DNA Shuffling of Biphenyl Dioxygenase Genes, *bphAE* or *bphE*, from *Comamonas*

testosteroni B-356 and *Burkholderia cepacia* LB400

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ABSTRACT

DNA Shuffling of Biphenyl Dioxygenase Genes, *bphAE* or *bphE*, from *Comamonas testosteroni* B-356 and *Burkholderia cepacia* LB400

Roch Aumont
Concordia University, 2002

Bacterial degradation of biphenyl and some polychlorinated biphenyls is initiated by and dependent on the ability of biphenyl 2,3-dioxygenase to dihydroxylate substrate . Biphenyl 2,3-dioxygenase is a multi-component enzyme requiring reductase (BphG) and ferredoxin (BphF) components that transport electrons from NADH to the terminal dioxygenase (BPDO). BPDO is composed of an iron-sulfur/mononuclear iron containing α-subunit (BphA), and a β-subunit (BphE) ($\alpha_3\beta_3$). To alter the PCB congener selectivity pattern of BPDO part of our strategy has focused on molecular evolution using DNA shuffling techniques.

Biphenyl dioxygenase genes (*bphA* and *bphE*, or *bphE* only) from two sources, *Comamonas testosteroni* B-356 and *Burkholderia sp.* LB400, were shuffled using random fragmentation of DNA templates followed by reassembly using cycles of template annealing and extension with DNA polymerase. To screen shuffled biphenyl dioxygenase *in vivo* biphenyl degradation genes *(bphFGBC)* were stably inserted into the genome of *Pseudomonas putida* using a mini-*Tn5* transposon. Co-expressing biphenyl dioxygenase from a separate plasmid, we were able to use this strain to detect metabolite accumulation indicative of biphenyl dioxygenase activity.

DNA shuffling of *bphAE,* using various techniques to create random fragments, resulted either in a very low yield of reassembled product and a high level of non-specific recombination, or in reassembly of wild type enzymes. Sequencing of parts of 13

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randomly chosen active (8) or inactive (5) shuffled *bphAE* genes revealed only two recombination events. Inability to shuffle both subunits α and β together may be caused by the highly variable inter-genic region, which may not allow homologous recombination to occur between DNA templates.

A second strategy was DNA shuffling of the β-subunit alone, as this subunit has been shown to affect enzyme specificity. Site directed mutagenesis was used to incorporate homologous restriction sites in the *bphE* genes of both bacterial strains allowing insertion of shuffled *bphE*, without affecting its expression, downstream of un-shuffled *bphA*. To optimize the expression and detection of BPDO, biphenyl degradation genes (*bphFGB* but not *bphC*) were also inserted into high transformation efficiency *E. coli* BL21-Gold. The resulting strain allowed direct colorimetric detection of 2,3-dihydroxybiphenyl, the product of biphenyl dioxygenase.

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BPDO, biphenyl dioxygenase

diClB, dichlorobiphenyl

DNA, deoxyribonucleic acid

dNTP, deoxynucleoside triphosphates; the letter N refers to any or all of the common

bases

EDTA, ethylenediamine-tetraacetic acid

HOPDA, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate

NADH, nicotinamide adenine dinucleotide

PAGE, polyacrylamide gel electrophoresis

PCB, polychlorinated biphenyl

PCR, polymerase chain reaction

R-PCR, reassembly-PCR

INTRODUCTION

Polychlorinated biphenyls (PCBs) constitute a family of xenobiotic compounds consisting of a biphenyl ring, carrying one to ten chlorine substituents and generating up to 209 different congeners, of which three are shown in Figure 1.1. The stability and viscosity of PCB mixtures have made them useful for a variety of applications, and until 1977 large amounts of PCB mixtures, typically containing 60-70 congeners (Arochlors), were produced (Erickson 1986). Arochlors were widely used in transformers, capacitors, printing inks, paints, and more, eventually leading to PCB accumulation in soil and aquatic environments (Erickson 1986). Although PCB manufacture has been banned for several years, contaminated sites still persist because of the thermodynamic stability of the biphenyl ring system. This characteristic, and the hydrophobicity of PCBs, have also contributed to PCB accumulation in fatty tissues of many animals, including humans, through amplification up the food chain (Ayotte *et al.*, 1997). As a result, PCBs are one of the most widely-distributed environmental pollutants and constitute a serious health risk (Longnecker *et al.*, 1997).

Bioremediation is a technology that utilizes the metabolic activities of microorganisms to degrade toxic chemicals *in situ*. For example, bacteria capable of degrading oil have been used to clean up after oil spills (Head and Swannell, 1999). Various species of bacteria, especially those belonging to the genera *Pseudomonas, Alcaligenes, and Rhodococcus,* have the capacity to aerobically metabolize some PCBs (Furukawa 1994, Bedard *et al.*, 1986). The genes encoding pathways responsible for the metabolism of biphenyl and some PCBs have been cloned (Erickson and Mondello 1992, *Ahmad et al.* 1990, Ahmad *et al.*, 1991), and the gene products have been characterized.

1
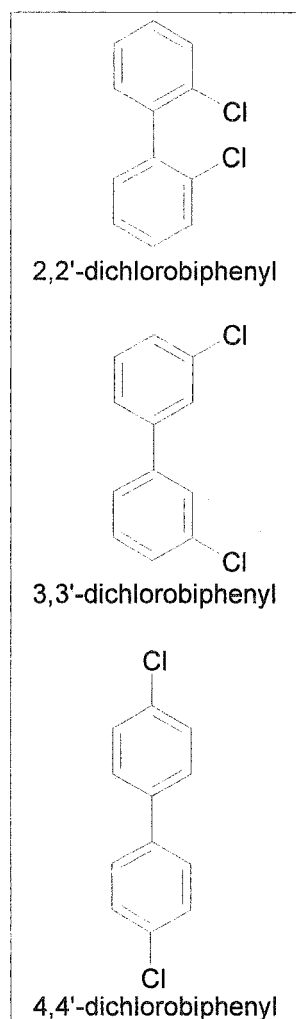
Figure 1.1 Three chlorinated biphenyls used in this study.

The enzymatic steps for the "upper" pathway, responsible for biphenyl metabolism (Fig. 1.2), are apparently the same in all isolates, including *Burkholderia cepacia* LB400 (formerly, *Pseudomonas sp.* LB400) (Seeger *et al.,* 1995), *Pseudomonas* KF707 (Furukawa and Miyazaki, 1986), *Rhodococcus globerulus* P6 (Asturias and Timmis, 1993) and *Comamonas testosteroni* B-356 (Hurtubise *et al.,* 1995). Although bacteria such as these can potentially be applied to bioremediation of PCB-contaminated material, the fact is that they are limited in terms of how many PCB congeners they can degrade.

Bacterial Metabolism of Biphenyl and Polychlorinated Biphenyls

Aerobic degradation of biphenyl and some PCBs is initiated by a multi-component biphenyl dioxygenase (BphAEFG) that dihydroxylates one of the phenyl rings to produce a *cis*-dihydrodiol (2,3-dihydro-2,3-dihydroxybiphenyl) (Fig. 1.2) (Haddock *et al.,* 1993). NAD^+-dependent 2,3-dihydro-2,3-dihydroxybiphenyl dehydrogenase (BphB) next produces 2,3-dihydroxybiphenyl, which is cleaved to the yellow *meta*-cleavage compound, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (HOPDA) by dihydroxybiphenyl dioxygenase (BphC) (Hofer *et al.,* 1993). Hydrolysis of HOPDA by a hydrolase (BphD) yields benzoic acid and 2-hydroxypenta-2,4-dienoic acid, which are further degraded to Krebs cycle intermediates to provide carbon and energy for the cell.

The genes encoding biphenyl degradation enzymes are organized in operons, of which two are shown in Figure 1.3. In *B. cepacia* LB400 (Fig. 1.3A), *bphAEFGBC* encode: the α-subunit (*bphA*) and β-subunit (*bphE*) of the oxygenase; ferredoxin (*bphF*)

3
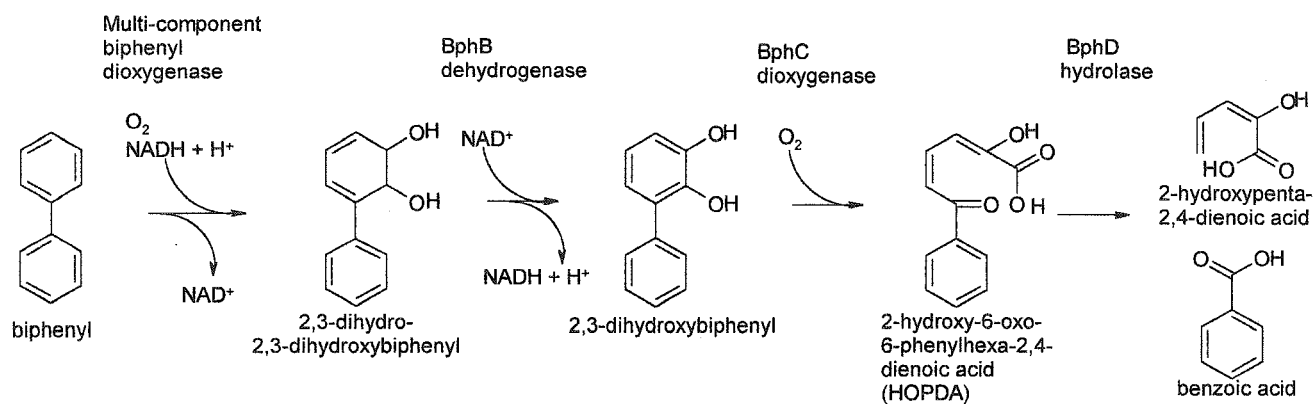
Figure 1.2 "Upper" biphenyl/polychlorinated biphenyl aerobic degradation

pathway.

and ferredoxin reductase (*bphG*), which supply electrons to BPDO from NADH; and the dihydrodiol dehydrogenase (*bphB*) and extradiol dioxygenase (*bphC*) enzymes (Erickson and Mondello, 1992). In *Comamonas testosteroni* B-356 (Fig. 1.3B), the genes are similarly organized although the reductase gene, *bphG*, is located outside the operon (Sylvestre *et al.*, 1996-B).

The degradation of biphenyl/PCBs is dependent on the ability of biphenyl dioxygenase (Fig. 1.4), the first enzyme of the pathway, to catalyze the dihydroxylation of one of the benzene rings. However, the chlorine substitution pattern of many PCB congeners interferes with this reaction, as biphenyl dioxygenases from various strains are able to oxidize only a selection, often narrow, of the congeners available (Ahmad 1990, Gibson *et al.*, 1993). The use of existing strains for bioremediation purposes is limited, in part, by this inability of their biphenyl dioxygenases to dihydroxylate many PCB congeners. Work in our laboratory focuses on ways to modify biphenyl dioxygenase to accept a wider range of PCB substrates

Properties of Biphenyl Dioxygenases

Biphenyl dioxygenases comprise 3 soluble components that interact to form an electron-transport chain, which transfers electrons from reduced pyridine nucleotide, NADH, via flavin and Rieske (2Fe-2S) redox centers to a terminal dioxygenase that interacts with the substrate and oxygen to catalyze the reaction (Fig. 1.4) (Haddock *et al.*, 1993, Hurtubise *et al.*, 1995). The FAD-containing oxidoreductase (BphG) initially oxidizes the reduced cofactor, NADH, and transfers the electrons one at a time to the

A

| bphA 1374 | 36 | bphE 561 | 41 | ORFX 429 | 82 | bphF 330 | 13 | bphB 846 | bphC 882 | bphG |

B

| bphA 1380 | 11 | bphE 566 | 37 | ORF1 420 | 74 | bphF 330 | bphG 1227 | 46 | bphB 834 | 21 | bphC 897 |

Figure 1.3 Organization of *Comamonas testosteroni* B-356 (A) and *Burkholderia cepacia* LB400 (B) *bphAEFGBC* genes. The sizes of each region represented are given in base pairs.

Rieske-type [2Fe-2S]-center containing ferredoxin, BphF (Colbert *et al.*, 2000, Couture *et al.*, 2001). BphF then transfers the electrons to a second Rieske-type [2Fe-2S] cluster located in the terminal oxygenase component of BPDO. The terminal oxygenase component, BphAE, uses these electrons to activate O_2 (via the mononuclear iron center) for insertion into the substrate. The BphA and BphE polypeptides assemble to form an active $\alpha_3\beta_3$ hexamer. The α-subunit, encoded by *bphA*, is 458 amino acids (51 kDa), and contains the Rieske iron-sulfur cluster and mononuclear iron-binding site. The β-subunit, encoded by *bphE*, is 187 amino acids (21 kDa)

Recently, highly purified BPDO from *C. testosteroni* was used to obtain a crystal structure of the $\alpha_3\beta_3$ hexamer (Imbeault *et al.*, 2000, Colbert C.L., Ph.D. thesis, Purdue University, IN, USA). The overall structure looks like a mushroom with the cap formed by the α_3-subunits, and the stem formed by the β_3-subunits (Fig. 1.5, left) a structure similar to that previously reported for naphthalene dioxygenase (Kauppi *et al.*, 1998). The interface between the α and β-subunits is in close proximity to the active-site mononuclear iron, shown as a blue or green ball (Fig 1.5, left). The top view of the $\alpha_3\beta_3$ complex (Fig. 1.5, right) shows the interactions between the α-subunits. Each α-subunit is composed of two domains: an iron-sulfur (2Fe-2S) domain and a mononuclear-iron site (Fe) domain. The iron-sulfur domain reaches out of the side of the complex such that its Fe-S cluster forms an active site with the Fe site of the adjacent α-subunit (Fig. 1.5, right).

Biphenyl
Dioxygenase

O_2

BphAE
Oxygenase$_{red}$
Rieske FeS, Fe
$\alpha_3\beta_3$
Oxygenase$_{ox}$

BphF
Ferredoxin$_{ox}$
Rieske FeS
Ferredoxin$_{red}$

BphG
Reductase$_{red}$
FAD
Reductase$_{ox}$

NAD^+

$NADH + H^+$

H
'''OH
'' OH
H
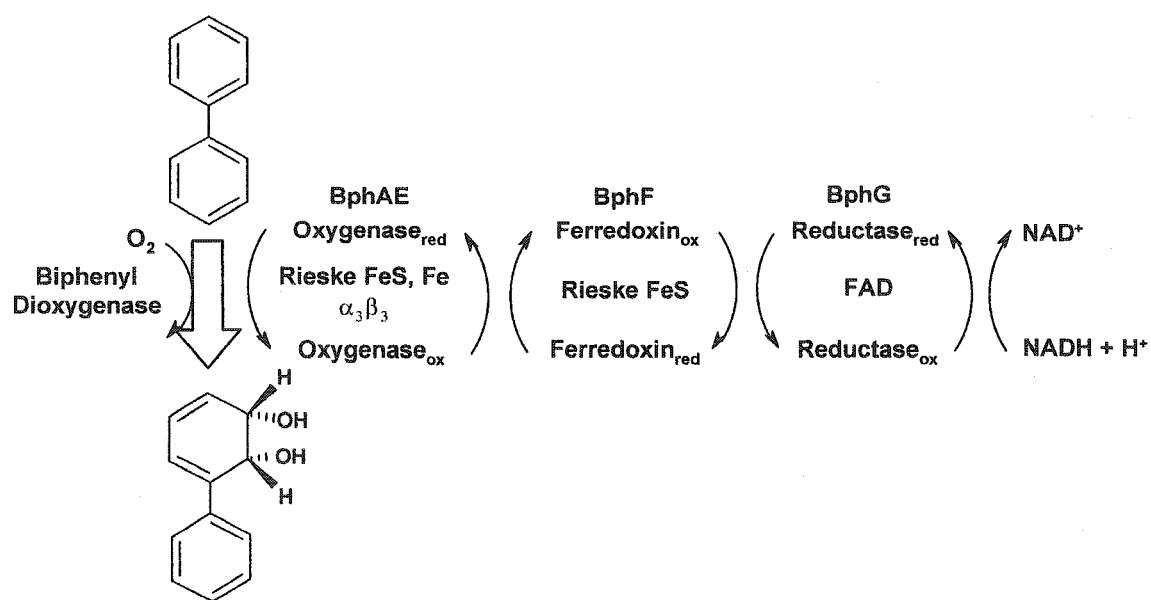
Figure 1.4 Transformation of biphenyl to *cis*-dihydrodiol by a biphenyl dioxygenase. Figure courtesy of N. Agar.
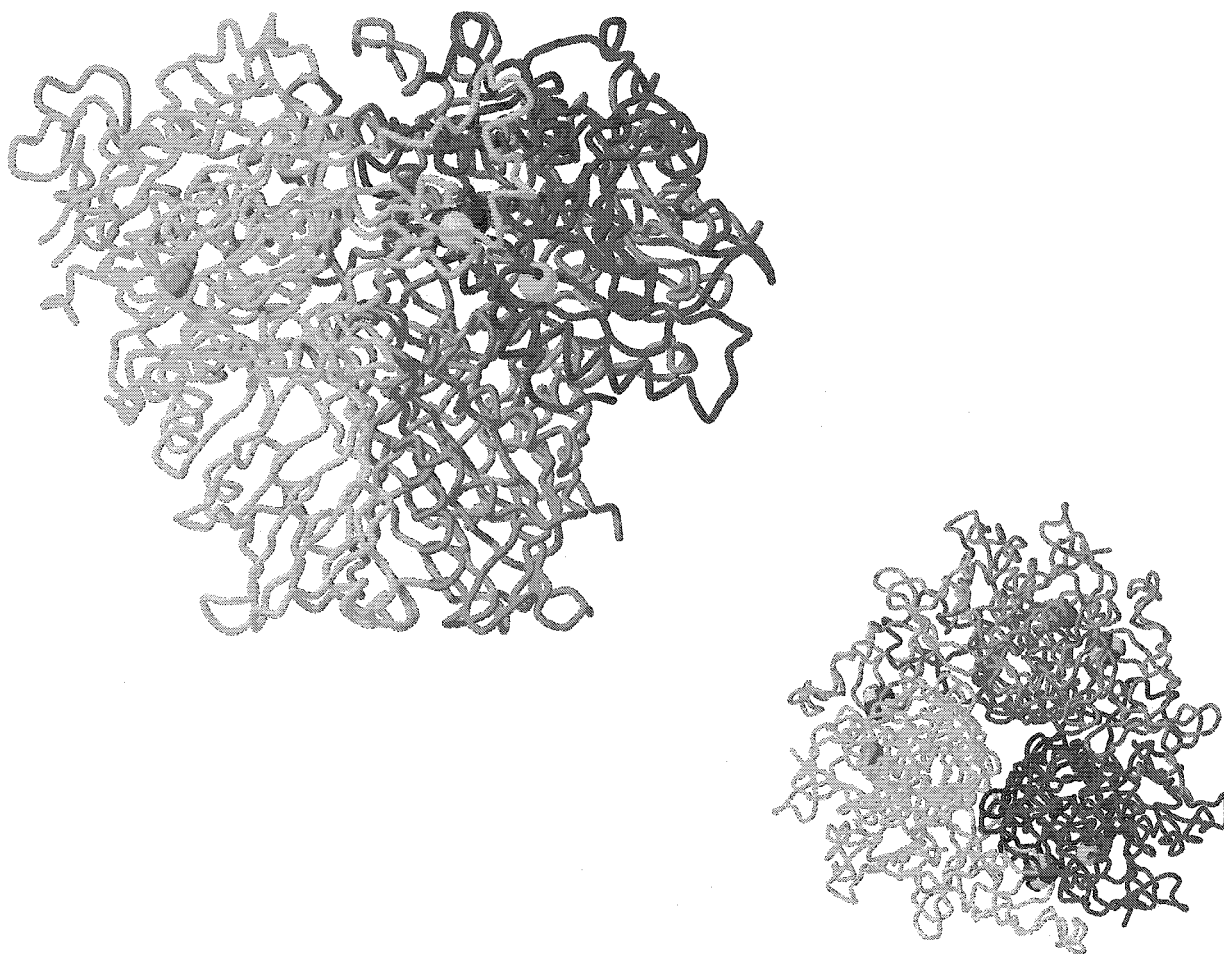
Figure 1.5 Two representations of BPDO from *C. testosteroni* **B-356.** Each α-β complex is either green, purple, or blue, with the corresponding β-subunits of a lighter shade. The iron-sulfur cluster, 2Fe-2S, is shown in red (Fe) and yellow (S). The mononuclear iron (a green, purple, or blue ball depending on the α-subunit it is located in can be seen interacting with the Fe-S cluster located on the adjacent α-subunit. Figure courtesy of Professor J. Bolin, Purdue University.

Substrate Specificities of Different Biphenyl Dioxygenases

The substrate specificities of a number of different biphenyl dioxygenases have been examined in whole cells, in crude extracts, or using purified enzymes. In some cases, it has been possible to deduce the molecular determinants of substrate specificity. The following discussion will focus on the three best-characterized enzymes: those from *Burkholderia cepacia* LB400, *Pseudomonas pseudoalcaligenes* KF707, and *Comamonas testosteroni* B-356.

BPDO from *Burkholderia cepacia* LB400 ($BPDO_{LB400}$) has been purified and studied in some detail (Gibson *et al.* 1993, Haddock *et al.* 1993, Haddock and Gibson 1996, Seeger *et al.* 1995) because it can dihydroxylate a relatively wide range of PCB congeners, including some that are hexachlorinated (Gibson *et al.*, 1993). The broad substrate specificity of $BPDO_{LB400}$ stems from its unique ability to dihydroxylate carbons 3 and 4 (*meta-para*) of one ring of some chlorinated biphenyls (Bedard *et al.*, 1986). This gives it an advantage in transforming *ortho*-substituted PCB substrates such as 2,2'-diClB, and in allowing transformation of congeners that lack unsubstituted *ortho*-carbons, such as 2,2',5,5'-tetraClB (Gibson *et al.*, 1993).

