad and *Cycloclasticus* species (Fig. 2). A consensus motif of the Rieske type [2Fe–2S] cluster (C-X-H-X_{17}-C-X_2-H, aa 80–105) and a mononuclear Fe^{2+}-binding domain were identified within the translated sequence of *bphA1f* [20]. *bphA2f* is 525 nt long and encodes a protein with a predicted MW of 20.757 kDa. The small subunit of the dioxygenase gene is most closely related to the corresponding gene in *Sphingomonas* sp. CHY-1 (BlastP = 93%) and clusters with bph/nap dioxygenase small subunits from related species (Fig. 3).

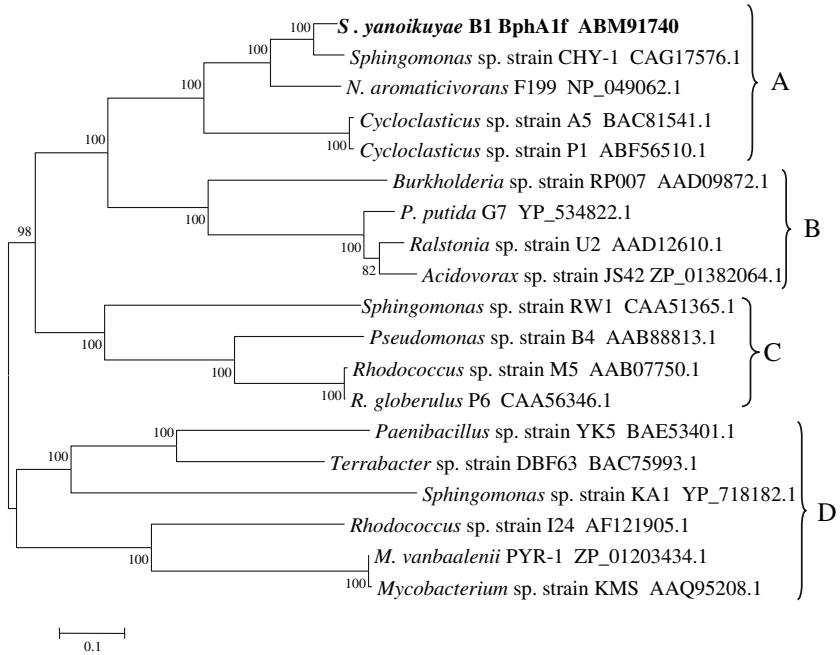

Fig. 2 Phylogenetic distribution of *S. yanoikuyae* B1 BphA1f and related PAH dioxygenase alpha subunits. The dendrogram was constructed from a ClustalW alignment of the alpha subunit amino acid sequences by neighbor-joining analysis using Mega 3.1. Bootstrap values greater than 50 are indicated at *branch nodes*

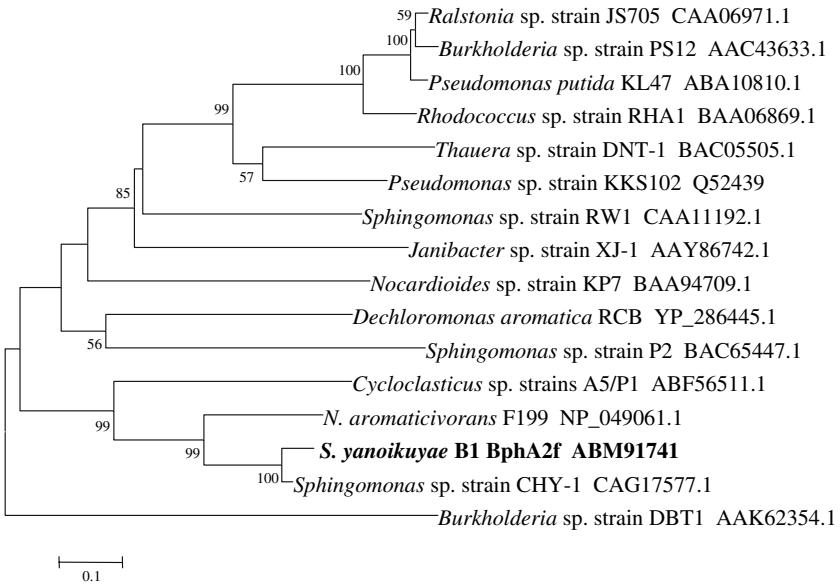

Fig. 3 Phylogenetic distribution of *S. yanoikuyae* B1 BphA2f and related PAH dioxygenase beta subunits. The dendrogram was constructed from a ClustalW alignment of the beta subunit amino acid sequences by neighbor-joining analysis using Mega 3.1. Bootstrap values greater than 50 are indicated at *branch nodes*

