POLYCHLORINATED BIPHENYL (PCB) METABOLISM IN PSYCHROTOLERANT AND MESOPHILIC BACTERIA: FROM SUBSTRATE UPTAKE TO CATALYSIS

By

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ABSTRACT

Bioremediation of soil contaminated with polychlorinated biphenyls (PCBs) is potentially a cost-effective cleanup strategy as it can be performed on-site. My general aims were to isolate PCB-degrading bacteria from PCB-contaminated Arctic soil and to determine if these bacteria are adapted for PCB degradation at low temperature. An Arctic soil bacterium and a mesophilic bacterium were compared on the basis of PCB degradation by whole cells, activities of purified biphenyl dioxygenases, regulation of genes encoding these enzymes and the mechanism of biphenyl uptake by these bacteria. In addition to these studies, I demonstrated bioremediation of weathered PCB-contaminated Arctic soil in a microcosm.

PCB-degrading bacteria were isolated at 7°C from PCB-contaminated Arctic soil. 16S rDNA sequences indicate that these isolates are members of the genus *Pseudomonas.* At 7°C, PCB congeners that were transformed by the Arctic bacteria and by the mesophilic PCB-degrader, *Burkholderia* sp. strain LB400, were transformed at higher initial rates by the Arctic soil isolates. Furthermore, in contrast to PCB transformation by LB400, PCB transformation by the Arctic bacteria was diminished at high temperature (50°C). These observations suggest that the Arctic soil bacteria are adapted to degrade PCBs at low temperature. The effect of temperature on the kinetics of biphenyl dioxygenase and the regulation of *bph* genes from LB400 and the Arctic soil bacterium *Pseudomonas* sp. strain Cam-1 were determined.

The biphenyl dioxygenase from Cam-1 ($BPDO_{Cam1}$) and LB400 ($BPDO_{LB400}$) were overproduced in *E.coli* and purified anaerobically. Consistent with observations using whole cells, $BPDO_{Cam1}$ displayed high catalytic activity with biphenyl (k_{cat}^{app}) at low

temperature and low thermal stability compared to $BPDO_{LB400}$. However, the respective catalytic efficiencies ($k_{cat}^{app} / K_m^{app}$) of $BPDO_{Cam1}$ and $BPDO_{LB400}$ were not significantly different at 15°C and 25°C. The constitutive expression of *bphA* in LB400 was diminished at low temperature. In contrast, the expression of *bphA* in Cam-1 was inducible, and was expressed at 7°C and 30°C. Thus, lower expression of BPDO in LB400 than in Cam-1 might contribute to the difference in PCB transformation activities by whole cells at 7°C. The induction of *bphA* in Cam-1 was highest with biphenyl although naphthalene, salicylate, 2-chlorobiphenyl and 4-chlorobiphenyl induced *bphA* in Cam-1 to levels above background.

To begin to elucidate how PCB-uptake affects PCB metabolism, biphenyl-uptake by Cam-1 and LB400 was investigated. In both microorganisms, biphenyl was actively transported and uptake did not depend on biphenyl catalysis. Moreover, biphenyl-uptake showed saturable kinetics with respect to biphenyl concentration. These data suggest that biphenyl-uptake occurs via an active biphenyl transport system.

To date, the enzymology of PCB degradation has been the main target for generating recombinant strains with improved ability to degrade PCBs. This thesis demonstrates that the regulation of *bph* genes and PCB uptake by bacteria are also important targets for improving the potential of PCB bioremediation. Thus, future investigations should include identifying potential biphenyl uptake systems and characterizing regulatory proteins involved in *bph* gene expression. As a result, it might be possible to design biphenyl uptake systems with improved ability to transport PCBs, and regulatory proteins that respond to different inducers, thereby optimizing *bph* gene expression in different environments.

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ABBREVIATIONS AND SYMBOLS

A	absorbance
bp	base pairs
BPDO	biphenyl dioxygenase
°C	degrees Celsius
Ci	Curie
cpm	counts per minute
DCCD	1,3-dicyclohexylcarbodiimide
DNA	deoxyribonucleic acid
DNP	2,4-dinitrophenol
dNTP	deoxynucleoside 5'-triphosphate
ε	extinction coefficient
E_a	activation energy
g	gram
ΔG^*	change in Gibbs free energy
GC	gas chromatography
Gm	gentamycin resistance gene
h	hour
ΔH^*	change in enthalpy
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
ht	his-tagged
ISP	iron sulfur protein
k_{cat}	catalytic constant
K_m	Michaelis constant
k_{cat}/K_m	specificity constant
kb	kilobase pair
kDa	kilodalton
lacZ	gene encoding beta-galactosidase
log	logarithmic
M	molar
MES	2-(4-morpholino)-ethane sulfonic acid
mg	milligram
min	minute
µl	microlitre
mM	millimolar
µM	micromolar
n	number of replicates
nm	nanometer
NADH	β-nicotinamide adenine dinucleotide
no.	number
ONPG	o-nitrophenyl-beta-D-galactopyranoside
PAS	phosphate-buffered mineral salts
PAGE	polyacrylamide gel electrophoresis
PCB	polychlorinated biphenyl
PCR	polymerase chain reaction
ppm	parts per million
rpm	revolutions per minute
s	second
S_{ab}	association coefficient
ΔS^*	change in entropy
SDS	sodium dodecyl phosphate
UV	ultraviolet
Wt	weight
x-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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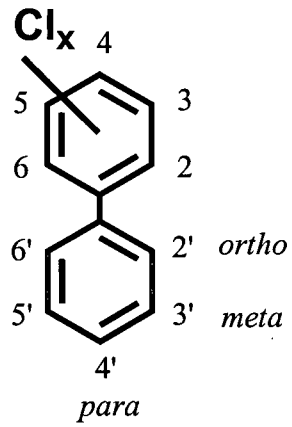
INTRODUCTION

1. Sources of PCB contamination and clean-up strategies.

Polychlorinated biphenyls (PCBs) are synthetically produced by the direct chlorination of biphenyl, resulting in 209 possible congeners (Fig.1). The chemical synthesis of PCBs for industrial use began approximately 80 years ago, and they were marketed under trade names such as Aroclor (Monsanto, USA), Clophen (Bayer, Germany) and Kanechlor (Kanegafuchi, Japan). PCB mixtures were classified with respect to the percent by weight of chlorine contained in the mixture. For example, Aroclor 1242 signifies the 12 carbon atoms of biphenyl and 42% chlorine by weight. PCBs have low water solubility, low electric conductivity and are extremely heat and chemical resistant. These physical and chemical properties meant that PCBs were ideal flame retardants, organic diluents, plasticizers and insulating fluids in transformers and capacitors. However, the extensive application of PCBs has resulted in widespread contamination of the environment. Due to the nature of PCBs, these compounds persist in the environment and have been shown to accumulate in the food chain and cause adverse health effects (reviewed in Abramowicz, 1990). As a result, the production of PCBs was banned in North America and Japan in 1977 and in Germany in 1983.

Fig.1. Structure of a polychlorinated biphenyl (PCB). The biphenyl nucleus may be chlorinated at any of 10 possible positions. Different PCB congeners vary in the number and position of the chlorine atoms.



Several military bases in the Canadian Arctic used PCBs in electrical equipment. The area surrounding many of these sites is now contaminated with PCBs. In some of these locations the concentration of PCBs exceeds 50 mg/kg of soil and so must be remediated in accordance with the Canadian Environmental Protection Act. In fact, certain land claim agreements specify remediation of soil that contains as little as 5 mg of PCBs/kg of soil. PCB-contaminated soil has been treated by incineration. However, incineration of contaminated Arctic soil would be costly since excavation and transport of soil to an incinerator, or construction of an incineration plant on-site, would be required. Alternatively, bioremediation of PCB-contaminated Arctic soil may be more cost effective since this technology can be used on-site with considerably less specialized equipment. Moreover, previous and current research of PCB biodegradation might enable mineralization of PCBs to carbon dioxide, chloride and water, whereas incineration can produce toxic byproducts.

Many commercial applications of PCBs used congeners that contain more than six chlorine substituents. However, most of the aerobic PCB-degrading bacteria that have been isolated so far degrade up to tetra-chlorobiphenyls, although some bacteria can degrade certain congeners with up to 6 chlorine substituents (Bedard *et al.*, 1987a; Bedard and Haberl, 1990). Consequently, much of the PCB content in contaminated sites is resistant to aerobic microbial metabolism. Several researchers have demonstrated anaerobic microbial reductive dehalogenation of PCBs (Bedard *et al.*, 1995; Kuipers *et al.*, 1999; Quensen *et al.*, 1988; Williams, 1997; Wu *et al.*, 1998 and 2000). At least six distinct microbial dechlorination patterns have been observed in different anaerobic aquatic sediments. These patterns are distinguished by congener selectivity and position

of the chlorine substituents that are removed (Bedard and Quensen III, 1995). None of these patterns show reductive dehalogenation of *ortho*-substituted chlorines. The anaerobic treatment of PCB-contaminated soil can potentially transform PCBs resistant to aerobic microbial attack to less chlorinated congeners that are susceptible to aerobic microbial mineralization (Abramowicz, 1990). Although a bacterium capable of dehalogenating hydroxylated PCBs has been isolated (Wiegel *et al.*, 1999), isolation of bacteria capable of PCB dehalogenation, has been unsuccessful to date. As a result, various anaerobic sediments containing mixed populations of anaerobic microorganisms have been used in experiments investigating PCB dehalogenation. It is currently believed that certain PCB congeners serve as catabolic electron acceptors under anaerobic conditions (Mohn and Tiedje, 1992).

Sequential anaerobic-aerobic degradation of PCBs has been previously demonstrated. Anid *et al.* (1993) demonstrated sequential anaerobic-aerobic treatment of soil samples spiked with Aroclor 1242. In their experiment, the soil was incubated anaerobically for 76 weeks, then aerobically for 96 days. Moreover, they showed that anaerobic soil can be aerated with H_2O_2 and that the aerobic step is enhanced by bioaugmentation. Shannon *et al.* (1994) also reported sequential treatment of soil contaminated with Aroclor 1242. Profiles demonstrating anaerobic transformation of highly chlorinated PCBs to less chlorinated congeners and subsequent aerobic degradation of these PCBs were shown. However, the details of their experimental method were not revealed. Finally, Evans *et al.* (1996) demonstrated treatment of soil containing weathered PCBs, probably from Aroclor 1248. In their experiment, the

anaerobic treatment continued for 19 weeks, followed by a 19-week aerobic treatment that included bioaugmentation.

Clearly, the potential for PCB bioremediation exists. However, more research is required to ensure the reproducibility and efficiency under variable conditions of this technology. For example, the optimal duration and physiochemical conditions for anaerobic dehalogenation should be established. In addition, parameters affecting the efficiency of aerobic PCB degradation require further elucidation. This thesis investigates how the catalytic activity of PCB-degrading enzymes, the regulation of genes encoding these enzymes, and the transport of substrate across bacterial cell membranes, affect the efficiency of aerobic PCB biodegradation.

2. Aerobic PCB Biotransformation.

Forest fires transform lignin to a variety of aromatic compounds, which has generated a selective advantage for microorganisms that use aromatic compounds as growth substrates (Harayama and Timmis, 1989). Microbial degradation of aromatic compounds generally occurs in two stages, which are often referred to as upper and lower pathways (Fig.2). Upper pathways are characterized by the destabilization of the aromatic compound, whereas lower pathways convert destabilized products to TCA cycle intermediates such as acetyl-CoA. Different microorganisms use a variety of upper pathways, which reflects the presence of different aromatic compounds in the environment and the metabolic diversity of microorganisms. However, upper pathways generally produce dihydroxylated aromatic intermediates such as protocatechuate,

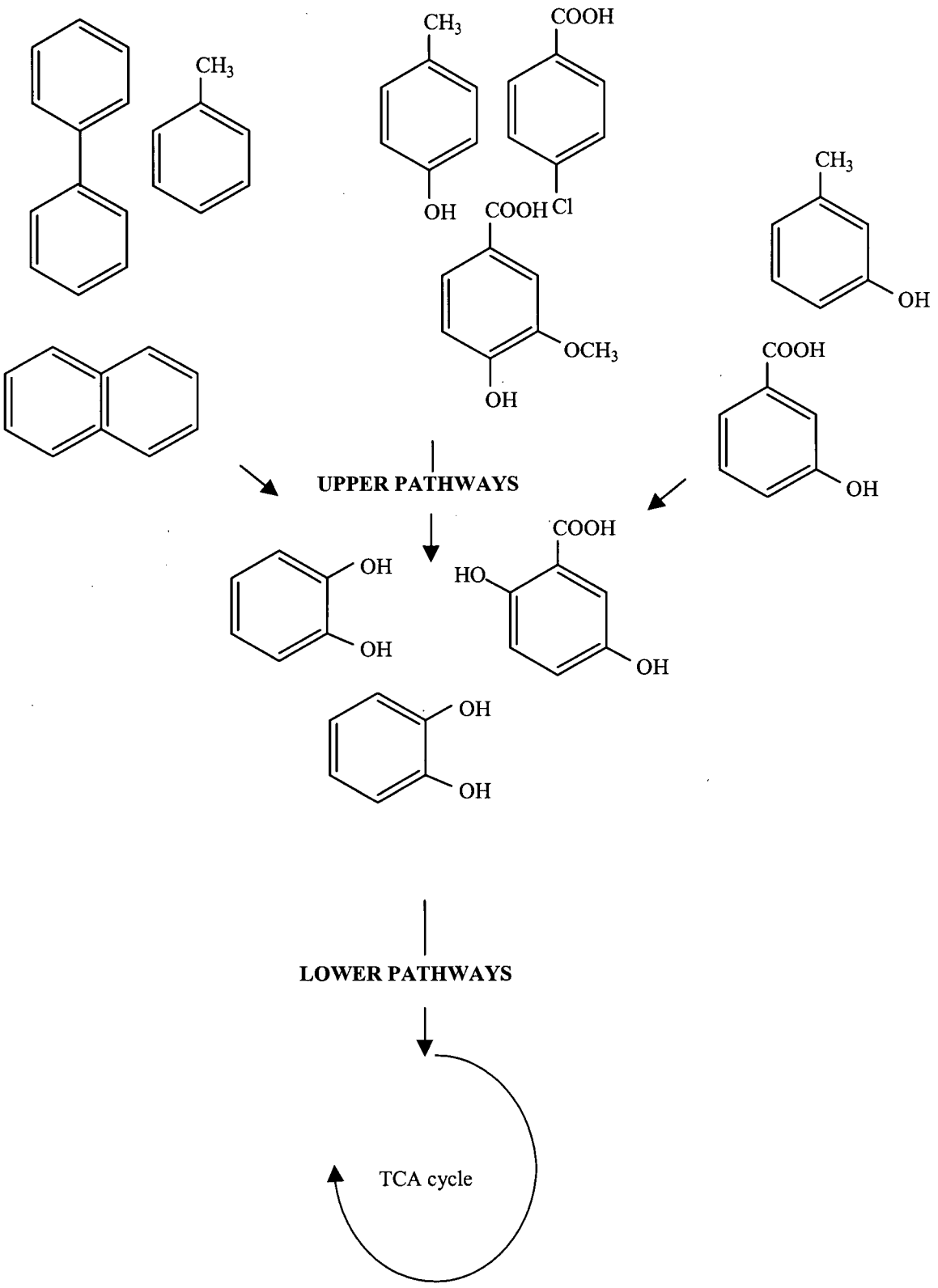


Fig. 2. Upper and lower pathways of aromatic compound degradation. Upper pathways convert diverse aromatic compounds to central intermediates such as catechol, protocatechuate and gentisate, which are further metabolized to CO_2 and H_2O through the TCA cycle.

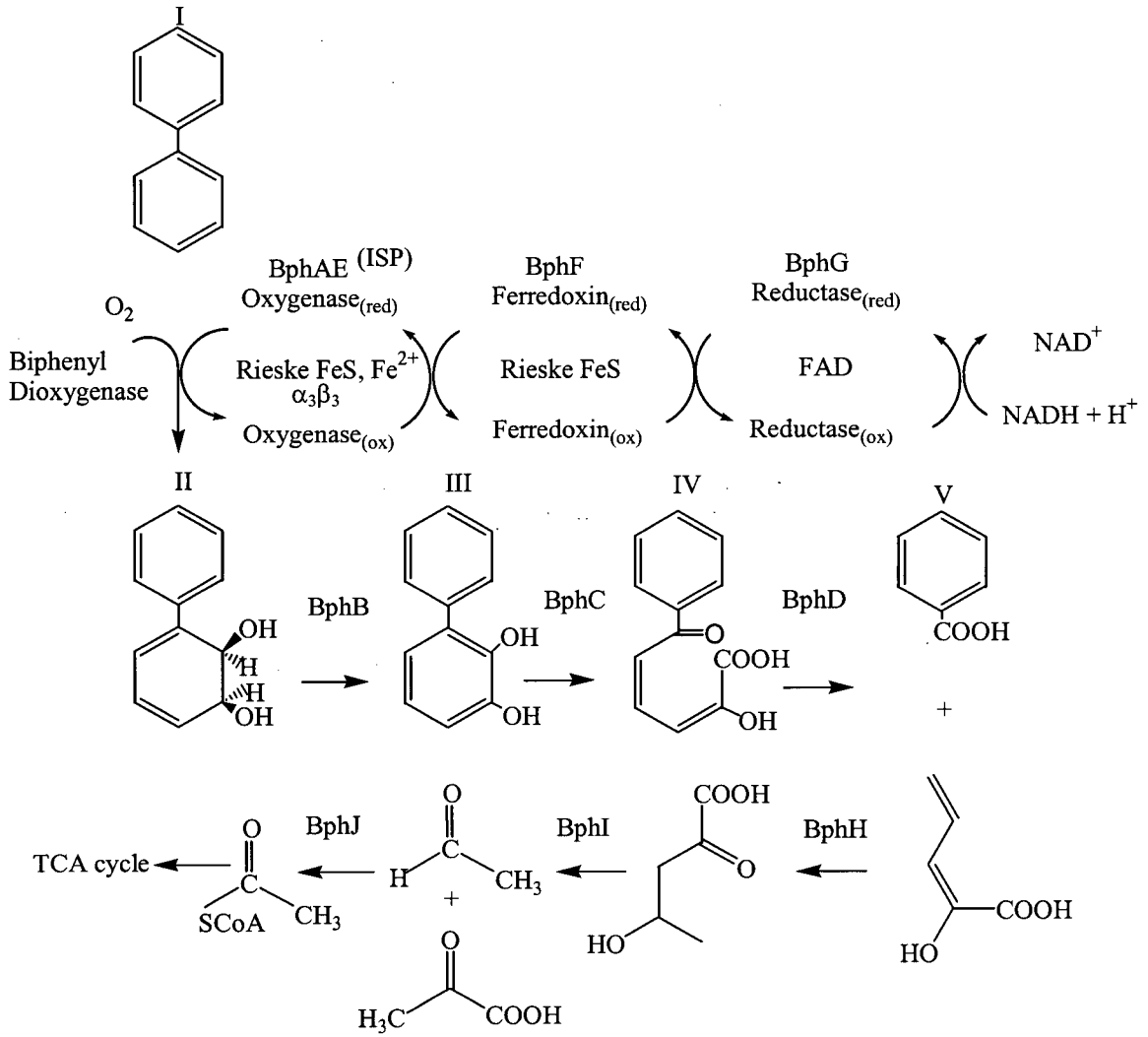


Fig. 3. The catabolic pathway of biphenyl and PCB biodegradation. Nomenclature used corresponds to *Burkholderia* sp. strain LB400. Compound I, biphenyl; compound II, *cis*-(2R,3S)-dihydrodihydroxy-1-phenylhexa-4,6-diene (dihydrodiol); compound III, 2,3-dihydroxybiphenyl; compound IV, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (yellow *meta*-cleavage product); compound V, benzoate; VI, 2-hydroxy-penta-2,4-dienoic acid. BphAE, terminal oxygenase; BphF, ferredoxin; BphG, reductase; BphB, dihydrodihydroxybiphenyl dehydrogenase; BphC, 2,3-dihydroxybiphenyl dioxygenase; BphD, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase; BphH, 2-hydroxy-6-oxo 6-phenylhexa-2,4-dienoate hydratase; BphI, 4-hydroxy-2-oxoalerate aldolase; BphJ, acetaldehyde dehydrogenase.

catechol or gentisate. These central intermediates are subsequently transformed to TCA cycle intermediates via lower pathways, such as the *ortho*-cleavage pathway, the *meta*-cleavage pathway or the gentisate pathway (Harayama and Timmis, 1989).

Aerobic PCB biotransformation is achieved using the biphenyl catabolic pathway (Furukawa, 1994; Furukawa and Miyazaki, 1986). The initial reaction is catalyzed by biphenyl dioxygenase (BPDO), a multicomponent enzyme consisting of a FAD-containing reductase (BphG), a Rieske-type ferredoxin (BphF) and a two-component oxygenase with $\alpha_3\beta_3$ constitution (BphAE) (Fig.3). BphG and BphF transfer electrons from NADH to BphAE, which transforms biphenyl to *cis*- (2*R*, 3S)-dihydrodihydroxy-1-phenylhexa-4,6-diene. The dihydrodiol is subsequently oxidized then cleaved via the successive activities of dihydrodihydroxybiphenyl dehydrogenase (BphB) and 2,3-dihydroxybiphenyl dioxygenase (BphC). The ring cleavage product is yellow and is hydrolyzed to benzoate and 2-hydroxy-2, 4-dienoic acid via the hydrolase BphD. The biphenyl lower pathway, which includes hydratase, aldolase and dehydrogenase enzymes, transforms 2-hydroxy-2, 4-dienoic acid to TCA cycle intermediates (Fig.3). Whereas, benzoate is catabolized to catechol by the benzoate upper pathway before being transformed to TCA cycle intermediates.

The mechanism for biphenyl metabolism was initially determined by analyzing metabolites of biphenyl transformation in wild type and mutant bacteria (Catelani *et al.*, 1971). Bacteria that aerobically degrade PCBs include members from the genera *Pseudomonas*, *Burkholderia*, *Alcaligenes*, *Ralstonia*, *Comamonas*, *Sphingomonas* and *Rhodococcus* (Ahmad *et al.*, 1990; Bedard *et al.*, 1987b; Commandeur *et al.*, 1996; Furukawa *et al.*, 1986; Masai *et al.*, 1995; Romine *et al.*, 1999). Some of these bacteria

can grow on monochlorinated and dichlorinated biphenyls, and most co-metabolize more highly chlorinated biphenyls while using biphenyl as a growth substrate (Abramowicz, 1990; Bedard *et al.*, 1986; Boyle *et al.*, 1992). In general, less chlorinated PCBs are more easily biodegraded than highly chlorinated PCBs, and the less chlorinated biphenyl ring is preferentially transformed (Furukawa, 1979).