Interestingly, $BPDO_{LB400}$ shares 98% amino acid sequence identity with BPDO from *Pseudomonas pseudoalcaligenes* KF707 ($BPDO_{KF707}$) (Taira *et al.*, 1992), although the range of PCB congeners metabolized by each enzyme is much different. $BPDO_{KF707}$ has a narrower substrate specificity as it can only hydroxylate carbons 2 and 3 (*ortho-meta*) of one ring, and is unable to transform 2,2',5,5'-tetraClB (Gibson *et al.*, 1993).

However, BPDO$_{KF707}$ has comparatively greater activity towards di-*para*-substituted PCBs such as 4,4'-diClB.

Mondello's group examined the primary structures of BphAs of closely-related BPDOs, and determined the substrate selectivity patterns for each enzyme (Mondello *et al.* 1997). Based on the ability to degrade 17 PCB congeners, BPDO's from different bacterial strains were divided into two groups, LB400-like or KF707-like, according to residues consistently associated with either broad (LB400) or narrow (KF707) substrate specificity. These residues of BphA were found to be grouped in regions denoted I to IV, and the effects of mutations in these regions on substrate selectivity were tested. Four amino acid residues in region III were mutated in *bphA*$_{LB400}$ to correspond to those found in *bphA*$_{KF707}$, which led to enhanced activity toward di-para-substituted congeners (as in BPDO$_{KF707}$) while conserving the broad substrate range of BPDO$_{LB400}$. Similar results were reported by Furukawa's group, which made hybrid BPDOs by exchanging 4 restriction fragments, or by DNA shuffling of *bphA*$_{LB400}$ and *bphA*$_{KF707}$ (Kimura *et al.*, 1997; Kumamaru *et al.* 1998). These experiments show how recombination between BPDOs (BphA) could be used to alter, and in some cases broaden, the PCB congener specificity. However, since all but one of the 21 amino acid differences between BPDO$_{LB400}$ and BPDO$_{KF707}$ are clustered within a 140-residue region in the carboxy-terminal half of the α-subunit, the importance of the β-subunit in determining substrate specificity was not revealed by these experiments.

Our laboratory and our collaborators study biphenyl dioxygenase from *Comamonas testosteroni* B-356 (BPDO$_{B-356}$), which shares 76% (α-subunit) and 70% (β-subunit) amino acid sequence identity with subunits from BPDO$_{LB400}$ (Sylvestre *et al.*,

1996-B). BPDO$_{B-356}$ has been purified to homogeneity (Hurtubise *et al.,* 1995; Imbeault *et al.,* 2000), and found to preferentially transform the di-*meta*-substituted congener, 3,3'-diClB, over 2,2'-diClB or 4,4'-diClB. It is unable to metabolize 2,2',5,5'-tetraClB (Hurtubise *et al.,* 1998). Therefore, BPDO$_{B-356}$ can be classified as having a 'narrow' substrate selectivity pattern, as described above for BPDO$_{KF707}$ although it differs from BPDO$_{KF707}$ in showing a preference for *meta*-substituted biphenyls over *para*-substituted biphenyls. The amino acid residues of regions I to IV, described above, that were found to influence substrate specificity in BPDO$_{KF707}$ versus BPDO$_{LB400}$ are identical, in BPDO$_{B-356}$, to those found in BPDO$_{KF707}$. However, substitution of Thr-375 of BphA$_{B-356}$ by Asn-375 (region IV), as in BphA$_{LB400}$, did not result in expansion of the range of biodegradable PCB congeners, (those requiring *meta-para* dihydroxylation) as observed when this substitution was made in BPDO$_{KF707}$ (Hurtubise *et al.,* 1998).

The work summarized so far was focused on determinants of BPDO substrate selectivity found in the active site-containing α-subunit. To investigate the role of the β-subunit in substrate selectivity, chimeric enzymes have been constructed by exchanging the α-subunit or β-subunit of BPDO$_{B-356}$ with the corresponding subunit of BPDO$_{LB400}$ (Hurtubise *et al.,* 1998). Amino acid sequences of the β-subunits of these two enzymes are 70 % identical. Interestingly, the α$_{B-356}$β$_{LB400}$ chimera was able to metabolize 2,2',5,5'-tetraClB, a characteristic of BPDO$_{LB400}$. This possibility would not be expected if the α-subunit were the sole determinant in substrate specificity, so the β-subunit must influence the ability to dihydroxylate *meta-para* carbons. In the same study, an *ortho*-substituted congener, 2,2'-diClB, was also transformed more efficiently by BPDO composed of α$_{B-356}$β$_{LB400}$ than BPDO$_{B-356}$. Conversely, the BPDO chimera with

$\alpha_{LB400}\beta_{B-356}$ was more active toward 3,3-diClB than parental $BPDO_{LB400}$, also a situation that would not be expected if the α-subunit were the sole determinant of substrate specificity. Thus, in both chimeras the β-subunit influenced the substrate selectivity pattern. The accumulated data, therefore, indicate that both subunits are important in determining the substrate selectivity pattern, and influence the capacity of BPDO to hydroxylate the *meta-para* carbons (Hurtubise *et al.,* 1998). This appears to be consistent with the recently-determined BPDO structure, which shows the involvement of residues from the β-subunit in forming the active site (Fig. 1.5).

Protein Engineering by Rational Design or Random Mutagenesis

As mentioned above, bioremediation of PCB-contaminated sites is one potential application of bacterial biphenyl degradation pathways. In contrast to chemical catalysts, enzymes are biodegradable, less toxic, and potentially cheaper to produce (Ness *et al.,* 2001). Since most enzymes have evolved to perform a specific biological function, modifications are often necessary to make them useful in practical applications. Alteration in protein function may be achieved by introducing mutations in the gene(s) encoding the protein of interest.

A typical approach to protein design is to make specific changes to a single enzyme based on what is known about its structure and function. However, structural data are often limited compared to the wealth of genomic data available, and even when structural data are available the challenge to rational design is huge. The range of effects caused by single mutations expected to change only one specific and simple property of an enzyme is often surprising. It can be very difficult to predict the long-range effects of

13

a single amino acid substitution on protein conformation, and the resulting changes in activity (Pluckthun *et al*, 2001).

It was Charles Darwin's idea that random variation in the population followed by selection of the fittest, by the forces of the environment, had given rise to the diversity of life. This view of evolution is evident all the way to the molecular level. For example, a major generator of diversity is thought to be the block-wise exchange of homologous regions of DNA in chromosomes (Ness *et al.*, 2001). Evolution creates new cellular functions by redesigning existing protein frameworks, rather than by starting over from scratch. This is revealed by sequence and structural comparisons that indicate the occurrence of gene duplication and recombination events (Ostermeier and Benkovic, 2001).

Random point mutagenesis has been used successfully to optimize specific enzyme properties. To this end, genes are mutated at random using error-prone PCR (Pluckthun *et al.*, 2001) or other mutagenesis methods, and screened *in vivo* for improved activity. The process can then be repeated with the best performer. Engineering of subtilisin, an enzyme used in laundry detergents, is one recent example of this approach (Wintrode and Arnold, 2001). However, this technique has inherent limitations because it can only introduce a limited number of changes per sequence since amino acid substitutions are limited to one or two per sequence to prevent the introduction of deleterious mutations. Also, only amino acid changes accessible through substitution of one nucleotide are allowed, limiting accessibility to some amino acid substitutions (Wintrode and Arnold, 2001). To expand the ways we can mimic evolutionary processes in the laboratory, methods of artificial evolution have been devised.

Directed Evolution of Enzymes and Pathways

DNA shuffling was introduced as an *in vitro* method to mimic homologous recombination, as observed in Nature, and produce libraries containing diverse sets of genes (Fig. 1.6) (Stemmer, 1994-A). The method, also termed 'sexual PCR', followed by *in vivo* selection, or screening, for the desired characteristics has been used successfully for the rapid directed evolution of proteins. DNA shuffling can be mutagenic, as well as recombinogenic, by adjusting the PCR conditions and the choice of DNA polymerase (Zhao and Arnold, 1997-A). Mutagenic conditions allow the incorporation of mutations into a single starting DNA sequence to create the initial diversity, and recombination shuffles those mutations to create genes with different combinations of those mutations. Thus, in DNA shuffling (Stemmer, 1994-A) a gene or pool of related genes is fragmented into random-sized pieces, and reassembled into full-length genes using self-primed PCR (Fig. 1.6). The reassembly reaction is recombinogenic because fragments from one DNA sequence can hybridize and prime DNA polymerization from homologous regions of different DNA sequences. Template switching between different cycles of hybridization and elongation, during the reconstruction PCR, results in crossovers in the regions of homology.

Improved function elicited by DNA shuffling was first demonstrated in experiments using the TEM-1 β-lactamase (Stemmer, 1994-B). Each cycle of DNA shuffling, using mutagenic PCR-reassembly as described in the previous section, was followed by *in vivo* selection in *E. coli* with increasing concentrations of the antibiotic, cefotaxime. The *E. coli* clones that showed the highest level of resistance were then used

in subsequent cycles of shuffling and selection, to yield, after three rounds, a clone exhibiting 16 000-fold greater resistance to cefotaxime (from 0.02 μg/ml to 320 μg/ml). In a control experiment, error-prone PCR was used without recombination, yielding a clone with only 20-fold greater resistance. To eliminate non-essential mutations, the best variant was shuffled with a molar excess of the parental gene. Two cycles of backcrossing removed four silent mutations and produced two new ones, and resulted in a mutant that was 32 000-fold more resistant than wild-type (Stemmer, 1994-B). These results are striking and demonstrate how DNA shuffling *in vitro,* coupled with selection *in vivo,* can effectively combine positive mutations and simultaneously remove negative mutations from the pool. Thus, selection was used to direct the evolution of an improved enzyme.

In part to determine if screening, rather than selection, is a viable approach to obtain functionally evolved enzyme, in terms of the numbers of clones that need to be screened, DNA shuffling was used to obtain improved green fluorescent protein (GFP), (Crameri *et al*, 1996). The GFP gene was subjected to three rounds of shuffling, each followed by visual screening of 10 000 colonies to pick the best 40 for the next cycle. After 3 cycles a variant was obtained with a 45-fold increase in fluorescence intensity over wild-type protein. In this case, mutations leading to the substitution of three hydrophobic amino acids with hydrophilic residues prevented formation of inclusion bodies that were observed with the wild-type protein (Crameri *et al.,* 1996).

DNA templates, a gene randomly mutated or
homologous genes.

Random Fragmentation

Pool of random DNA fragments

Gene reassembly using multiple
cycles of annealing and
DNA polymerization (primer-less
PCR)

Shuffled set of genes

Select or screen for desired properties, *in vivo.*
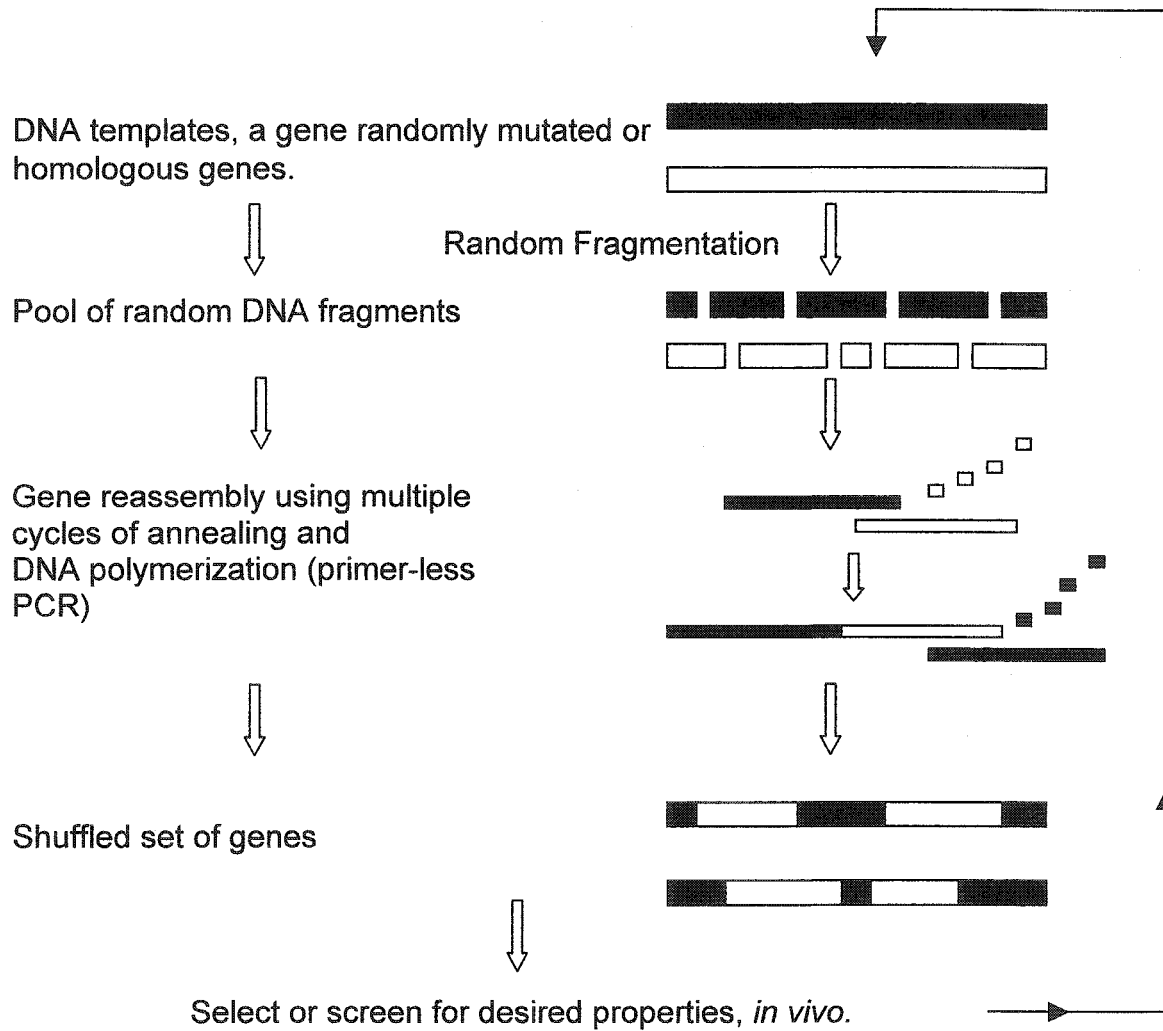
Figure 1.6 Overview of DNA shuffling

DNA shuffling also was used to obtain rapid functional improvement of the arsenic detoxification pathway from *Staphylococcus aureus*, illustrating the technique's usefulness in manipulating multi-component systems. When cloned in *E. coli*, the 2.7-kb operon confers resistance to 4-10 mM concentrations of the toxic anion, arsenate. Following three cycles of shuffling followed by selection on increasing concentrations of arsenate (Crameri *et al.* 1997), one colony that was resistant to 500 mM arsenate (a 40-fold increase) was isolated and characterized further. Sequencing of the *ars* operon from this evolved strain revealed 13 point mutations. Seven mutations were found in the *arsB* gene, coding for the arsenite membrane pump, but only three of these resulted in amino acid substitutions. Two silent mutations were found in *arsR*, encoding a transcriptional repressor, and one was found downstream from *arsC*, the arsenite reductase gene. In this example the molecular mechanism leading to the observed increase in resistance to arsenate is unclear, and would have been impossible to design rationally. These examples show how DNA shuffling followed by selection or screening can provide solutions to a complex biochemical problem independently of a detailed understanding of the mechanism, and often through more than one route.

DNA shuffling also makes it possible to 'breed' any number of evolutionary related genes independent of species. Naturally-occurring homologous genes provide sequence space that is rich in functional diversity, as recombination and random mutation of these genes become fixed in populations over million of years, withstanding the tests of time and functionality. The first example of DNA shuffling using natural diversity as the starting material was done with four cephalosporinase genes that shared from 58% to 82% amino acid identity (Crameri *et al.*, 1998). One cycle of shuffling of four

cephalosporinase genes together, followed by selection of 50 000 clones, produced a variant that was different from its closest parent at more than 25 % of its amino acids residues. This variant conferred a 270- to 540-fold increase in resistance to moxalactam compared to the wild-type enzymes. Shuffling of the four genes individually yielded clones that only were resistant up to an 8-fold higher moxalactam concentration. Thus, 'family' shuffling with molecular recombination to produce multiple enzyme properties is similar to the techniques of classical breeding of plants and animals, and is referred to as "molecular breeding" (Ness *et al.*, 2001)

Thesis Goal: DNA shuffling of Biphenyl dioxygenase

Some progress is being made in understanding how biphenyl dioxygenase works using a combination of structural and functional data, (Imbeault *et al.*, 2000; Agar, Ph.D Thesis, Concordia University 2002). However, engineering enzymes that can use a broader range of PCBs cannot yet be done rapidly using a rational approach. Because DNA shuffling and screening for improved variants has proven an effective method, we wanted to apply it to engineer BPDO with altered substrate specificity.

Recently (Barriault *et al.*, 2002) used a new strategy to reduce the size of the library produced and explore the different regions of the α-subunit, BphA. By DNA shuffling only portions of the *bphA* genes, additional combinations of amino acid residues (to those already shown to be involved in substrate specificity and selectivity, as discussed above), but also located in the carboxyl terminal of the α-subunit, were selected. Some enzymes with improved performance toward several congeners were

obtained. The variant BPDOs obtained by family shuffling justify using this strategy to explore the large sequence-structural space otherwise too complex for rational design.

Since it has been shown that the substrate selectivity pattern was influenced by the β-subunit in the $BPDO_{LB400}$ and $BPDO_{B-356}$ association, we specifically chose to carry out DNA shuffling of the genes encoding the β-subunit, *bphE*, to complement the *bphA* shuffling experiments of Sylvestre's group. We also attempted to shuffle both genes encoding the αβ-subunits, *bphAE*, of BPDO from *Comamonas testosteroni* B-356 and *Burkholderia cepacia* LB400 simply because by using more sequence space a more diverse set of recombinant molecules can be generated.

MATERIALS AND METHODS

Materials

Oligonucleotide primers were designed with the help of PCGENE or *Primerfinder* v0.07 software and were synthesized by BioCorp Inc. (Montreal, Que.) Native *Pfu* DNA polymerase, and ultracompetent *E. coli* BL21 Gold cells were purchased from Stratagene. PCR dNTP mix, DNase I, and kanamycin were purchased from Roche Applied Science. Gibb's reagent (2,6-dichloro-*p*-benzoquinone), and carbenecillin were purchased from Sigma Chemical Co.. GFX Band Prep kit was purchased from Pharmacia. Wizard Mini-prep DNA purification kits, restriction enzymes, and T4 DNA ligase were all obtained from Promega. Microcon YM-10 and Micropure–EZ Enzyme remover devices were purchased from Millipore.

Broad host range expression vector pJB658 (Blatney *et al.,* 1997), transposon mutagenesis related plasmids (pUC19Not, pCNB1), and *E. coli* strains CC118(λ pir) and S-17(λ pir) (de Lorenzo *et al.,* 1993) were provided by Dr. Lindsay Eltis, University of British Columbia.

Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are described in Table 1. *E. coli* strains (DH5α, BL21Gold, or XL2) harboring pJB658 and its derivatives were grown in LB broth or LB agar (*Sambrook et al.,* 1989) containing carbenicillin (200 μg/ml), unless following a transformation in which case a lower concentration of carbenicillin (100 μg/ml) was used. *E. coli* BL21 (*-bphFGBC and –bphFGB)* were grown in the presence of: tetracycline (20 μg/ml) to distinguish *E. coli* BL21 from the

donor strain (*E. coli* S-17(λ pir)) used during transposon mutagenesis; or carbenicillin (200 μg/ml) and kanamycin (40 μg/ml) to select for the presence of the chromosomal insert (*bphFGBC* or *bphFGB*). *E. coli* S-17(λ pir) and *E. coli* CC118(λ pir) harboring pCNB1 and its derivatives were grown in the presence of kanamycin (40 μg/ml) and carbenicillin (200 μg/ml). All *E. coli* strains were grown at 37 °C.

P. putida KT2442 harboring pJB658 and its derivatives were grown in the presence of carbenicillin (800 μg/ml), unless following a transformation, in which case a lower concentration of carbenicillin (400 μg/ml) was used. Rifampicin (40 μg/ml) was used to distinguish *P. putida* from the donor strain (*E. coli* S-17(λ pir)) used during transposon mutagenesis, and kanamycin (40 μg/ml) was used to select *P. putida* cells with the chromosomal *bphFGBC* insert. All *P. putida* strains were grown at 30 °C.