In addition to the biphenyl dioxygenase the cosmid clone contains other genes potentially involved in PAH degradation (Table 2). Immediately downstream of *bphA1fA2f*, and transcribed in the same direction, is an ORF encoding an aromatic alcohol dehydrogenase (XylB). Previous work which identified two extradiol dioxygenases and many other genes involved in aromatic hydrocarbon degradation in *S. yanoikuyae* B1 on a 40 kb region of DNA failed to locate this gene. This gene is most closely related to XylB from *Sphingomonas* sp. CHY-1 and *N. aromaticovorans* F199 (BlastP = 93%) [9, 33] (Table 2, Fig. 4). Immediately upstream of *bphA1fA2f* and *xylB*, and transcribed in the opposite direction, are ORFs encoding a permease of the drug/metabolite transporter (DMT) superfamily and an AMP dependent synthetase. Two transposase genes which are transcribed in opposite directions are located upstream of these genes (Table 2). A pyruvate phosphate dikinase gene, two hypothetical proteins, and a gene encoding isopropyl malate dehydrogenase are located downstream of *xylB* (Table 2).

Arrangement of genes compared to other *Sphingomonad* strains

The arrangement of the *S. yanoikuyae* B1 biphenyl dioxygenase genes and their closest neighbors was compared to the arrangement of these genes in other PAH degrading strains (Fig. 5). Previous work showed that in *S. yanoikuyae* B1 the previously characterized PAH degradation genes are located on the chromosome [22]. In other Sphingomonads PAH degradation clusters have been found on both large catabolic plasmids or on the chromosome [22, 33]. In *S. yanoikuyae* B1 *bphA1f* and *bphA2f* were found upstream of a *xylB* gene and downstream of a putative DMT permease gene. In *Sphingomonas* sp. strain CHY-1, the *bphA1fbphA2f* genes are also found between similar genes [9]. In *N. aromaticovorans* F199, genes bearing 79 and 69% identities to *S. yanoikuyae* B1 *bphA1f* and *bphA2f* are located on a catabolic plasmid (pNL1) between *xylB* and a protein of unknown function, which is most closely related to DMT permeases. It is interesting that in all organisms the *bphA1fbphA2f* genes, which are thought to catalyze the initial step in the degradation of PAHs are located in a separate part of the plasmid/chromosome to the genes catalyzing the subsequent steps. Most significantly, in all the strains described above, genes encoding the ferredoxin and ferredoxin reductase (*bphA3*, *bphA4*) necessary for electron transport are located some distance away from the initial dioxygenase genes. This contrasts markedly with the arrangement in *Cycloclasticus* sp. strain A5, a PAH degrading gamma proteobacterium [19], which contains a closely related terminal dioxygenase gene (BlastP identity of 63%), found closely associated with the ferredoxin and ferredoxin reductase genes. Furthermore, it is interesting that the synteny between the genes from *S. yanoikuyae* B1 and those in *N. aromaticovorans* F199 ends at the DMT transporter gene. In strain B1 upstream of this location we find an AMP dependent synthetase/ligase gene that best matches a gene from the chromosomal sequence of *N. aromaticovorans* DSM 12444 and two transposase genes whereas in F199 we find 3 genes encoding a multidrug efflux pump [33] (Fig. 5).

Biotransformation of PAHs by E. coli clones expressing bphA1fA2f

In order to confirm that the *bphA1fA2f* genes encode a PAH dioxygenase component biotransformation assays were