The biphenyl pathway was confirmed by the discovery of genes that encode necessary enzymes for biphenyl metabolism. The *bph* operon was first cloned from the bacterium *Pseudomonas pseudoalcaligenes* KF707 (Furukawa and Miyazaki, 1986; Taira *et al.*, 1992). Since then, *bph* genes from several Gram-negative and Gram-positive bacteria have been cloned, and shown to be present on bacterial chromosomes, plasmids and transposable elements (Bergeron *et al.*, 1994; Hayase *et al.*, 1990; Kikuchi *et al.*, 1994; Masai *et al.*,1995; Masai *et al.*,1997; Merlin *et al.*, 1997; Nishi *et al.*, 2000; Mondello,1989; Peloquin and Greer, 1992; Springael *et al.*, 1993) (Fig.4).

Although the PCB degradation pathway is similar in Gram-positive and Gram-negative PCB-degrading bacteria, several Gram-positive bacteria encode multiple BphC enzymes (Austrias and Timmis, 1993; Hauschild *et al.*, 1996; Kosono *et al.*, 1997; Maeda *et al.*, 1995). BphC is an important target of inhibitory metabolites. As a result, the expression of multiple BphC enzymes with distinct functionality may increase the range of substrates that can be transformed by the *bph* pathway (Brenner *et al.*, 1994). Interestingly, terminal oxygenases in Gram-positive bacteria are more similar to terminal toluene dioxygenases than to the corresponding *bph* genes in Gram-negative bacteria. In addition, the codon usage of *bph* genes in *Rhodococcus* sp. strain P6 is more similar to

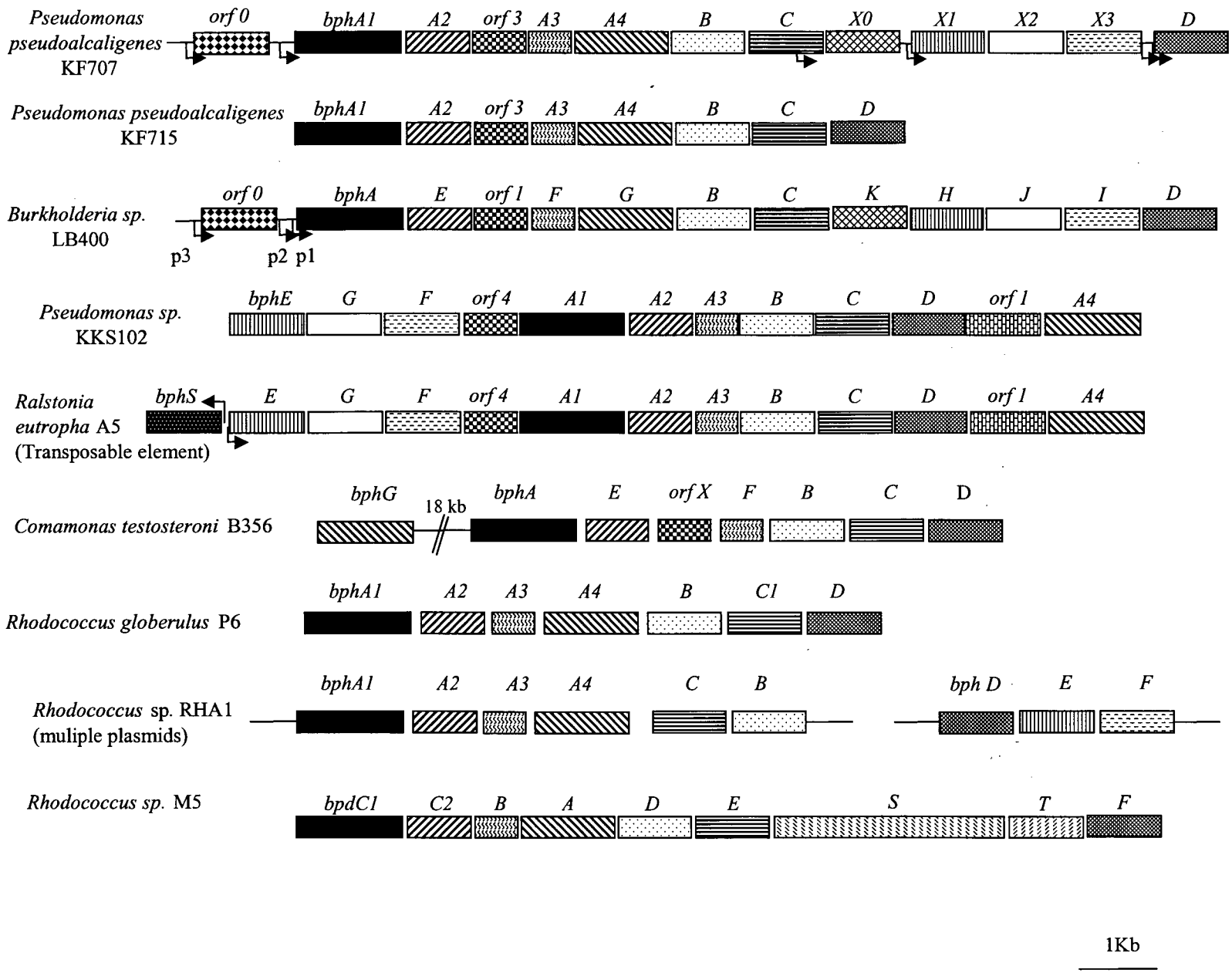
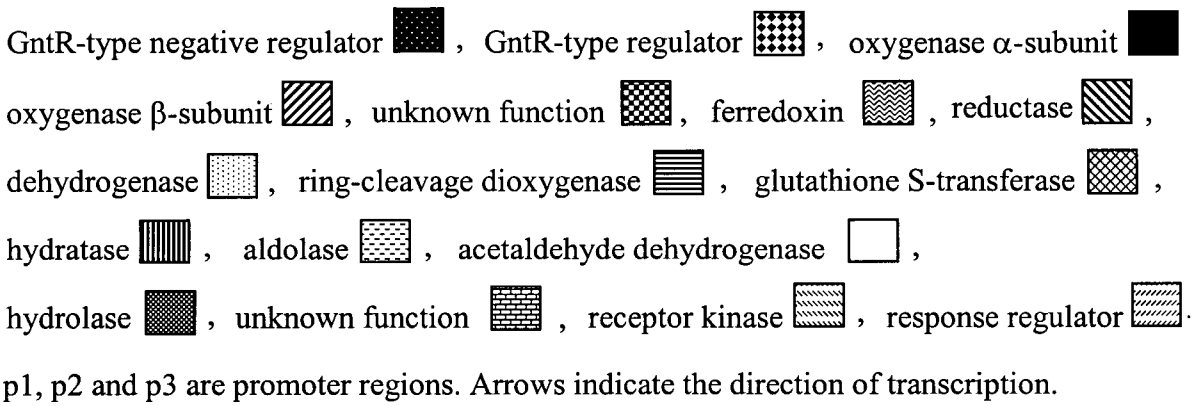

Pseudomonas pseudoalcaligenes KF707
orf 0
bphA1
A2
orf 3
A3
A4
B
C
X0
X1
X2
X3
D
Pseudomonas pseudoalcaligenes KF715
bphA1
A2
orf 3
A3
A4
B
C
D
Burkholderia sp. LB400
orf 0
bphA
E
orf 1
F
G
B
C
K
H
J
I
D
p3
p2
p1
Pseudomonas sp. KKS102
bphE
G
F
orf 4
A1
A2
A3
B
C
D
orf 1
A4
Ralstonia eutropha A5 (Transposable element)
bphS
E
G
F
orf 4
A1
A2
A3
B
C
D
orf 1
A4
Comamonas testosteroni B356
bphG
18 kb
bphA
E
orfX
F
B
C
D
Rhodococcus globerulus P6
bphA1
A2
A3
A4
B
C1
D
Rhodococcus sp. RHA1 (muliple plasmids)
bphA1
A2
A3
A4
C
B
bph D
E
F
Rhodococcus sp. M5
bpdC1
C2
B
A
D
E
S
T
F
1Kb

Fig. 4. Organization of *bph* genes in different PCB-degrading bacteria



p1, p2 and p3 are promoter regions. Arrows indicate the direction of transcription.

that of *Pseudomonas* genes than to other *Rhodococcus* genes. These observations suggest that the genes encoding enzymes for biphenyl and toluene degradation are ancestrally related, and initially evolved in Gram-negative bacteria (Asturias *et al.*, 1995). Recently, plasmid encoded *bph* genes from *Sphingomonas aromaticivorans* F199 were described (Romine *et al.*, 1999). F199 contains 7 homologues to the large and small subunits of the terminal oxygenase component and only 1 homologue to the ferredoxin and the reductase components of BPDO (Romine *et al.*,1999). Furthermore, the *bph* genes from F199 have low similarity to *Pseudomonas bph* genes, and are distributed among 8 different putative transcripts (Romine *et al.*,1999). These data suggest that *bph* genes from *Sphingomonas* species represent a unique lineage.

3. Biphenyl Dioxygenase.

Many bacterial aromatic dioxygenase enzymes have evolved in response to the enormous variety of aromatic compounds that exist in the environment. Recently, it has become clear that aromatic biotransformations can be applied for the production of enantiomerically pure compounds as well as the degradation of harmful contaminants. Dioxygenases are typically soluble multicomponent enzymes that catalyze the addition of dioxygen to the aromatic nucleus forming dihydroxydiols. Bacterial dioxygenase systems transfer electrons from NADH or NADPH to a terminal oxygenase component via a short electron transport chain composed of a FAD or FMN reductase and a [2Fe-2S] ferredoxin. Dioxygenase systems that include a reductase component containing a [2Fe-2S] cofactor may not have a ferredoxin component. The variation in number and type of dioxygenase components led to the classification of three main

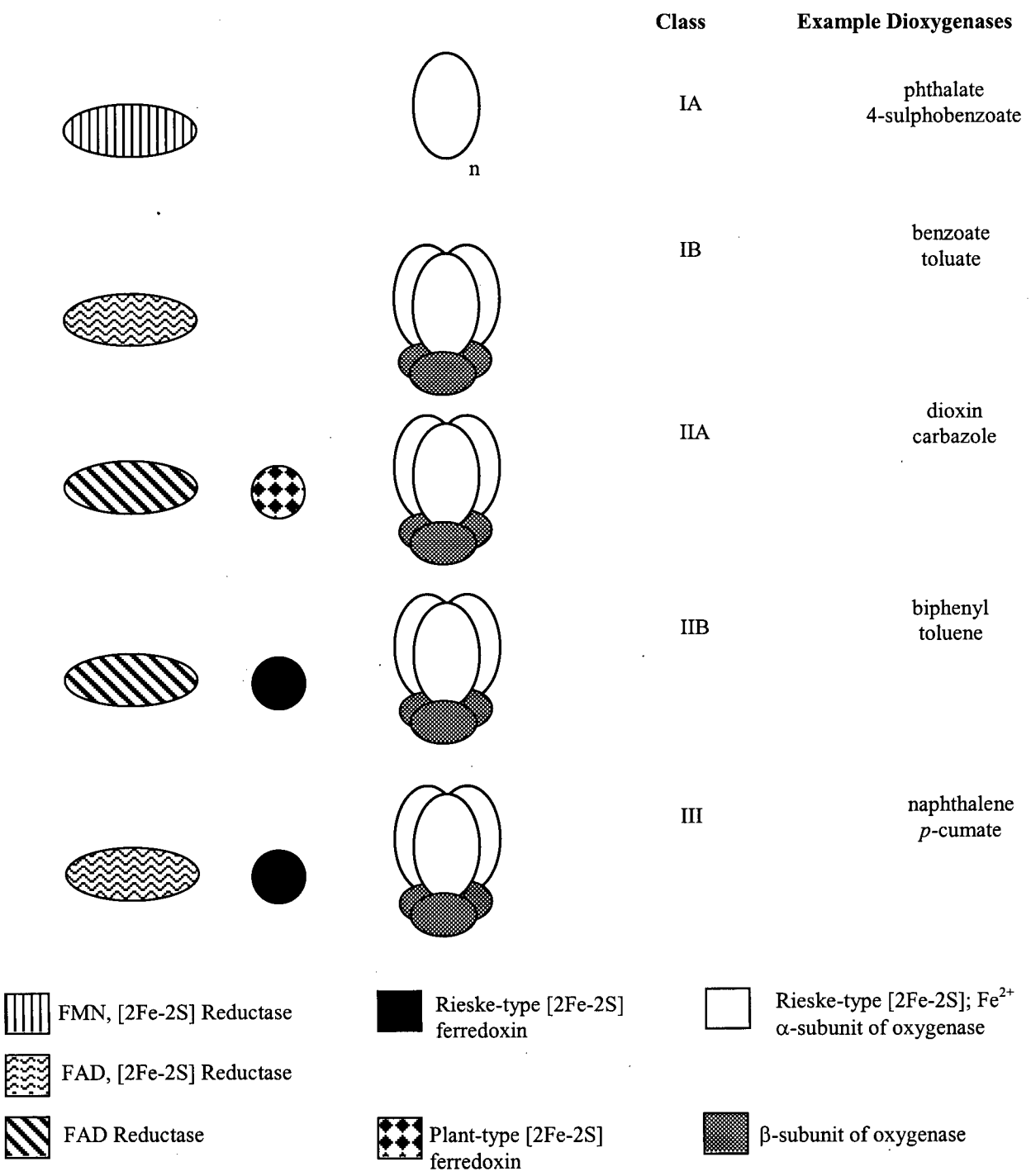


Fig. 5. Classes of Dioxygenases. Rieske-type [2Fe-2S] clusters are characterized by the coordination of one iron atom by two cysteine residues, and the other iron atom by two histidine residues, whereas Plant-type [2Fe-2S] clusters are characterized by the coordination of both iron atoms by cysteine residues.

groups of dioxygenase systems: class I, class II and class III (Fig.5) (Butler and Mason, 1997). Since then, investigators have shown that the electron transport components of dioxygenase systems can complement multiple terminal oxygenases. Consequently, it has been argued that each component of a dioxygenase system should be classified separately (Butler and Mason, 1997). For example, Rieske-type, mononuclear iron oxygenase components generally cluster into families corresponding to the substrates oxidized (Gibson and Parales, 2000). The toluene/biphenyl family of oxygenases catalyzes the hydroxylation of aryl compounds, the naphthalene family of oxygenases hydroxylates fused aromatic compounds as well as nitroarenes, and the benzoate family of oxygenases hydroxylates aromatic acids (Gibson and Parales, 2000). Terminal oxygenases belonging to the toluene/biphenyl, naphthalene and benzoate families are heteromultimers consisting of α- and β-subunits. The phthalate group comprises a fourth family of Rieske-type, mononuclear iron oxygenases, and are characterized by homomultimeric terminal oxygenases. Thus, BPDO is a class IIB multicomponent dioxygenase, and the oxygenase component of BPDO belongs to the toluene/biphenyl family of Rieske-type, mononuclear iron oxygenases.

The reaction mechanism of BPDO has been postulated on the basis of reaction mechanisms of similar enzymes, such as naphthalene 1,2-dioxygenase (Harayama *et al.*, 1992; Kauppi *et al.*, 1998; Que and Ho, 1996). The exact nature of the oxygen species that activates naphthalene is unknown (Wolfe *et al.*, 2001). However, the activation of dioxygen by naphthalene 1,2-dioxygenase requires binding of naphthalene and the reduction of both the mononuclear Fe^{2+} centre and the Rieske-type cluster (Wolfe *et al.*, 2001). Similarly, biphenyl catalysis is thought to proceed via aromatic substrate binding

followed by the activation of dioxygen. The solved structures of BPDO and naphthalene dioxygenase show that the catalytic Fe^{2+} centre in BPDO is coordinated by Asp386, His233 and His239, whereas one iron of the Rieske cluster is coordinated by Cys100 and Cys120, and the other by His102 and His123 (Imbeault *et al.*, 2000; Kauppi *et al.*, 1998). The structures of these dioxygenases also reveal that the distance between a catalytic Fe^{2+} centre and the Rieske cluster of an adjacent α-subunit (BphA) is less than the distance between these cofactors within BphA (Kauppi *et al.*, 1998). In BPDOs, Asp230 connects the Fe^{2+} centre and the Rieske cluster of neighboring BphAs through hydrogen bonding, and may facilitate electron transfer from the Rieske cluster of one BphA to the Fe^{2+} centre of an adjacent BphA (Kauppi *et al.*, 1998; Parales *et al.*, 1999). The mononuclear Fe^{2+} centre and the Rieske cluster are reduced using electrons from NADH. However, these cofactors only accept single electrons, whereas NADH can transfer electrons via hydride. Importantly, the reductase component of BPDO (BphG) mediates the transition of two electrons from NADH to two single electron transfers via the semiquinone redox state of the FAD cofactor (Butler and Mason, 1997; Mason and Cammack, 1992). Moreover, the ferredoxin component of naphthalene 1,2-dioxygenase is not required for the catalysis of naphthalene by the reduced enzyme, which suggests that the electron transfer components of this enzyme, and possible BPDO, do not facilitate substrate binding (Wolfe *et al.*, 2001).

The terminal oxygenase components of BPDOs that have been studied so far share between 61% to over 99% amino acid sequence identity, and have different substrate specificity (Table 1) (Sylvestre *et al.*, 1996b). *Burkholderia* sp. strain LB400 (Viallard *et al.*, 1998) (originally identified as a member of the genus *Pseudomonas*

[Bopp, 1986]) oxidizes more congeners than most other PCB-degrading bacteria that have been isolated (Abramowicz, 1990; Bedard *et al.*, 1990). LB400 preferentially transforms *ortho*-substituted biphenyls and oxidizes PCBs with up to six chlorine substituents (Gibson *et al.*, 1993). In contrast, PCB-degrading bacteria such as

Table. 1. Amino acid sequence identities of α-subunits of biphenyl dioxygenases from different bacteria.

α-subunit[a]	Cam-1	LB400	KF707	B-356	P6	RHA1	KKS102
Cam-1	100	95.4	95.6	76.8	62.1	64.0	75.1
LB400		100	99.7	75.8	61.7	64.2	73.6
KF707			100	76.8	62.1	63.8	74.9
B-356				100	61.8	63.1	81.6
P6					100	76.3	60.8
RHA1						100	61.2
KKS102							100

[a] Cam-1, *Pseudomonas* sp. strain Cam-1; LB400, *Burkholderia* sp. strain LB400; KF707, *Pseudomonas pseudoalcaligenes* KF707; B-356, *Comamonas testosteroni* B-356; P6, *Rhodococcus globerulus* P6; RHA1, *Rhodococcus* sp. strain RHA1; KKS102; *Pseudomonas* sp. strain KKS102

Pseudomonas pseudoalcaligenes KF707 and *Comamonas testosteroni* B-356 preferentially transform *para*-substituted biphenyls or *meta*-substituted biphenyls respectively, and oxidize PCBs with up to four chlorine substituents (Barriault *et al.*, 1997, Gibson *et al.*, 1993). The ability of BPDO from LB400 ($BPDO_{LB400}$) to transform a comparatively broad range of PCB congeners is due to 2,3- and 3,4- dihydroxylation activities and the ability of $BPDO_{LB400}$ to dechlorinate certain *ortho*-substituted PCBs

upon 2,3-dihydroxylation (Haddock *et al.*, 1995; Seeger *et al.*, 1995b). Interestingly, $BPDO_{LB400}$ catalyses dehalogenation as well as denitration and dehydroxylation of *ortho*-substituted biphenyls (Seeger *et al.*, 2001), however dechlorination of *meta*- or *para*-substituted carbons has not been demonstrated. The ability of $BPDO_{LB400}$ to hydroxylate *ortho*-substituted carbons is influenced by the remaining chlorine atoms on the PCB molecule. For instance, $BPDO_{LB400}$ hydroxylates 2,4,2'-, 2,4,4'-, and 2,4,2'4'-chlorobiphenyls predominantly at positions 2 and 3 of the 2,4-dichlorinated ring (Seeger *et al.*, 1999). In contrast, the dichlorinated ring of 2,5,2'-, 2,5,3'-, and 2,5,2', 5'-chlorobiphenyls is hydroxylated at positions 3 and 4 (Haddock *et al.*, 1995). The chlorine substituents on the non-oxidized ring also affect the hydroxylation pattern of PCBs by $BPDO_{LB400}$. In contrast to 2,4,2'-trichlorobiphenyl, 2,4,3'-trichlorobiphenyl is attacked at positions 5 and 6 (Seeger *et al.*, 1999). Likewise, the major transformation products of 2,2'- and 2,4'-dichlorobiphenyl are 2,3-dihybroxybiphenyls, whereas 2,3'-dichlorobiphenyl is attacked at positions 5 and 6 (Seeger *et al.*, 1995a; Haddock *et al.*, 1994; Seeger *et al.*, 1999). Thus, *meta*-chlorines of non-oxidized rings appear to promote 5,6-dihydroxylation of PCBs. Adding to the complexity of predicting the hydroxylation pattern of PCB transformations, $BPDO_{LB400}$ may generate more than a single reaction product (Arnett *et al.*, 2000; Seeger *et al.*, 1999; Seeger *et al.*, 2001). Taken together, analyses of metabolites from $BPDO_{LB400}$ catalyzed reactions suggest that $BPDO_{LB400}$ has relaxed regiospecificity of attack of substrates, and that steric and electronic effects of chlorine substituents determine the hydroxylation pattern of PCBs. Importantly, most other BPDOs that have been studied have 2,3-dihydroxylation activity only, and are unable to dechlorinate *ortho*-substituted biphenyls. As a result, the hydroxylation pattern

of PCBs transformed by most BPDOs may be easier to predict than that resulting from $BPDO_{LB400}$ activity.