General molecular biology methods

Plasmid Manipulations

Plasmid DNA was purified using the Wizard Miniprep kit. DNA was digested using standard protocols (Sambrook *et al.*, 1989). Restriction enzyme digests with multiple enzymes were carried out consecutively with their preferred buffers, as recommended by the manufacturer. Phenol-chloroform extraction followed by ethanol precipitation from 0.3 M sodium acetate was used to clean up the DNA between steps. DNA fragments separated on agarose gels were purified from gel slices using the GFX Band Prep kit. Unless otherwise noted, the same kit was used to recover DNA fragments from 2% agarose gel slices for use in primerless PCR. An alternative method used Micropure-EZ Enzyme Removers and Microcon YM-10 concentrator, as described by

Table 1 Strains and plasmids used in this study

Species and strains	Relevant genotype/properties	Reference
E. coli CC118(λ pir)	λpir lysogen; recipient of pCNB1	deLorenzo *et al.* (1993)
E. coli S-17(λ pir)	λpir, *recA, thi, pro, hsdR*-M+, RP4:2-Tc:Mu:km Tn7Tp^{R}Sm^R; mobilizing strain for pCNB derivatives	deLorenzo *et al.* (1993)
P. putida KT2442	HsdR, Rif^R prototrophic	Herrero and deLorenzo (1990)
P. putida (*bphFGBC*)	KT2442 with chromosomal mini-Tn5 *xylS*/Pm::*bphFGBC* insertion	This work
E. coli DH5α		Hanahan (1983)
E. coli XL2-Blue		Stratagene
E. coli BL21-Gold	Tc^R	Stratagene
E. coli (*bphFGBC*)	BL21Gold with chromosomal mini-Tn5 *xylS*/Pm::*bphFGBC* insertion	This work
E. coli (*bphFGB*)	BL21G with chromosomal mini-Tn5 *xylS*/Pm::*bphFGB* insertion	This work

Plasmids	Characteristic(s)	References
pYH*bphFGBC*	*bphFGBC* insert (cut out with *BamHI* and *HindIII*)	Dr. Sylvestre Lab.
pUC19 Not	Ap^R Identical to pUC19 but with *NotI* sites flanking the MCS.	deLorenzo *et al.* (1993)
pUC-*bphFGB(C)*	pUC19 NotI containing *bphFGBC* or *bphFGB*	This work
pCNB1*	Ap^R, Km^R, ori R6K, mobRP4, pUT/mini-Tn5 *xylS*/Pm	deLorenzo *et al.* (1993)
pCNB-*bphFGB(C)*	pCNB1 derivative with *bphFGBC* or *bphFGB* (LB400) inserted in the *NotI* site	This work
pJB658	Ap^R, RK2 expression vector containing the Pm promoter and the gene encoding the regulatory protein, XylS, 6.7-kb	Blatny *et al.* (1997)
pJB*bphAE*$_{LB-400}$	pJB658 with *bphAE* $_{LB-400}$ inserted in *NdeI-BamHI* site, downstream from Pm,	This work
pJB*bphAE*$_{B-356}$	pJB658 with *bphAE* $_{B-356}$ genes inserted *NdeI-BamHI*, downstream from *Pm*	This work

Table 1 continued

pJB*bphAE* LB-400xhoI	pJB*bphAE*LB-400 with point mutation (T63C *bphE*) to introduce an *XhoI* restriction site.	This work
pJB*bphE*B-356	pJB658 with *bphE*B-356 inserted *KpnI-BamHI*	This work
pJB*bphE*LB400xhoI	pJB658 with *bphE*LB400 (XhoI) inserted *KpnI-BamHI*	This work

Ap^R, ampicillin resistance; Km^R kanamycin resistance; Tc^R, tetracycline resistance.

pCNB1 utilizes a suicide delivery system. The *tnp* product promotes the transposition of any DNA segment flanked by the 19-bp termini of Tn*5*.

the manufacturer. Buffer exchange was accomplished by repeated concentration and dilution with the new buffer. The GFX Band Prep kit was also used to purify PCR product from solution. Ligations were done using T4 DNA ligase (Promega) and the supplied buffers and protocols. Ligation mixtures were extracted with phenol-chloroform, and DNA was precipitated by ethanol in the presence of 0.3 M sodium acetate (Sambrook *et al.*, 1989), and re-dissolved in distilled water prior to electroporation into bacterial cells.

Plasmid DNA was introduced into *P. putida bphFGBC* by electroporation (Cho *et al.*, 1995). Cells grown to OD_{600} of 0.8-1.0 in 50 ml of LB, following inoculation with 0.5 ml of an overnight culture, were chilled on ice for 15 minutes, harvested by centrifugation at 2500 x g, and washed twice in ice-cold electroporation buffer (1 mM HEPES, pH 7.0). Cells were finally resuspended in 2 ml electroporation buffer. In a previously-chilled Gene Pulser cuvette (0.1 cm electrode gap), cold cells (60 µl) and DNA (1 µl) from a ligation mixture were electroporated at 1.25 kV, 400-600 Ohm, 25 µF, using a Gene-Pulser apparatus (BioRad) and immediately re-suspended in SOC medium (400-1000 µl) (Sambrook *et al.*, 1989). Following shaking at 190 RPM, 30°C, for 30 minutes, the cells were plated on selective medium and then incubated overnight at 30 °C.

Introduction of plasmid DNA into *E. coli bphFGBC* or *E. coli bphFGB* was also done by electroporation as described (Ausubel *et al.*, 2001). Cells grown in LB were harvested by centrifugation (as above) when they reached an OD_{600} of 0.5. The resulting pellet was resuspended with a volume of filter-sterilized water equal to that of the cell

pellet. Ice-cold cell suspension (79 µl) and 1 µl DNA (10-20 ng) were mixed in a pre-chilled tube prior to electroporation using the procedure described above.

Permanent cell stocks were prepared by diluting an overnight cell culture 1:1 with a solution of 25 mM Tris-HCl (pH 8.0) containing 0.1 M MgSO$_4$ and 65 % glycerol (Sambrook *et al.*, 1989).

Polymerase chain reaction (PCR) and site-directed mutagenesis

The PCR conditions for amplification of DNA using primers contained template DNA (50-100 ng), primers (100 pmol of each), dNTPs (200 µM of each), native *Pfu* DNA polymerase (2.5 U), and 1X Native Plus *Pfu* buffer, in 100 µl. These reaction mixtures were heated to 95 °C for 3-5 min and allowed to proceed for 30 cycles of: 45 sec at 95 °C, 60 sec at 57 °C (for primers *atm, bpm, tm, Pm*) or 60 °C (*ndeI, bamHI, dstrm bphA, upstrm bphE*), and 3.5 min (*bphAE* template) or 1 min (*bphE* template) at 72 °C, followed by 1 final cycle of 10 min at 72 °C. Primers are described in the appropriate sections, below. Following amplification by PCR, product size was checked by agarose gel electrophoresis of an aliquot and the DNA was cleaned using the GFX Band Prep kit.

Reconstruction (primer-less) PCR mixtures contained purified DNA fragments from each parent (up to 2 µg/reaction), dNTPs (0.4-1 mM of each), native *Pfu* DNA polymerase (1.75 U), and 1X Native Plus *Pfu* buffer in a 50 µl reaction volume. These reactions were allowed to proceed for various numbers of cycles (40 to 60 or more) of: 45 sec at 95 °C (denaturation), 60 sec at 48-55 °C (for hybridization), and for 30-60 sec + 5 sec per cycle at 72 °C for DNA polymerization. The number of cycles, the hybridization

temperature, and elongation time were all varied to optimize reconstruction of recombinant *bphAE* or *bphE* genes, as described in *Results*.

Site-directed mutagenesis was done according to the QuickChange protocol (Stratagene). Briefly, DNA template (10 ng of pJB*bphAE*$_{LB400}$), primers (125 ng of each), native *Pfu* DNA polymerase (2.5 units), 1X *Pfu* Plus buffer, and dNTPs (0.2 mM of each), in a total volume of 50 μl were cycled, following 3 min at 95 °C, 18 times: 50 sec at 95 °C, 50 sec at 60 °C, and 20 min at 68 °C. Following this, *Dpn*I digestion was used to remove parental plasmid DNA, and the product was transformed into competent *E. coli* XL2 Blue cells, which were then plated on LB agar containing carbenecillin (100 μg/ml).

DNA Sequencing

Plasmid DNA (pJB*bphAE* $_{shuffled}$) was recovered from *P. putida bphFGBC* using the Wizard Mini Prep kit and following the modified protocol for *EndA*$^{+}$ strains described by the manufacturer (Promega). To further reduce DNA degradation by endonucleases, the DNA was eluted from the column with 0.3 M sodium acetate into a tube already containing a solution of phenol and chloroform. Following extraction with phenol-chloroform, the DNA was precipitated with ethanol (95%), washed twice with 70% ethanol, dried, and redissolved in water (Sambrook *et al.*, 1989).

Oligonucleotide primers *pm* and *tm*, (described below in the section *Shuffling Methodology*), were used for sequencing of shuffled product, as described in the *Results* section. Three additional oligonucleotides were used for sequencing shuffled *bphAE*:

Primer *Mlu:* 5'-CGACAAGGCCGACTGGGGGCCGTTGC-3' was designed by Nathalie

Agar and corresponds to bases 461-486 of *bphA*$_{B-356}$ (or 458-484 of *bphA*$_{LB400}$). Only 4-

bp differ in this region of *bphA* of strain LB400 as compared to strain B-356, so this

primer was expected to work for both genes. Primers *Bphae3:* 5'-

ATGTTCGGCCAGCACATGA-3' and *Bphae4:* 5'-GACACGCTCAAACCCTGA-3'

anneal to the sequence corresponding to bases 958 to 977 and 1356 to 1374 of *bphA*$_{B-356}$,

respectively, or 953 to 972 and 1361 to 1379 of *bphA*$_{LB400}$.

DNA Shuffling Methodology

Plasmid construction for shuffling/expression of bphAE

To obtain BPDO genes from *C. testosteroni* B-356 and *B. cepacia* LB400 with

the desired flanking restriction sites for cloning into pJB658, oligonucleotide primers

were designed for PCR amplification of these genes. Oligonucleotide pairs,

ndeI $_{LB400}$: 5'-CGGAGACGTTAA<u>CATATG</u>AGTTCAGC-3',

bamHI $_{LB400}$: 5'-GCG<u>GGATCCC</u>TAGAAGAACATGCTCAGGT-3'

and

ndeI $_{B-356}$: 5'-GGAGCCAGT GAA <u>CATATG</u>AGTTCGAC-3',

bamHI $_{B-356}$: 5'-ACG<u>GGATCC</u>TCAAAAGAACACGCTCAGGTT-3'

were designed based on known sequences: *bphAEFGB* from *B. cepacia* LB400,

accession number M86348 (Erickson and Mondello 1992), and accession number

U47637 for *bph* genes from *C. testosteroni* B-356 (Sylvestre *et al.,* 1996-B). These

primers were used to amplify the coding region of *bphAE* from each strain, incorporating

an *Nde*I recognition site (underlined in *ndeI* oligos) corresponding to the start codon

(bold) of *bphA,* and a *Bam*HI restriction site (underlined) just downstream from the stop

codon (bold) of *bphE.* The PCR conditions used in each case are described above, or in

Results.

The resulting PCR products were digested with *Nde*I and *Bam*HI and cloned into

the corresponding sites of pJB658. The resulting plasmids were maintained in *E. coli*

DH5α and transformed into the appropriate expression/screening host as required.

DNA shuffling techniques

DNA shuffling was done using both genes, *bphAE*, from each strain, or only with

bphE from each strain. Several different shuffling strategies were used, as outlined in the

sections below.

Shuffling bphAE *using restriction enzymes to create fragments.*

The method employed here uses restriction enzymes to generate fragments,

essentially as described by Kikuchi *et al.,* 1999. Template DNA was PCR amplified (as

described in the *PCR* section) using pJB*bphAE*$_{B-356}$ or pJB*bphAE*$_{LB400}$, and their

respective oligonucleotide primers, *ndeI and bamHI* (see above). PCR product from each

parent was cleaned and digested to completion with two or more of the restriction

enzymes, *Fok*I, *Nci*I, or *Bsp*1286I (see Table 2 for restriction sites). Following agarose

gel electrophoresis, restriction fragments were recovered by cutting out the bands from

the gel and purifying the DNA using the GFX Band Prep kit.

Gene reassembly using purified restriction fragments from *bphAE* was done in

two stages. A number of PCR reassembly reactions were created, by combining 2 or 3

Template:	*bphAE*~LB400~ (2063 base pairs)	*bphAE*~B-356~ (1972 base pairs)
Enzyme	(number of sites); positions	
*Nci*I	(9); 73, 210, 608, 795, 841, 1086, 1617, 1695, 1953	(5); 244, 822, 1271, 1603, 1735
*Fok*I	(9); 592, 790, 1078, 1186, 1398, 1572, 1837, 1963, 2074	(11); 202, 315, 649, 1025, 1120, 1338, 1568, 1579, 1586, 1685, 1925
*Bsp*1286I	(8); 33, 188, 477, 814, 846, 867, 931, 1354	(7); 78, 188, 410, 815, 1213, 1377, 1937

Table 2 Restriction sites in the parental genes *bphAE* (LB400 and B-356).

Positions are relative to the start codon.

digests of each parental DNA, generating 4 or 9 different reactions, respectively. These were cycled 15 times. Each assembly reaction was then pooled and cycled 30-40 more cycles, (PCR conditions are specified in the *Results* section). PCR product from these reassembly reactions was then amplified for cloning using PCR with the same oligonucleotide primers, *ndeI and bamHI* (LB-400 and/or B-356) used to amplify the parental genes. PCR product from this final step was checked by agarose gel electrophoresis for the correct size and amount. Shuffled product, cleaned from the PCR mixture using the GFX Band Prep kit, and pJB658 vector were digested using *NdeI* and *BamHI*, gel purified, and electroporated into *P. putida bphFGBC* following ligation of vector and *bphAE* fragments. Cells were plated on LB agar containing kanamycin (40 μg/ml), rifampicin (40 μg/ml), and carbenicillin (400 μg/ml).

Shuffling bphAE *using DNase I to create fragments.*

This method uses DNase I for random fragmentation of *bphAE*, essentially as described by Stemmer, 1994-A. Template DNA for shuffling was obtained by PCR amplification of pJB*bphAE* (LB400 or B-356) using the primers *ndeI* and *bam*H1 (described above), or with the following primers that hybridize to flanking plasmid DNA:

pm: 5'- CAACAGAAACAATAATAATGGAGTC-3' and

tm: 5'-AACGGTTTACAAGCATAAAGC-3'

which anneal 10 to 35 bp upstream of the *NdeI* restriction site and 16 to 37 bp downstream from the *BamHI* restriction site, respectively, of the MCS in pJB658. These primers, therefore, allow amplification of *bphAE* sequences inserted into the MCS.

Purified PCR-amplified parental template DNA was then digested with DNase I. For the sake of reproducibility from one digestion experiment to the next, a stock solution of DNase I (3 U/μl), was prepared in 20 mM Tris-HCl, (pH 7.5) containing 1 mM $MgCl_2$, quick-frozen in aliquots in a dry ice-ethanol bath, and stored at –80 °C until use (Ausubel *et al.*, 2001). Prior to the reaction DNase I stock solution was thawed on ice, and, to avoid oxidation of manganese upon storage, 10X reaction buffer (500 mM Tris-HCl, 100 mM $MnCl_2$, pH 7.6) was prepared fresh. DNase I stock solution was diluted 1000-fold in 1x reaction buffer just before initiating the reaction. All solutions included in the reaction mixtures were pre-equilibrated at 15 °C in a water bath. The reaction mixtures (50 μl) contained DNA template, 2.5 μg (*bphAE* or *bphE*) from each strain, and 20 mU DNase I in reaction buffer (50 mM Tris-HCl, 10 mM $MnCl_2$, pH 7.6). Digestion was allowed to proceed at 15 °C for various lengths of time, from 5-15 min. To stop digestion, aliquots removed from the reaction mixture were added to an ice-cold microfuge tube containing enough stop buffer, (50 mM EDTA, pH 8.0, containing 30% glycerol) to give a final concentration of 15 mM EDTA and 10% glycerol (Volkov and Arnold, 2000). Following electrophoresis in a 2 % agarose gel, gel pieces containing DNA in the desired size range were cut out and purified from the gel using the GFX Band Prep kit.

Purified DNA fragments were reassembled using primer-less PCR with conditions as described in *Results*. Samples from these PCR reactions ("reassembly PCR") were then used as template DNA in a final PCR amplification step with oligonucleotide primers, *pm* and *tm,* to amplify full-length, shuffled, DNA for cloning into pJB658 and subsequent screening for BPDO activity.

Primers *atm* and *bpm* were designed following reports (Volkov and Arnold, 2000) recommending the use of nested primers for the PCR amplification step following gene reassembly: *atm:* 5'-TCGCAACATCCGCATTA-3', and *bpm:* 5'-AGCCAGCATA ACCAGCAT-3' also hybridize to plasmid DNA, 137 to 155 bp upstream from the *Nde*I site and 163 to 180 bp downstream from *Bam*HI site, respectively, in the MCS of pJB658. Thus, the primers *pm* and *tm* (see above) are nested inside these primers on parts of the vector flanking the MCS.

Plasmid construction for shuffling bphE *only, and its expression downstream of* bphA.

Two methods (denoted 1 and 2) were employed for shuffling *bphE* independently of *bphA*. The second method was devised because the expression vectors described in the first method did not successfully produce active biphenyl dioxygenases. In *Method 1*, four new oligonucleotide primers were designed to be used with *ndeI and bamHI* (LB-400 and B-356) primers, described above, to PCR amplify both *bphA* and *bphE* separately. These include:

dstrm bphA LB400: 5'-AGAGGTACCGATTGCGTCTTGATCAG-3' and

dstrm bphA B-356: 5'-CATCTGGTACCTTGGTCGGGCTCAG-3',

which are used with their respective *nde*I primers, described above, to PCR amplify *bphA* from each strain and incorporate a *Kpn*I site (underlined) downstream of *bphA*. To PCR amplify and incorporate a *Kpn*I site (underlined) upstream of each *bphE* gene, primers:

upstrm bphE LB-400: 5'-CGAGGTACCGTCCCCTGATATTTACTTGG-3' and

upstrm bphE B-356: 5'-AATCCAGGTACCGCCGCAGGAGATTT-3'

were used with their respective *bamHI* primers, described above.

Plasmid constructs were generated by inserting *bphA* (LB-400 or B-356), into the *NdeI-KpnI* sites, and *bphE* (LB-400 or B-356) into the same *KpnI* site, and the *BamHI* site of pJB658. These constructs were not successful (See *Results*) and a new strategy (Method 2) was adopted.

Method 2 involved making a mutant such that *bphE* from each parent would have a common restriction site, early in the gene, to allow exchange of most of *bphE* with shuffled sequences. Site-directed mutagenesis was used to introduce an *XhoI* site into *bphE$_{LB400}$* at the same position as in *bphE$_{B-356}$*. This would allow removal and replacement of most of *bphE* with shuffled sequence, without modifying the intergenic region, and affecting its expression downstream of *bphA*. Oligonucleotides used for mutagenesis were: *xhofor*: 5'-CAAGGCGGCTGGCCT<u>C</u>GAGTTGCAGAACGAG-3' and its reverse complement, *xhorev*. These oligonucleotides were designed to substitute thymine 63 of *bphE$_{LB400}$* with cytosine (underlined). This silent mutation creates an *XhoI* restriction site (C/TCGAG) in the LB400 sequence at the same position as the one already present in *bphE$_{B-356}$*. The resulting plasmid is pJB*bphAE$_{LB400xho}$*. Plasmid DNA was extracted and transformed into a screening strain (*E. coli bphFGB*) to show that pJB*bphAE$_{LB400xho}$* produces active BPDO (see *Results*).

New DNA constructs were made by inserting *bphE* from each strain into the MCS of pJB658. These plasmids (pJB*bphE$_{LB-400}$* and pJB*bphE$_{B-356}$*) then could be used as template in a PCR amplification step (using primers that anneal to pJB658) to obtain parental DNA, flanked by identical sequence upstream and downstream, for use in DNA shuffling of *bphE* only. To insert *bphE$_{LB-400}$* and *bphE$_{B-356}$* into pJB658, PCR was used to obtain both genes with a *KpnI* site upstream and a *BamHI* site downstream of the *bphE*

sequence. Each *bphE* gene was ligated into the *Kpn*I and *Bam*HI sites in pJB568, and transformed by electroporation into *E. coli* DH5α.

To obtain large quantities of parental *bphE* DNA from each strain for shuffling, the resulting vectors (pJB*bphE*$_{LB400Xho}$ and pJB*bphE*$_{B-356}$) were used as templates in PCR reactions with primers *atm* and *bpm* (see above). Procedures for digestion of parental DNA were as those described for shuffling *bphAE* using DNase I. Fragments (40 to 200-bp or 40 to 100-bp) produced by digestion with DNase I were purified from a 2% agarose gel and reassembled by primer-less PCR. The shuffled product was amplified by PCR with primers *pm* and *tm* (see above).

Strains harbouring shuffled plasmids were then subjected to one of the screening methods described in the next section.

Screening Methods

The dihydroxydihydrodiol products formed by biphenyl dioxygenase are normally converted first to the dihydroxybiphenyl derivative by the gene product of *bphB*, and then to 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate (HOPDA) by BphC (Hofer *et al.* 1993; Sylvestre *et al.*, 1996-A; Hein *et al.*, 1998). *In vivo* screening methods for biphenyl dioxygenase activity were, therefore, based on chemical or enzymatic detection of dihydroxybiphenyl, the product of BPDO and BphB, on an accumulation of HOPDA in the presence of BphB and BphC. Genes encoding the coupling enzymes and proteins were, therefore, inserted into the chromosome of *P. putida* using transposon mutagenesis methods (Herrero and deLorenzo, 1990). These include *bphFG*, encoding the reductase components of BPDO, *bphB* (dihydrodiol dehydrogenase), and *bphC*

(dihydroxybiphenyl dioxygenase), which are required to convert the initial BPDO products into more easily detectable compounds.

Construction of DNA elements for transposon mutagenesis of P. putida *using* bphFGBC *or* bphFGB.