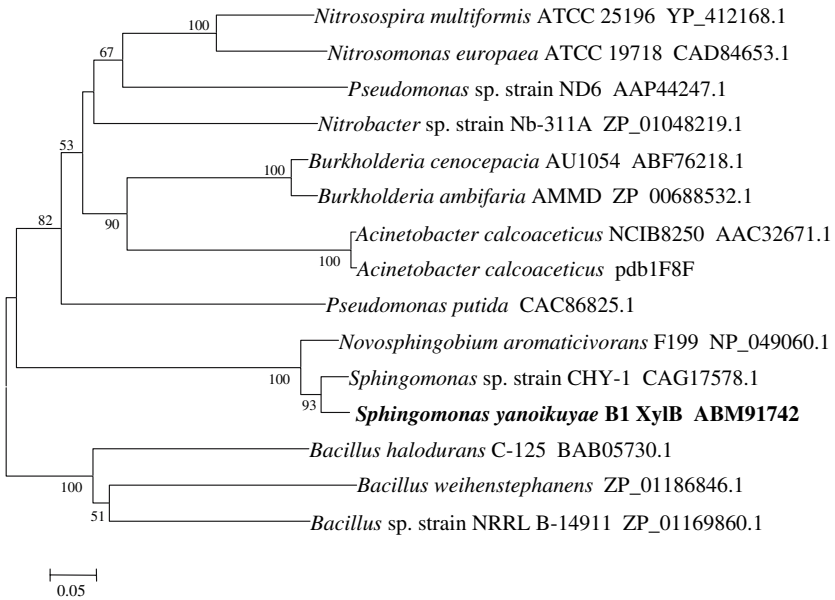

Fig. 4 Phylogenetic distribution of *S. yanoikuyae* B1 XylB and related aromatic alcohol dehydrogenases. The dendrogram was constructed from a ClustalW alignment of the XylB amino acid sequences by neighbor-joining analysis using Mega 3.1. Bootstrap values greater than 50 are indicated at *branch nodes*

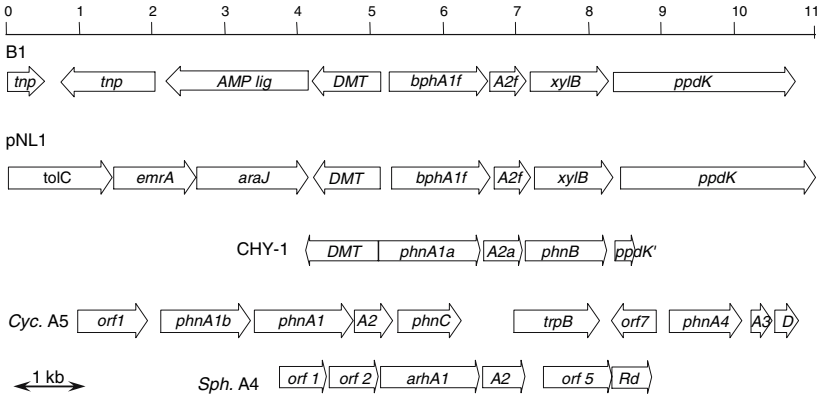

Fig. 5 Arrangement of *bphA1fA2f* and neighboring genes in *S. yanoikuyae* B1 and related species. *Tnp*, transposase; *AMP-lig*, AMP-dependent synthetase and ligase; *DMT*, MFS drug metabolite transporter; *bphA1f*, *phnA1a*, *b*, *arhA1*, ring-hydroxylating dioxygenase alpha subunit; *bphA2f*, *phnA2a*, *arhA2*, ring-hydroxylating dioxygenase beta subunit; *xylB*, *phnB*, aryl-alcohol dehydrogenase; *ppdK*, pyruvate phosphate dikinase; *tolC*, *emrJ*, *araJ*, putative outer membrane proteins; *phnC*, extradiol dioxygenase*; trpB*, tryptophan synthase *β* subunit; *orf7*, hypothetical protein; *phnA4*, ferredoxin reductase; *phnA3*, ferredoxin; *phnD*, isomerase

performed using *E. coli* BL21 (DE3) pLysS (Invitrogen) containing the oxygenase (pB1Af) and electron transfer components (pB1FdRd). Results of incubation of the induced clone with naphthalene and biphenyl are shown in Fig. 6. Control experiments were performed with *S. yanoikuyae* B8/36, a *cis*-dihydrodiol accumulating mutant of *S. yanoikuyae* B1. The MS traces were identical (data not shown). Incubation of clones containing the genes encoding the oxygenase alone did not result in the transformation of biphenyl or naphthalene or in the production of indigo on L agar plates. This confirms that the previously identified genes encoding a ferredoxin and reductase are needed for PAH dioxygenase activity.

Complementation of the bphA1f mutation in S. yanoikuyae EK110

The *bphA1fbphA2f* genes were cloned into the broad host range vector pRK415 and mobilized into *S. yanoikuyae* B1 by triparental mating. The cloned *bphA1fbphA2f* genes restored the ability of *S. yanoikuyae* B1 to grow on biphenyl and to produce indigo on L agar, indicating that the cloned genes are indeed responsible for the initial step in the degradation of biphenyl.

Discussion

The study of sphingomonads continues to attract interest because of the remarkable catabolic diversity found within these bacteria. The species described to date are able to grow on mono- and polycyclic hydrocarbons [16, 42, 43], tetralin [18], naphthalene sulfonate [38], dibenzo-*p*-dioxin [15, 40], dibenzothiophene, and methylated PAHs [10, 17]. However, while the catabolic diversity of the sphingomonads has been well described, the description of the genes encoding their metabolic capabilities reported has lagged