As the initial enzyme in the pathway, BPDO is a major determinant of the specificity and the kinetics of PCB transformation by different bacteria. Consequently, engineering BPDOs is a common strategy for developing more efficient PCB-degrading bacteria (Furukawa, 2000). The effective design of recombinant BPDOs should optimize the specificity and kinetics of PCB transformation. However, thus far, most engineering attempts have increased the range of PCBs transformed by BPDOs rather than improve the kinetics of PCB biotransformation. Much of this work has involved $BPDO_{LB400}$ and BPDO from *P. pseudoalcaligenes* KF707 ($BPDO_{KF707}$). Site-directed mutagenesis of $BPDO_{LB400}$ and $BPDO_{KF707}$ (Erickson and Mondello, 1993) and exchanging domains of the genes that encode these enzymes (Kimura *et al.*, 1997; Mondello *et al.*, 1997; Suenaga *et al.*, 1999), indicate that the major determinants of congener preference are located in the carboxyl-terminal region of BphA. Moreover, shuffling the genes that encode $BphA_{KF707}$ and $BphA_{LB400}$ generated BPDOs with broader substrate range than the parent enzymes (Bruhlmann and Chen, 1999; Furukawa, 2000; Kumamaru *et al.*, 1998, Suenaga *et al.*, 2001a). In particular, the substitution of Thr376 (KF707) to Asn376 (LB400) in $BphA_{KF707}$ is sufficient for this enzyme to gain 3,4-dihydroxylation activity (Kimura *et al.*, 1997; Mondello *et al.*, 1997). In addition, the substitution of Phe336 (LB400) to Ile336 (KF707) in $BphA_{LB400}$ enhances activity towards di-*para*-substituted PCBs (Mondello *et al.*, 1997; Bruhlman and Chen, 1998). Monocyclic aromatic compounds are poorly attacked by $BPDO_{KF707}$ or $BPDO_{LB400}$. However, exchange of four amino acids (H225Q, V258I, G268A and F277Y) in $BphA_{KF707}$ to those

of $BphA_{LB400}$, generated recombinant enzymes with enhanced capacity to degrade toluene, benzene or alkylbenzenes (Suenaga *et al.*, 2001b). Furthermore, changing Thr-376 to valine in $BphA_{KF707}$, particularly if Ile-24, His-66 and lys-89 are changed to valine, tyrosine and arginine respectively, improves the degradation capacity of this enzyme towards dibenzofuran and dibenzo-p-dioxin (Suenaga *et al.*, 2001a). These results indicate that by changing only a few amino acids, BPDOs can be engineered with different substrate ranges. However, the amino acid substitutions described may not be sufficient to impart new activities to other BPDOs since the specificity of amino acids that are identical in $BPDO_{KF707}$ and $BPDO_{LB400}$ may also be important. Finally, exchanging β-subunits (BphE) of distantly related BPDOs clearly affects substrate preference (Chebrou *et al.*, 1999; Hurtubise, 1998), which suggests that BphE plays a role in determining the substrate specificity of BPDOs. However, structural analyses of naphthalene dioxygenase reveal that β-subunits of this enzyme do not contribute to the substrate binding site (Carredano *et al.*, 1999).

Highly active preparations of BPDOs and a continuous assay would facilitate the detection of recombinant BPDOs with higher specificity constants for certain PCBs than parental enzymes. The comparatively few attempts to optimize the kinetics of PCB transformation then, may result from the difficulty to purify active BPDOs, and the lack of a continuous activity assay. Imbeault *et al.* (2000) reported an improved method for anaerobic purification of $BPDO_{B356}$, and described an oxygraph assay to continuously monitor biphenyl and PCB transformation in vitro. As a result, Imbeault *et al.* (2000) revealed that the transformation of certain PCB congeners is poorly coupled to O_2 consumption. Such uncoupling depleted reducing equivalents from the cell, with

concomitant production of reactive oxygen species (Imbeault *et al.*, 2000). Furthermore, the $k_{cat}^{app} / K_m^{app}$ for oxygen of $BPDO_{B356}$, was lower in the presence of PCBs than in the presence of biphenyl. Thus, Imbeault *et al.* (2000) suggest that PCB congeners might prevent the displacement of active site solvent species, thereby inhibiting O_2 binding. Alternatively, the chlorine substituents of PCBs bound to the aromatic substrate binding site might directly hinder O_2 binding (Imbeault *et al.*, 2000). The kinetics of biphenyl and PCB transformation by $BPDO_{LB400}$ have also been investigated (Arnett *et al.*, 2000). $BPDO_{LB400}$ transforms 3-chlorobiphenyl (3-CB) and 2,5-CB at higher initial rates than biphenyl, and the rate of transformation of 2-CB was similar to that of tri- and tetra-CBs. Moreover, PCB congeners that are significantly less soluble than biphenyl were transformed at rates almost equal to biphenyl. These results suggest that the electronic effect of chlorine substituents, rather than steric hindrance of chlorine atoms or relative bioavailability of PCB congeners, influences rates of PCB transformation. Understanding the kinetic behavior of BPDOs will reveal additional criteria for the effective design of recombinant BPDOs.

4. Defining Characteristics of Cold-Adapted Bacteria

PCB-degrading bacteria isolated from Arctic soil samples may express cold-adapted BPDO enzymes, thereby increasing the efficiency of PCB degradation at low temperatures compared to mesophilic bacteria. As a result, on-site bioremediation of PCB-contaminated Arctic soil may be improved if cold-adapted, rather than mesophilic, PCB-degrading bacteria or enzymes are used. To determine if certain Arctic soil bacteria express cold-adapted BPDOs, a clear definition of cold-adapted enzymes is required.

Cold-adapted bacteria are able to grow at temperatures near 0°C and are classified as psychrophilic if the microorganism is unable to grow above 20°C or psychrotolerant if the organism grows above 20°C (Morita, 1975). The ability of psychrophilic and psychrotolerant bacteria to grow at low temperatures is the result of phenotypic and genotypic adaptations. Phenotypic responses to growth at low temperature may be regulated by cold-shock proteins, and include synthesizing shorter and less saturated membrane lipids to maintain membrane fluidity, synthesizing antifreeze glycoprotein and peptides to avoid freezing, or entering a state of dormancy to tolerate freezing (Aguilar et al, 1998; Feller and Gerday, 1997; Gounot, 1991; Xu *et al.*, 1998).

In 1867, A.V. Harcourt noted that lowering the temperature by 10°C typically diminishes the rate of enzyme catalyzed reactions by a factor between two and three (Cornish-Bowden, 1995). A more accurate description of the effect of temperature on enzyme activity is given by the Arrhenius equation (Equation 1) (Cornish-Bowden, 1995). The Arrhenius equation predicts that the rate of enzyme catalyzed reactions will increase exponentially with increasing temperature until the enzyme is thermally denatured. Consequently, the effect of temperature on enzyme activity depends

$$k = Ae^{-Ea/RT}$$

Equation 1. The Arrhenius equation. k, reaction rate; E_a, activation energy; R, gas constant (8.314 J $mol^{-1}K^{-1}$); T, temperature (Kelvin); A, constant equal to the product of the collision frequency (Z) and the probability that the reactants are in the correct orientation to react (p).

on the activation energy (E_a) and the thermostability of the enzyme. The dependence of enzyme catalyzed reactions on temperature is also described using the transition state theory. Thus, the effect of temperature on the thermodynamic properties of the catalyzed reaction can be determined.

$$k = (k_B T/h)e^{-\Delta G^*/RT} \therefore k = (k_B T/h)e^{-((\Delta H^* - T\Delta S^*)/RT)} \therefore k = (k_B T/h)e^{-((\Delta H^*/RT)-(\Delta S^*/R))}$$

Equation 2. The dependence of thermodynamic parameters on temperature. k_B, Boltzmann constant (1.3805×10^{-23} J K^{-1}); T, temperature (Kelvin); h, Planck constant (6.6256×10^{-34} J s); ΔG^*, free energy of activation; ΔH^*, change in enthalpy; ΔS^*, change in entropy.

Despite predicted decreases in enzyme activity at low temperatures, cold-adapted microorganisms maintain similar rates of metabolic flux as related species in temperate environments (reviewed in Feller and Gerday, 1997). Strategies that cold-adapted microorganisms might use to maintain levels of metabolic flux at low temperature include synthesizing more enzymes and evolving cold-adapted enzymes. Synthesizing more enzymes would be costly if adopted as a general strategy. Therefore, genotypic responses to growth at low temperature have probably evolved enzymes that have high catalytic activity under these conditions.

Due to the potential application of cold-adapted enzymes, initiatives to define and characterize such enzymes have increased over the past decade (Gerday *et al.*, 1997; Marshall, 1997). Enzymes expressed by psychrophilic and psychrotolerant organisms maybe at various stages of evolutionary adaptation to low temperature. Thus, it is not

useful to define cold-adapted enzymes simply as enzymes expressed by cold-adapted microorganisms. An important characteristic of cold-adapted enzymes is higher specific activity at low temperature than mesophilic homologues (Feller *et al.*, 1997, 1996, Gerday *et al.*, 1997). In environments where substrate is limiting, the catalytic efficiency (as measured by k_{cat}/K_m) may be a better indicator of thermal adaptation since substrate binding as well as catalytic activity is affected by temperature (Feller and Gerday, 1997). Ideally, cold-adapted enzymes will have values of ΔG^* near 0, such that enzyme activity is not affected by decreased temperature (Equation 2). Indeed, cold-adapted enzymes generally display lower values of ΔG^* then mesophilic homologues (reviewed in Lonheinne, 2000). In addition, the contribution of enthalpic and entropic energies to ΔG^* may differ between homologous mesophilic and cold-adapted enzymes. Cold-adapted enzymes often exhibit reduced enthalpy of activation of the enzyme-substrate complex, thereby rendering the reaction less temperature dependent. Reduced enthalpy of activation is achieved by decreasing the number of heat dependent bonds of formation or dissociation that are required to form the enzyme-substrate complex. Thus, reduced enthalpies of activation of reactions catalyzed by cold-adapted enzymes may account for the limited thermal stability of many cold-adapted enzymes.

Structural comparisons of homologous cold-adapted and mesophilic enzymes indicate that the modification of many weak interactions is the prevailing mechanism of enzyme adaptation to temperature (Feller and Gerday, 1997). Relative to mesophilic homologues, cold-adapted enzymes may contain fewer salt bridges, fewer arginine or proline residues, and may display reduced affinity for ions that contribute to increased enzyme rigidity (Feller *et al.*, 1997). Furthermore, cold-adapted enzymes are often less

compact than mesophilic homologues due to amino acid substitutions that result in increased solvent interactions or decreased clustering of hydrophobic residues within the protein core. Importantly, only a selection of these alterations may appear in any particular cold-adapted enzyme (Table 2).

Table 2. Kinetic parameters of selected cold-adapted enzymes and mesophilic homologues, and structural adaptations identified in the cold-adapted enzyme.

Enzyme	Source	°C	k_{cat} (s^{-1})	ΔG*	Characteristics of the cold-adapted enzyme[a]	Ref.
α-amylase	*Alteromonas haloplanctis*,	15	495.2	55.6	↓ Pro and Arg residues; ↓ ion binding, ↓disulfide bonds, ↓core hydrophobicity	Feller *et al.* (1994); Aghajari *et al.*., 1998; Lonhienne *et al.*, 2000
	Bacillus		90.5	59.7		
Subtilisin	*Bacillus* TA41	15	25.4	62.7	↓ ion binding, ↓ salt bridges, ↓ aromatic interactions, ↑ solvent interactions	Narinx *et al.*; 1997 Lonhienne *et al.*, 2000
	Bacillus subtilis		5.4	66.4		
Trypsin	Antarctic cod	15	0.97	70.5	↓ salt bridges, ↓ aromatic interactions, ↑ solvent interactions	Genicot *et al.*, 1996; Lonhienne *et al*, 2000
	Bovine		0.68	71.4		

[a] "↑" and "↓" signify an increase or decrease respectively of the parameter described.

In summary, cold-adapted enzymes are characterized by high catalytic activity at low temperature. Unless the substrate converted is present at concentrations well above the K_m of the enzyme, high k_{cat}/K_m at low temperature is a better indicator of cold-adaptation than k_{cat}. These criteria will be used in subsequent sections of this thesis to evaluate whether PCB-degrading bacteria from Arctic soil express cold-adapted, PCB-degrading enzymes.

5. Regulation of the *bph* operon.

The presence of biphenyl can be important for maintaining PCB-biodegradation activity in soil (Barriault and Sylvestre, 1993, Focht and Brunner, 1985). However, adding biphenyl to soil to stimulate PCB-degradation activity is problematic due to the low water solubility of biphenyl and its possible adverse health effects (Abramowicz, 1990; Focht, 1995). Since biphenyl is rare in natural environments, it is possible that alternative, more common constituents of soil also induce genes encoding biphenyl-degrading enzymes. Such inducers may be less toxic and more water-soluble than biphenyl, better enabling their addition to soil to stimulate PCB-degradation activity in bioremediation projects.

Several studies have investigated the induction of PCB transformation in cell suspensions of PCB-degrading bacteria by compounds other than biphenyl. Notably, cell suspensions of *Arthrobacter* sp. strain B1B grown on fructose medium supplemented with *l*-carvone, limonene, *p*-cymene or isoprene remove Aroclor 1242 (Gilbert and Crowley, 1997). Likewise, PCB transformation by *Pseudomonas stutzeri* was higher when growing on xylose supplemented with *l*-carvone than on xylose alone (Tandlich *et al.*, 2001). *Alcaligenes eutrophus* H850 and *Corynebacterium* sp. strain MB1 grown on plant phenolic compounds and *Burkholderia* sp. strain LB400 grown on plant phenolic compounds, glucose or glycerol degrade certain PCB congeners (Billingsley *et al.*, 1997; Donnelly *et al.*, 1994). Also, *Alcaligenes eutrophus* H850 grown on fructose plus *l*-carvone produces a *bphC* mRNA transcript, which suggests that monoterpenes can induce *bph* genes in certain Gram-negative bacteria (Park *et al.*, 1999). Finally, *Cellulomonas* sp. strain T109 and *Rhodococcus rhodochrous* T100 grown on cymene and

limonene, respectively, remove over 80% more Aroclor 1242 than when grown on glucose (Hernandez *et al.*, 1997). These studies support the hypothesis that certain compounds other than biphenyl may be used to stimulate PCB biodegradation. However, investigations so far have not shown that bacteria grown on substrates other than biphenyl remove PCBs as a result of induction of *bph* genes above constitutive levels. Moreover, it is possible that the compounds used to induce bacterial PCB degradation activity did not induce *bph* genes but instead induced genes that encode other enzymes that also degrade PCBs or stimulate PCB degradation via mechanisms other than genetic regulation.

Although the genetic organization of *bph* genes and the biochemistry of the biphenyl degradation pathway are well documented, the regulation of the *bph* operon is less characterized. S1 nuclease mapping studies identified three promoter regions upstream of *bphA* in *Burkholderia* sp. strain LB400 (Mondello, 1989) (Fig.4). Two of the promoters (p1 and p2) are constitutively active; transcription from p3 increased in the presence of biphenyl, and generates *orf0* transcripts (Mondello, 1989). Despite constitutive expression of the *bph* genes from p1 and p2, Mondello (1989) showed that LB400 grown on biphenyl is able to degrade di-*para* substituted PCBs and tetra- and penta-chlorobiphenyls more effectively than LB400 grown on succinate or on biphenyl plus succinate. Using a *lacZ* reporter construct, Brazil *et al.* (1995) also demonstrated that p1 and p2 from LB400 are constitutively active. Moreover, Brazil *et al* (1995) observed that expression of *bphC* was similar in LB400 grown on mineral medium supplemented with succinate and mineral medium supplemented with succinate and biphenyl. Taken together, these results suggest that *bphC* is transcribed with *bphA*, and

that transcription of *orf0* is necessary for transformation of certain PCB congeners. In contrast, Beltrametti *et al.* (2001) report that the expression of *bphA* in LB400 is induced by biphenyl and that biphenyl has little affect on the expression of *orf0*. It is possible that mutations in the LB400 strains used in these studies have affected the promoter regions of corresponding *bph* genes. Accordingly, the promoter of *bph* genes from LB400 strains used in these studies should be sequenced.

Recently, other investigators found that in the PCB-degrader *Pseudomonas pseudoalcaligenes* KF707 the translation product of *orf0* is autoregulated and is necessary for the expression of genes encoding enzymes in the biphenyl degradation pathway downstream of *bphC* (Watanabe *et al.*, 2000). Promoter sequences in KF707 are located upstream of *orfO*, *bphA*, *bphX0* and *bphX1*, and two promoter sequences are found upstream of *bphD* (Watanabe *et al.*, 2000). Genes *orf0* and *bphA1* are independently transcribed, and *bphA1A2A3A4BC* are constitutively co-expressed. In contrast, growth of KF707 on biphenyl is required to express *orf0*, and *orf0* is necessary for the transcription of *bphX0XX1X2X3D* (Watanabe *et al.*, 2000). Orf0 is a GntR-type transcriptional activator that appears to bind the *meta*-cleavage product of BphC (Watanabe *et al.*, 2000). O*rf0* from LB400 and KF707 are 86% similar. Therefore, Orf0 may also be necessary for expression of genes downstream of *bphC* in LB400. Induction of genes downstream of *bphC* allows cells to grow on biphenyl and minimizes the accumulation of metabolites resulting from biphenyl and PCB catabolism. Decreased accumulation of metabolites from PCB transformation could explain why LB400 grown on biphenyl degrades di-*para* substituted PCBs and tetra- and penta-chlorobiphenyls more effectively than LB400 grown on other substrates (Mondello, 1989).

Alternative strategies have evolved to regulate *bph* genes in other bacteria. For instance, a putative histidine kinase receptor (*bpdS*) and a response regulator (*bpdT*) may regulate *bph* genes in *Rhodococcus* M5 (Labbe *et al.*, 1997) (Fig.4). Disruption of *bpdS* inhibits growth of M5 on biphenyl. The histidine kinase TodS regulates toluene degradation in *Pseudomonas putida* F1 (Lau *et al.*, 1997). However, BpdS is more similar to leucine-rich receptors-like kinases found in eucaryotes, than to TodS (Labbe *et al.*, 1997). In *Ralstonia eutropha* A5, *bph* genes are negatively regulated (Mouz *et al.*, 1999). Genes encoding the upper and lower *bph* pathways in A5 are co-transcribed and their expression is repressed by BphS (Fig.4) (Mouz *et al.*, 1999). Although BphS negatively regulates *bph* genes, BphS displays over 40% identity with Orf0 from LB400.

The evolution of different strategies for regulating *bph* genes may not be surprising given the diversity of *bph* gene organization in different bacteria. Moreover, analyses of other catabolic pathways suggest that regulatory and structural genes may have evolved independently (de Lorenzo and Perez-Martin, 1996). For instance, the phenol catabolic pathway in *Pseudomonas* CF600 is activated by a NtrC-type regulator in response to phenol, whereas the phenol catabolic pathway in *Pseudomonas putida* PaW85 is activated by a LysR-type regulator in response to the metabolite *cis,cis*-muconate (de Lorenzo and Perez-Martin, 1996). Thus, inducers of *bph* genes in one PCB-degrading bacterium may not induce *bph* genes in other PCB-degrading bacteria, and bacteria that express structural genes ideal for PCB bioremediation may be difficult to regulate. Further investigations of *bph* gene regulation in different bacteria will reveal whether a general method can be applied to induce PCB bioremediation.

6. Uptake of aromatic compounds.

Little is known about biphenyl and PCBs movement across bacterial membranes to reach cytoplasmic catabolic enzymes. However, an understanding of the mechanism of biphenyl and PCB uptake in different bacteria can be used to enhance the bioremediation of PCB-contaminated soils. It is generally believed that aromatic compounds are available for intracellular metabolism only when dissolved in aqueous solution, and that uptake of these compounds is due to passive diffusion (Sikkema *et al.*, 1995). Conversely, recent reports have demonstrated that certain bacteria can access aromatic compounds, including biphenyl, that are sorbed to surfaces (Bastian *et al.*, 2000; Bouchez et al, 1997; Cavillo and Alexander, 1996; Feng *et al.*, 2000; Tang et al, 1998). Moreover, active transport of aromatic acids has been demonstrated. Permease proteins that actively transport benzoate (BenK), 4-hydroxybenzoate and protocatechuate (PcaK) have been identified (Collier *et al.*, 1997; Nichols and Harwood, 1997), and putative transport proteins for 3-hydroxyphenyl propionate (HppK) and 2,4-dichlorophenoxyacetate (TfdK) have been found (Leveau et al, 1998; Pao *et al.*, 1998). All of these transport proteins were discovered in corresponding pathway regulons of Gram-negative bacteria and are members of the major facilitator superfamily (Collier *et al*, 1997; Leveau *et al.*, 1998; Nichols and Harwood, 1997; Pao *et al.*, 1998).

The active transport of non-ionic aromatic compounds has also been described. Although naphthalene uptake is passive in *Pseudomonas putida* PpG1 (Bateman *et al.*, 1986), Whitman *et al* (1998) suggest that naphthalene uptake is active in a *Pseudomonas fluorescens* isolate. Notably, naphthalene uptake by *Pseudomonas fluorescens* was disrupted by metabolic inhibitors and analogs of naphthalene (Whitman *et al.*, 1998).

Certain genes belonging to pathways for the degradation of styrene (*porA*), isopropylbenzene (*ipbH*), toluene (*todX* and *tbuX*), cumene (*cumH*), *p*-cymene (*cymD*) and phenol (*phlX*) share low but significant sequence similarity with *fadL*, which encodes an outer membrane protein involved in the transport of long-chain fatty acids in *E.coli* (Kahng *et al.*, 2000; Velasco *et al.*, 1998). Furthermore, deleting *tbuX* inhibits toluene degradation by *Ralstonia pickettii* PKO1 and reduces the expression of enzymes involved in toluene utilization (Kahng *et al.*,2000). These results are consistent with *tbuX* encoding a membrane protein involved in toluene uptake.

Kim *et al.* (2001) recently demonstrated that 2,2'5,5'-tetrachlorobiphenyl increases the fluidity of the cytoplasmic membrane and that growth on biphenyl increases the saturation of membrane fatty acids. Although biphenyl uptake by *Ralstonia eutropha* H850 was not studied, Kim *et al.* (2000) found that 2,2'5,5'-tetrachlorobiphenyl enters *Ralstonia eutropha* H850 via passive diffusion. Since PCBs are structurally diverse, passive uptake of 2,2',5,5'-tetrachlorobiphenyl in H850 may not model the transport of biphenyl or other PCB congeners into H850. Also, the mechanism of biphenyl and PCB uptake may differ between different bacteria. Importantly, the investigation of biphenyl uptake in different PCB-degrading bacteria may identify a new transport protein of nonionic aromatic compounds, and thereby reveal an additional target for improving PCB bioremediation.