A 4.3 kbp DNA fragment carrying *bphFGBC*$_{LB400}$ was cut from *pYHbphFGBC* using *Bam*HI and *Hind*III, ligated into *Bam*HI/*Hind*III-digested pUC19NotI, and then cloned into *E. coli* DH5α to obtain pUC19N-*bphFGBC*. Purified pUC19N-*bphFGBC* was digested with *Not*I, fragments were separated by agarose gel electrophoresis, and the fragment corresponding to *bphFGBC* was isolated for cloning into pCNB1.

In some cases, it was desirable to screen for BPDO activity in strains expressing only *bphFGB* (i.e., no *bphC*): strains expressing active BPDO would thus accumulate dihydroxybiphenyl, rather than HOPDA. Constructs for the insertion/expression of *bphFGB* were made by digesting pUC19*bphFGBC* with *Cla*I and *Hind*III to remove 800 bp of 896 bp of the coding region of *bphC*. DNA fragments were separated using agarose gel electrophoresis and the 5 kbp DNA fragment corresponding to pUC-*bphFGB* was cut out and purified from the gel using the GFX Band Prep kit. The protruding 5'- and 3'-ends of pUC-*bphFGB* were filled in using *Klenow* fragment (Sambrook et al., 1989). Blunt ends were ligated using T4 DNA ligase and the resulting plasmid construct was transformed into *E. coli*. The plasmid, pUC-*bphFGB*, was subsequently isolated from overnight cultures and digested using *Not*I to obtain *bphFGB* with flanking *Not*I restriction sites for cloning into pCNB1.

*Not*I-flanked fragments of either *bphFGBC* or *bphFGB* were ligated into *Not*I-digested pCNB1. To prevent self-ligation of vector DNA, the 5'-ends were dephosphorylated using CIP. After ligation, plasmids were transformed into *E. coli* CC118 (*λpir*), and plated on LB containing kanamycin (40 µg/ml) and carbenecillin (200 µg/ml).

Plasmid DNA was isolated from a few *E. coli* CC118 colonies harboring pCNB1-*bphFGBC* or pCNB1-*bphFGB*. The presence of an insert was verified using *Not*I digestion followed by agarose gel electrophoresis. To increase the likely-hood of having at least one DNA construct with the *bph* genes oriented correctly, DNA was isolated from four colonies of each strain harboring pCNB1-*bphFGBC* or pCNB1-*bphFGB*, and transformed into mobilizing strain *E. coli* S-17(*λ pir*). Cells were plated on LB agar containing carbenicillin (100 µg/ml) and kanamycin (40 µg/ml) and tested further as described in *Results*.

Transposon mutagenesis to insert bphFGB *or* bphFGBC *into recipient chromosomes*

Transposon mutagenesis was carried out as described by de Lorenzo *et al.*, 1990. In preparation for the mating, colonies of *E. coli* S-17(λ pir) harboring pCNB1-*bphFGBC* (the donor strain) were picked from plates and grown overnight in LB broth containing carbenicillin (200 µg/ml) and kanamycin (40 µg/ml). Recipient cells, *P. putida* KT2442 or *E. coli* BL21-Gold, were grown overnight in LB broth containing rifampicin (40 µg/ml) or tetracycline (20 µg/ml), respectively.

Overnight cultures of the donor and recipient strains were used to inoculate fresh media (5 ml) supplemented with their respective antibiotics, and allowed to grow until an

optical density at 600 nm of 1-1.2 was reached. Donor and recipient cells (approx. 200 μl total) were then mixed at a ratio of 4:1 into sterile 10 mM $MgSO_4$ (5 ml), and collected onto sterile filters (13 mm, 0.45 μm nitrocellulose) using a syringe. Mating was allowed to proceed by incubating the filters with the cells on LB agar plates for 8 to 16 hours at 37 °C. Mating cells were then re-suspended in 10 mM $MgSO_4$ (10 ml) and plated on LB agar with kanamycin (40 μg/ml), and rifampicin (40 μg/ml) to select *P. putida* (*bphFGBC*) or kanamycin (40 μg/ml) and tetracycline (20 μg/ml) to select *E. coli* (*bphFGBC*) *or E. coli (bphFGB)*.

To test *P. putida (bphFGBC)* strains, selected colonies were grown overnight in LB broth (5-10 ml). Overnight cultures were used to inoculate (1/100) fresh LB broth (5 ml). When cell cultures reached an OD_{600} of 0.2, *m*-toluate (2 mM) was added to induce protein expression from the promoter, *Pm*. After an additional 8 hours at 37°C, a substrate for *BphC*, catechol, was added to the growth media. One strain that showed BphC activity, by turning bright yellow within 1-2 min due to the accumulation of *meta* cleavage product, was picked and grown overnight in LB containing kanamycin (40 μg/ml). A permanent culture was prepared, and stored at –80 °C for later use in screening for BPDO activity.

To test *E. coli* (*bphFGB*) strains, 5 selected colonies were transformed with pJB*bphAE*$_{B-356}$ by electroporation and plated on LB plates containing carbenicillin (100 μg/ml). BPDO activity was detected as explained below.

Screening for BPDO activity in P. putida (bphFGBC)

Ligation of *Nde*I-*Bam*HI digested *bphAE* shuffled sequence into *Nde*I-*Bam*HI digested pJB658 was followed by electroporation into *P. putida (bphFGBC)*. Cells were subsequently plated on LB agar containing carbenicillin (400 µg/ml), kanamycin (40 µg/ml), and rifampicin (40 µg/ml), and incubated overnight at 37 °C. To induce protein expression, cells were picked one by one or replica plated onto expression medium (LB) plates containing: *m*-toluate (2 mM); kanamycin (40 µg/ml); carbenicillin (400 µg/ml); and added nutrients solubilized in HCl, including $FeSO_4$ (40 µM), $CaCl_2$ (10 mM), $MgSO_4$ (10 mM), and thiamine (0.1 mM) (Vaillancourt *et al.,* 1998). To more easily distinguish the yellow color characteristic of HOPDA accumulation, agar plates as described above, but with reduced LB content (50%) and supplemented with succinate (3%), were also used. After plating, cells were incubated at 30 °C for 18-24 hours, and then exposed to the biphenyl substrate. This was accomplished by placing biphenyl crystals in the lids of these plates, making them airtight with parafilm, and incubating them at 37 °C for various lengths of time to allow accumulation and visual detection of the yellow metabolite HOPDA.

Screening for BPDO Activity in E. coli *(bphFGB(C))*.

Screening for BPDO activity in *E. coli-bphFGB* was done using a protocol described for the *in vivo* detection of catechols (Joern *et al.,* 2001) with some modifications. Following electroporation of plasmids containing shuffled *bphAE* genes (pJB*bphAE$_{SH}$*) into *E. coli (bphFGB)*, cells were plated on LB agar containing

carbenicillin (100 µg/ml), kanamycin (40 µg/ml), and tetracycline (20 µg/ml), and incubated overnight at 37 °C.

To allow expression of *bphAEFGB,* colonies from transformation plates were replica plated, using velvet, onto expression medium (LB) agar plates containing *m*-toluate (2 mM), tetracycline (20 µg/ml), kanamycin (40 µg/ml), and carbenicillin (200 µg/ml). For exposure to biphenyl substrate, colonies were lifted onto 0.45 µm filters that were then placed on M9 plates (Sambrook *et al.,* 1989) containing *m*-toluate (2 mM), glucose (1.6 %), carbenicillin (200 µg/ml), kanamycin (40 µg/ml), and added nutrients (described above). Biphenyl crystals were placed in the lids of these plates, which were then made airtight with parafilm and incubated at 37 °C for various lengths of time. When screening for BPDO activity in *E. coli (bphFGBC),* the yellow color, HOPDA, produced as a result of BPDO activity, was detected directly on these plates.

In *E. coli (bphFGB)* strains, colorimetric reaction with Gibb's reagent to reveal the presence of catechols was done by transferring filters exposed to substrate, as described in the previous section, onto 3% agarose containing 0.025 % Gibb's reagent (added as a 2 % solution in ethanol) (Joern *et al.,* 2001). Reaction with Gibb's reagent was allowed to proceed at room temperature for up to 40 min. Using a desktop scanner, images of the plates were recorded: colonies with active BPDO were expected to turn a blue color from the reaction of Gibb's reagent and the dihydroxybiphenyl product of BPDO.

Since dioxygenase activity was difficult to detect using the methods described above when either 2,2'-diClB or 3,3-diClB was added to the plates, some changes were made in the detection method when these compounds were used. Following published

results (Barriault *et al.*, 2002), *E. coli (bphFGB)* cells on nitrocellulose filters were treated, as above, but they were incubated with substrate(s) for extended periods of time up to 72 hours. Also, the cells were exposed to each substrate vapour by incubating substrate and cells together in Petri dishes placed at 30 °C, without additional wrapping, to help ensure air circulation: oxygen is required for cell activity and dioxygenase activity.

Screening for BPDO Activity in E. coli (bphFGB) in liquid cultures.

Screening of BPDO activity in liquid culture, in contrast to the solid media used above, allows spectrophotometric detection of levels of activity otherwise undetectable by eye. To test the method, larger (50 ml) cultures of *E. coli* BL21-Gold (*bphFGB*) containing pJB*bphAE*$_{B-356}$ or pJB*bphAE*$_{LB400}$ were grown with or without the inducer, *m*-toluate. Following a 4- or 6-hour induction period, aliquots were iced, and centrifuged to remove the original medium. The pellet was washed once with M9 medium (Sambrook *et al.*, 1989), and then resuspended in minimal medium (M9) containing substrates. Following one or three hour(s) incubation at 30 °C with biphenyl or 2,2'diClB, respectively, with shaking at 180 RPM, cells were removed by centrifugation and the supernatant was incubated for 40 min with Gibb's reagent (0.03 %) before scanning the spectrum between 400-800 nm. The Gibb's reagent was prepared as a 0.4% solution in ethanol. This method was used in 96 well microtiter plate format, as described previously (Joern *et al.* 2001), and in larger volumes using a conventional spectrophotometer.

Since, BPDO is not expressed well in cells grown in minimal medium, the cells were first grown and induced in LB broth. Then, because the presence of LB broth interferes with the Gibb's reagent, cells were washed once with M9 medium before they

were incubated further in M9 medium containing BPDO substrates. Colonies of *E. coli-bphFGB* containing shuffled *bphE* genes were picked from the transformation plates (or cell stocks streaked for single colonies) and used to inoculate the wells of a 96 well, V-bottom micro-titer plate (Corning no. 3894) containing 200 µl of LB broth supplemented with carbenicillin (300 µg/ml), kanamycin (50 µg/ml), and tetracycline (20 µg/ml), and incubated overnight at 37 °C with shaking at 225 RPM. The overnight cultures (2 or 10 µl) were used to inoculate the wells of one or more micro-titer plates containing expression medium (200 µl/well of LB broth supplemented with added nutrients (Vaillancourt *et al.*, 1998), *m*-toluate (2mM), carbenicillin (300 µg/ml), and kanamycin (50 µg/ml)). Following 3 or 4 hours of incubation at 30°C, the plates were centrifuged at 3250 RPM for 7 min, washed with minimal media, centrifuged again, and the supernatant was removed from each well and replaced with minimal medium (200 µl/well M9 salts, glucose (3%), added nutrients (Vaillancourt *et al.*, 1998), carbenicillin (300 µg/ml), and kanamycin (50 µg/ml)), and containing substrate: biphenyl (45 µM), or 2,2'-diClB (4.5 µM), or 3,3'-diClB (1.5 µM)). The cell cultures were then incubated overnight at 30°C with shaking at 180 RPM, and finally centrifuged (3250 RPM, 10 min). The supernatant (160 µl) was transferred to the well of an optically clear, flat-bottom, micro-titer plate (Corning no. 3596) containing 20 µl of Gibb's reagent (0.4 % in ethanol). After a 40 min incubation at room temperature the plates were analyzed using a plate reader.

RESULTS

1. Expression of recombinant BPDO and detection of its activity *in vivo*.

1.1 *Expression of recombinant BPDO*

Genes encoding the two subunits of BPDO (*bphAE*) from *Comamonas testosteroni* B-356 and *Burkholderia cepacia* LB400 were cloned into pJB658 to generate pJB658*bphAE*$_{B-356}$ and pJB658*bphAE*$_{LB400}$, respectively (Fig. 2.1). After electroporation into *P. putida* KT2442 or *E. coli* BL21G, cells harboring these plasmids were exposed to *m*-toluate to induce expression of *bphAE* from the promoter, *Pm*. Two polypeptides with molecular weights corresponding to the oxygenase components, BphA (51 kDa) and BphE (21 kDa), were detected in crude extracts obtained from induced cell cultures (Fig. 2.2, lanes 1 and 2). BPDO thus appeared to be expressed in a soluble form in this expression system.

1.2 *Assay of biphenyl dioxygenase (BPDO) activity in* P. putida (bphFGBC)

Previous experiments showed that expression of some biphenyl dioxygenase genes was more efficient in *Pseudomonas putida* KT2442, a more closely-related host to the parental strains, than in *E. coli* (Imbeault *et al.*, 2000). To increase the chances of obtaining expressed, soluble, and active enzymes after shuffling, *Pseudomonas putida* KT2442 was chosen initially as the screening host.

In addition to the BPDO genes, *bphAE*, genes that encode the reductase components of BPDO (BphFG), as well as two additional enzymes, BphB and BphC, are

GGAGTCATGAACA/*TATGA*GTTCAGCAAT-*bphAE*_{LB400}-ACAACCTGAGCATGTTCTTCT**AGG**/GATCCTCT

GGAGTCATGAAC*A/TATG*AGTTCGACTAT-*bphAE*B-356 AAAACCTGAGCGGTGTTCTTT**GAG**/GATCCTCT
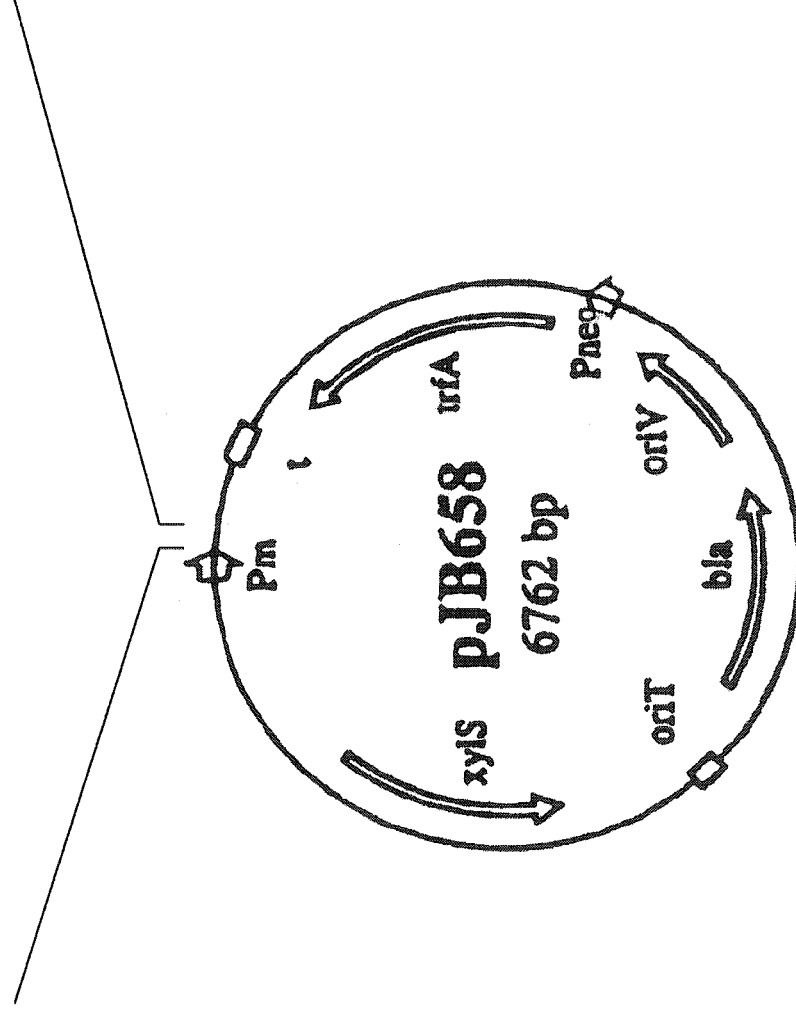

Figure 2.1 Expression vector for BPDO genes (*bphAE*_{B-356} and *bphAE*_{LB400}): pJB*bbphAE*_{LB-400} and pJB*bbphAE*_{B-356} were constructed as described in *Material and Methods*

required to convert the dihydrodiol products of BPDO to HOPDA (See Fig 1.2, *Introduction*): HOPDA is easily detected by virtue of its intense yellow colour.The genes (*bphFGBC*) encoding these additional proteins were cloned downstream from the promoter, *Pm*, and inserted into the chromosome of *Pseudomonas putida* KT2442 by using mini-*Tn5* transposon mutagenesis to generate *Pseudomonas putida* (*bphFGBC*) (as described in *Materials and Methods*). The resulting strain was tested by exposure to catechol: the appearance of a bright yellow product confirmed that that *bphC* had been successfully introduced into the chromosome of *P. putida* KT2442 (data not shown).

Assay of wild-type recombinant $BPDO_{LB400}$ and $BPDO_{B-356}$ expressed from pJB*bphAE$_{LB400}$* or pJB*bphAE$_{B-356}$* in *Pseudomonas putida* (*bphFGBC*) confirmed that *bphAE*, as well as *bphFGBC*, were expressed and functional. Upon exposure to biphenyl vapour, the distinctive yellow colour associated with HOPDA accumulation was visible in cells expressing *bphAEFGBC* (data not shown). *Pseudomonas putida (bphFGBC)* harbouring pJB658*bphAE$_{B-356}$* turned yellow within 5 min using plates with reduced LB content (see *Material and Methods*), while colonies of *Pseudomonas putida bphFGBC* harbouring pJB658*bphAE$_{LB400}$* only turned yellow after a 90-120 min or more incubation. The lower activity of $BPDO_{LB400}$ relative to $BPDO_{B-356}$ is consistent with the kinetic parameters observed using purified preparations of: $BPDO_{B-356}$ $k_{cat} = 7.3$ s^{-1}, and $BPDO_{LB400}$ $k_{cat} = 0.5$ s^{-1} (N. Agar, PhD thesis, 2002)

Detection of low levels of activity such as those observed with $BPDO_{LB400}$, using the *P. putida* KT2442 host strain was prone to interference from other pigments that were produced upon prolonged incubation. First, the *P. putida* colonies themselves were

Figure 2.2 SDS-PAGE: Expression of biphenyl dioxygenase in *P. putida* KT2442.

Cells were grown and prepared as described *Material and Methods*. In gel A, crude extracts were from cells that were harboring: pJB$bphAE_{B-356}$ cloned *Nde*I-*Bam*HI (lane 1); pJB$bphA_{LB400}bphE_{LB400}$ cloned *Sac*I-*Kpn*I and *Kpn*I-*Bam*HI, respectively (lane 2); pJB$bphA_{LB400}bphE_{B-356}$, cloned *Sac*I-*Kpn*I and *Kpn*I-*Bam*HI, respectively (lane 3); pJB$bphA_{B-356}$ $bphE_{LB400}$ cloned *Nde*I-*Kpn*I and *Kpn*I-*Bam*HI, respectively (lane 4); or pJB$bphAE_{shuffled}$ cloned *Nde*I-*Bam*HI (lanes 5-8) (from *Experiment 2, Table 2*). Expression of BPDO$_{LB400}$ from pJB$bphAE_{LB400}$ cloned *Nde*I-*Bam*HI is shown in B (lane 2) versus extracts from cells not harboring the plasmid (lane 1).

Arrows indicate the positions of the α and β-subunits. The β-subunit of LB400 migrates at a slightly higher position (Dr. Sylvestre, personal communication). The α-subunit of LB400 was poorly expressed when *bphA* was cloned *Sac*I-*Kpn*I (A: lanes 2 and 3). The β-subunit of B-356 was not expressed when $bphE_{B-356}$ was cloned downstream from $bphA_{LB400}$ (lane 3), while the β-subunit of LB-400 was expressed well with its gene, $bphE_{LB400}$, cloned downstream from bphA $_{B-356}$ (lane 4).

46
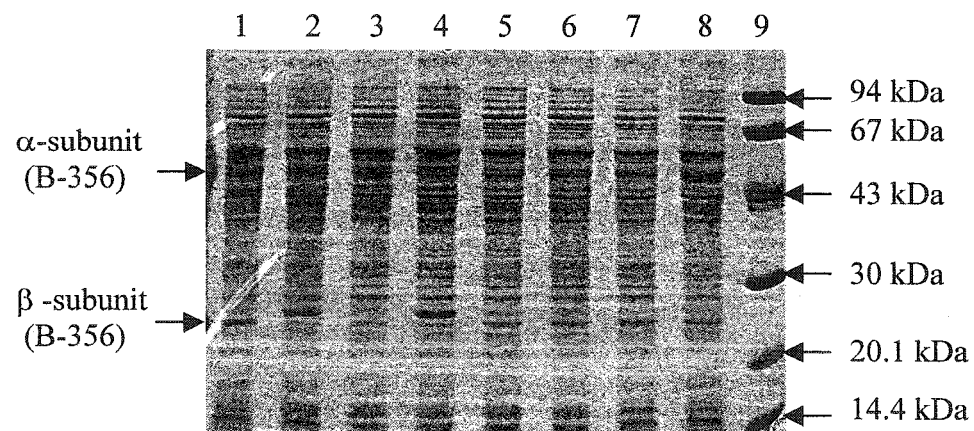
A
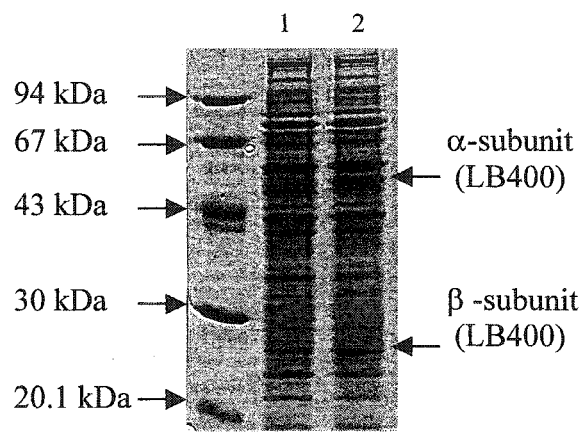
B

yellowish, making it difficult to detect lower amounts of HOPDA characteristic of BPDO$_{LB400}$. This problem was compounded by the production of an unidentified pink substance that diffused out into the growth medium following a 2-3 hour incubation period (not shown). It is possible that the formation of this substance is caused by endogenous toluate dioxygenase activity that metabolizes the protein expression inducer, *m*-toluate, present in the growth medium. In any event, the presence of these pigments made it very difficult to detect anything but the most active BPDOs.