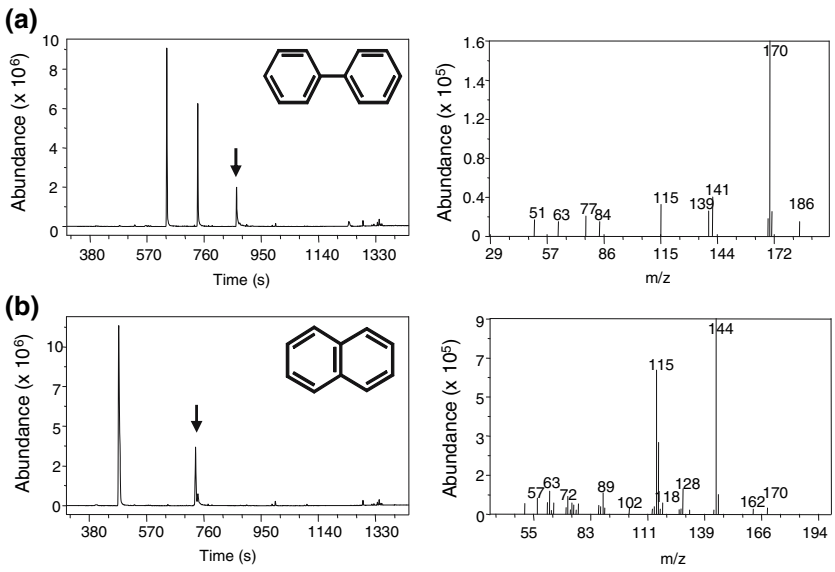

Fig. 6 GC-MS trace of (**a**) biphenyl and (**b**) naphthalene biotransformation by *E. coli* BL21 (DE3) pLysS (pB1Af)(pB1FdRd). The *cis*-dihydrodiol peak is indicated with an *arrow* in each panel

behind. Previous work reported the remarkable catabolic range of *S. yanoikuyae* B1 and many of the genes encoding the mono- and polycyclic hydrocarbon degradation pathways were described [4, 7, 23, 25, 36]. The catabolic genes have an interesting arrangement in that genes predicted to be involved in the degradation of monoaromatic compounds are interspersed with genes involved in PAH and biphenyl degradation [7, 23, 25, 36, 43]. Multiple copies of genes that could potentially catalyze the initial oxidation of biphenyl were detected on overlapping cosmid clones, however, the gene encoding the dioxygenase which catalyzes the initial attack on biphenyl and other PAHs by *S. yanoikuyae* B1 was not among them [23].

In many of the sphingomonad strains reported to date the genes for aromatic hydrocarbon degradation are found in a mosaic arrangement, whether located on the chromosome or on a plasmid [9, 16, 23, 29, 31, 32, 38, 39, 43]. In contrast, related genes necessary for the initial attack on aromatic substrates including the dioxygenase and the electron transfer components, are located together in *Cycloclasticus* sp. strain A5 and *Cycloclasticus* sp. strain P1. It is interesting that genes displaying such a high degree of homology to the sphingomonad initial dioxygenases are found in such a different gene neighborhood. Phylogenetic analysis showed that the large subunit of the B1 dioxygenase gene is closely related to the initial dioxygenase gene from the chrysene degrading strain *Sphingomonas* sp. CHY-1 and the putative naphthalene/biphenyl dioxygenase gene from *N. aromaticovorans* F199 pNL1 and more distantly related to dioxygenase genes from *Cycloclasticus* species. Together they form a distinct cluster (Group A, Fig. 2) that groups away from previously described naphthalene dioxygenases from pseudomonads and the phenanthrene dioxygenase of *Burkholderia* sp. strain RP007. This pattern is conserved to a lesser degree in the small subunit and the XylB phylogenetic trees, suggesting that these genes share a common lineage. The large subunits of the sphingomonad dioxygenase genes amino acid sequences share 79–93% identity. The shared identity falls to 62–93% when the *Cycloclasticus* sequences are included in the analysis. However, this is still significantly higher than the identity shared by the other groups included in the analysis. Group B, which includes naphthalene/biphenyl dioxygenases from Gram-negative bacteria share only 50–90% amino acid identity. It appears that the initial dioxygenase genes from the sphingomonads form a distinct cluster that may explain the wide metabolic capacity of these organisms.

In this study we identified the dioxygenase genes, *bphA1fA2f* from *S. yanoikuyae* B1, which catalyze the first step in the degradation of biphenyl and naphthalene and expressed them in *E. coli*. Successful expression of the initial dioxygenase was facilitated by the use of a two-plasmid system where the terminal dioxygenase genes were carried on one plasmid and the electron transfer components were carried on a second compatible plasmid. While very low levels of dioxygenase activity could be detected in *E. coli* containing the dioxygenase alone, maximal activity was obtained using the two-plasmid system. This approach allows for the testing of the many dioxygenases from *S. yanoikuyae* B1 and potentially from other sphingomonad species.

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