THESIS OBJECTIVES

There are several advantages to bioremediation of PCB-contaminated Arctic soil using indigenous PCB-degrading bacteria. First, Arctic soil bacteria may be adapted for PCB degradation at low temperatures. As a result, heating costs for on-site bioremediation of Arctic soil may be reduced. Second, obtaining permission to use foreign bacteria for on-site bioremediation of contaminated soil is difficult, since the ecological effects of introducing new bacteria to soil are difficult to predict.

The general objectives of my thesis were to establish that PCB-degrading bacteria exist in Arctic soil, then to determine if these bacteria are adapted to degrade PCBs at low temperature. My general approach was to compare an Arctic soil bacterium and a mesophilic bacterium on the basis of PCB degradation by whole cells, activities of purified BPDOs, the regulation of genes encoding these enzymes, and the mechanism of biphenyl uptake by these bacteria. In addition, I determined if substrates other than biphenyl induce genes that are required for PCB degradation, and I demonstrated quantitative aerobic PCB degradation of anaerobically treated PCB-contaminated soil.

MATERIALS AND METHODS

1. Bacterial strains, plasmids and culture conditions.

The bacterial strains and plasmids used in this study are listed in Table 3. Unless otherwise specified, *Escherichia coli* strains were cultured at 37°C in Luria-Bertani (LB) medium. *Pseudomonas* sp. strain Cam-1 was cultured at 7°C, 22°C and 30°C and *Burkholderia* sp. strain LB400 were cultured at 15°C, 22°C and 30°C on Tryptic Soy Broth, or mineral medium (PAS) (Bedard *et al.*, 1986) containing 1 g/L (9 mM) pyruvate or 200 mg/L (150 mM) biphenyl as the growth substrate.

2. Chemicals.

Biphenyl (99%), ^{14}C-biphenyl (7.7 Ci/mol), anthracene (99%), benzoate (99%), (±)-camphor (96%), (s)-(+)-carvone (96%), beta-citronellol (95%), cumene (99%), *p*-cymene (99%), 1,3-dicyclohexylcarbodiimide (DCCD) (99%), fluorene (99%), 2-methylnaphthalene (97%), naphthalene (99%), 1,4-dimethylnaphthalene (95%), phenanthrene (99.5%), (+)-Limonene (97%), linoleic acid (60%), myricetin (85%), naringenin (95%), β-nicotinamide adenine dinucleotide (β-NADH), 2,4-dinitrophenol (90%), (+)-(α)-pinene (99%), salicylic acid (99%) and o-nitrophenyl-beta-D-galactopyranoside (ONPG) were obtained from Sigma-Aldrich Canada (Oakville, Ontario). Benzene (99.9%) and toluene (99.8%) were obtained from Fisher Scientific. 2-Chlorobiphenyl (99%), 3-chlorobiphenyl (99%), 4-chlorobiphenyl (99%), 2,2'-dichlorobiphenyl (99%), 4,4'-dichlorobiphenyl (99%), Aroclor 1221 (99%), Aroclor 1242 (99%), Aroclor 1254 (99%) and Aroclor 1260 (99%) were obtained from Accu Standard (New Haven, CT, USA). Oligonucleotide primers were synthesized at the

Table 3. Strains and plasmids used in this study

Strain or plasmid	Genotype or description	Reference or source
Strains		
Pseudomonas sp. Cam-1	Wild type	this study
Pseudomonas sp. Sag-1	Wild type	this study
Pseudomonas sp. Iqa-1	Wild type	this study
Pseudomonas sp. Sag-50G	Wild type	Mohn *et al.*(1997)
Pseudomonas sp. Cam-10	*bphA-lacZ*-Gm^R	this study
Burkholderia sp. LB400	Wild type	Bopp (1986)
Burkholderia sp. LB400-1	*bphA-lacZ*-Gm^R	this study
Escherichia coli XL1-Blue MR	△(*mcrA*)183 △(*mcrCB-hsdSMR-mrr*)173 *endA1 supE44 thi-1 recA1 gyrA96 relA1 lac*	Stratagene
Escherichia coli DH5α	*EndA1 hsdR17*(r_k^- m_k^-) *supE44 thi-1 recA1 gyrA*(Nal^r) *relA1* △(*lacZYA-argF*) U169 *deoR* (ϕ80*dlac* (*lacZ*)M15)	Gibco BRL
Escherichia coli S17-1	*recA pro thi hsdR* with integrated RP4-2-tc::Mu-kan::Tn7; Tra^+, Tr^R, Sm^R	Schweizer (1993)
Escherichia coli C41(DE3)	mutant derived from strain BL21(DE3) (Studier *et al.*, (1990))	Miroux and Walker (1996)
Plasmids		
pUC19	Cloning vector; Amp^R	Yanisch-Perron *et al.*(1985)
pT7-7	Cloning vector; T7 promoter; Amp^R	
pT7-6a	*bphAEFG* from *Burkholderia* sp. strain LB400 into multiple cloning site of pT7-6	Studier *et al.*(1990) Hofer *et al.* (1993)
pEM1	SuperCosI cosmid library clone containing *bphAEFGBC* gene cluster from Cam-1	this study
pEM10	*bphAEFGBC* containing 8 kb *Sac*I fragment from pEM1 into *Sac*I of pUC19	this study
pT7-7a	4 kb *Bam*HI fragment PCR amplified from pEM10 into *Bam*HI of pT7-7	this study
pPAISC	*isc* gene cluster from *Pseudomonas putida* KT2442 cloned into pRKNMC (Nakamura *et al.*(1999))	provided by Pascal D. Fortin and Yong Ge
pEX100T	*sac*B conjugable plasmid for gene replacement; Amp^R	Schweizer (1995)
pX1918GT	Plasmid containing the xylE_gentamicin resistance cassette; Amp^R, Gm^R	Schweizer (1995)
pUCGm	Plasmid containing the gentamicin resistance cassette; Amp^R, Gm^R	Schweizer (1993)
pIND/lacZ	Plasmid containing *lac*Z, Amp^R, Neo^R	Invitrogen
pEM2	4 kb *Bam*HI fragment from pT7-7a into *Sma*I of pEX100T	this study
pEM20	877 bp *Xba*I fragment from pUCGm into *Xba*I of pIND/lacZ	this study
pEM21	4 kb *Pme*I fragment from pEM20 into *Eco*RI of pEM2	this study

Nucleic Acid and Protein Services unit at the University of British Columbia. Soil extracts were prepared using standard methods (Gerhardt *et al.*, 1994).

3. Characterization of PCB-degrading bacteria from Arctic Soil

3.0. Isolation of PCB-degrading bacteria from Arctic soil samples.

Soil samples were obtained from a Vancouver location, as well as from the following PCB-contaminated (5-100 μg/g dry soil) or pristine Arctic locations: Saglek, Labrador (57°N, 63°W); Cambridge Bay, Northwest Territories (69°N, long. 105°W); Iqaluit, Northwest Territories (lat. 63°N, 69°W). Enrichment cultures were prepared with 1.0 g soil suspended in 100-ml PAS mineral medium containing 200 mg/L biphenyl (Aldrich) as the sole organic carbon source. The enrichment cultures were incubated on a shaker for 3 weeks at 7°C. Subsequent enrichment cultures had 1% inocula. If growth occurred, 0.1% of the culture was transferred to homologous medium. Enriched cultures were diluted in PAS medium then spread on PAS with 1.5% purified agar and 200 mg/L biphenyl on the agar surface. Distinct colonies were picked, suspended in 2.5-ml PAS medium with 200 mg/L biphenyl and incubated at 7°C. Isolates that grew were stored at –70°C with 8% dimethylsulphoxide.

To test PCB transformation activities, isolates were inoculated into solvent washed tubes containing 2.5-ml PAS medium with 100 mg/L biphenyl, 200 mg/L Aroclor 1221 (Accu Standard), and 10 mg/L 2,2',4,4',6,6'-hexachlorobiphenyl (Accu Standard) as an internal standard, then incubated for 5 weeks at 7°C on a shaker (n=2). The tubes contained approximately twice the amount of oxygen required to completely mineralize the added biphenyl. After incubation, the remaining PCBs were extracted

from the cultures with hexane, and the percent transformation of the PCB mixture was determined. Uninoculated controls were used as negative controls.

3.1. PCB degradation by cell suspensions.

Arctic soil isolates were grown at 7°C, and LB400, at 15°C, in 1-L cultures of PAS mineral medium containing 200 mg/L biphenyl. At late logarithmic phase, the remaining biphenyl crystals were allowed to precipitate, then cultures were decanted and centrifuged at 8000 x g for 10 min and washed with PAS buffer. Final cell suspensions contained 600 mg - 800 mg of cell protein per ml of buffer. Cell samples of 2.5-ml were transferred to solvent washed screw cap tubes (n = 3). Control cell suspensions were boiled. PCBs (100 mg/L Aroclor 1242 and 10 mg/L 2,2', 4,4', 6,6'-hexachlorobiphenyl) were added to each tube, then the reaction tubes were incubated at 7, 37 or 50°C. Reaction tubes were transferred to –20°C at regular time intervals over 24 h to stop PCB degradation activity. Control tubes were incubated for 0, 4, and 24 h. Initial rates of transformation of selected PCB congeners were calculated from the slopes of initially linear curves generated by plotting percent of the PCB congener remaining versus time and were standardized for protein concentration.

3.2. Analysis of PCBs.

Remaining PCBs from batch cultures and cell suspensions were extracted twice with an equal volume of hexane. The extracts were pooled then mixed with Na_2SO_4 to dry the organic phase. The extracts were analyzed using a gas chromatograph fitted with a DB5-ms column (30 m x 0.25 mm x 0.25 μm) and coupled to a mass spectrometer (Varian Saturn model 4D ion trap). The sample volume injected was 2.5 μl, the temperature of the injector was maintained at 260°C, and the temperature of the transfer

line was 280°C. The column temperature program used was as follows: 104°C for 3 min, increased at 20°C/min to 160°C, increased at 2.5°C/min to 260°C, increased at 50°C/min to 290°C, and held at 290°C for 3 min .

The mass spectrum of each GC peak reported verified that it corresponded to a PCB congener with a particular number of chlorine substituents. PCB congeners were identified (Table 4) by comparing the relative retention times of peaks corresponding to PCBs with published chromatographs of Aroclor 1221 and 1242 (Frame *et al.*, 1996). Relative amounts of PCB congeners were determined by integrating the area under each peak and dividing by the peak area of the internal standard. The percent transformation of each PCB congener was calculated by subtracting from 100 the percentage values obtained by dividing the relative peak area of a PCB congener in the test sample by the corresponding relative peak area in a control sample, then multiplying by 100.

3.3. Characterization of isolates.

Gram staining, cell size and motility tests were performed on liquid cultures in late-logarithmic phase. Colony morphology determination, oxidase tests and catalase tests were performed on colonies grown on PAS agar with biphenyl at 7°C (Smibert and Kreig, 1994). Anaerobic respiration with nitrate by cultures using glucose as a carbon source was tested as previously described (Wilson *et al.*, 1996). The abilities of each isolate to use a range of substrates for growth at 7°C (Table 6) and to oxidize substrates contained in GN microplates (Biolog, Hayward, Calif.) were also determined. Percent transformation of 100 mg/L Aroclor 1242 at 7°C by Arctic soil isolates growing on co-substrates other than biphenyl was determined by extracting and analyzing remaining

Table 4. Peak number assignment and percentage of each congener in Aroclors 1221 and 1242.

PCB Congener	Aroclor 1221		Aroclor 1242	
	Peak no.	Wt %[a]	Peak no.	Wt %[a]
2	1	42.24	1	0.5
3	2	2.62		
4	3	20.66		
2,2' / 2,6	4	6.34 / 0.05	2	2.74 / 0.05
2,5	5	2.49	3	1.02
2,3'	6	2.99	4	1.34
2,4'	7	12.88	5	6.4
2,6,2'			6	0.96
3,4	8	1.34		
4,4' / 2,5,2'	9	3.66 / 0.34	7	3.19 / 6.98
2,4,2'			8	4.57
2,6,3'	10	0.5	9	0.83
2,3,2' / 2,6,4'	11	0.89 / <0.05	10	5.14
2,5,3' / 2,4,3'			11	1.23 / 0.89
2,5,4' / 2,4,4'	12	0.87 / 0.99	12	6.75 / 8.01
3,4,2' /2,5,2',6'	13	0.89 / 0	13	5.95 / 0.33
2,3,4' /2,4,2',6'	14	0.79 / 0	14	2.79 / 0.09
2,3,6,2'			15	1.1
2,5,2',5'			16	3.32
2,4,2',5'			17	2.63
2,4,6,4'			18	1.48
2,3,2',5'			19	3.26
3,4,4' /2,3,2',4'			20	1.94 / 1.38
2,3,6,4'			21	3.11
2,3,2',3'			22	1.01
2,4,5,4'			23	1.6
2,5,3',4'			24	3.41
2,4,3',4'			25	3.47
2,3,4,4'			26	2.51
2,3,6,2',3'			27	0.76
2,4,5,2',5'			28	0.88
2,4,5,2',4'			29	0.74
2,4,5,2',3'			30	0.72
2,3,4,2',5'			31	0.78
2,3,4,2',4'			32	0.71
2,3,6,3',4'			33	1.0
2,4,5,3',4'			34	0.8
2,3,4,3',4'			35	0.74

[a] Percent composition of each PCB congener was obtained from Frame (1996)

PCBs from batch cultures after 5 weeks of incubation (n=2).

3.4. 16S rDNA PCR and DNA sequencing.

Cell pellets from 500-µl aliquots of late-logarithmic-phase cultures grown at 7°C on 200 mg/L biphenyl were washed with sterile 0.8% (w/v) NaCl, suspended in 90 µl of sterile, de-ionized water, and boiled for 10 min. Cell debris was pelleted by brief centrifugation. The 16S rRNA gene was amplified from each isolate by PCR using the reagents and procedures of Gibco BRL Life Technologies, Inc., Gaithersburg, Md. and primers hybridizing to positions 8 to 27 and 1541 to 1525 (Lane, 1991). Thermal cycling was performed in a PowerBlock II™System (ERICOMP) according to the following program: initial denaturation at 94°C for 3 min; 30 cycles of 94°C for 30 s, 43°C for 1 min, 72°C for 2 min; and a final extension at 72°C for 10 min.

PCR products were cloned using a TOPO TA cloning kit according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Resulting transformants were screened by alkaline lysis (Ausubel *et al.*, 1992), then digesting recovered plasmids with *EcoR1* and checking for 1.6 Kb fragments on a 0.8% agarose gel. Nearly complete 16S rDNA sequences were determined using FS Taq terminator chemistry and primers between bases 27 to 1518 (Lane, 1991). DNA sequences were determined by the Nucleic Acid and Protein Services unit by using AmpliTaq FS DyeDeoxy terminator cycle sequencing chemistry (Applied Biosystems) and Centri-Sep columns (Princeton Separation, Adelphia, N.J.) to purify the extension products.

3.5. Phylogenetic analysis.

Each 16S rDNA sequence that was determined was compared to other prokaryotic 16S rDNA sequences using the Similarity_Rank analysis service of the Ribosomal

Database Project (Larsen *et al.*, 1993). The 16S rDNA sequences of the closest relatives to the Arctic soil isolates env. JAP501 (Genbank U09827), env. JAP412 (Genbank U09773) and *Pseudomonas syringae* str. A501 (GenBank L24787), as well as representative α-proteobacteria, *Acetobacter aceti* (ATCC 15973), *Agrobacterium tumefaciens* (ATCC 4720), *Sphingomonas capsulata* (GIFV 11526), *Sphingomonas paucimobilis* (ATCC 29837), *Sphingomonas terrae* (IFO 15098), β-proteobacteria (*Alcaligenes denitrificans* (ATCC 15173), *Burkholderia cepacia* (ATCC 25416), *Comamonas testosteroni* (ATCC 11996), *Stenotrophomonas maltophilia* (ATCC 13637), *Zoogloea ramigera* (ATCC 25935) and γ-proteobacteria, *Pseudomonas aeruginosa* (ATCC 10145), *Pseudomonas fluorescens* (ATCC 13525), *Pseudomonas putida* (ATCC 12633), *Pseudomonas stutzeri* (ATCC 17588), *E. coli* (GenBank J01695), were retrieved from RDP and aligned with the 16S rDNA sequences of the Arctic soil isolates using ClustalX. One hundred bootstrapped data sets of the aligned sequences were obtained using SEQBOOT. Phylogeny estimates for each of these data sets were obtained using the default parameters of DNADIST. A phylogenetic tree was obtained by analyzing the resulting distance matrices with the default parameters of NEIGHBOR and CONSENSE. The 16S rDNA sequences determined for Cam-1, Iqa-1, Sag-50G and Sag-1 have been deposited in the EMBL Nucleotide Database and have the following accession numbers: AF098464, AF098465, AF098466, AF098467, respectively.

4. Comparison of Biphenyl Dioxygenases from Psychrotolerant and a Mesophilic PCB-degrading Bacteria

4.0. Cloning *bph* genes from strain Cam-1.

Total genomic DNA was isolated from *Pseudomonas* sp. strain Cam-1 using hexadecyltrimethyl ammonium bromide (CTAB) (Ausubel *et al.*, 1992) and was partially digested with *Sau3A*. Partially digested DNA was size fractionated using a 10 to 40% linear sucrose gradient. DNA fragments of approximately 20 Kb were cloned into SuperCos following the manufacturer's instructions (Stratagene). In vitro packaging of the recombinant molecules was performed using GigapackII Gold packaging extract (Stratagene), and packaging reactions were used to infect *E.coli* XL1-Blue MR. The resulting cosmid library was amplified and screened for the production of 2-hydroxy-6-oxo-6-phenyl-hexa-2,4-dienoic acid (a yellow meta-cleavage product) from biphenyl (Kiyohara *et al.*, 1982). Removal of biphenyl by yellow colonies was confirmed by adding 25 mg/L biphenyl to cell suspensions of these clones, then extracting remaining biphenyl with hexane after incubation, and analyzing the extracts by gas chromatography-mass spectrometry as previously described. Cosmid pEM1 was obtained from constructs that transformed biphenyl. Restriction fragments of cosmid pEM1 were separated on an agarose gel, transferred to maximum strength Nytran Plus nylon membrane (S&S Nytran Plus®), then hybridized with ^{32}P- labeled *bphA*, *bphF* and *bphG* from pT7-6a (Table 3). The Nick Translation System from Life Technologies was used to label *bphA* and *bphG* with [α-^{32}P]deoxycytidine triphosphate(dCTP). The Oligolabelling Kit from Pharmacia Biotech (Uppsala, Sweden) was used to label *bphF* with [α-^{32}P]dCTP. A *SacI* restriction fragment that hybridized to all three probes was

subcloned into pUC19, giving pEM10. The sequence of the cloned DNA from Cam-1 was obtained by generating successive unidirectional deletions of pEM10 using the double stranded nested-deletion system from Pharmacia Biotech (Uppsala, Sweden). Oligonucleotide primers were used to sequence DNA regions not covered by the deletions. DNA sequences were determined as described in "methods section 3.5". The nucleotide sequence obtained was submitted to the GenBank database under the accession no. AY027651. ClustalX was used to align cloned Cam-1 DNA sequence with the *bph* operon sequence from LB400. Vent polymerase (New England Biolabs) and PCR primers with *BamHI* containing 5'-extensions were used to subclone *bphAEFG* from pEM10 into pT7-7, yielding pT7-7a.

4.1. Expression of recombinant biphenyl dioxygenases in *E.coli*.

The terminal oxygenase components of BPDO from *Burkholderia* sp. strain LB400 (ISP_{LB400}) were overexpressed in *E.coli* strain C41(DE3) (Miroux and Walker, 1996) from pT7-6a, which contains *bphAEFG* genes from LB400 (Hofer *et al.*, 1993, Studier *et al.*, 1990). The terminal oxygenase components of BPDO from strain Cam-1 (ISP_{Cam1}) were overexpressed in *E.coli* strain C41(DE3) from pT7-7a. The expression of iron-sulfur cluster (*isc*) genes from *E.coli* enhances the expression of active iron-sulfur containing enzymes (Nakamura *et al.*, 1999). Accordingly, *isc* genes from *Pseudomonas putida* KT2442 were cloned into pRKNMC (Nakamura *et al.*, 1999) forming pPAISC, and pPAISC was coexpressed in *E.coli* strain C41(DE3) containing pT7-6a or pT7-7a. For expression of ISP_{LB400} and ISP_{Cam1}, recombinant *E.coli* strains were grown at 37°C on Terrific broth (12 g/L bacto-tryptone, 24 g/L bacto-yeast extract, 4 ml/L glycerol, 2.31 g/L KH_2PO_4, 12.54 g/L K_2HPO_4) containing 100 μg/ml ampicillin, 10 μg/ml tetracycline,

an HCl-solubilized solution of minerals (10 ml/L) (Vaillancourt *et al.*, 1998), and ferric ammonium citrate (0.1 mg/ml). When the OD_{610} of the culture reached approximately 0.7, the culture was cooled to 22°C, then isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 1 mM. After addition of IPTG, cultures were incubated for an additional 18 h at 22°C before harvesting by centrifugation. The harvested cell pellet was washed with PAS buffer then stored at –70 °C until use.

4.2. Purification and handling of proteins.

Proteins were purified and handled anaerobically in a glovebox maintained at less than 2 ppm O_2 unless otherwise specified. High resolution chromatographic procedures were performed using an ÄKTA Explorer (Amersham Pharmacia Biotech, Baie d'Urfé, Québec, Canada) interfaced with a Labmaster Model 100 glovebox (MBraun, Peabody, MA) (Vaillancourt *et al.*, 1998). In this way, anaerobic buffers were delivered to the ÄKTA Explorer and column eluates were collected in the glovebox. Buffers were prepared using water purified on a Barnstead NANOpure UV apparatus to a resistivity of greater than 17 MΩcm. Buffers for anaerobic procedures were filtered (Ahlstrom Filtration), bubbled with argon for 20 min, and equilibrated in the glovebox for 24 h prior to addition of dithiothreitol and iron. Buffer A contained 25 mM HEPES (pH 7.3), 10 % glycerol, 2 mM dithiothreitol and 0.5 mM ferrous ammonium sulfate; buffer B was buffer A with 1 M NaCl; and buffer PS was buffer A containing 5% saturated ammonium sulfate (0.205 M). Protein-containing fractions were concentrated by ultrafiltration using an Amicon stirred cell equipped with a YM30 membrane.