Several other properties of this strain made it less than ideal as an expression and screening host. High transformation efficiency is crucial to efficient screening of limited quantities of shuffled DNA. Electroporation of *Pseudomonas putida* (*bphFGBC*) using ligation mixtures typically resulted in 0.1 transformants/ng DNA or less. An additional concern was that this *Pseudomonas* strain is somewhat resistant to carbenicillin, necessitating the use of large quantities: despite this, growth of a resistant background was often observed (data not shown). Although this strain was used in some initial screening experiments, a high transformation efficiency *E. coli* strain, BL21-Gold (Greener *et al.*, 1996,1997), was modified by chromosomal insertion of *bphFGBC* for further screening experiments.

1.3 *Assay of biphenyl dioxygenase (BPDO) activity* in E. coli bphFGBC

Using the methods described for the *P. putida* KT2442 experiments, the additional genes (*bphFGBC*) required for the conversion of biphenyl to HOPDA were inserted into the chromosome of *E. coli* BL21-gold (see *Materials and Methods*). Following mini*Tn5*-mediated chromosomal insertion of *pm* promoter-controlled

bphFGBC, cells were subjected to stringent selection on antibiotics to obtain *E. coli* BL21-gold potentially containing the insert, as described in *Materials and Methods*. Several colonies were picked, grown in liquid medium, exposed to *m*-toluate to induce *bphFGBC* expression, and then tested for BphC activity, as described above for *P. putida (bphFGBC)*. One clone that turned bright yellow following the addition of catechol was tested further by co-expressing BPDO genes from pJB658*bphAE*$_{B-356}$ or pJB658*bphAE*$_{LB400}$. As with *P. putida* (*bphFGBC*), colonies of *E. coli* (*bphFGBC*) co-expressing wild-type BPDO showed the characteristic yellow color of HOPDA accumulation when exposed to biphenyl. This occurred with BPDO$_{B-356}$ after a 3-5 min exposure to biphenyl vapour, but when BPDO$_{LB400}$ was expressed, a faint yellow colour was visible only after a 50-60 min of exposure to biphenyl (Fig. 2.3). The results shown in Fig. 2.3 were obtained after transferring *E. coli* (*bphFGBC*)-pJB*bphAE*$_{LB400}$, induced overnight, onto a nitrocellulose filter, which was placed in a minimal LB agar plate (see *Materials and Methods*). This allowed better visualization of the yellow product, HOPDA, than when the colonies were on LB agar plates (data not shown). HOPDA accumulation is visible in *E. coli* cells expressing BPDO$_{B-356}$ after just 5 min in the presence of biphenyl vapour, and the colour is much more pronounced after a 90 min time period in the presence of substrate. Cells expressing BPDO$_{LB400}$ showed only a comparatively small amount of the yellow pigment (HOPDA) after the same 90 min incubation period (Fig 2.3). Unlike *P. putida* KT2442, this *E. coli* strain formed beige colonies, and no diffusible pink substance was produced even after prolonged incubation of the colonies in the presence of *m*-toluate and/or biphenyl. The *E. coli* BL21G (*bphFGBC*) strain, therefore, allowed more sensitive detection of BPDO activity, but the

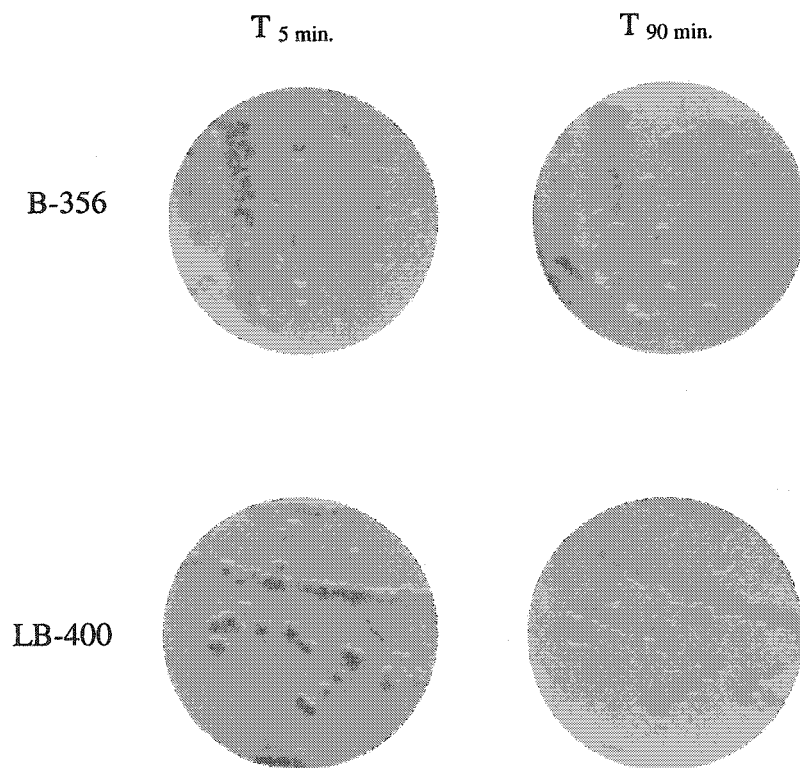$T_{5\ min.}$ $T_{90\ min.}$

B-356

LB-400

Figure 2.3 BPDO Assay in *E. coli bphFGBC* with biphenyl as substrate.

E. coli bphFGBC colonies expressing $BPDO_{B\text{-}356}$ (top) or $BPDO_{LB400}$ (bottom) were

lifted onto a filter and exposed to biphenyl vapor as described in *Materials and Methods*.

faint yellow colour produced by BPDO$_{LB400}$ only after prolonged incubation was still a concern, since activities were likely to be even lower in the presence of poorly-metabolized chlorinated congeners. Indeed, yellow colour could not be detected in colonies of *E. coli* (*bphFGBC*) expressing wild-type BPDO following exposure for up to 12 hours to either 2,2'-diCB or 3,3'-diClB, which are normally metabolized by BPDO.

1.4 *Assay of biphenyl dioxygenase (BPDO) activity* in E. coli (bphFGB)

Difficulties detecting low levels of BPDO activity in the *P. putida* (*bphFGBC*) and *E. coli* (*bphFGBC*) strains prompted a search for more sensitive alternatives.

Recent reports of *in vivo* methods to detect products of dioxygenases utilize chemical and/or enzymatic reactions with the aromatic dihydrodiol products to produce coloured derivatives (Arisawa *et al.*, 1999, Joern *et al.*, 2001). For example, Gibb's reagent has been shown to react rapidly with catechols, allowing quantitative and reproducible detection of as little as 30 μM of product (Joern *et al.*, 2001). Application of such a method to BPDO screening would eliminate the need for BphC to convert 2,3-dihydroxybiphenyls to HOPDA. This is actually an advantage because the ring-cleaving dioxygenase, BphC, is not capable of transforming *cis*-3,4-diols produced by the action of BPDO$_{LB400}$ (see *Introduction*). Also, since the BphC-catalyzed step is eliminated it is possible that the sensitivity would be improved by detecting the dihydroxy products more directly, especially when they are substituted with chlorine.

To use Gibb's reagent for *in vivo* screening of shuffled biphenyl dioxygenases, *bphFGB*$_{LB400}$ was inserted into the chromosome of *E. coli* BL21-Gold, as described in *Materials and Methods*. A few potential *E. coli* (*bphFGB*) colonies were recovered from

the antibiotic selection plates, transformed with pJB*bphAE*$_{B-356}$ by electroporation, and tested for activity using Gibb's reagent as described in *Materials and Methods*. One strain that tested positive by rapidly turning a deep crimson colour was saved for use in further experiments.

E. coli (bphFGB) was further tested by transforming with either pJB*bphAE*$_{LB400}$ or pJB*bphAE*$_{B-356}$, which express the wild-type BPDO genes. Cells expressing BPDO$_{B-356}$ rapidly turned a deep crimson colour (Fig. 2.4). Although cells expressing BPDO$_{LB400}$ turned colour more slowly, it was nevertheless still readily distinguishable from background after 60 min (Fig. 2.4). In comparison to the BPDO$_{LB400}$ assay in strains relying on HOPDA accumulation (Fig. 2.3), the colour produced by the lower-activity enzyme was much more intense.

2. DNA shuffling

Two types of DNA shuffling strategies were used: shuffling of *bphAE*, and shuffling of *bphE* alone. Although greater diversity is possible by shuffling both genes together, it proved to be difficult to reassemble intact *bphAE*. The successful techniques developed for *bphAE* shuffling were therefore applied to shuffling *bphE* alone.

2.1 *DNA shuffling of bphAE using restriction enzymes to create fragments*

DNA corresponding to BPDO genes, *bphAE*$_{B-356}$ (1972 bp) and *bphAE*$_{LB400}$ (2063 bp), was obtained by PCR amplification from pJB658*bphAE*$_{B-356}$ or pJB658*bphAE*$_{LB400}$ using their respective *ndeI* and *bamHI* primers, as described in *Materials and Methods*. Agarose gel electrophoresis confirmed the presence of bands corresponding to

$$T_{5\ min} \qquad\qquad T_{40\ min}$$
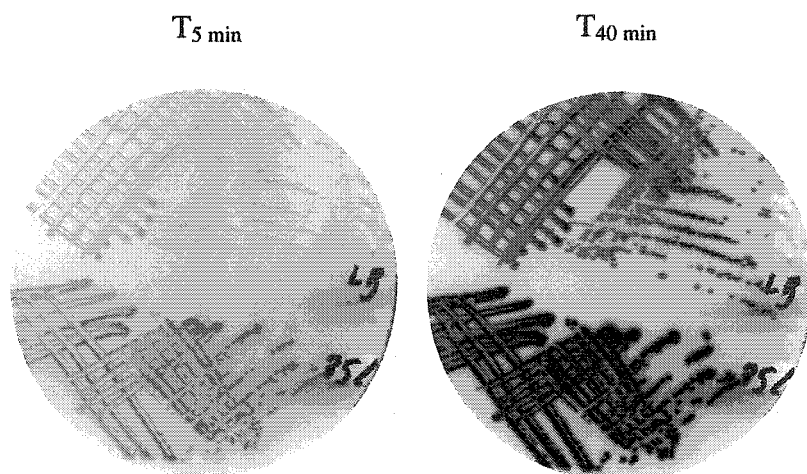
Figure 2.4 Assay of BPDO activity in *E. coli* (*bphFGB*) using Gibb's reagent. *E. coli* (*bphFGB*) colonies expressing $BPDO_{B-356}$ or $BPDO_{LB400}$ were lifted onto a nitrocellulose filter and were exposed to biphenyl vapour for 60 minutes (see *Materials and Methods*). The colonies were then allowed to react with Gibb's reagent by placing the filter in a Petri dish containing 0.025 % Gibbs reagent, added as a 3 % solution in ethanol.

*bphAE*_{LB400} at 2.1 kbp (Fig. 2.5A, lane 1) and *bphAE*_{B-356} at 2 kbp (Fig. 2.5A, lane 2), which are the expected sizes. After purification from the gel, these fragments were used as the parental DNA for shuffling in experiments 1-3 (Table 2). In experiment 4 (Table 2), parental DNA was prepared in a similar way, but using the primers *pm* and *tm* to introduce short identical sections of vector DNA, on each end of the genes. This was to ensure that the whole gene could be completely reassembled by PCR.

The two parental DNA templates were digested separately with various combinations of *Bsp*1286I, *Fok*I and *Nci*I (Table 3). The expected cleavage sites for these restriction enzymes within *bphAE* are shown in Table 2 (*Material and Methods*). Complete digestion was verified by using agarose gel electrophoresis and comparison with the expected fragments and sizes: an example is shown in Fig. 2.5B for experiment 2, Table 3. The gel (Fig. 2.5B) contains *bphAE*_{LB400} DNA digested with *Bsp*1286I, *Fok*I and *Nci*I, lanes 1-3, respectively, and *bphAE*_{B-356} digested with the same enzymes in lanes 4-6. Restriction fragments such as these (Fig. 2.5B) were purified as described in *Materials and Methods* either using centrifugal devices (Experiment 1, Table 3) or agarose gel electrophoresis (Experiments 2-4, Table 3). In experiments after the first series, purification following agarose gel electrophoresis was preferred to eliminate the possibility of contamination of the subsequent R-PCR step with residual intact wild-type DNA.

After purification, fragments were combined in reassembly PCR (R-PCR) reactions: R-PCR is PCR done without primers, relying on overlap between homologous fragments during the annealing step to generate substrates for extension by DNA

Table 3   DNA shuffling of *bphAE* using restriction enzymes to create DNA fragments. In the left-hand column, parental DNA and amplification primers are in a), restriction enzymes used in digest are in b), and fragments purification method is in c).

Parental DNA, Digestion, Purification	Reconstruction PCR	A-PCR; Results
Experiment 1 a) *bphAE*B-356 and LB400 primers *ndeI-bamHI* b)-*NciI* or *Bsp*1286I and *NciI* or FokI, respectively c) EZ, Ym-10 microconcentrators	DNA (approx. 15 ng/μl), dNTPs (250 μM of each) -1 R-PCR: 40 cycles: Hybridization temp: 55°C Elongation time: 30 s + 5 sec/cycle + 30 cycles: Hybridization temp: 58°C Elongation time: 60 s + 5 sec/cycle	- no product using 10μl R-PCR after 40 cycles of R-PCR - some product after 70 cycles See Fig. 2.5C.

Experiment 2
a) *bphAE*B-356 and LB400 primers *ndeI-bamHI*
 	
b) *Bsp*1286I, *NciI, FokI*
 	
c) Agarose gel/Band prep purification
	DNA (approx. 20 ng/μl), dNTP (285 μM)
	
	-9 R-PCR; 15 cycles:
	Hybridization temp: 55°C
	Elongation time: 60 sec + 5 sec/cycle
	-1 R-PCR, 25 cycles:
	same program as previous
		-Some A-PCR product. See Fig. 2.5D
		-Screening
		-Sequencing of *bphAE* from
		  -Active colonies 8
		  -Inactive colonies 5

Experiment 3 a) *bphAE*B-356 and LB400 primers *ndeI-bamHI* b) *Bsp*1286, *and NciI* c) Agarose gel/Band prep purification	DNA(approx. 15 ng/μl) dNTP (180 μM) -4 R-PCR, 15 cycles: Hybridization temp: 48°C Elongation time: 25 sec + 5 sec/cycle -1 R-PCR, 30 cycles: same program	Some product (2 kbp) is amplified with B-356 primers only. See Fig. 2.5 E
Experiment 4 a) *bphAE*B-356 and LB400 (primers *pm-tm*) b) *Bsp*1286, *and NciI* c) Agarose gel/Band prep purification	Fragments (approx. 90 ng/μl), dNTP (200 μM) -4 R-PCR, 15 cycles: Hybridization temp: 48°C Elongation time: 25 s + 5 s/cycle +1 R-PCR, 30 cycles: same as previous + 30 cycles: Hybridization temp: 48°C Elongation time: 3 min 30 sec	After 45 cycles of R-PCR: only a smear After 75 cycles of R-PCR: band at 2 kbp See Fig. 2.5F

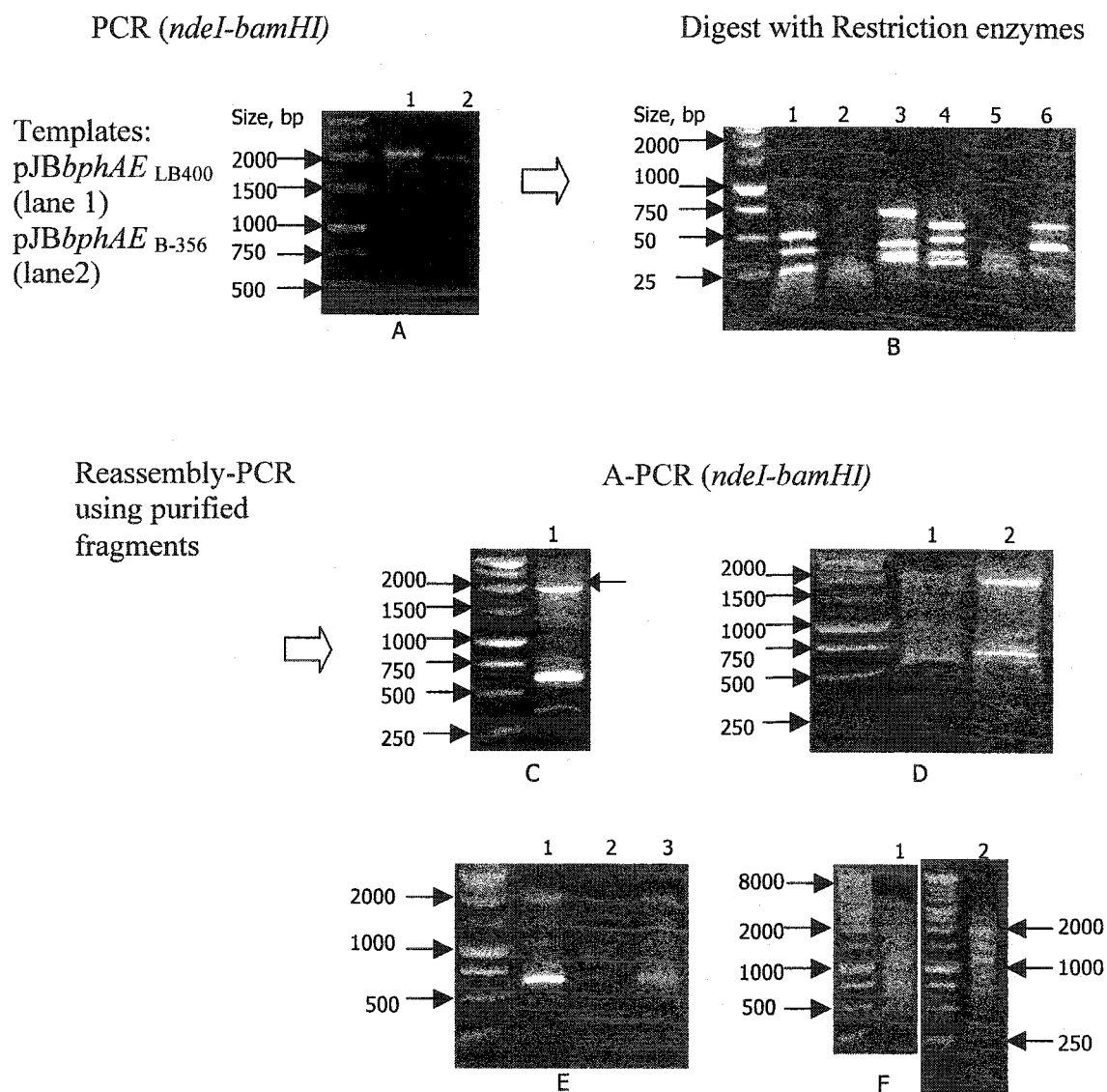**Figure 2.5 Agarose gel electrophoresis at various steps of DNA shuffling of *bphAE* using restriction enzymes.** Refer to Table 3 and text for details.

polymerase. R-PCR conditions are shown in Table 3, column 3. In experiment 1 (Table 3), all fragments were combined in a single R-PCR reaction, whereas in subsequent experiments initial R-PCR reactions were run using combinations of the different fragments to allow dimerization (hence recombination) to occur between DNA segments that may otherwise dimerize preferentially with only one of the other fragments. The number of initial R-PCR reactions for each experiment is indicated in Table 3. After usually 15 cycles of R-PCR, the individual R-PCR mixtures were pooled and subjected to an additional R-PCR. The initial hybridization temperature (55 °C) was chosen by the degree of sequence conservation of the genes to be shuffled and the temperatures used in previous work (Kikuchi *et al.*, 1999). In experiments 3 and 4 (Table 3), the hybridization temperature was lowered from 55 °C to 48 °C to encourage annealing between segments of DNA that were not identical in sequence.

In the final step, an aliquot of the R-PCR reaction mixture was used in a final PCR amplification step (A-PCR) with appropriate primers to obtain full-length *bphAE* flanked by *Nde*I and *Bam*H1 sites for cloning into the corresponding sites of the expression vector, pJB658 (Table 3, column 3). All four primers, nde_{B-356}, $bamHI_{B-356}$, $ndeI_{LB400}$ and $bamHI_{LB400}$ (described in *Material and Methods*) were used to PCR-amplify the product of the R-PCR. The primer set(s) used for particular experiments are noted in the text referring to the products from the A-PCR reactions, which were analyzed by agarose gel electrophoresis (Fig. 2.5C-2.5F).