For purification of ISPs, washed cell pellets from 4 L of culture were suspended in 80 ml 25 mM HEPES (pH 7.3) and 10% glycerol containing 2 mM dithiothreitol and

0.1 mg/ml DNase I. Cell suspensions were divided into two batches and sonicated using an ultrasonic liquid processor (Misonix Incorporated, Farmingdale, NY) with 10 X 12 s pulses adjusted to 30% of maximum output. The cell debris was removed by ultracentrifugation at 300 000 g for 70 min at 4°C . Centrifuge tubes were transferred to the glove box and the clear supernatant was decanted, passed through a 0.45 μm filter, then diluted with 40 ml buffer A. Samples of 20 ml crude extract were loaded onto a Mono Q HR 10/10 anion-exchange column (Pharmacia Biotech) equilibrated with buffer A and operated at a flow rate of 3 ml/min. Bound proteins were eluted using a linear gradient of 0 to 20% buffer B over 10 column volumes. Brown-colored ISP-containing fractions (*i.e.*, absorbing at 323 nm and 455 nm, characteristic of the [2Fe-2S] Rieske-type centre, and containing subunits of the expected size as seen by denaturing gel electrophoresis), were pooled and concentrated to less than 50 ml by ultrafiltration. The samples were brought to 5% saturation with ammonium sulfate, filtered, then loaded onto a phenyl-Sepharose column (1 X 9 cm; Amersham Pharmacia Biotech) equilibrated with buffer PS. The column was operated at a flow rate of 5 ml/min. Bound proteins were eluted using a linear gradient of 5 to 0% ammonium sulfate over 5 column volumes. ISP_{Cam1} eluted at 0% ammonium sulfate. ISP_{LB400} eluted as a comparatively broad peak at 1% ammonium sulfate concentration. ISP-containing fractions were concentrated to between 20 and 30 mg/ml and flash-frozen as beads in liquid nitrogen.

His-tagged ferredoxin from *Burkholderia* sp. LB400 (ht-$BphF_{LB400}$) and his-tagged reductase from *Comamonas testosteroni* B-356 (ht-$BphG_{B356}$) were prepared using the QIAexpress system from Qiagen (Couture *et al.*, 2001; Hurtubise *et al.*, 1995).

4.3. Steady-state Kinetic Measurements.

Enzymatic activity was measured by following the consumption of O_2 using a Clark-type polarographic O_2 electrode (Yellow Springs Instruments Model 5301, Yellow Springs, OH) (Vaillancourt, 1998). The activity assay was performed in a thermojacketted Cameron Instrument Co. model RCI respiration chamber (Port Aransas, TX) connected to a Lauda Model RM6 circulating bath. Initial velocities were determined from progress curves by analyzing the data using Microsoft Excel.

The standard activity assay was performed in a total volume of 1.4 ml of air-saturated 50 mM MES, pH 6.0 (25°C) containing 160 μM biphenyl, 320 μM NADH, 1.8 μM ht-$BphG_{B356}$, 3.6 μM ht-$BphF_{LB400}$, and 0.6 μM of ISP. The assay was initiated by adding the oxygenase after equilibrating the assay with all other components for 30 s. The reaction buffer and stock solutions used in the assay were prepared fresh daily. Stock solutions and protein samples were prepared anaerobically, stored under argon on ice, and withdrawn using a gas-tight syringe. On the day of use, the O_2 electrode was zeroed by adding excess of sodium hydrosulfite to buffer in the reaction chamber, and calibrated using standard concentrations of 2,3-dihydroxybiphenyl and excess of dihydroxybiphenyl dioxygenase (BphC). One unit of enzyme activity was defined as the quantity of enzyme required to consume 1 μmol of O_2/min.

For assays performed at other temperatures, the temperature of the respiration chamber was regulated using an attached circulating water bath. The reaction buffer was pre-equilibrated at the desired temperature using ice or a hot plate, as appropriate. Apparent steady-state kinetic parameters were determined by using biphenyl concentrations from 0.5 μM to 160 μM. The solubility of biphenyl in 50 mM MES

buffer (pH 6.0), 44 μM, is similar to that in water (Foreman and Bidleman, 1985; Imbeault *et al.*, 2000). Initial velocities determined at different substrate concentrations were fitted to the Michaelis-Menten equation using the least-squares fitting and dynamic weighting options of LEONORA (Cornish-Bowden, 1995).

4.4. Half-life Measurements.

The half-life of ISP_{Cam1} and ISP_{LB400} at 57°C was determined spectrophotometrically by continuous monitoring of absorbance at 323 nm. The oxygenase components were diluted in MES buffer (pH 6.0) pre-equilibrated to 57°C, and the temperature of the spectrophotometer was maintained at 57°C using an attached circulating water bath.

4.5. Analytical Methods.

SDS-PAGE analysis of ISP preparations were performed using 12% resolving gels and Coomassie Blue staining according to standard protocols (Ausubel *et al.*, 1992). The concentration of ISP was determined spectrophotometrically using $\varepsilon_{450} = 10.1$ $mM^{-1}cm^{-1}$ (Haddock and Gibson, 1995) and by performing a bicinchoninic acid protein assay (Pierce) after removing interfering substances (Brown, 1989). Iron assays were performed using Ferene S and $FeCl_3$ as a standard (Haigler and Gibson, 1990). The content of acid-labile sulfur in the ISP preparations was determined using *N,N*-dimethyl-*p*-phenylenediamine and Na_2S as a standard (Chen and Mortenson, 1977).

Concentrations of ht-$BphF_{LB400}$ and ht-$BphG_{B356}$ were determined spectrophotometrically using $\varepsilon_{326} = 9.0$ $mM^{-1}cm^{-1}$ and $\varepsilon_{450} = 11.8$ $mM^{-1}cm^{-1}$, respectively (Couture et al, 2001; Hurtubise *et al.*, 1995).

5. Regulation of the *bph* operon

5.0. Insertion of a *lacZ*-Gm^R cassette into the *bph* operon.

The pEX100T gene replacement vector (Schweizer, 1993; 1995) was used to insert a selectable *lacZ* reporter gene cassette between *bphA* and *bphE* genes of *Pseudomonas* sp. strain Cam-1 and *Burkholderia* sp. strain LB400 (Fig.6). Plasmids pEM2, pEM20 and pEM21 were selected in *E.coli* DH5α grown on LB medium containing appropriate antibiotics and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). Plasmid pEM21 was transformed by electroporation into the mobilizer strain *E.coli* S17-1 and conjugally transferred into Cam-1 and LB400 (Gerhardt *et al.*, 1994). Cam-1 and LB400 grow on pyruvate, however *E.coli* S17-1 does not. Therefore, exconjugants were plated on minimal medium containing 9 mM pyruvate and 10 μg/ml gentamycin and colonies that appeared on this medium after 48 h incubation at 30°C were streaked onto LB agar containing 10 μg/ml gentamycin. Colonies of Cam-1 and LB400 in which double homologous recombination had occurred were selected on LB medium containing 10 μg/ml gentamycin and 5% sucrose and were designated Cam-10 and LB400-1 respectively. Sucrose resistant colonies were also sensitive to ampicillin, indicating that these colonies had lost the pEX100T vector associated sequences. Gene insertions in Cam-10 and LB400-1 were also confirmed by colony PCR with 20-mer primers annealing to the 3'-region of *bphA* (5'-GACCTGGCAGAACAGCGACT) and the 5'- regions of *bphE* (5'-TCTGCACATGCACGTCCAGC-3') or the *lacZ* reporter gene (5'-GTATCGCTCGCCACTTCAAC-3') (Zon *et al.*, 1989).

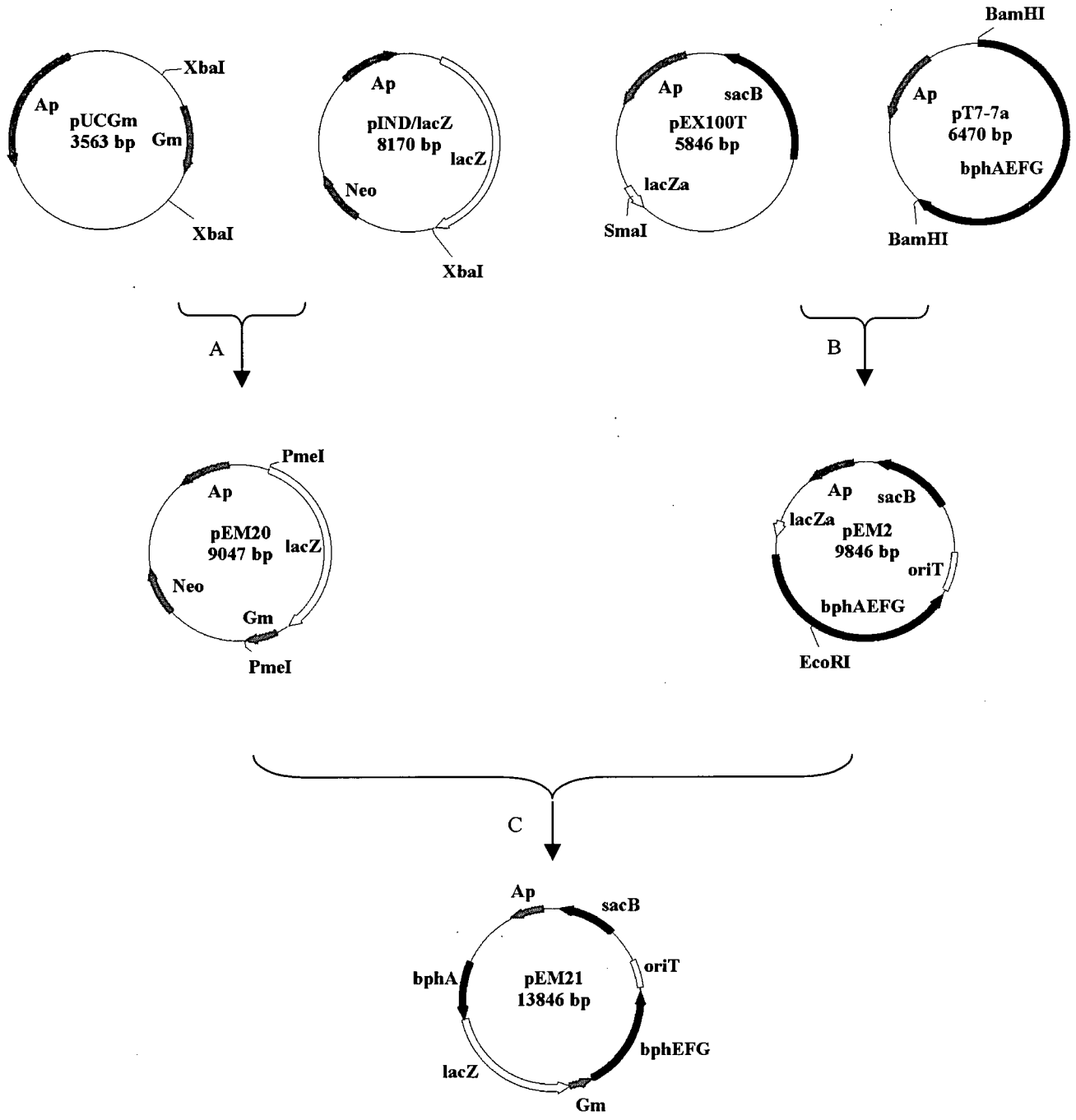

XbaI
Ap
pUCGm
3563 bp
Gm
XbaI
Ap
pIND/lacZ
8170 bp
lacZ
Neo
XbaI
Ap
sacB
pEX100T
5846 bp
lacZa
SmaI
BamHI
Ap
pT7-7a
6470 bp
bphAEFG
BamHI
A
B
PmeI
Ap
pEM20
9047 bp
lacZ
Neo
Gm
PmeI
Ap
sacB
lacZa
pEM2
9846 bp
oriT
bphAEFG
EcoRI
C
Ap
sacB
oriT
bphA
pEM21
13846 bp
bphEFG
lacZ
Gm

Fig. 6. Construction of a selectable *lacZ* reporter cassette, and pEM21. Step A, construction of pEM20. The 877 bp *Xba*I fragment from pUCGm was gel-purified and ligated into the *Xba*I site of pIND/lacZ. Restriction digests using *Eco*RV were used to isolate plasmids containing *lacZ* and *Gm* in the same transcriptional orientation. Step B, The 4kb *BamH*I fragment from pT7-7a was treated with the large fragment of DNA polymerase I (PolK) then ligated into the *Sma*I site of pEX100T. Restriction digests using *Sca*I and *Sac*II were used to isolate plasmids containing *lacZ*α and *bphAEFG* in the same transcriptional orientation. Step C, construction of pEM21. The 4 kb *Pme*I fragment of pEM20 was gel-purified and ligated into the PolK-treated *EcoR*I site of pEM2. Colonies containing *bphAEFG* and *lacZ*-Gm^R in the same transcriptional orientation were detected by formation of blue color when grown on Luria-Bertani medium supplemented with gentamycin, ampicillin and X-gal. The location of restriction sites, genes and their transcriptional orientation are shown. *Ap*, β-lactamase encoding gene; *Gm*, gentamycin acetyltransferase-3-1 encoding gene; *Neo*, neomycin resistance gene; oriT, origin of transfer.

To verify that Cam-1 requires the *bph* genes for biphenyl degradation, I inserted the *xylE*_Gm^R cassette from pX1918GT between chromosomal *bphA* and *bphE* genes in Cam-1 to form Cam-20. The *xylE*_Gm^R cassette contains a transcriptional termination sequence downstream of the gentamycin resistance gene. Consequently, transcription of genes downstream of the cassette is inhibited. The method used to generate Cam-20 was similar to that used to generate Cam-10, except pEM20 was substituted by pX1918GT, and the *xylE*_Gm^R cassette was ligated as an *EcoR1* fragment to the *EcoR1* site in pEM2. The resulting plasmid was transformed into the mobilizer strain *E.coli* S17-1 and conjugally transferred into Cam-1 (Gerhardt *et al.*, 1994). Exconjugants were selected as described above. Gene insertions in Cam-20 were confirmed by colony PCR with primers annealing to the 3'-region of *bphA* (5'-GCCGGCACAACATCC) and the 5'-regions of *bphB* (5'-CCAGCTCTGCAAGGCGC-3') (Zon *et al.*, 1989).

5.1. Beta-galactosidase assays.

Unless otherwise specified, Cam-10 and LB400-1 were grown at 30°C on 9 mM pyruvate, in the presence of 10 μg/ml gentamycin, to mid-log phase then cooled on ice for 15 min. Cultures were centrifuged at 5000 x g for 15 min at 4°C and washed with mineral buffer. Washed cells were suspended in mineral medium with 1 mM pyruvate and adjusted to a final A_{600} of 0.6. Cell suspensions (20 ml) were prepared in 125-ml Erlenmeyer flasks, then were inoculated with 0.001 mM to 1 mM potential inducers of the *bphA* gene. Unless otherwise specified, triplicate cell suspensions were incubated with potential inducers for 3 h at 30°C on a rotary shaker at 200 rpm. Beta-galactosidase assays were performed as described by Miller (1972). Precise volumes of chloroform (20 μl) and 0.1% SDS (10 μl) were used to permeabilize cells (Giacomini *et al.*, 1992). Test

samples without ONPG were used as negative controls. Protein concentrations of cell suspensions were determined using a bicinchoninic acid protein assay (Brown *et al.*, 1989).

5.2. Biphenyl transformation by Cam-10 and LB400-1.

Cell suspensions of Cam-10 and LB400-1 were prepared as described above, then 2.5-ml aliquots were transferred to Teflon-lined screw-capped tubes. Duplicate cell preparations were inoculated with 0.1 mM biphenyl then incubated on a tube roller at 30°C for 3 h or 6 h. Boiled cells and mineral medium containing 0.1 mM biphenyl were used as negative controls. Remaining biphenyl was extracted from cell suspensions with hexane, and extracts were analyzed by gas chromatography as described in "Materials and Methods" section 3.3.

6. Biphenyl Uptake

6.0. Uptake Assay.

Pseudomonas sp. strain Cam-1 and *Burkholderia* sp. strain LB400 were grown at 22°C on 200 mg/L biphenyl or 1 g/L pyruvate until late logarithmic phase. Cultures were centrifuged at 6000 x g for 10 min then washed with PAS mineral buffer. Final cell suspensions contained approximately 2 mg of cell protein/ml. Biphenyl transport was assayed at 22°C, and separate reaction mixtures were prepared in teflon-lined screw capped vials for each timed interval. [^{14}C]-biphenyl was supplied at 150 μM (0.67 Ci/ mol) to 50 μl mineral buffer, and was equilibrated for 10 min. The assay was initiated by adding 50 μl of concentrated cells to the reaction mixture using a gas-tight Hamilton syringe. At timed intervals, biphenyl uptake was terminated by diluting the reaction

mixture with 1.0 ml of ice-cold mineral buffer, and filtration through a nitrocellulose membrane (0.22 μm pore size, Millipore GS). The filter membranes were then washed for 1 min with 5% Tween and 50% ethanol, and transferred to scintillation cocktail for counting. Kinetic parameters for biphenyl transport were determined by performing an uptake assay using different concentrations of biphenyl. The time course experiments revealed that the rate of biphenyl uptake decreased after 1 min. Thus, in the kinetic experiments, biphenyl uptake was terminated after 1 min. The effect of metabolic inhibitors on biphenyl uptake was investigated by adding DCCD or DNP at final concentrations of 2.0 mM and 16 mM, respectively to cell suspensions 10 min before adding cells to the transport assay medium. Killed cell treatments and treatments without cells were used as negative controls.

6.1. Effect of biphenyl transformation on biphenyl uptake.

Biphenyl transformation by Cam-1 and LB400 requires oxygen. Therefore, to determine if biphenyl uptake was driven by biphenyl metabolism, biphenyl uptake was assayed under anaerobic conditions. Cell suspensions Cam-1 and LB400 grown on biphenyl were prepared as described above, then were flushed with Argon gas. Reaction mixtures were prepared as described above except anaerobic mineral buffer was used, and reaction vials were flushed with Argon gas. Biphenyl metabolism under anaerobic and aerobic reaction conditions was monitored by trapping $^{14}CO_2$ in 1 M NaOH.

7. Sequential anaerobic-aerobic treatment of soil contaminated with weathered Aroclor 1260.

7.0. Preparation and sampling of aerobic treatments

Weathered PCB-contaminated soil samples from Saglek, Canada, were incubated anaerobically with sediment that contained reductive dechlorinating activity (Kuipers *et al.*, 1999). This initial anaerobic treatment was performed by graduate students from the Department of Chemistry at U.B.C. as previously described (Kuipers *et al.*, 1999). In preliminary experiments, anaerobically treated soil was washed with mineral medium (PAS) (Bedard *et al.*, 1986) to remove potentially inhibitory soluble compounds (e.g., sulfide), aerated, or washed and aerated before bioaugmentation with LB400 grown on biphenyl. The effect of adding biphenyl to the aerobic treatments was also tested. These preliminary tests showed that washing the anaerobically treated soil or adding biphenyl did not improve the aerobic treatment. However, the amount of biphenyl added might not have been significant compared to the amount of biphenyl that was carried over in the inoculum. As a result, for this experiment the anaerobically treated soil and sediment slurries were pooled in a 500 ml Erlenmeyer flask, then aerated on an orbital shaker. Slurry aliquots of 15 g were transferred into 6 125-ml serum bottles then 10 ml of mineral medium was added to each bottle. Six additional slurries were prepared in 125-ml serum bottles by mixing 15 g of the above soil, which was not anaerobically treated, and 10 ml of mineral medium. Biphenyl (100 μg/g) was added to each treatment. LB400 was added to 3 of the 6 anaerobically treated and 3 of the 6 non-anaerobically treated slurries, to 2 x 10^6 cells/g. Serum bottles were crimp-sealed with Teflon-faced rubber septa and incubated at 21°C on an orbital shaker at 200 rpm. Following 0, 3, 7, 14, and 28 days of incubation, 2-g samples from each aerobic treatment were transferred to Teflon-lined screw capped glass tubes. Samples were stored at -20°C before extraction. Each sampling permitted equilibration of culture headspace with air.

7.1. Extraction of PCBs from soil.

The slurries were extracted by shaking and vortexing soil twice with an equivalent volume of acetone, then twice with an equivalent volume of hexane. Tubes were centrifuged (2 min. at 1,500 x g) between extractions. The extracts were pooled and evaporated under N_2(g) to approximately 0.5 ml, then passed through a Pasteur pipette packed with hexane washed Florisil topped with sodium sulfate. PCB congeners were eluted from the Florisil column using hexane, and eluates were collected in glass chromatographic vials.

Samples were analyzed as described in section 3.3.

Congener assignments were made by comparison of the retention times with those of Aroclor 1221, 1242, 1254 and 1260 and comparison of the relative retention times with published results (Frame, 1997). Individual congener standards were used to quantify major product congeners, which are present in traces or not present in the Aroclor standards. Linear three-point calibration curves were generated for all congeners using either pure congeners or using the weight percent contributions of the components present in Aroclor standards (Williams, personal communication). Amounts of PCBs (μg) per g of dry soil plus sediment were calculated, using as an internal standard, 2,3,4,5,6-2,3,4,5-nonachlorobiphenyl, which is present in the contaminated soil, to correct for variability in sample size and PCB recovery. Importantly, 2,3,4,5,6-2,3,4,5-nonachlorobiphenyl is not significantly degraded after 16 weeks of anaerobic incubation (Kuipers *et al.*, 1999), and aerobic biodegradation of this PCB congener has not been demonstrated.

RESULTS

CHAPTER ONE: Psychrotolerant bacteria isolated from Arctic soil that degrade polychlorinated biphenyls at low temperature

1.0. Introduction.

Several mesophilic bacteria capable of co-metabolizing PCBs via the biphenyl catabolic pathway have been described. Well-characterized PCB-degrading bacteria include *Pseudomonas pseudoalcaligenes* KF707 (Furukawa and Miyazak, 1986), *Pseudomonas* sp. strain KKS102 (Kimbara *et al.*, 1989), *Burkholderia* sp. strain LB400 (Bopp, 1986), *Comamonas testosteroni* B-356 (Ahmad *et al.*, 1990); *Rhodococcus globerulus* P6 (Peloquin and Greer, 1993), and *Rhodococcus* sp. RHA1 (Seto *et al.*, 1993). Bioremediation strategies in Arctic and temperate areas that use cold-adapted PCB-degrading bacteria may be more efficient than strategies employing mesophilic PCB-degrading bacteria, since heating requirements for degradation activity may be reduced. In addition, PCB-degrading bacteria indigenous to Arctic soil have presumably adapted to various soil characteristics that limit the survival or activity of foreign PCB-degrading microorganisms. In this chapter, the isolation and characterization of PCB-degrading psychrotolerant bacteria from PCB-contaminated Arctic soil is described. The effect of temperature on the extent and rate of PCB transformation by Arctic soil isolates and the mesophile *Burkholderia* sp. strain LB400 are also compared.