The agarose gels presented in Fig. 2.5, C to F, shows the DNA produced by each experiment described in Table 3, 1 to 4, respectively. For the 1st experiment, both sets of primers were used in the A-PCR (Fig 2.5C, lane 1), whereas in experiment 2 each primer

set was used individually (Fig 2.5D, lane1; $ndeI_{LB400}$ and $bamHI_{LB400}$ primers while in lane2; nde_{B-356}, $bamHI_{B-356}$ primers were used). The A-PCR product represented in Figure 2.5E was obtained using either each set of primers individually. In Fig.2.5E, lane 1 the use of the nde_{B-356}, $bamHI_{B-356}$ primers resulted in a product of the correct size (2000 bp), while in lane 2, using the $ndeI_{LB400}$ and $bamHI_{LB400}$ primers did not result in any DNA of the correct size. Using both sets of primers (Fig. 2.5E, lane 3) resulted in the same discreet bands as observed for lane 1 (Fig 2.5E) but with decreased intensity.

A-PCR product was present at approximately 2000 bp when nde_{B-356} and $bamHI_{B-356}$ were used (Fig. 2.5D, lane 2, or Fig. 2.5E, lane 1, but not when $ndeI_{LB400}$ and $bamHI_{LB400}$ primers were used (Fig. 2.5E, lane 2), except in experiment 2 (Table 3) where some product was obtained with these primers. Visible on the gels also are some DNA of odd sizes (650 bp), which may be due to 'non-specific' hybridization during the reconstruction step. Since no shuffled product could be amplified with the $ndeI_{LB400}$-$bamHI_{LB400}$ primers, and because the patterns of bands observed on this agarose gel (Fig. 2.5E) are similar to those observed for the two previous experiment (Fig.2.5C, 2.5D) the shuffled product obtained in this experiment was not cloned.

For the 4th experiment parental DNA was obtained by PCR with primers *pm* and *tm* as described in *Materials and Methods*, then digested with restriction enzymes *Bsp* 1286 and *Nci*I, and reassembled by PCR. Following amplification with primers *pm* and *tm* primers, one sharp but faint band was observed at the expected size (2000 bp) of *bphAE* (Fig. 2.5F, lane 2). The shorter-length DNA fragments seen in Figs. 2.5A, 2.5B, and 2.5C were not observed here, which is probably due to the amplification of parental

DNA templates using primers *pm* and *tm*. However because little 2 kbp product was obtained few transformants were obtained for screening.

2.1.1 *Screening of BPDO in* Pseudomonas putida(bphFGBC)

The screening host *Pseudomonas putida* (*bphFGBC*) was transformed with plasmid DNA containing shuffled *bphAE* genes reconstructed from fragments generated by restriction enzymes, as described above (Experiment 1 and 2, Table 3). Approximately 1000 colonies were screened for biphenyl dioxygenase activity by exposing them to biphenyl vapour, as described in *Materials and Methods*. Colonies showing activities with biphenyl that were intermediate between the wild-type parental enzymes were picked, and plasmid DNA isolated from them was sequenced.

2.1.2 *Sequencing of shuffled* bphAE *obtained with the restriction enzyme method*

Sequence data was obtained as described in *Materials and Methods*. Approximately 12 000 base pairs of *bphAE* DNA, which was recovered from 8 different clones expressing active BPDO were sequenced and compared to the parental genes, *bphAE*$_{B-356}$ and *bphAE*$_{LB400}$. Only one recombination event was found for a total of 12 000 bases of DNA sequenced, as DNA from 6 of 7 was found to be identical to *bphAE*$_{B-356}$. One *bphAE* gene was mostly identical to *bphAE*$_{B-356}$ but had one section from base pair 580 to 640 that was clearly from *bphAE*$_{LB400}$, (data not shown).

DNA was also recovered from 5 colonies that did not show BPDO activity *in vivo,* and sequenced. Approximately 5000 base pair were sequenced to reveal only one recombination event. These enzymes were probably inactive, or poorly expressed,

because of deleterious mutations present within the coding and/or the inter-genic regions of *bphAE*.

2.1.3 *Conclusions from DNA shuffling using restriction enzymes to create fragments*

DNA shuffling of *bphAE* using the restriction enzyme method as detailed above, produced full length genes primarily when the $ndeI_{B-356}$ and $bamHI_{B-356}$ primers were used in the final amplification PCR. This was an indication that $bphAE_{LB400}$ sequence was not present in the shuffled product, at least in the regions to which the primers annealed. In addition, sequencing of shuffled BPDO genes that produced both active and inactive enzymes revealed that very little shuffling had occurred between the two parental genes. Crossing-over between parental DNA during the reconstruction PCR did not occur with significant frequency under the conditions used. The conditions used for the reconstruction PCR must favor the formation of homoduplex DNA molecules, which in turn precludes the formation of heteroduplex DNA and results in reconstruction of wild-type enzymes (mostly $BPDO_{B-356}$) rather than recombined genes. Since the number and variety in size, as well as sequence similarity of the DNA fragments produced, from each parental strain influences the outcome of the shuffling procedure DNase I was used to digest parental genes and create a wider range DNA fragments than that produced with specific restriction enzymes.

2.2 *DNA Shuffling of* bphAE *Using DNAase I*

To increase the likelihood of producing shuffled product, parental DNA templates were digested with DNase I (Stemmer, 1994-A) instead of restriction enzymes.

Increasing the size diversity and number of the DNA fragments should increase the potential for heteroduplex formation during the reconstruction PCR, leading to crossovers and highly shuffled final product.

For these experiments (Table 4), parental DNA was obtained by PCR amplification of pJB*bphAE*$_{LB400}$ and pJB*bphAE*$_{B-356}$ using oligonucleotide primers *pm* and *tm*, as described in *Materials and Methods*. PCR product was checked for the correct size by agarose gel electrophoresis: *bphAE*$_{B-356}$ (Fig. 2.6A, lane 1) and *bphAE*$_{LB400}$ (Fig. 2.6A, lane 2). The sizes of the products (2 and 2.1 kb, respectively) corresponded to those expected of the genes amplified. After purification of the PCR products, each of the parental DNA templates was fragmented separately with DNase I. The resulting fragments were purified using EZ-enzyme removers and Microcon YM-10 devices to exchange the buffer, as described in *Materials and Methods*. The fragments produced by DNase I digestion were then digested with *NciI* and an aliquot was analyzed by agarose gel electrophoresis to verify that the DNA templates were completely digested, and to estimate the sizes of the fragments produced (e.g. Fig. 2.6B). Fragments in the range 20 to 600 bp (Table 4) were purified using EZ-enzyme removers and Microcon YM-10 devices, and subjected to reassembly PCR.

The parameters for the reassembly PCR reactions were varied in an effort to obtain full length *bphAE* genes, as detailed in Table 4, column 2. Experiment 1 contained purified DNA fragments (80-120 ng/μl) that varied in size from 100 to 600 bp with most around 250 bp. No increase in the size of the DNA fragments was observed following R-PCR (Fig. 2.6C). The fragments from the R-PCR were recovered and cleaned using the

Parental DNA, Digestion, Fragments sizes	Reassembly-PCR	A-PCR; Results
Experiment 1 a)*bphAE*B-356 and LB400 (*pm-tm*) b)DNase I, *Nci*I c)100-600 bp (avg: 250 bp)	DNA (approx. 80-120 ng/μl), dNTPs (300 μM of each) - R-PCR, 35 cycles: Hybridization temp: 52°C Elongation time: 30 s + 5 sec/cycle + 55 cycles: same as above + 30-55 cycles: same as above	See Fig. 2.6C, 2.6D, and 2.6E
Experiment 2 a)*bphAE*B-356 and LB400 (*pm-tm*) b) DNase I, *Nci*I c)20-250 bp (avg: 100 bp)	DNA (approx. 140ng/μl), dNTP (300 μM) R-PCR; 13 cycles: Hybridization temp: 42°C Elongation time: 30 sec + 5 sec/cycle R-PCR, 30 cycles: Same as above	See Fig 2.6F
Experiment 3 a) *bphAE*B-356 and LB400 (*pm-tm*) b)DNase I, *Nci*I c) 250-1000 bp (avg: 500 bp)	DNA(approx. 50 or 100 ng/μl) dNTP (1 mM of each) R-PCR, 40 cycles: Hybridization temp: 50 °C Elongation time: 60 sec + 5 sec/cycle	See Fig. 2.4G

Table 4 Summary of DNA shuffling experiments

PCR (*pm-tm*)

Templates:
pJB*bphAE* B-356
(lane 1) and
pJB*bphAE*
LB400 (lane 2)

DNase I digest

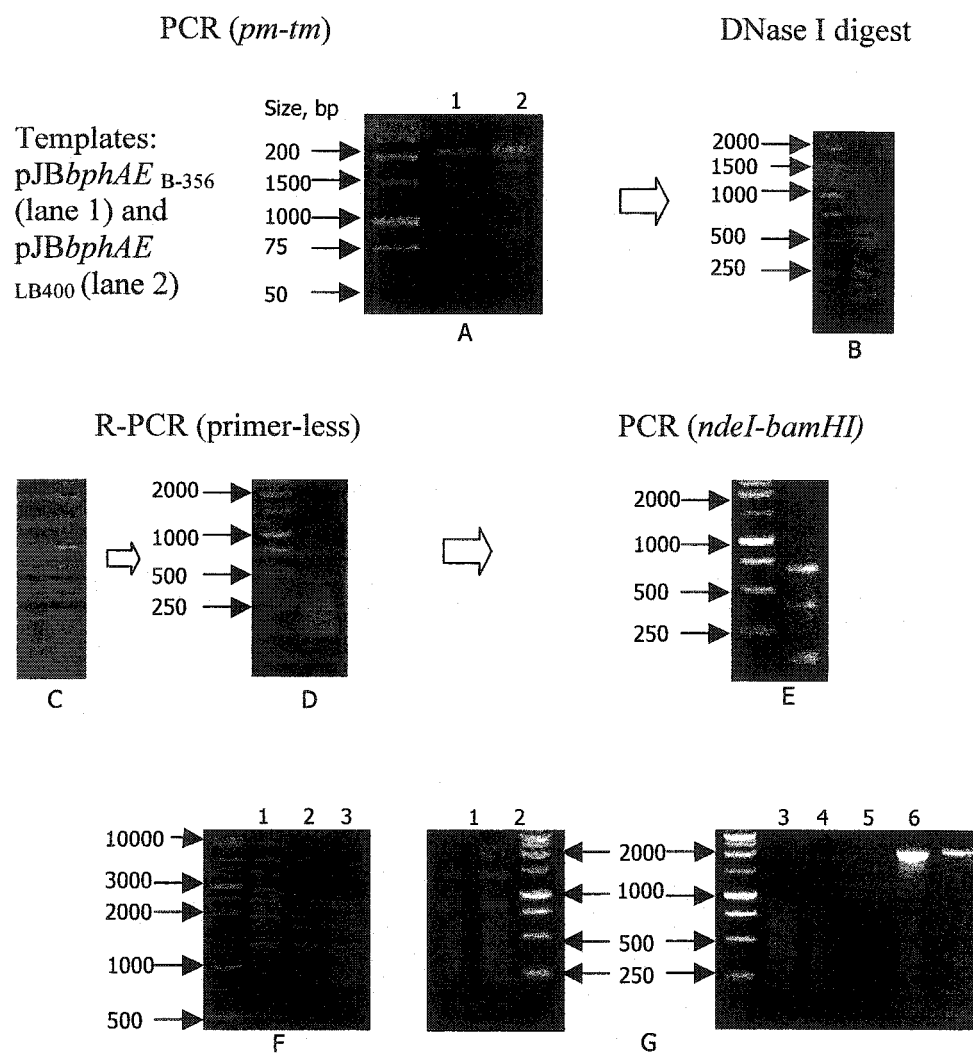**R-PCR (primer-less)**

PCR (*ndeI-bamHI*)

Figure 2.6 DNA shuffling of *bphAE* using DNase I (*Nci*I) to create fragments. See Table 4 and text for details.

GFX Band Prep kit, and included in a second reassembly PCR reaction, which was run for 55 cycles. Despite the increased number of cycles, no reconstruction was observed (Fig. 2.6D). After recovery of the DNA from the gel, followed by 30 or 55 additional cycles of R-PCR, products were used as template in amplification PCR reactions with *ndeI* and *bamHI* primers. This resulted in unexpectedly small products (700, 400, and 75-bp), and no product corresponding to full-length (2000 bp) *bphAE* genes (Fig. 2.6E).

A similar experiment using a $10°$ C lower hybridization temperature gave similar results (Table 4, Experiment 2). In this case, reassembly PCR contained DNA fragments ranging in size from 20 to 250-bp (most around 100-bp). Following 43 cycles of reassembly PCR, as described in Table 4, the product was amplified by PCR with primers *ndeI* and *bamHI*, (Fig. 2.6F). No bands corresponding to the size of *bphAE* were produced when both sets (LB400 and B-356) of *ndeI* and *bamHI* primer were used (Fig. 2.6F, lane 1), or when either primer set was used individually (Fig. 2.6F, lanes 2 and 3). Only a non-specific smear, representing DNA of all sizes, was observed.

Template concentration was another parameter that was varied in an attempt to generate full-length shuffled products. Experiment 3 (Table 4) reassembly PCR reactions contained purified DNA fragments ranging in size from 100 to 1000-bp, but mostly 500-bp and smaller, that were cycled 40 times as described in Table 4. Different volumes of the reconstruction PCR reaction then were used as template in amplification PCR reactions with primers *ndeI* and *bamHI*. Lower template concentrations should reduce the formation of non-specific product and favour amplification of the target, shuffled *bphAE*. The results after the final PCR reaction are shown for different volumes of the R-PCR reaction (Fig. 2.6G): with 3 µl (lane 1), 5µl (lane 2), 1 µl (lane 3), or 2 µl (lane 4)

of the R-PCR (containing 100 ng/μl DNA fragments) as template, or using 1 μl of R-PCR

(containing 50 ng/μl DNA fragments) (Fig. 2.6G, lane 5). Plasmid DNA ($pJBbphAE_{B-356}$

and $pJBbphAE_{LB400}$) was used as template in PCR reactions with the *ndeI* and *bamHI*

primers carried out as positive controls, and products of the correct sizes were observed

(Fig. 2.6G, lanes 6 and 7). Some shuffled product corresponding to *bphAE* in size (2000

bp) is visible in lanes 1 to 4 (Fig. 2.6G), when more of the DNA fragments were used in

the R-PCR (100 ng/μl versus 50 ng/μl). In addition to the full-length fragment, a smear

of DNA, and in some cases (lanes 3 and 4) the same non-specific 200 bp and 700 bp

bands observed in earlier experiment were present. Despite the limited amount of 2000

bp product, it was purified from the gel and cloned into pJB658. However, few

transformants were obtained for subsequent screening.

In an effort to reduce non-specific product formation observed when amplifying

shuffled product, and as suggested in the literature (Volkov and Arnold, 2000), parental

DNA fragments were amplified from their respective pJB*bphAE* plasmids using primers

(*bpm* and *atm*) that anneal further upstream and downstream from the target genes than

do primers *pm* and *tm* (Table 5). This should result in fragments consisting of the *bphAE*

coding regions from each strain, B-356 and LB400, flanked by plasmid sequence.

Consistent with this, the sizes of products following PCR amplification were 2.4-kbp

($bphAE_{LB400}$) and 2.3-kbp ($bphAE_{B-356}$) (Fig. 2.7A, lanes 1 and 2, respectively). DNase I

digestion of these parental templates (5 μg total, mixed equally) for various lengths of

time was followed by agarose gel (2%) electrophoresis. In Figure 2.7B, digestion of

bphAE with DNase I was stopped following 7, 8, 9, or 10 min (lanes 1 to 4, respectively).

Fragments of the desired size, generally 100-250 bp, were excised and purified from the agarose gel as described in *Material and Methods*.

The first reassembly PCR experiment (Table 5, column 2) followed by amplification with primers *pm* and *tm*, did not yield full-length shuffled *bphAE* genes (Fig. 2.7C, lane 1). A positive control PCR done using pJB*bphAE*$_{B-356}$ as template is shown in Fig. 2.7C, lane 2. In Experiment 2 (Table 5) reassembly PCR was carried out using more of the DNA fragments (30-40 ng/μl compared to 10-20 ng/μl), and at a lower hybridization temperature (50 °C compared to 53 °C) for the first 40 cycles of 70. The increase in DNA concentration and the lowering of the hybridization temperature are less stringent conditions, which are expected to help self-priming of the DNA fragments during the reassembly PCR. The final product was obtained by using 1.5 μl of the R-PCR reaction in an amplification PCR reaction with primers *pm* and *tm*. Some product of the correct size (2000-bp) was obtained (Fig. 2.7D, lane 1), but could not be cloned. A positive control PCR reaction was successfully done using pJB*bphAE*$_{LB-400}$ with the same primers (Figure 2.7D, lane 2).

2.3 *DNA Shuffling of* bphE

Since DNA shuffling of both subunits, *bphAE*, eliminates the need to insert *bphE* independently of *bphA*, part of this research has focused on shuffling *bphAE* together. DNA shuffling of both subunits also increases the potential for recombination simply by using more sequence space. Unfortunately, DNA shuffling of *bphAE*, as presented in the previous sections, resulted either in reconstruction of wild-type enzymes (using restriction enzymes to produce fragments), or in low yields of correctly-sized product

PCR (*bpm-atm*)

Templates:
pJB*bphAE* B-356
pJB*bphAE*LB400

Size, bp

2500
2000
1500
1000
750
500

A

DNase I digest

2000
1000
500
250
100

B

R-PCR

See *Summary Table 5*.

PCR (*pm-tm*)

3000
2000
1500
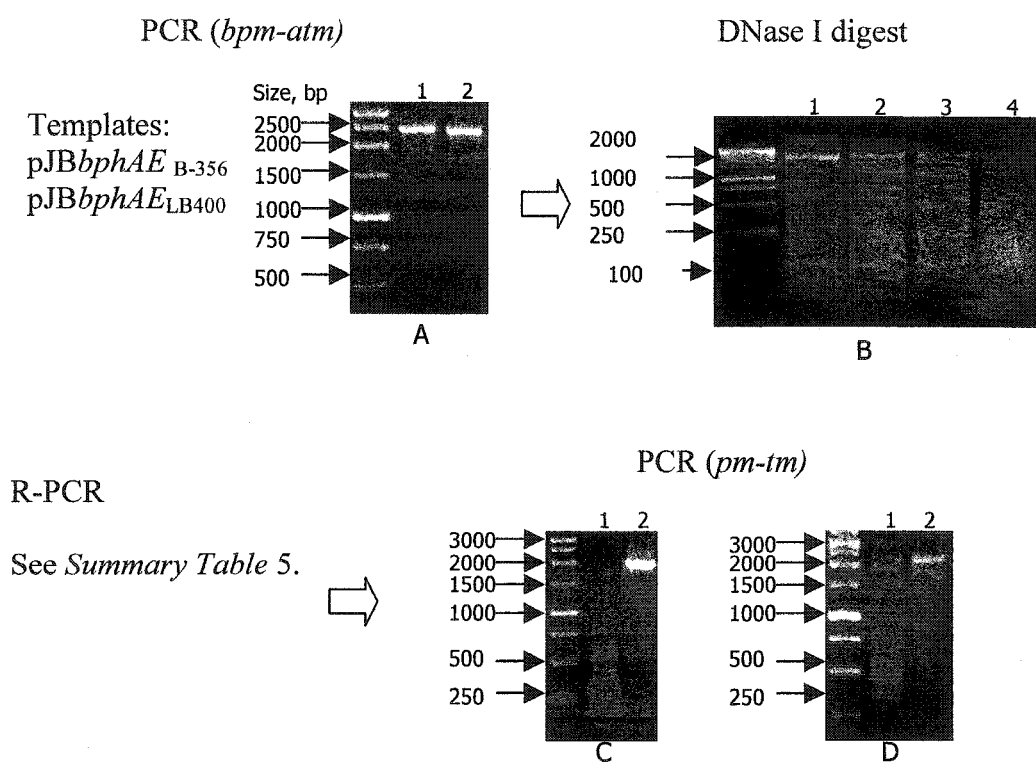1000
500
250

C

3000
2000
1500
1000
500
250

D

Figure 2.7 DNA shuffling of *bphAE* using DNAase I to create fragments.

Parental DNA, Digestion, Fragments sizes	Reassembly-PCR	A-PCR; Results
Experiment 1 a)*bphAE*B-356 and LB400 (*bpm-atm*): b)DNase I c)100-600 bp	DNA (approx.10-20 ng/μl), dNTPs (2 mM of each) R-PCR: 43 cycles: Hybridization temp: 53 °C Elongation time:10 s + 4 sec/cycle + 40 cycles: Hybridization temp: 55 °C Elongation time: 60 s + 5 sec/cycle	See Fig. 2.7C, lane 1
Experiment 2 a)*bphAE*B-356 and LB400 (*bpm-atm*): b)DNase I c)100-600 bp	DNA (approx.30-40 ng/μl), dNTP (1 mM of each) R-PCR; 40cycles: Hybridization temp: 50 °C Elongation time: 30 sec + 5 sec/cycle R-PCR, 30 cycles: Hybridization temp: 55 °C Elongation time: 90 sec + 5 sec/cycle	See Fig. 2.7D, lane 1

Table 5 Summary of DNA shuffling experiments where template DNA, *bphAE*, was obtained using the *bpm-atm* primer pair.

(using DNase I to fragment the templates). In either case very few colonies that could be screened were produced. Therefore, shuffling *bphE* alone was attempted.

The small subunit of BPDO, encoded by *bphE*, has been shown to influence the range of substrates that the enzyme can use. This was demonstrated using hybrid enzymes made by exchanging the α-subunit (encoded by *bphA*) and β-subunit (encoded by *bphE*) of BPDO from *Comamonas testosteroni* B-356 and *Burkholderia cepacia* LB400. (Hurtubise *et al.* 1998) (see *Introduction*). In their study (Hurtubise *et al.* 1998) had to express *bphA*$_{LB400}$ and *bphE*$_{B-356}$ from two different plasmids inside the same cell because none of their chimeras *bphA*$_{LB400}$/*bphE*$_{B-356}$, would produce active (α$_3$β$_3$) chimeric BPDOs. In this study, two different methods were used to try and clone *bphE*$_{B-356}$ downstream of *bphA*$_{LB400}$. In the first, *bphE* was cloned as a *Kpn*I-*Bam*HI fragment downstream from *bphA* (inserted *Sac*I -*Kpn*I) in pJB658. No α-subunit$_{B-356}$ could be detected by SDS-PAGE of the cell extract (Fig. 2.2A, lane 3), nor could activity be detected by HOPDA accumulation when expressed in *P. putida* (*bphFGBC*). (not shown). The second method is described below.

Our collaborators have successfully used restriction sites in *bphA* to replace selected regions of the gene with shuffled DNA (Barriault *et al.*, 2002). A similar approach could be used to allow shuffling of most of *bphE*. Common restriction sites early in the coding regions of *bphE* from each of *C. testosteroni* B-356 and *B. cepacia* LB400 would allow removal of wild-type sequence and insertion of shuffled product, without making adverse changes to DNA sequence that appear to affect its expression downstream of *bphA*.

Since common restriction sites early in the coding regions of $bphE_{LB400}$ and

$bphE_{B-356}$ are not present, one was inserted. To place an *Xho*I recognition sequence in

$bphE_{LB400}$, as already present in $bphE_{B-356}$, site-directed mutagenesis was used to produce

pJB*bphAE*$_{LB400xhoI}$ (see *Materials and Methods*). This would allow cloning of shuffled

bphE (*Xho*I-*Bam*HI) downstream of unshuffled $bphA_{LB400}$, in pJB*bphAE*$_{LB400xhoI}$, since an

*Xho*I site is already present in *pJBbphAE*$_{B-356}$. Following site-directed mutagenesis

mutant plasmid DNA was recovered from colonies of *E. coli* XL2, and the presence of

the *Xho*I site was confirmed by digestion with *Xho*I and *Bam*HI and analysis of the

fragment sizes by agarose gel electrophoresis (data not shown).

To obtain parental *bphE* DNA with *Xho*I sites present in both genes, prior to

shuffling, two more plasmids were constructed, pJB*bphE*$_{B-356}$ and pJB*bphE*$_{LB400xho}$ (see

Materials and Methods). In summary, each gene, $bphE_{LB400xhoI}$ and $bphE_{B-356}$, was PCR

amplified using primers that incorporate flanking restriction sites for cloning into

pJB658: *Kpn*I and *Bam*HI, upstream and downstream of the coding region, respectively.

Parental DNA fragments coding for $bphE_{LB400xhoI}$ and $bphE_{B-356}$, and flanked with

plasmid sequence upstream and downstream, were obtained by PCR using pJB*bphE*$_{LB-}$

$_{400xhoI}$ or pJB*bphE*$_{B-356}$ as templates, together with the primers *bpm* and *atm* (*Materials*

and Methods). The sizes of the PCR products were checked using agarose gel

electrophoresis (Fig. 2.8A, lanes 1 and 2): products correspond to the expected size of

bphE flanked with plasmid DNA (970 bp). Fragments of 50-125 bp produced by DNase

I digestion were recovered from agarose gels (Fig. 2.8B, DNA in lanes 1, 2, and 3, was

digested 10, 12, or 15 min, respectively), purified, and used to reconstruct full length

bphE. Reconstruction of *bphE* from DNA fragments is relatively easy as full-length

product has been obtained using a range of conditions. To maximize recombination, R-PCR reactions contained *bphE* DNA at relatively high concentration (60 ng/µl), and dNTPs (1 mM of each), and were initially subjected to 15 cycles of amplification with a relatively low hybridization temperature of 53°C and elongation times of 30 sec + 5 sec /cycle. To maximize yield, another 15 or 30 additional cycles of R-PCR with hybridization temperature of 55 °C and elongation time of 60 sec/cycle were used. The final amplification PCR step was carried out with 1 or 2 µl of the R-PCR reaction mixture and primers *pm* and *tm* (see *Materials and Methods*). Agarose gel electrophoresis of an aliquot of the final amplification step revealed a band corresponding to the expected size of 590 bp (Fig. 2.8C, lane 1)). Digestion of reconstructed *bphE* with *Xho*I and *Bam*HI produced a 500 bp fragment of DNA, as expected (Fig 2.8C, lane 2). The *Xho*I-*Bam*HI fragment was cloned downstream from $bphA_{LB400}$ in pJB$bphAE_{LB400xhoI}$ previously digested with the same enzymes to remove 500 of 567 bp of $bphE_{LB400}$. After ligation, plasmids were electroporated into screening host *E. coli (bphFGB)*.

3.1. Screening of BPDO containing shuffled bphE in E. coli BL21-Gold (bphFGB)

The basis for the most sensitive assay developed was detection of the 2,3-dihydroxybiphenyl product of BphB using Gibb's reagent, as described in *Materials and Methods,* and in sections of *Results* below. Electroporation of *E. coli* BL21G-Gold using ligation mixtures, as described in *Materials and Methods* typically resulted in approximately 120 transformants/ng DNA which also justified using this screening host.
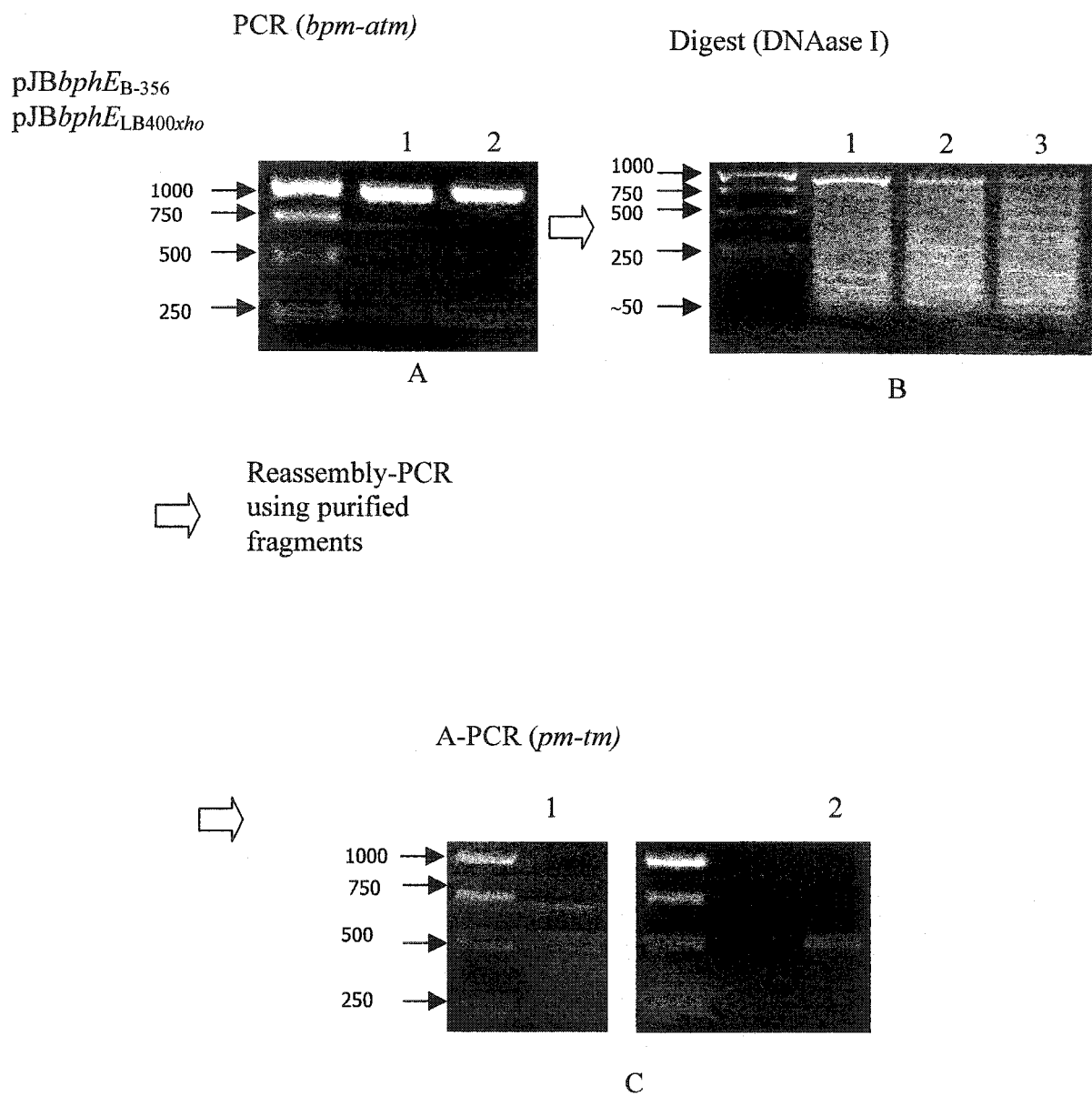
PCR (*bpm-atm*)

Digest (DNAase I)

pJB*bphE*B-356
pJB*bphE*LB400xho

Reassembly-PCR
using purified
fragments

A-PCR (*pm-tm*)

Figure 2.8 Agarose gel electrophoresis at various steps in the DNA shuffling of

bphE **using DNase I.** See text for details.

E. coli BL21G*(bphFGB)* cells harboring the mutant plasmid, pJB*bphAE*$_{LB400XhoI}$, were expected to produce active BPDO since the nucleotide substitution introducing the *Xho*I site is silent. This was confirmed by screening using the reaction of the product, 2,3-dihydroxybiphenyl, produced by BPDO (and BphB), with Gibb's reagent on agar plates as described in *Materials and Methods*. The colour formed by *E. coli BL21G (bphFGB)*-pJB*bphAE*$_{LB400XhoI}$ was of similar intensity to that formed when the same cells express wild-type BPDO$_{LB400}$ (see Fig. 2.11).

Somewhat surprisingly, hybrid enzymes made by exchanging the *Xho*I-*Bam*HI fragment of *bphE*$_{LB400}$ in *bphAE*$_{LB400XhoI}$ with the same fragment of *bphE*$_{B-356}$ to produce pJB*bphA*$_{LB400XhoI}$-*bphE*$_{B-356}$ as described in *Materials and Methods*, did not test positive in the Gibb's assay on agar plates, see Fig. 2. 11). Expression tests were conducted to determine whether or not the hybrid enzyme is soluble. It was found that *bphA*$_{LB400XhoI}$-*bphE*$_{B-356}$ is insoluble, as it is found primarily in the cell pellet (Fig. 2.9, lane 9) and not in the soluble fraction (Fig. 2.9, lane 8).

An estimated 10 000 colonies of *E. coli* BL21-Gold (*bphFGB*) containing pJB*bphA*$_{LB400xhoI}$ –*bphE*, with *bphE*$_{LB400}$ and *bphE*$_{B-356}$ shuffled together, were screened using the Gibb's method applied to agar plates. Colonies that had active biphenyl dioxygenase, using biphenyl as substrate, were picked for further experiments. Some examples are shown in Fig. 2.10. However, no metabolite accumulation could be distinguished easily using the plate assay when dichlorobiphenyls were supplied as the substrate (Fig. 2.10).
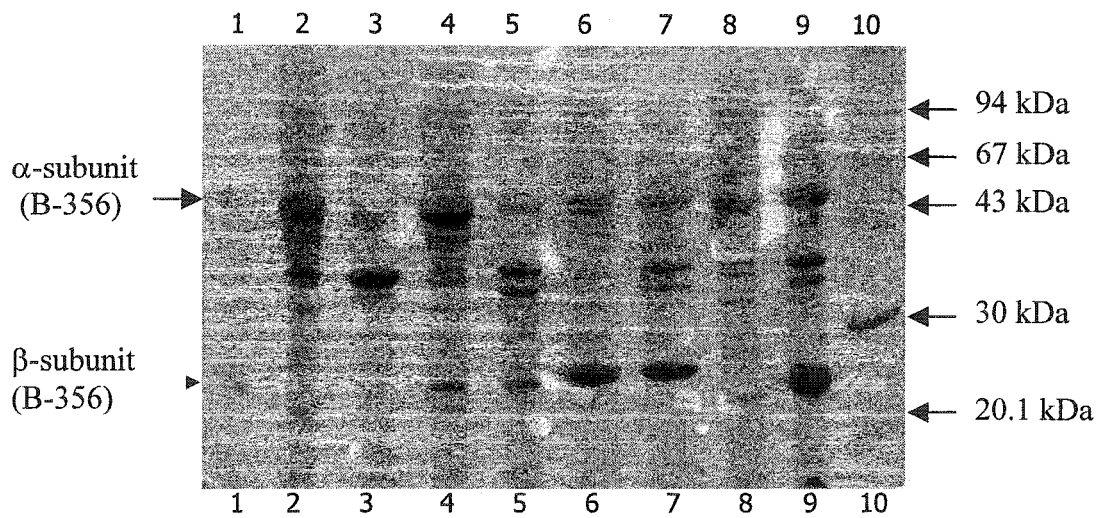
Figure 2.9 SDS-PAGE used to monitor expression of BPDO in *E. coli* (*bphFGB*).

E. coli (*bphFGB*)-pJB*bphAE*$_{B-356}$ uninduced cell fractions: soluble fraction in lane 2, and insoluble (pellet) fraction in lane 3. *E. coli* (*bphFGB*)-pJB*bphAE*$_{B-356}$ induced cell fractions: soluble (lane 4) and insoluble (lane 5) fractions. *E. coli* (*bphFGB*)-pJB*bphAE*$_{LB400}$ fractions were loaded in lanes 6 (soluble) and 7 (insoluble). *E. coli* (*bphFGB*) cell fractions, harboring the hybrid plasmid, pJB*bphA*$_{LB400xhol}$*bphE*$_{B-356}$, are in lanes 8 (soluble) and 9 (insoluble). Lane 1 contains purified BPDO$_{B-356}$ and lane 10 contains low-molecular weight markers. Arrows on the left indicate the position of α and β-subunits, as well as the sizes of the markers on the right.
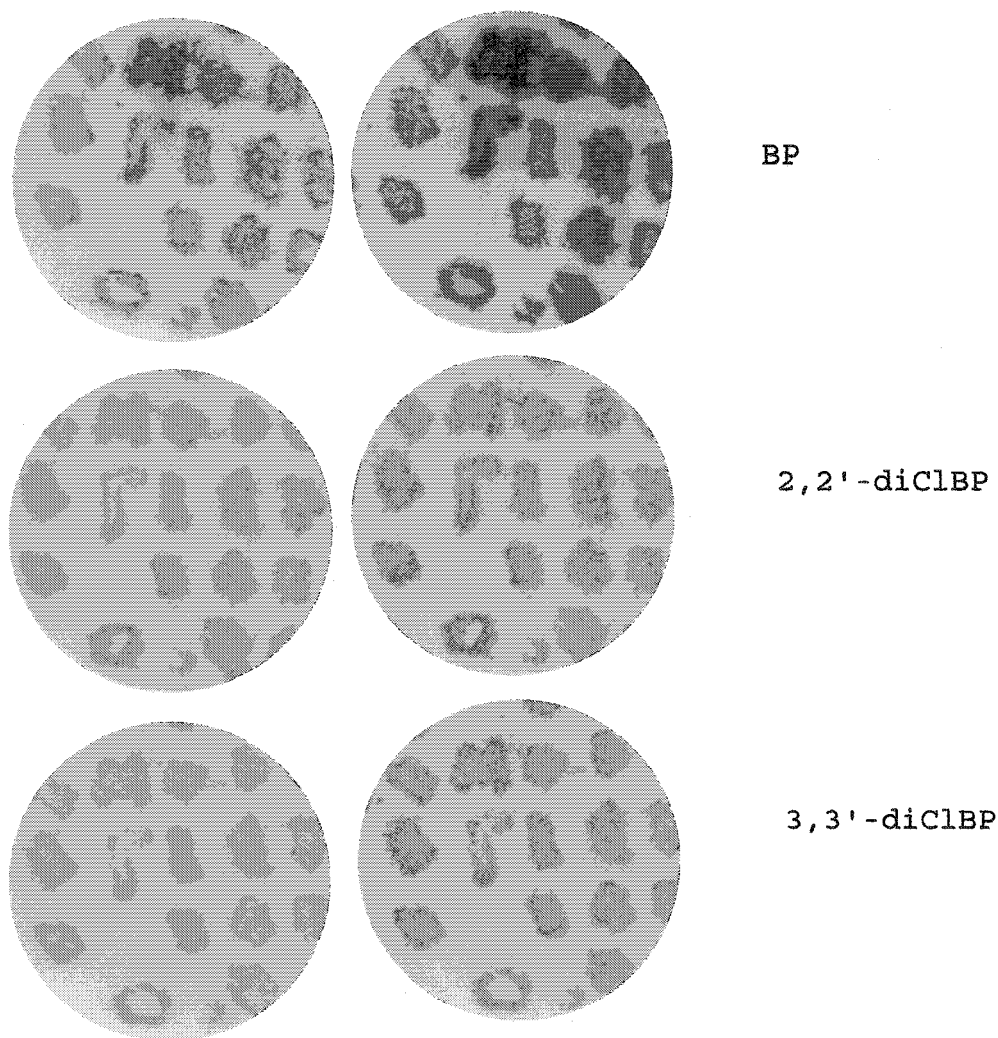
Figure 2.10 **Assay of BPDO activity using Gibb's reagent.** *E. coli* BL21 *(bphFGB)* expressing $BPDO_{B-356}$, $BPDO_{LB400}$, $BPDO_{LB400xhol}$ or several mutants with *bphE* shuffled were exposed to biphenyl or 2,2'-diClB or 3,3'-diClB vapors. Following exposure for 1 hour to biphenyl or 3 hours to the dichlorobiphenyls, the nitrocellulose filters were transferred to an agarose plates containing Gibb's reagent. The resulting plate was scanned after 5 min (plates on the left) and after 40 min (plates on the right).

3.2 *Modified screening of BPDO containing shuffled* bphE *in* E. coli *BL21-Gold* (bphFGB).

To screen mutant BPDOs, Barriault *et al.*, 2002, used extended incubations of *E. coli* cells, expressing *bphFGB*$_{LB400}$ in addition to *bphAE*, in the presence of chlorobiphenyls to let catechols accumulate. Catechols then oxidized slowly to produce brown pigments. This method was tested using the system described for screening in *E. coli* BL21-Gold (*bphFGB*), in *Materials and Methods*. Since little pigmentation was observed following 72-hour incubation of cells with 2,2-diClB, the cells were subsequently treated with Gibb's reagent, as shown in Fig. 2.11A and 2.11B. Transformation plates with approximately 500 *E. coli* BL21-Gold (*bphFGB*) colonies harboring distinct pJB*bphA*$_{LB400xhoI}$ –*bphE*$_{shuffled}$ constructs were tested by exposure to biphenyl or 2,2'-diClB. About 90% of the colonies displayed some activity towards biphenyl, while 36 colonies tested positive with 2,2'-diClB. These colonies were re-picked, and tested again using the same method to obtain the results shown in Figure 2.11.

BPDO activity towards biphenyl was monitored by simply letting catechols accumulate and oxidize to visible levels (Fig 2.11, top and left). However, it was still difficult to distinguish colonies with BPDO active on 2,2'-diClB or 3,3'-diClB (Fig. 2.11A, middle and bottom row). Following treatment with Gibb's reagent, cells exposed to 2,2'-diClB for 72 hours showed activity that appeared to vary from colony to colony presumably a result of different BPDO variants. This modification appears to improve the sensitivity of the published procedure. Unfortunately, it was not possible to identify dioxygenase activity towards 3,3'-diClB with this method (Fig 2.11, bottom).