1.1. Isolation of PCB degraders

Of approximately 50 biphenyl degrading bacteria isolated from PCB-contaminated or pristine (< 5 mg PCBs/ kg of soil) Arctic soil samples or pristine Vancouver soil samples, three (Cam-1, Sag-1 and Iqa-1) could remove PCBs. Interestingly, all of the PCB-degrading isolates were obtained from PCB-contaminated Arctic soil samples; no PCB-degrading bacteria were isolated from pristine soil samples. Most of the PCB congeners present in Aroclor 1221 were transformed at 7°C by batch cultures of the above three Arctic soil isolates, albeit to different extents (Fig.7, Table 5). The congener range of these isolates was similar to the congener range of Sag-50G, which was previously reported (Mohn *et al.*, 1997). None of the isolates could remove 2,2'-chlorobiphenyl, though Cam-1 and Sag-1 did remove 4,4'-chlorobiphenyl. This result is consistent with other PCB-degrading bacteria that express 2,3-biphenyl dioxygenase activity and not 3,4-biphenyl dioxygenase activity (Bedard and Haberl, 1990). Transformation of Aroclor 1242 at 7°C and at 15°C by batch cultures of each isolate were also studied. The total transformation of Aroclor 1242 by Cam-1 and Sag-1 was approximately 30% and 15% higher at 15°C than at 7°C, respectively (Table 5). However, the total transformation of Aroclor 1242 by Iqa-1 was approximately 30% higher at 7°C than at 15°C. Differences in relative transformation (per unit final biomass) were consistent with differences in absolute transformation. The range of Aroclor 1242 congeners transformed by each isolate at 7°C and 15°C was similar to the range of congeners transformed by Cam-1 at 7°C (Fig.8) and to that of most mesophilic PCB-degrading bacteria that have been reported. Cam-1 also removed some tetrachlorobiphenyls (peak no.18, 21, 26) at 15°C. The transformation

Table 5. Relative and absolute transformation of Aroclors 1221 or 1242 by batch cultures incubated at 7°C or 15°C for 5 weeks.

Soil Isolate	Relative transformation (absolute transformation)[a] of:		
	Aroclor 1221 at 7°C (n=2)	Aroclor 1242 at:	
		7°C (n=3)	15°C (n=3)
Cam-1	10.3 (56.0)	2.0 ± 0.36 (11.3 ± 2.0)	2.6 ± 0.52 (14.4 ± 3.0)
Sag-1	11.7 (82.1)	1.2 ± 0.34 (8.1 ± 2.4)	1.4 ± 0.41 (10.2 ± 3.4)
Iqa-1	8.7 (87.3)	0.80 ± 0.10 (7.1 ± 1.0)	0.56 ± 0.10 (5.3 ± 1.0)

[a] Relative transformation is expressed as μg of PCBs transformed per mg of cell protein; absolute transformation is expressed as a percentage of total PCBs removed. Values are means ± standard deviations

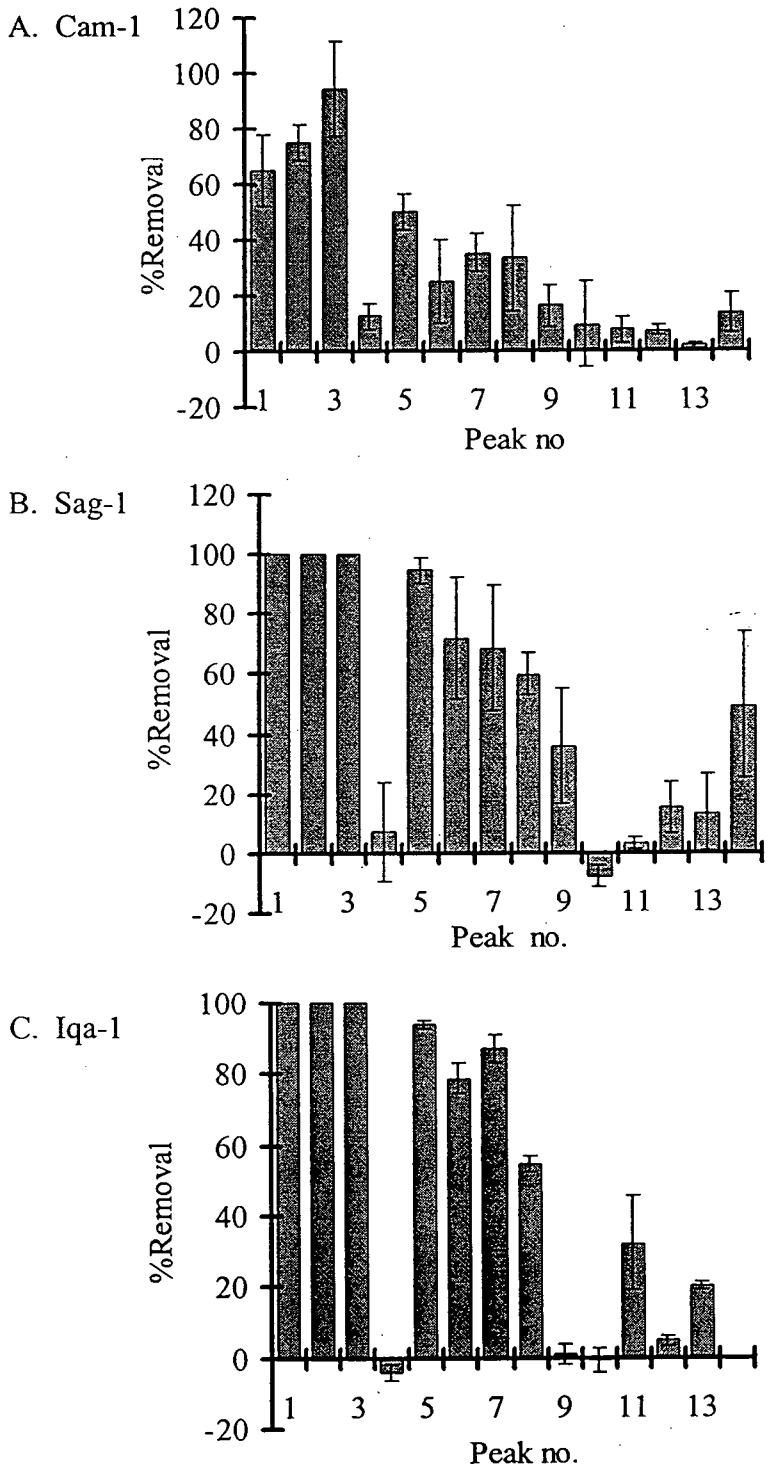


Fig. 7. Percent removal of major congeners in Aroclor 1221 by batch cultures of Arctic soil isolates incubated at 7°C for 5 weeks (n=2; error bars indicate range). Peaks are identified in Table 4.

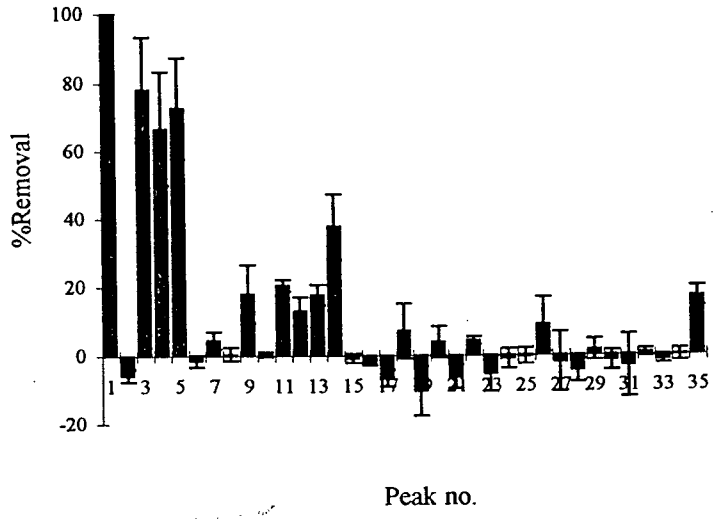


Fig. 8. Percent removal of major congeners in Aroclor 1242 by a batch culture of Cam-1 incubated at 7°C for 5 weeks (n=3; error bars indicate standard deviation). Peaks are identified in Table 4.

of 2,3,4,3',4'-pentachlorobiphenyl by Cam-1 is unlikely since transformation of 2,5,3',4'- and 2,4,3',4'-tetrachlorobiphenyl was not observed. However, the position of chlorine substituents, and not only the number of chlorine substituents, can determine whether a PCB congener is degraded (Arnett *et al.*, 2000; Seeger *et al.*, 1999).

1.2. Morphology, physiology and phylogeny

All of the PCB-degrading bacteria isolated were members of the genus *Pseudomonas*. Both physiological analyses and 16S rDNA sequence analyses support this conclusion. All isolates were Gram-negative, catalase-positive, motile rods. Cam-1 and Iqa-1 were oxidase-positive; Sag-1 was oxidase-negative. The isolates were obligately aerobic and failed to grow anaerobically on glucose, fermentatively or with nitrate. When grown on biphenyl at 7°C, the dimensions of Cam-1 cells were 1.5-3.0 by 1.0 μm; Sag-1 cells were 1.5-4.0 by 1.0 μm and Iqa-1 cells were 1.2-4.0 by 1.0 μm. On PAS agar with biphenyl, colonies of each isolate were circular with a slight undulate edge, convex, butterous, smooth, grey and opaque, except Sag-1 which was translucent. The use of different primary growth substrates and oxidation of test substrates in GN Biolog plates (Table 6) confirmed that these isolates are distinct from each other and from Sag-50G. All of the Arctic bacterial isolates used glucose and galactose as sole carbon sources. None of the Arctic bacterial isolates used 2-chlorobiphenyl, 3-chlorobiphenyl, camphor, citronellol, cymene, dehydroabietic acid, limonene, methanol, *n*-hexadecane, pentachlorophenol, phenol or (+)-(α)-pinene as sole carbon sources.

The 16S rDNA sequences of Cam-1 and Sag-50G were most similar to that of *Pseudomonas* sp. strain JAP501 (S_{ab} = 0.931 and 0.935, respectively), the 16S rDNA

Table 6. Substrate use or oxidized by Arctic soil isolates.

Substrate (mg/L)	Results[a] for			
	Cam-1	Sag-1	Iqa-1	Sag-50G[b]
Fatty Acid				
Linoleic acid (1000)	G	G	G	NG
Sugars				
L-arabinose (1000)	G	G	NG	G
D-galactose (1000)	G	G	G	G
D-glucose (1000)	G	G	G	G
D-sorbitol	NO	NO	NO	O
D-trehalose	NO	NO	NO	O
Aromatic compounds				
Benzoic acid (240)	G	G	G	NG
Biphenyl (200)	G	G	G	G
2-chlorobiphenyl (200)	NG	NG	NG	NG
3-chlorobiphenyl (200)	NG	NG	NG	NG
4-chlorobiphenyl (200)	G	G	NG	G
Naphthalene (200)	G	NG	NG	NG
Other compounds				
α-keto-valerate	O	NO	NO	NO
Acetate (1000)	G	G	G	G
D-galacturonate	NO	O	NO	NO
D-saccharate	O	O	NO	O
Ethanol (200)	NG	NG	G	NG
Glycerol (1000)	G	G	G	G
Hydroxy L-proline	NO	NO	NO	O
Methyl pyruvate	O	O	NO	O
Pyruvate (1000)	G	G	NG	G

[a]G, supports growth as sole organic substrate; NG, does not support growth; O, oxidized in Biolog assay; NO, not oxidized in Biolog assay.

[b] Isolated previously from a PCB-contaminated Arctic soil sample (Mohn *et al.*, 1997).

sequence of Sag-1 was most similar to that of *Pseudomonas* sp. strain JAP412 (S_{ab} = 0.858), and the 16S rDNA sequence of Iqa-1 was most similar to that of *Pseudomonas syringae* A501 (S_{ab} = 0.845). JAP501 and JAP412 are 16S rDNA clones obtained from deep marine environments (Rochelle *et al.*, 1994). The microorganisms represented by these clones may be psychrotolerant. A501 was studied to investigate expression of an ice nucleation gene (Edwards *et al.*, 1994), however its physiology was not described. Importantly, the reported S_{ab} values are too low to indicate the species of the Arctic soil isolates. Moreover, when using a different algorithm to generate a phylogenetic tree, all four isolates cluster together and are closest to *P. syringae* (Fig. 9). Thus, the phylogenetic analysis of the 16S rDNA sequences of the Arctic soil isolates suggests these microorganisms are new species within the genus *Pseudomonas*.

1.3. Temperature optimum for growth

With biphenyl as their organic substrate, Cam-1 and Iqa-1 grew optimally at 15°C, and Sag-1 grew optimally at 7°C (Fig.10). Up to 2 weeks of incubation at 7°C were required before growth was detected, and exponential phase continued for up to 3 days. The mesophile, *Burkholderia* sp. strain LB400, did not grow at 7°C but did grow from 15°C to 37°C. Temperatures higher than 37°C were not tested. Psychrophilic and psychrotolerant bacteria are defined by optimal growth temperatures below 20°C and growth at temperatures as low as 0°C. Psychrotolerant bacteria are distinguished by growth at temperatures above 20°C (Morita, 1975). Since Cam-1, Iqa-1 and Sag-1 grew at 22°C, these microorganisms are psychrotolerant rather than psychrophilic bacteria.

As described in "Introduction" section 4, physiological adaptations of psychrophilic and psychrotolerant bacteria include increased fluidity of cell membranes

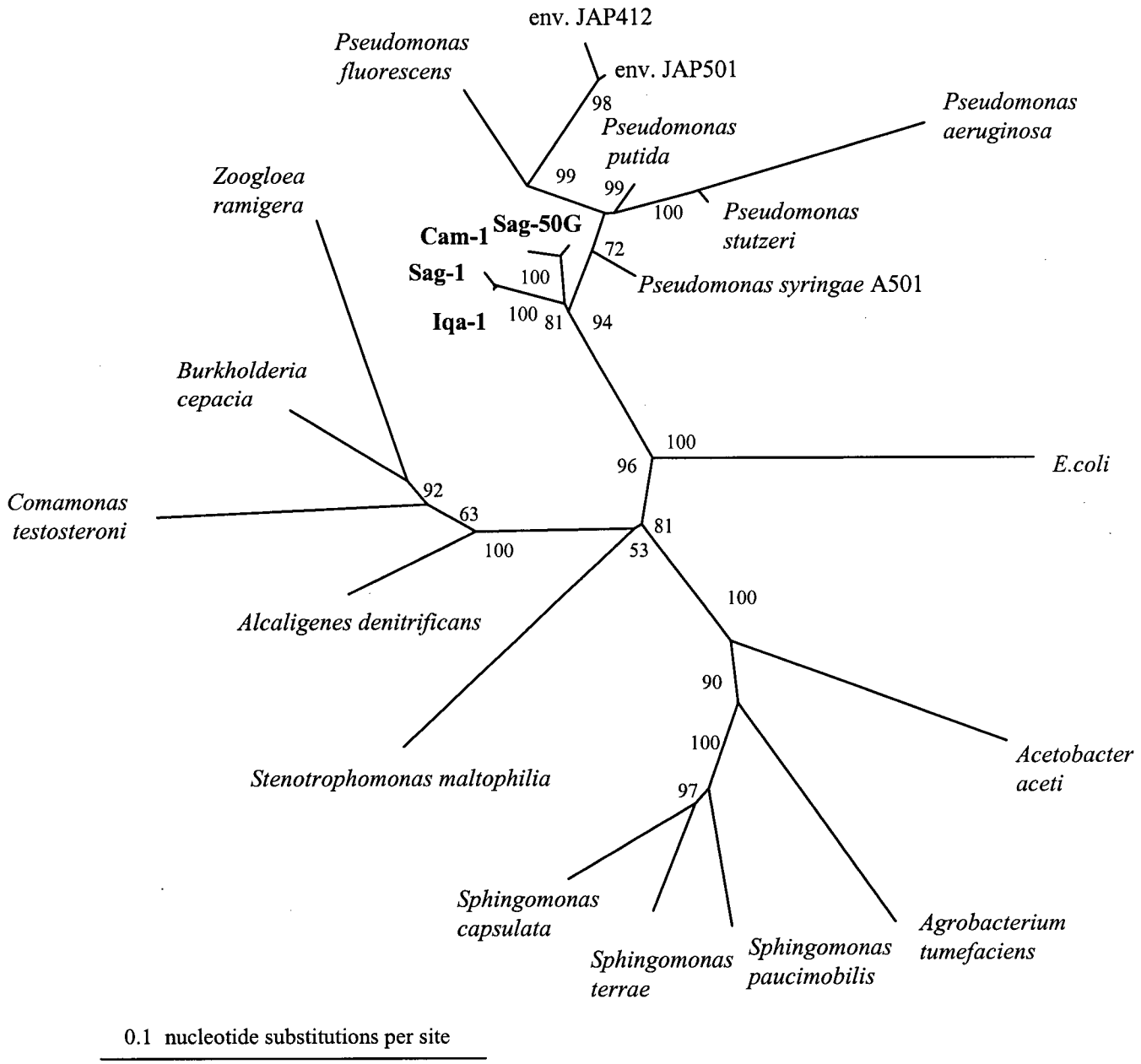


Fig. 9. Unrooted tree showing phylogenetic relationships of Arctic soil bacteria (in bold) and representative members of α-, β-, and γ-*Proteobacteria*. The phylogenetic tree was generated with nearly complete 16S rDNA sequences. The scale shows evolutionary distance. Numbers indicate bootstrap values.

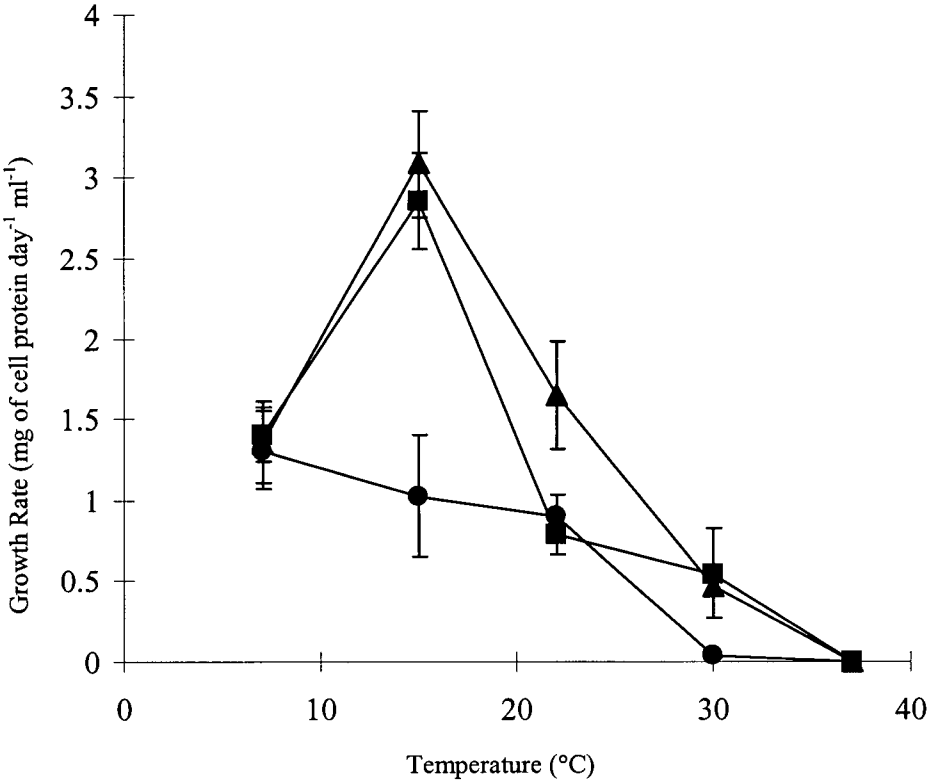


Fig. 10. Growth rates of Arctic soil isolates grown on biphenyl at various temperatures (n=3, error bars indicate standard deviation). ■, Cam-1; ●, Sag-1; ▲, Iqa-1.

resulting from decreased saturation and shortening of phospholipids. Cold adaptation of enzymes encoded by psychrophilic and psychrotolerant organisms may also occur to compensate for decreased kinetic energy at low temperatures. Such physiological and genetic changes may exist in the bacteria that I isolated from Arctic soil and may affect PCB transformation by these organisms. To test this possibility, I compared transformation of PCBs by cell suspensions of the Arctic isolates and LB400 at several temperatures.

1.4. PCB degradation by cell suspensions

High rates of PCB transformation at low temperature, and temperature sensitivity of PCB transformation activity, suggest that PCB degradation enzymes expressed by the Arctic soil bacteria are cold adapted. The ranges of PCBs transformed by cell suspensions of Arctic soil isolates at 7°C (not shown) did not differ from those of batch cultures growing at 7°C (Fig.8). Although no significant transformation of peak 10 by Cam-1 was detected in growing cultures, an initial rate of transformation was evident (Table 7). Cam-1 is probably removing 2,6,4'-trichlorobiphenyl, which is a minor component of peak 10, rather than 2,3,2'-trichlorobiphenyl, which is the major component of peak 10. The inability of Cam-1 to remove 2,3,2'-trichlorobiphenyl seems consistent with its inability to remove other 2,2'- substituted biphenyls. The range of PCBs transformed by LB400 differed from those of the Arctic soil isolates, since LB400 removes 2,2'-dichlorobiphenyl. No significant differences in relative areas of peaks corresponding to PCBs on gas chromatographs were detected between psychrotolerant killed cells and LB400 killed cells, nor between killed cell controls incubated for different time points.

Table 7. Initial rates of transformation of selected Aroclor 1242 PCB congeners by cell suspensions incubated at 7°C (n=2).