Figure 2.11 Assay of BPDO activity in *E. coli (bphFGB)* using extended incubation in the presence of substrates, with or without Gibb's reagent. *E. coli (bphFGB)* cells are expressing either: *bphAE*$_{\text{B-356}}$ (B-356); *bphAE*$_{\text{LB400}}$ (LB); *bphAE*$_{\text{LB400xhoI}}$ (LBx); *bphA*$_{\text{LB400xhoI}}$-*bphE*$_{\text{B-356}}$ (ALBx/EB-356); several shuffled genes, pJB*bphA*$_{\text{LB400xhoI}}$-*bphE*$_{\text{shuffled}}$, that were shown, using 2,2'-diClB, to produce active BPDO (labelled 1-36); or several shuffled genes, pJB*bphA*$_{\text{LB400xhoI}}$-*bphE*$_{\text{shuffled}}$, that were randomly picked (unlabelled). The colonies were grown and treated as described in *Materials and Methods*. Each of the triplicate plates made was incubated at 30 °C with either: biphenyl (top); 2,2'-diClB (middle); or 3,3'-diClB (bottom). The scans in the left column (A) were obtained following a 72 hour incubation at 30 °C in the presence of their respective substrates. To obtain the results shown in column B, the colonies (filters) shown in column A were transferred to plates containing 0.025 % Gibb's reagent for 40 minutes before they were scanned. The first plate, shown below, is a reference plate.
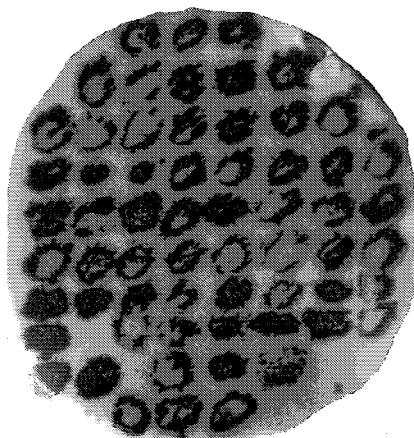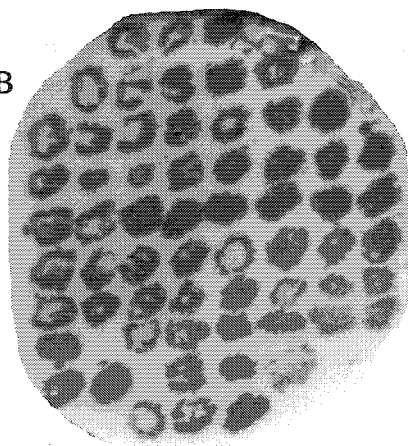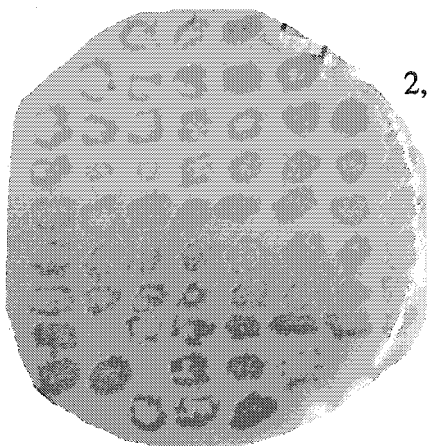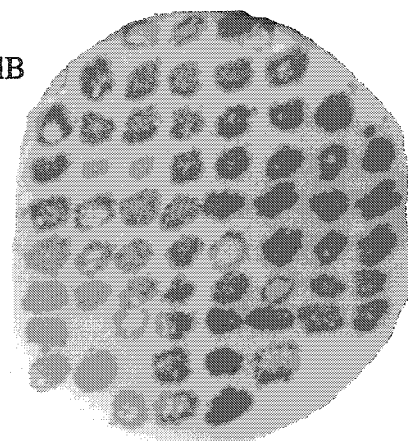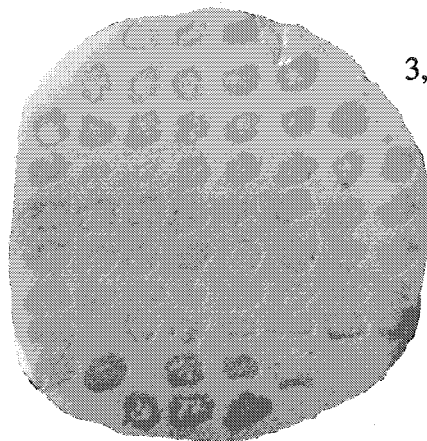
A   B

biphenyl

2,2'-diClB
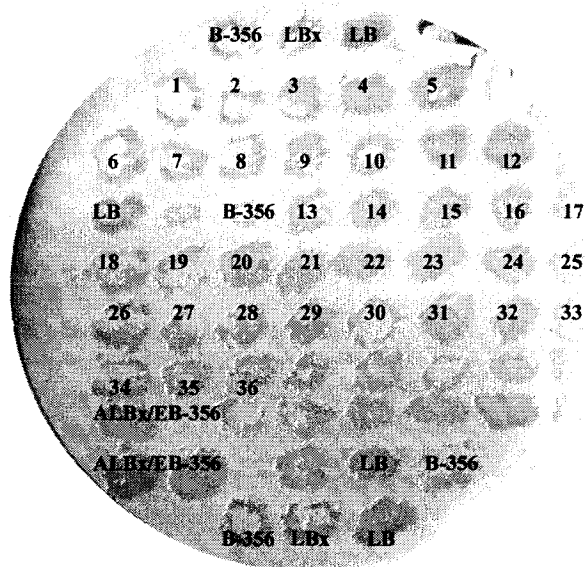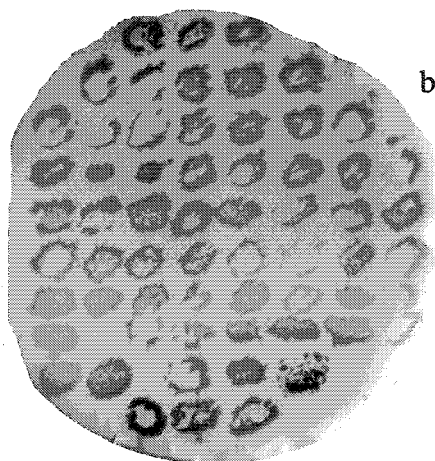
3,3'-'diClB

3.3 *Screening of biphenyl dioxygenase activity in liquid cultures*

Since it was difficult to distinguish BPDO activity on dichlorobiphenyls using the agar plate-based Gibb's assay, the liquid-based version of the Gibb's assay was evaluated using these substrates. *E. coli* BL21G (*bphFGB*) cells harboring pJB*bphAE*$_{B-356}$ or pJB*bphAE*$_{LB400}$ were grown and exposed to biphenyl substrates as described in *Materials and Methods*. As shown in Fig. 2.12, a general increase in absorbance was observed from 440 to 720 nm for cells expressing *bphAEFGB*, compared to uninduced cells. As expected when the substrate was biphenyl, cells expressing *bphAE*$_{B-356}$ showed the greatest activity, followed by those expressing *bphAE*$_{LB400}$, (Fig. 2.12A). BPDO$_{LB400}$ is reportedly better at degrading 2,2'-diClB than BPDO $_{B-356}$ but this was not observed in the experiment shown in Fig. 2.12B.

To screen cells for improved BPDO activity toward selected chlorobiphenyls the procedure was adapted for 96-well microtiter plates, as described in *Materials and Methods*. Colonies of *E. coli (bphFGB)*-pJB*bphAE* (shuffled, LB400, or B-356) were tested using the substrate 2,2'-diClB (Fig. 2.13). The signals produced, albeit small, are consistent with the ability of BPDO$_{LB400}$, but not of BPDO$_{B-356}$, to dihydroxylate 2,2'-diClB. Also as expected, most colonies expressing shuffled BPDOs exhibited activities that lie between those observed for the two parents.

Figure 2.12 Spectrophotometric assay of BPDO activity using liquid cultures of *E. coli* BL21G (*bphFGB*) and Gibb's reagent. Biphenyl was the substrate in A, whereas 2,2'-diClB was the substrate in B. Media only treated with Gibb's reagent is the bottom trace in both A and B. Induced (356+) cultures of *E. coli* (*bphFGB*)-pJB*bphAE*$_{B-356}$ were used, as well as one induced culture of *E. coli* (*bphFGB*)-pJB*bphAE*$_{LB400}$ (LB400xhoI). With biphenyl as the substrate the largest observed increase over media only was for the cells expressing BPDO$_{B-356}$ ($\Delta A_{460\ nm}$ of 0.3), followed by those expressing BPDO$_{LB400}$ ($\Delta A_{460\ nm}$ of 0.2), as expected. The uninduced cells (B-356-) also showed a higher signal than expected in A. With 2,2'-diClB as substrate, the spectral increase produced is similar for cells expressing BPDO$_{LB400}$ or BPDO$_{B-356}$ ($\Delta A_{500\ nm}$ of 0.1). The results shown in Figure 2.11 were taken after a 4-hour induction period. Data were also obtained after a 6-hour induction period but a prolonged induction period resulted in a decrease of detectable BPDO activity (not shown).

81

A

B
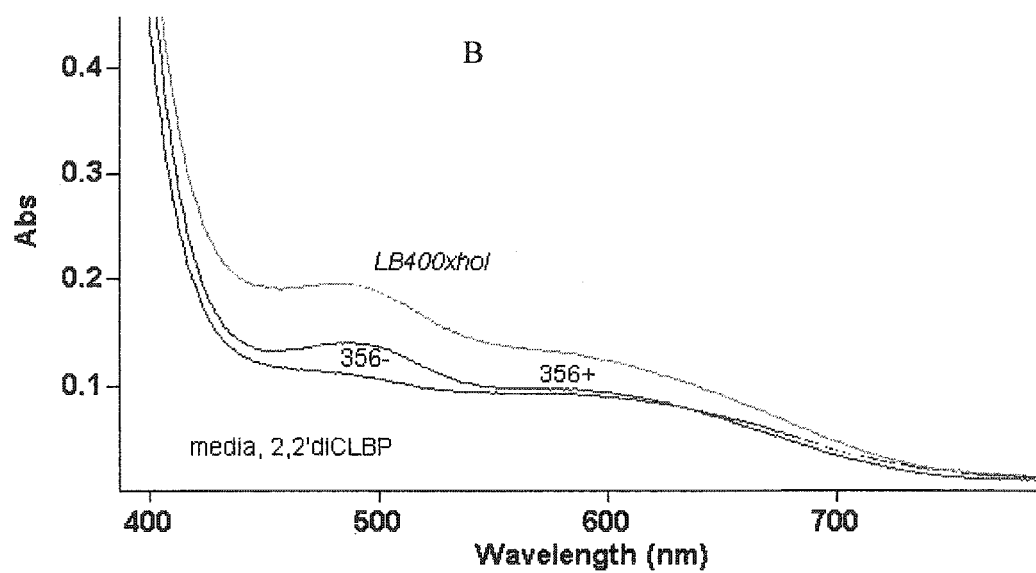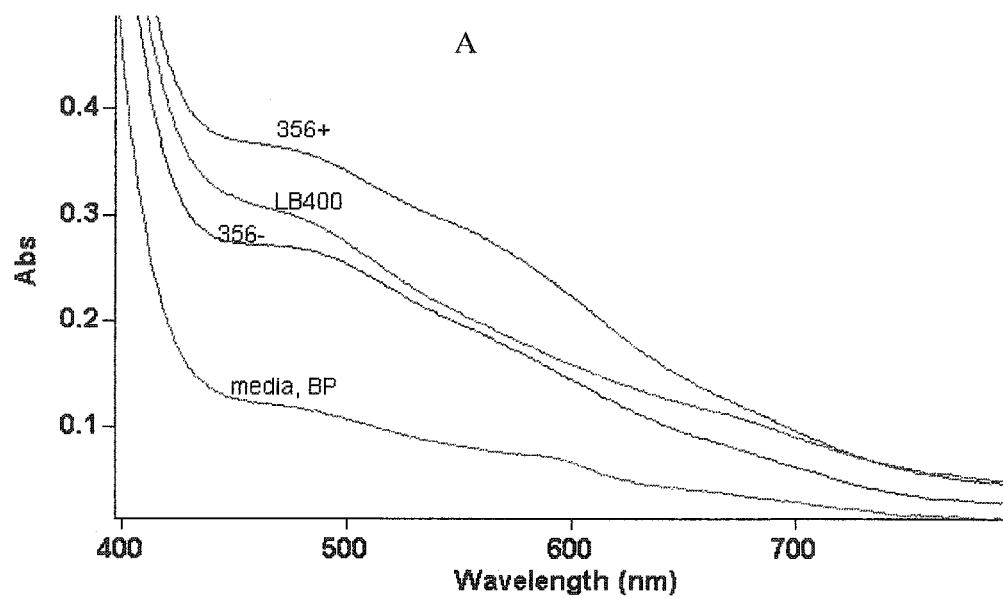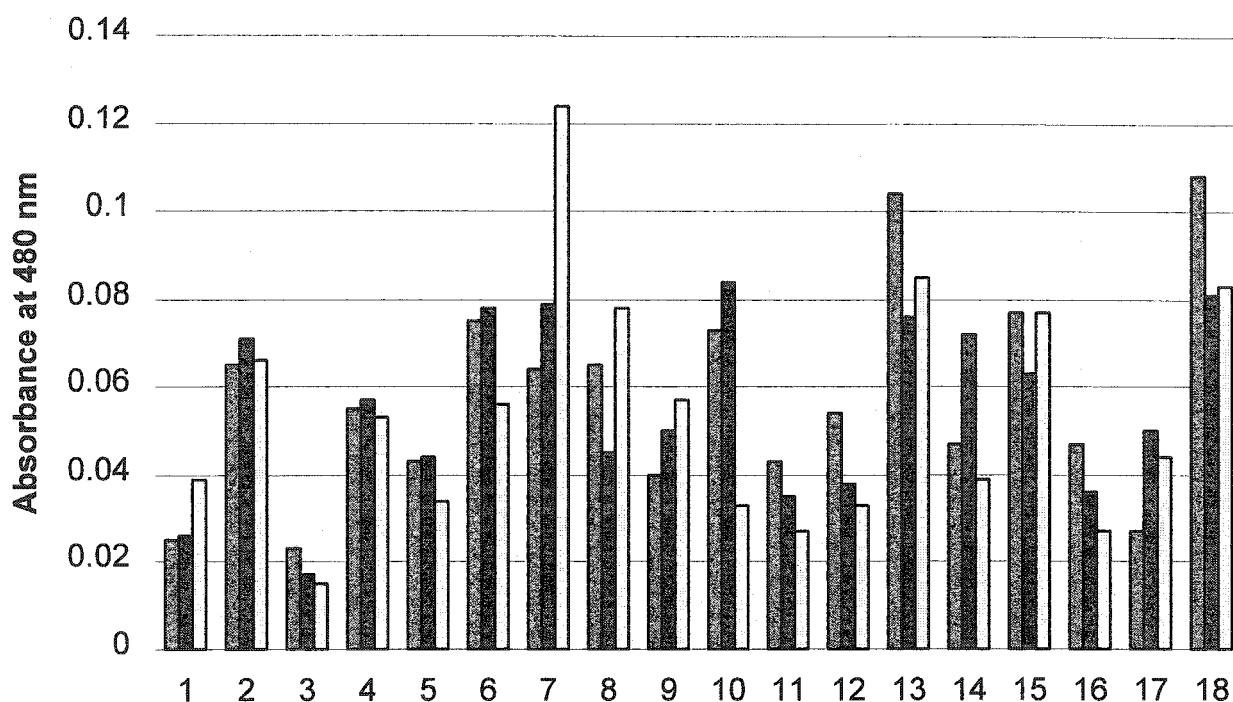
Figure 2.13 BPDO assay in *E. coli bphFGB* using the microtiter plate based assay
with Gibb's reagent. The absorbance at 480 nm following reaction with Gibb's reagent
is shown for 16 randomly picked colonies recovered from transformation plates, 1-16
(triplicate cultures and measurements were done for each as shown by the different
colours). Numbers 17 and 18 represent cells expressing $bphAE_{B-356}$ and $bphAE_{LB400}$,
respectively. The cells were incubated with 2,2'-diClB for 18 hours, as described in the
Materials and Methods.

SUMMARY

Different methods of DNA shuffling of biphenyl dioxygenases (BPDO) from two bacterial strains, *C. testosteroni* B-356 and *B. cepacia* LB400, were used to produce libraries that were screened using one of two systems: visual detection of HOPDA accumulation, or detection of the catechols produced by complex formation with Gibb's reagent.

Screening of mutant BPDO in vivo.

Different strategies were used to create the tools necessary to allow efficient screening of substrates specificities of large numbers of shuffled biphenyl dioxygenases. The screening host (*P. putida versus E. coli*), transformation method, and the method for the detection of the metabolites produced as a result of BPDO activity were all tested and evaluated.

Despite its superiority as an expression host, *P. putida* turned out to be a poor host for screening shuffled BPDOs, mainly because if its low transformation efficiency, and because it produced interfering pigments. *E. coli* BL21-Gold was chosen as the new screening host because of its superior transformation efficiency (Greener *et al.*, 1997). Transposon mutagenesis (de Lorenzo *et al*, 1990) allowed the chromosomal insertion of the necessary genes: $bphFGB_{LB400}$ or $bphFGBC_{LB400}$, encoding the electron transfer proteins (BphF and BphG), 2,3-dihydro-2,3-dihydroxybiphenyl dehydrogenase (BphB) and extradiol dioxygenase (BphC), all under the control of the *m*-toluate-induced promoter, *Pm*. The biphenyl dioxygenase genes *bphAE* were cloned into pJB658 under

the control of the promoter, *Pm*. Thus, all of the necessary genes, *bphAEFGB/C*, required for the conversion of BP/diClBs could be induced from the promoter, *Pm*, using *m*-toluate.

The screening of BPDO activity with the substrate biphenyl and using the accumulation of the yellow ring-cleavage compound, HOPDA, was used with some success, especially in *E. coli*. In the screening host, *E. coli* BL21 (*bphFGBC*), dioxygenase activity of $BPDO_{LB400}$, which has been shown to be less efficient at using biphenyl than $BPDO_{B-356}$ (Hurtubise *et al.*, 1998; N. Agar PhD thesis, 2002) could be detected reliably. However, activities on dichlorobiphenyls were not detected in this system. Recent work by Joern *et al.*, 2001 using Gibb's reagent to detect the product of dioxygenases (catechols) allowed this group to screen large numbers of colonies (10,000/day) directly from agar plates. The method was adapted in this work to screen shuffled BPDOs produced on solid and in liquid media. In *E. coli* BL21Gold (*bphFGB*) the catechols formed by the combined action of BPDO and BphB (dehydrogenase) on the aromatic substrate are detected by forming a colored complex with Gibb's reagent.

Screening for BPDO activity on solid media in E. coli *BL21-Gold (*bphFGB)

Since more colonies can be screened using solid rather than the liquid media, screening directly on agar plates is advantageous. Although a method using Gibb's reagent was more rapid and sensitive than HOPDA-based method for screening colonies for metabolites from the substrate biphenyl, it was still difficult to detect products from the di-chlorinated substrates used: 2,2'-diClB and 3,3'-diClB (Fig. 2.10, *Results*). Although preliminary screen of colonies on agar plates using biphenyl was useful to

determine if the shuffled BPDOs were active, it provides no insight into the altered specificity of the shuffled BPDOs. A more sensitive method was needed to screen mutant BPDOs for activity on chlorinated biphenyls.

BPDO's (B-356, LB400, and shuffled) were screened for activity on chlorinated biphenyls with somewhat more success using liquid cultures of *E. coli (bphFGB)*-pJB*bphAE*. The results shown in Fig. 2.12 demonstrate that the method can be used to determine with accuracy the levels of BPDO activity of variants relative to the wild-type(s) using 2,2'-diClB. As mentioned above, the disadvantage of this method is the time-consuming task of inoculating individual wells with the colony to be screened. Also, this method requires more materials and is, therefore, less-cost effective. These factors are important considerations since large (10 000-20 000 clones) libraries must be screened to increase the likely-hood of obtaining variants with interesting functional-structural features.

Recently, Barriault *et al.*, 2002, used prolonged incubation of *E. coli* cells expressing *bphAEFGB* on solid media (minimal agar plates and filters at 37°C) to detect the darkening of colonies due to the accumulation of catechols produced from biphenyl, 2,2'-diClB and/or 2,2',5,5'-tetraClB. Cells expressing $bphAE_{LB400}$ darkened within 24 hours exposure to 2,2'-diClB, while no darkening was observed for cells expressing a control plasmid or $bphAE_{B-356}$. The incubation period of the cells with di-chlorinated substrates was extended to 72 hours or more, in our experiments, which allowed metabolite detection from 2,2'-diClB, but not from 3,3'-diClB. Detection of activity was best when the cells were subsequently treated with Gibb's reagent (Fig 2.11). This

method can be used to screen a larger library of shuffled BPDOs on 2,2'-diClB, allowing interesting variants to be picked for further characterization.

DNA shuffling

This research was focused on family shuffling of both genes, *bphAE*, encoding the α and β-subunits, and on shuffling of *bphE* alone, encoding the β-subunit.

As discussed in *Results*, DNA shuffling of *bphAE* was not successful in producing libraries that could be screened. The DNA shuffling was done either using restriction enzymes or DNase I to create the fragments of parental DNA used in the reassembly-PCR process. Progeny of the shuffling process using the restriction enzyme method were shown to be mainly identical to either parent. On the other hand, family shuffling of *bphAE* using DNase I to create the fragments used in reassembly PCR did not result in appreciable quantities of full-length product. There are many factors that influence the reassembly PCR and the outcome of the shuffling process, some of which were varied in an effort to obtain shuffled *bphAE*. However, none of the conditions used allowed for the proper reconstruction of these two genes together.

Family shuffling of *bphE* from *C. testosteroni* B-356 and *B. cepacia* LB400 was done since the β-subunit has been shown to influence the selectivity of BPDO towards chlorobiphenyls (Hurtubise *et al.*, 1998, Chebrou *et al.*, 1999). Unlike DNA shuffling of both subunits, shuffling of *bphE* resulted in large quantities of shuffled product that could be screened. The inability to obtain full-length shuffled *bphAE* may be due, in part, to the different inter-genic regions, 36 bp and 117 bp, in $bphAE_{B-356}$ and $bphAE_{LB400}$, respectively. In addition to impeding the reassembly-PCR process by not allowing

hybridization between those DNA segments that are missing in DNA from one strain, B-356, most changes in the inter-genic region of *bphAE* are likely to affect the expression of *bphE*, downstream.

Further screening of the *bphE* variants produced by DNA shuffling should lead to enzymes with improved and/or altered specificity/selectivity toward specific PCB congeners. Further characterization of *bphE* variants obtained by DNA shuffling will hopefully lead to insight into the structural features responsible for the specificity/selectivity of BPDO and eventually to improvements in the efficiency of biphenyl dioxygenase to metabolize all, including the most persistent, PCBs.

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