Peak no.	Congener	Rate of transformation ($\mu g\ h^{-1}$ g of cell protein^{-1}) by cell suspensions of:				
		LB400	Cam-1	Sag-1	Iqa-1	Sag50G
1	2	105	323	196	360	151
2	2,2'/2,6	362	0	0	0	0
3	2,5	55	108	123	147	109
4	2,3'	45	209	162	173	116
5	2,4'	229	982	772	1150	1048
6	2,6,2'	34	0	0	0	0
7	4,4'/2,5,2'	73	150	0	0	315
8	2,4,2'	78	0	0	0	0
9	2,6,3'	36	961	13	18	0
10	2,3,2'/2,6,4'	24	303	0	0	0
11	2,5,3'/2,4,3'	27	200	64	160	113
12	2,5,4'/2,4,4'	106	784	222	106	0
13	3,4,2'/2,5,2',6'	76	389	189	564	0
14	2,3,4'/2,4,2',6'	33	247	174	176	308

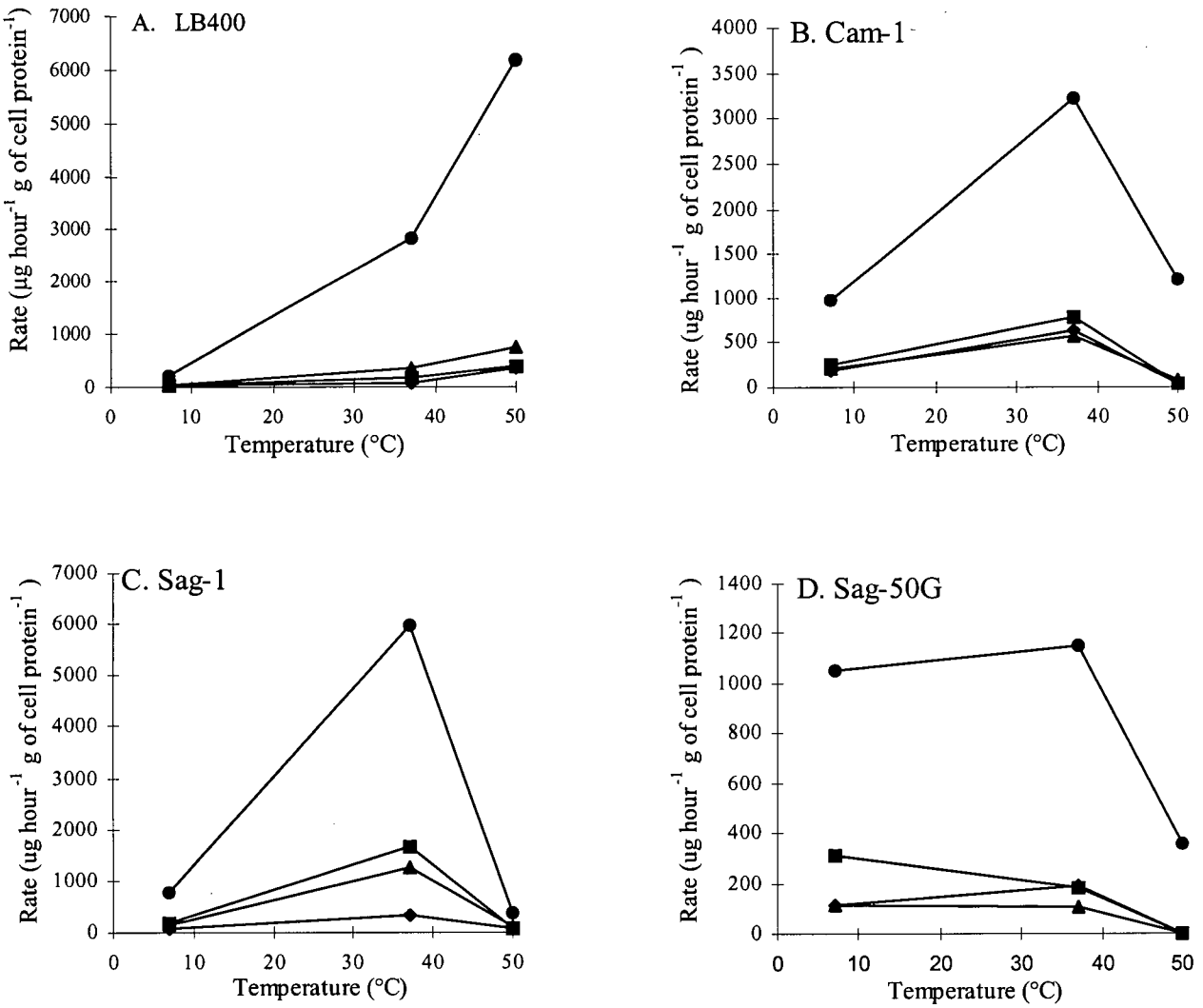


Fig. 11. Rate of degradation of individual PCB congeners in Aroclor 1242 by cell suspensions at various temperatures (n=2). ▲, Peak 4; ●, Peak 5; ◆,Peak 12; ■,Peak 14. Peaks are identified in Table 4.

The initial rates of transformation of selected PCB congeners at 7°C were generally higher in the Arctic soil isolates than in LB400 (Table 7). Corresponding rates were higher at 37°C than at 7°C for Cam-1 and Sag-1 (Fig. 11B and C). The rates of transformation of corresponding congeners at 37°C by Sag-1 were higher than those of Cam-1. The rates of transformation of corresponding congeners at 37°C by Sag-1 and Cam-1 were higher than that of LB400 (Fig. 11A through C). The increase in corresponding rates was less for Sag-50G and decreased from 7°C to 37°C for certain congeners (Fig. 11D). At 50°C, initial rates of PCB transformation by the Arctic soil isolates significantly decreased, to zero for some congeners (Fig. 11B through D). However, the initial rates of transformation of selected PCB congeners by LB400 continued to increase (Fig 11A). The rates of transformation of selected congeners at 7°C, 37°C and 50°C by Iqa-1 was similar to those of Sag-1 (data not shown). The extent of PCB congener transformation by the Arctic soil isolates increased or did not change from 7°C to 37°C, but decreased by up to 90% at 50°C (Table 8). Differences in relative transformation were consistent with differences in absolute transformation. These results suggest that PCB transformation by the psychrotolerant bacteria is temperature sensitive. At 7°C, the Arctic soil isolates transformed PCB congeners at higher initial rates than LB400 (Fig. 11). Thus, similar total transformation of Aroclor 1242 after 24 h by these bacteria under these conditions may reflect the relatively broad substrate range of LB400 or may suggest that PCB transformation by LB400 is less susceptible to inhibiting metabolites, cell starvation and depletion of reductant (Table 8). Also, higher initial rates of transformation of PCB congeners at 37°C by the Arctic soil isolates compared to those

by LB400, but higher total transformation of Aroclor 1242 by LB400 after 24 h suggest that the PCB-degrading enzymes in the Arctic soil isolates are temperature sensitive.

In summary, psychrotolerant bacteria, which at low temperature degrade PCBs at higher initial rates than the mesophile LB400, were isolated from PCB-contaminated Arctic soil. Several factors may contribute to the differences in PCB removal activities of the psychrotolerant bacteria and LB400. These include the possibility that cold adapted PCB-degrading enzymes are expressed in the Arctic soil isolates. Alternatively, the comparatively high rates of PCB degradation at low temperature by psychrotolerant bacteria may result from differences in *bph* gene expression or differences in rates of PCB transport into the cell. The following chapters compare mesophilic LB400 and psychrotolerant Cam-1 on the basis of BPDO activities, *bph* gene regulation and biphenyl uptake.

Table 8. Relative and absolute transformation of Aroclor 1242 by cell suspensions incubated at various temperatures for 24 h.

Isolate	Relative transformation (absolute transformation)[a] at:		
	7°C	37°C	50°C
LB400	3.6 ± 0.52 (10.1 ± 1.5)	11.1 ± 3.6 (31.0 ± 9.5)	10.0 ± 0.25 (27.8 ± 0.7)
Cam-1	2.7 ± 0.58 (9.1 ± 2.0)	5.2 ± 0.50 (17.6 ± 1.7)	0.43 ± 0.05 (1.4 ± 0.2)
Sag-1	2.3 ± 0.10 (7.5 ± 0.3)	1.7 ± 0.60 (5.5 ± 2.0)	0.63 ± 0.33 (2.0 ± 1.1)
Iqa-1	2.2 ± 0.45 (6.2 ± 1.3)	2.2 ± 0.33 (6.2 ± 0.9)	1.3 ± 0.43 (3.6 ± 1.2)
Sag-50G	0.88 ± 0.40 (3.2 ± 1.5)	2.5 ± 0.48 (8.8 ± 1.7)	0.28 ± 0.43 (1.0 ± 1.5)

[a] Relative transformation is expressed as micrograms of PCBs transformed per milligram of cell protein; absolute transformation is expressed as a percentage. Values are means ± standard deviations (n = 3).

CHAPTER TWO: Comparison of Activities of Biphenyl Dioxygenase from Psychrotolerant *Pseudomonas* sp. Strain Cam-1 and Mesophilic *Burkholderia* sp. Strain LB400

2.0. Introduction

PCB congeners that were transformed by both Cam-1 and LB400 at 7°C were transformed up to 10 times faster by Cam-1 (see chapter 1). Moreover, the rates of PCB transformation by LB400 were higher at 50°C than at 37°C, whereas the rates of PCB transformation by Cam-1 were significantly decreased at 50°C (see chapter 1). Higher rates of PCB transformation at 7°C by Cam-1 than LB400, and decreased PCB transformation at high temperatures by Cam-1 suggested that Cam-1 may express a cold-adapted BPDO (Feller *et al.*, 1997; Feller and Gerday, 1997). The terminal oxygenase components of BPDO (ISP) from LB400 (ISP_{LB400}) and Cam-1 (ISP_{Cam1}) were purified and then oxygraph assays were performed to compare the activities and stability of these enzymes at various temperatures. Furthermore, the deduced amino acid sequences of ISP_{Cam1} and ISP_{LB400} were compared to identify the determinants of the differences in activities and thermostability of these enzymes.

2.1. Purification of ISP components.

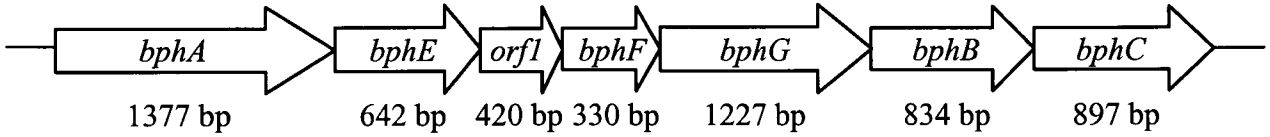
The respective yields of purified ISP_{LB400} and ISP_{Cam1} were approximately 1.6 and 2.5 mg/g cell paste (wet weight), respectively. Oxidized preparations of ISP_{LB400} and ISP_{Cam1} had R-values (A_{280}/A_{323}) of 10.8 and 8.6, respectively. Purified ISP_{LB400} contained 3.4 ± 0.5 mol of iron and 3.6 ± 1.1 mol of labile sulfur per mol of αβ-heterodimer. Purified ISP_{Cam1} contained 2.5 ± 0.4 mol of iron and 3.9 ± 0.5 mol of sulfur

per mol of αβ-heterodimer. The R-values and Fe- and S-content of these preparations are similar to those reported for anaerobically purified preparations of ISP_{B356} (Imbeault *et al.*, 2000), and indicate that the ISPs contain a full complement of Rieske-type [2Fe-2S] clusters and mononuclear Fe^{2+} centres. The specific activities of ISP_{LB400} and ISP_{Cam1} were 0.27 ± 0.01 U/mg and 3.1 ± 0.4 U/mg, respectively.

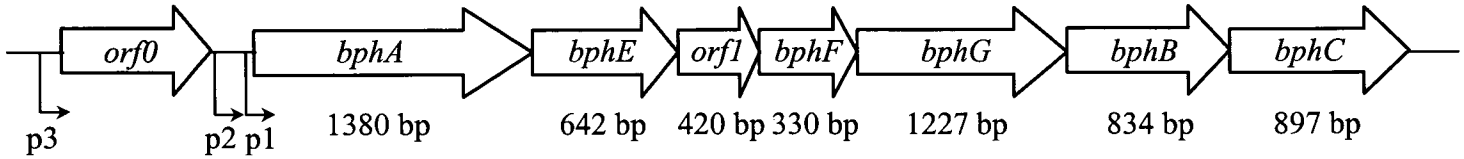
2.2. Oxygen Uptake Assays.

Steady-state kinetics of BPDO were performed using ht-$BphG_{B356}$ and ht-$BphF_{LB400}$. The sequences of $BphF_{LB400}$ and $BphF_{Cam1}$ are identical (Fig.12). Moreover, steady-state experiments performed using recombinant BphF in which the his-tag was removed established that the tag did not detectably affect steady state kinetic parameters of BPDO (data not shown). The activity of ISP_{LB400} was previously investigated using ht-$BphG_{B356}$ (Hurtubise *et al.*, 1998). In their study, Hurtubise *et al.* (1998) demonstrated that exchanging the electron transfer components of different BPDOs does not affect enzyme activity. It was concluded that differences in steady-state kinetic parameters of $BPDO_{LB400}$ and $BPDO_{Cam1}$ as determined using the conditions described in "Materials and Methods", reflect differences in the respective ISPs of these systems. Under all conditions studied, the steady-state consumption of O_2 by BPDO as a function of biphenyl concentration obeyed classical Michaelis-Menten kinetics (*i.e.*, random trends in the residuals were observed; Cornish-Bowden, 1995). As observed for $BPDO_{B356}$, the steady-state kinetic parameters of $BPDO_{LB400}$ and $BPDO_{Cam1}$ depended on the concentrations of BphG and BphF. The determined kinetic parameters are thus referred to as apparent parameters as they were determined at fixed concentrations of O_2, ht-$BphG_{B356}$ and ht-$BphF_{LB400}$.

A. Cam-1 *bph* operon



B. LB400 *bph* operon



% DNA Sequence Similarity	94	99.6	100	100	100	99.8	99.8

Fig.12. Organization and similarity of the *bph* gene cluster in *Pseudomonas* sp. Cam-1 (A) and *Burkholderia* sp. LB400 (B). *bphA*, terminal dioxygenase large subunit; *bphE*, terminal dioxygenase small subunit; *bphF*, ferredoxin; *bphG* ferredoxin reductase; *bphB*, dihydrodiol dehydrogenase; *bphC*, 2,3-dihydroxybiphenyl dioxygenase.

2.3. Temperature dependence of BPDO activity.

The dependence of BPDO activity on temperature was investigated by performing standard activity assays at temperatures from 4°C to 60°C. Under these conditions, BPDO is saturated with biphenyl, thus the determined activities reflect k_{cat}^{app}. Consistent with what is observed in enzymatic reactions, the k_{cat}^{app} of each BPDO approximately doubled with each 10°C increase in temperature (Cornish-Bowden, 1995) (Fig. 13). Moreover, at temperatures below 50°C, the activity of $BPDO_{Caml}$ was higher than that of $BPDO_{LB400}$ (Fig. 13). The measured activity of $BPDO_{Caml}$ was maximal at 47°C and decreased to almost zero at 60°C. In contrast, the activity of $BPDO_{LB400}$ was maximal at 55°C, and at 60°C was still 90% of this maximum. Significantly, the kinetic parameters of $BPDO_{Caml}$ were determined at 4°C, however $BPDO_{LB400}$ had no detectable activity at this temperature. Determination of the apparent steady-state parameters of the two BPDOs at 10°C revealed that their respective $k_{cat}^{app} / K_m^{app}$ were not significantly different, as observed at 25°C (Table 9). Finally, these data were used to construct Arrhenius plots for $BPDO_{Caml}$ and $BPDO_{LB400}$ from which thermodynamic parameters were derived (Table 10) (Hoyoux *et al.*, 2001; Lonhienne *et al.*, 2000). The lower free energy of activation (ΔG^*) of $BPDO_{Caml}$ is consistent with the enzyme's higher k_{cat}^{app}.

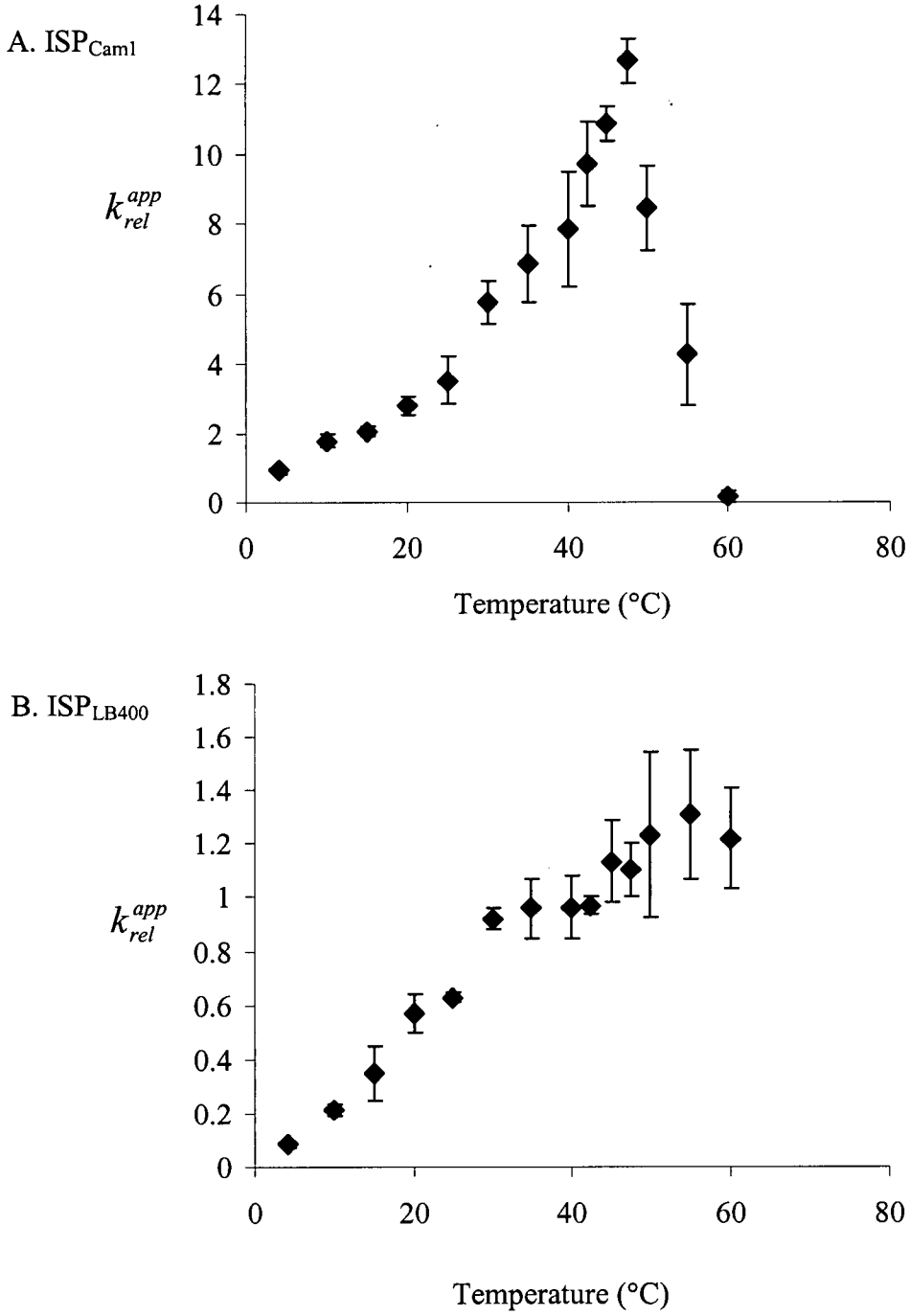


Fig. 13. The effect of increasing temperature on the activities of ISP$_{Cam1}$ (A) and ISP$_{LB400}$ (B) (n=3, error bars indicate standard deviation)

Table 9. Apparent steady-state kinetic parameters of BPDOs for biphenyl at various temperatures.

Temperature (°C)	ISP	k_{cat}^{app}	K_m^{app}	$k_{cat}^{app} / K_m^{app}$
		s^{-1}	μM	X 10^6 $M^{-1}s^{-1}$
4	Cam-1	1.1 (0.1)	18.1(2.9)	0.06 (0.001)
	LB400	-[a]	-	-
10	Cam-1	2.2 (0.06)	10.8 (1.1)	0.20 (0.02)
	LB400	0.26 (0.01)	1.6 (0.3)	0.16 (0.03)
25	Cam-1	4.6 (0.2)	28.8 (2.6)	0.17 (0.05)
	LB400	0.51 (0.03)	3.5 (1.4)	0.13 (0.07)
	B-356[b]	7.3 (0.2)	6.2 (0.5)	1.2 (0.1)

Experiments were performed using air-saturated 50 mM MES, pH 6.0. Values in parentheses represent standard errors. (n=3 or 4) . [a]No detectable O_2 utilization. [b]Data from Imbeault *et al.* (2000).

Table 10. Thermodynamic parameters of ISP_{Cam1} and ISP_{LB400} at 25 °C with biphenyl. Values in parentheses represent standard errors. (n=3)

ISP	E_a (kJ mol^{-1})	Δ G* (kJ mol^{-1})	Δ H* (kJ mol^{-1})	T Δ S* (kJ mol^{-1} K^{-1})
Cam-1	40.2 (1.8)	69.9 (0.05)	37.7 (2)	-32.1 (2)
LB400	50.9 (5.4)	74.2 (0.07)	48.4 (5)	-25.7 (5)

2.4. Thermostability.

The effect of temperature on BPDO activity may result from alterations of interactions involved in substrate binding or thermal denaturation of the three dimensional structure of the enzyme. In order to distinguish between these effects, the half-lives of ISP_{Caml} and ISP_{LB400} were determined spectrophotometrically by continuously monitoring decreasing absorbance at 323 nm. At 57°C, the half-lives of ISP_{Caml} and ISP_{LB400} were 16 ± 2 min and 38 ± 8 min, respectively (n=3). Thus, the decreased activities of ISP_{Caml} at temperatures above 47°C probably resulted from heat-induced unfolding of the enzyme.

2.5. Preparation dependence of BPDO activity.

At 25°C, the k_{cat}^{app} of $BPDO_{B356}$ and $BPDO_{Caml}$ for biphenyl are similar (Table 9). Likewise, previous reports concluded that $BPDO_{LB400}$ and $BPDO_{B356}$ have similar activities under these conditions (Hurtubise *et al.*, 1998). As a result, the difference in k_{cat}^{app} of $BPDO_{Caml}$ and $BPDO_{LB400}$ for biphenyl at 25°C was not expected (Table 9). The *bphAE* genes that were used to express ISP_{LB400} in *E.coli* were sequenced, however no mutations in these genes were found. Furthermore, incubating ISP_{LB400} anaerobically with Fe^{2+} and dithiothreitol did not increase the activity of the enzyme. As mentioned, the preparation of ISP_{LB400} contained a full complement of Rieske-type [2Fe-2S] clusters and mononuclear Fe^{2+} centres. Nevertheless, the anaerobic purification of ISP_{LB400} was repeated, however the specific activity of the second preparation of ISP_{LB400} was not different from that of the first. Moreover, the specific activity of the preparation of ISP_{LB400} increased following each purification step, confirming that the purification

procedure did not inhibit ISP_{LB400} activity. The difference in activities of ISP_{Cam1} and ISP_{LB400} then, are not preparation dependent. Notably, previous comparisons of ISP_{LB400} and ISP_{B356} were performed using aerobic preparations of the oxygenases and a discontinuous enzyme assay (Hurtubise *et al.*, 1998). As a result, differences in activities of ISP_{LB400} and ISP_{B356} may not have been detected.

2.6. Comparison of ISP Sequences.

The deduced amino acid sequence of the α-subunit (BphA) and β-subunit (BphE) of ISP_{Cam1} show 95.6% and 97% identity with corresponding subunits of ISP_{LB400} (Fig. 14). The alignment of $BphA_{Cam1}$ and $BphA_{LB400}$ showed that all but 2 of the 21 amino acid differences are located within the carboxyl-terminal half of the subunit (Fig. 14A). All of the amino acid differences between $BphE_{Cam1}$ and $BphE_{LB400}$ occur between 26 amino acids within the amino-terminal region of the subunit (Fig. 14B). Since ISP_{Cam1} and ISP_{LB400} share in excess of 70% identity with ISP_{B356}, the structure of ISP_{B356} was used to model ISP_{Cam1}, and to identify particular amino acids that may contribute to the difference in activities and thermostability of ISP_{Cam1} and ISP_{LB400} (Imbeault *et al.*, 2000). Notably, amino acids 237 and 238 of BphA are located near the probable active site of the oxygenase and are, respectively, methionine and serine in $BphA_{Cam1}$, and threonine residues in $BphA_{LB400}$. Furthermore, amino acid differences at positions 58 and 59 of BphE are located at a possible interface of BphA and BphE, whereas amino acid differences at positions 65 and 104 of BphE are located at a possible interface of two BphE subunits.

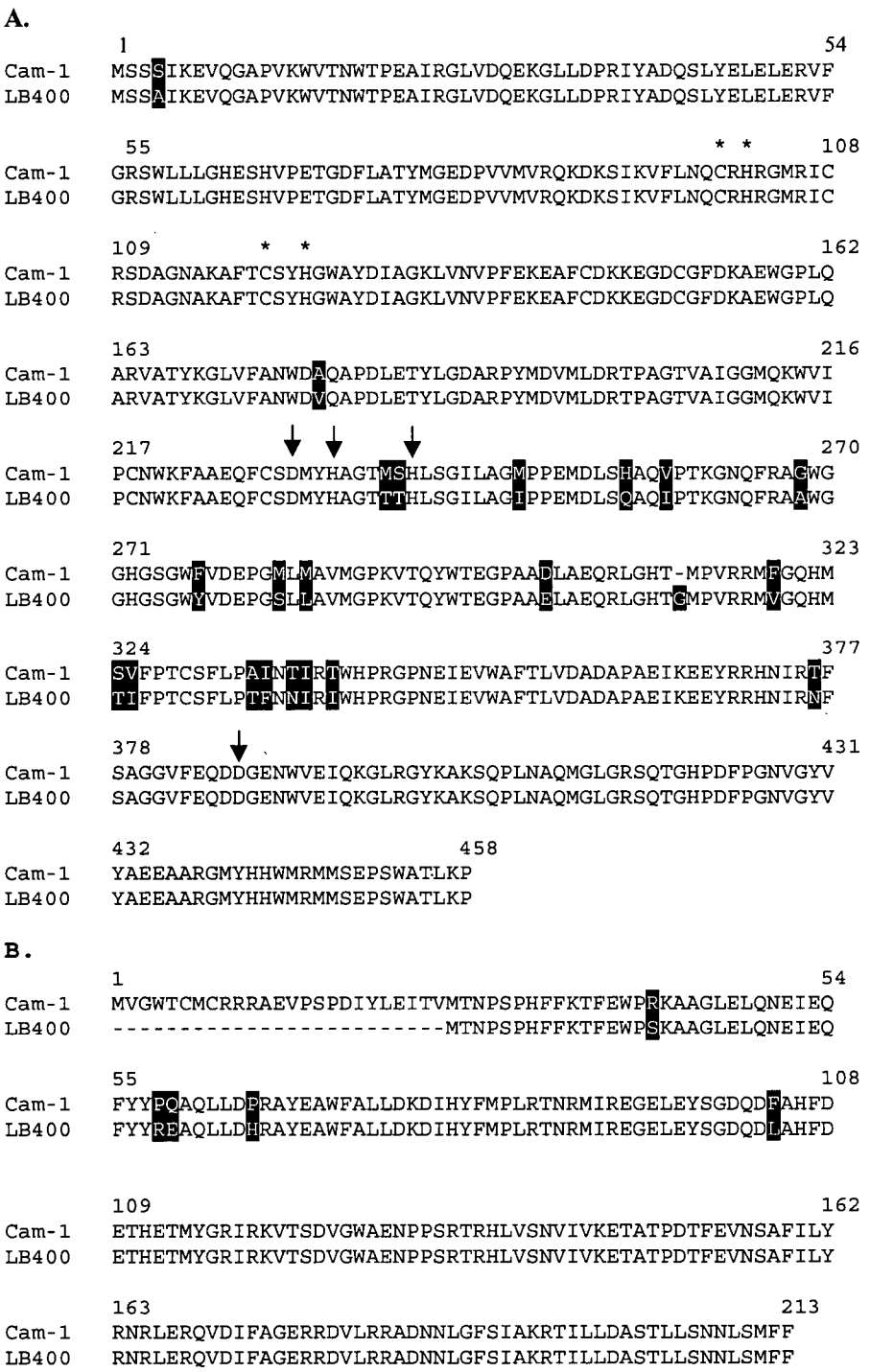


Fig. 14. Alignment of amino acid sequences of terminal oxygenase subunits BphA (A) and BphE (B) from $BPDO_{Cam1}$ and $BPDO_{LB400}$. Differences in amino acid sequences are highlighted. Arrows indicate residues that coordinate the mononuclear Fe^{2+} centre; (*) indicate residues that coordinate the Rieske-type [2Fe-2S] cluster.

In summary, $BPDO_{Caml}$ is more active than $BPDO_{LB400}$ at low temperatures, and is less thermostable that $BPDO_{LB400}$. However, at 10 and 25°C the values of $k_{cat}^{app} / K_m^{app}$ for $BPDO_{Caml}$ and $BPDO_{LB400}$ were not significantly different. In addition, the steady-state kinetic parameters of $BPDO_{Caml}$ for biphenyl at 25°C were similar to those reported for $BPDO_{B356}$. The significance of the kinetic data and of the amino acid differences between $BPDO_{Caml}$ and $BPDO_{LB400}$ will be interpreted in the "Discussion".

CHAPTER THREE: Induction of *bphA* Encoding Biphenyl Dioxygenase in Two Polychlorinated Biphenyl-degrading Bacteria, Psychrotolerant *Pseudomonas* sp. Strain Cam-1 and mesophilic *Burkholderia* sp. Strain LB400

3.0. Introduction.

The presence of biphenyl as a potential growth substrate and inducer of PCB metabolism is considered important for maintaining PCB-degradation activity in soil (Barriault and Sylvestre, 1993; Focht and Brunner, 1985). However, since biphenyl is poorly soluble in water (44 μM, Foreman and Bidleman, 1985), and can cause adverse health effects, it is important for field applications to determine whether other compounds can support PCB biodegradation. This chapter describes the induction of PCB transformation activity in batch cultures of the Arctic soil isolates Cam-1, Iqa-1, Sag-1 and Sag-50, which were incubated with substrates other then biphenyl. To determine if induction of PCB transformation results from induction of *bph* genes, beta-galactosidase assays were performed using chromosomal *bphA-lacZ* reporter constructs of *Pseudomonas sp.* strain Cam-1 and *Burkholderia sp.* strain LB400 (Cam-10 and LB400-1, respectively). As described in chapter one, at 7°C Cam-1 transformed PCBs at higher rates than LB400. To investigate the role of *bph* gene induction in the efficiency of PCB transformation at low temperature, beta-galactosidase activity in cell suspensions of Cam-10 and LB400-1 incubated with biphenyl at 7°C were also performed.

3.1. Effect of growth substrates on PCB degradation.

Arctic soil isolates growing on carbon sources other than biphenyl also partially transformed Aroclor 1242 (Table 11). Compared to the relative transformation of

Table 11. Relative and absolute transformation of Aroclor 1242 by batch cultures grown on different primary substrates[a]

Substrate (mg/L)	Relative transformation (absolute transformation)[b] by:			
	Cam-1	Sag-1	Iqa-1	Sag-50G
Linoleic acid (1000)	1.2 (3.5)	3.0 (9.9)	1.3 (14.6)	NG (0)
Glucose (1000)	0.42 (7.4)	0.20 (2.6)	0.17 (4.1)	0 (0)
Glycerol (1000)	1.0 (16.2)	1.2 (7.2)	0.1 (3.2)	1.0 (4.3)
Benzoate (240)	0 (0)	0 (0)	0.1 (0.6)	0.03 (0.16)
Acetate (1000)	0.42 (2.3)	0.39 (1.6)	0.15 (0.9)	0.20 (0.7)
Pyruvate (1000)	1.0 (8.4)	1.4 (13.8)	NG (0)	0.47 (3.9)
Biphenyl (200)	2.2 (11.3)	1.1 (8.1)	1.3 (11.3)	0.45 (2.2)

[a]All cultures were incubated at 7°C for 5 weeks (n = 2).

[b]Relative transformation is expressed as μg of PCBs transformed per mg of cell protein; absolute transformation is expressed as a percentage of the total PCB mixture that was transformed by the batch cultures. NG, no growth on primary substrate.

Aroclor 1242 in cultures growing on biphenyl, relative transformation was generally lower in cultures growing on other primary substrates (Table 11). Exceptions were linoleic acid-grown and to a lesser extent, pyruvate-grown cultures of Sag-1 whose relative transformations were higher than that of biphenyl grown cultures. Partial transformation of mono-, and some di- and tri-chlorobiphenyls was detected in cultures of each isolate growing on linoleic acid, glucose, glycerol, acetate, or pyruvate. Cultures of Cam-1 and Sag-1 growing on benzoate failed to remove PCBs. Cultures of Sag-50G growing on benzoate transformed comparatively small amounts of the PCB mixture. It is possible that benzoate inhibits induction of the biphenyl operon in these microorganisms because benzoate is an end product of the biphenyl degradative pathway. In some cases,

substrates supported similar absolute transformation, but different relative transformation, of Aroclor 1242 by a particular isolate. Therefore, primary substrates affect PCB transformation by affecting both cell density and the efficiency of PCB transformation by a constant amount of biomass.

To determine if induction of PCB transformation results from induction of *bph* genes, I constructed a chromosomal *bphA-lacZ* reporter in *Pseudomonas* sp. strain Cam-1, to generate Cam-10. I also constructed a chromosomal *bphA-lacZ* reporter in the mesophile, *Burkholderia* sp. strain LB400, to generate LB400-1.

3.2. Optimization of *lacZ* reporter gene expression in Cam-10 and LB400-1.

Maximum expression of the *lacZ* reporter gene in cell suspensions of *Pseudomonas* sp. strain Cam-10 was observed 3 h after addition of 1 mM biphenyl (Fig.15A). Other compounds that were studied for their ability to induce beta-galactosidase activity in Cam-10 were tested using these conditions. Expression of the *lacZ* reporter gene in Cam-10 did not increase with increasing amounts of biphenyl above 1 mM. At concentrations of biphenyl less than 0.1 mM, beta-galactosidase activity was consistently higher when 1 mM pyruvate was also supplied. The addition of pyruvate may provide cells with energy that allows greater beta-galactosidase production. Thus, unless otherwise stated, 1 mM pyruvate was added to all subsequent treatments testing potential inducers of *bphA*.

At each concentration of biphenyl tested, beta-galactosidase specific activity in Cam-10 initially increased with time then decreased (Fig. 15A). This result suggested

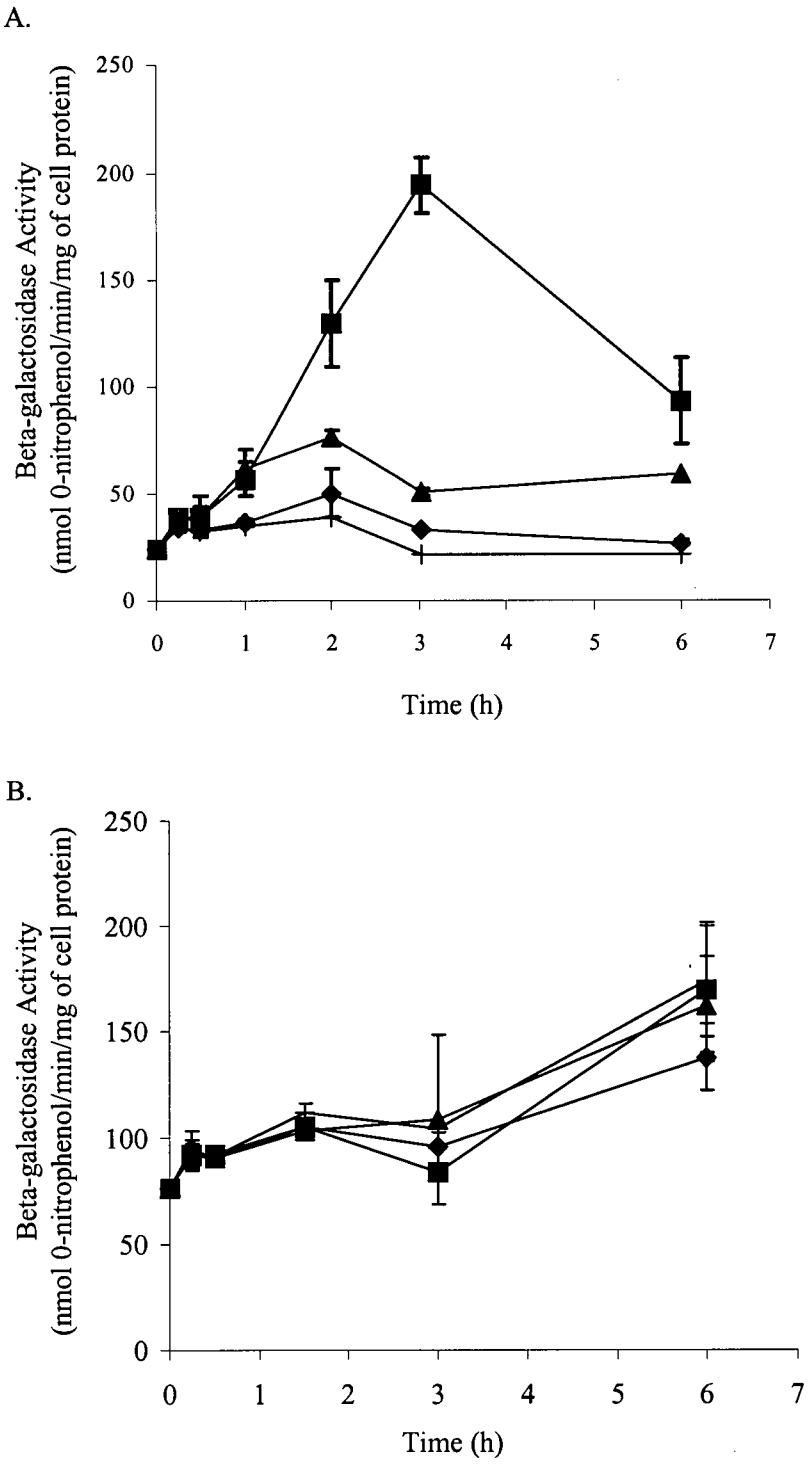


Fig.15. Induction of beta-galactosidase activity at 30°C in Cam-10 (A) and LB400-1 (B); n = 3; error bars indicate standard deviation; Treatments contained 1 mM pyruvate (+) plus biphenyl at 0.01 mM (◆), 0.33 mM (▲), 1mM (■).

that biphenyl was depleted by Cam-10, thereby diminishing the concentration of inducer. The utilization of biphenyl by Cam-10 was not surprising since the *lacZ*-Gm cassette does not contain a transcription termination sequence (Schweizer, 1993) and was inserted between *bphA* and *bphE* genes in Cam-1 without disrupting either gene. To verify that Cam-10 transformed biphenyl, 0.1 mM biphenyl was added to cell suspensions of pyruvate-grown Cam-10, then cell suspensions were incubated at 30°C. After 3 h and 6 h of incubation, 30% and 100% of the biphenyl added to cell suspensions of Cam-10 was removed, respectively. Biphenyl was not removed by killed cells or from medium without cells. Cam-10 also grew on 1 mM biphenyl. Biphenyl degradation by Cam-10 requires the *bph* gene products, since insertion of the transcription termination sequence containing *xylE*-Gm cassette from pX1918GT between *bphA* and *bphE*, resulted in cells unable to grow on biphenyl. Thus, there does not appear to be any additional enzyme system in Cam-1 catalyzing biphenyl degradation.

In contrast to induction of beta-galactosidase activity in Cam-10, the level of beta-galactosidase activity in *Burkholderia* sp. strain LB400-1 did not depend on the presence of biphenyl (Fig. 15B). This suggests that expression of the *bphA* gene in LB400 is constitutive. Like Cam-10, LB400-1 completely transformed 0.1 mM biphenyl after 6 h. Biphenyl degradation by LB400-1 is believed to require the *bph* gene products, since many attempts by others to find more than one BPDO in LB400 have not been successful (Mondello, 1989).

3.3. Inducers of beta-galactosidase activity in Cam-10.

Biphenyl induced beta-galactosidase activity in Cam-10 approximately 6 times above the basal level of expression in cells with pyruvate (Fig. 16A). At 1 mM

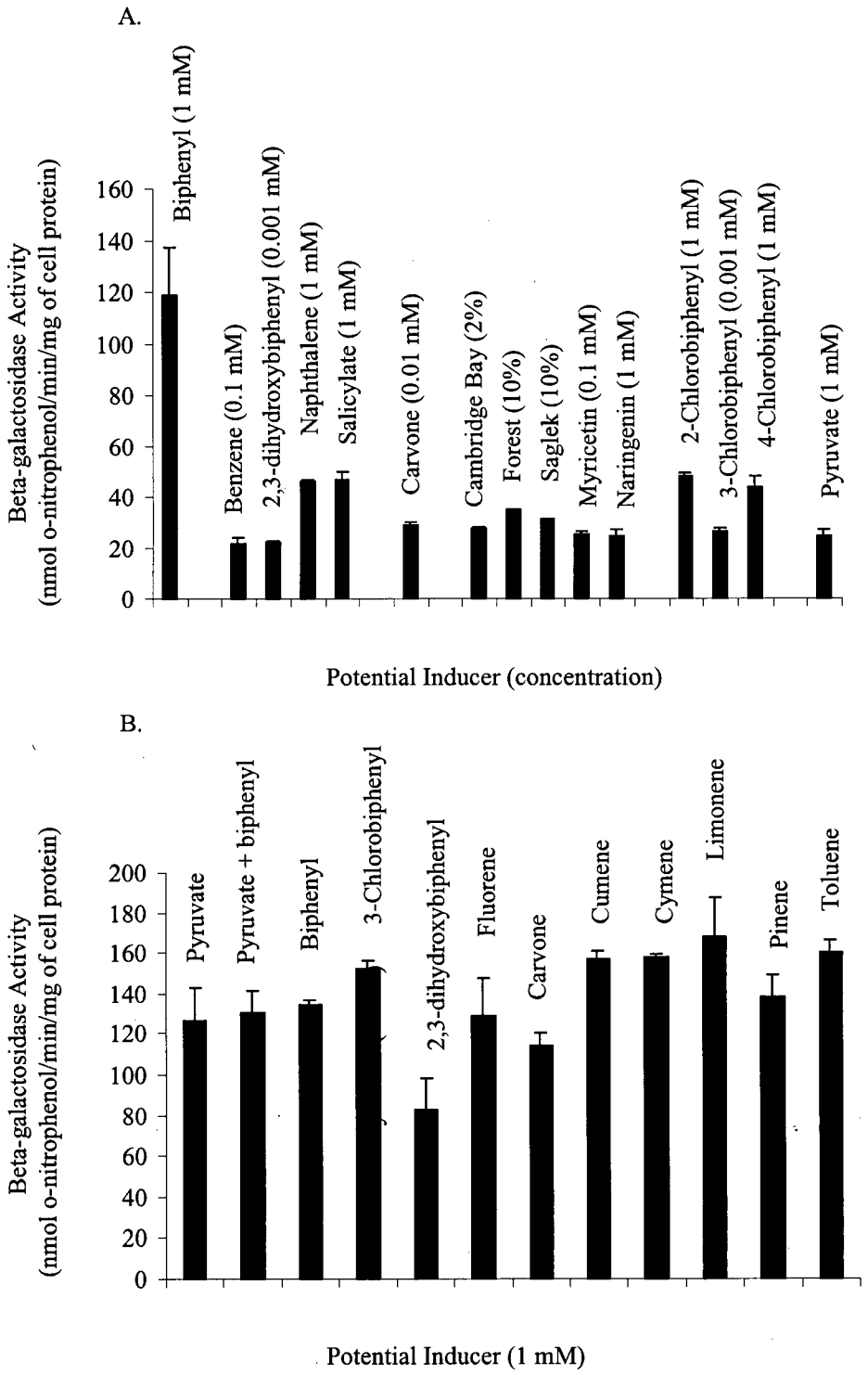


Fig.16. Induction of beta-galactosidase activity for 3 h at 30°C in Cam-10 (A) and LB400-1 (B); n = 3; error bars indicate standard deviation. The concentrations of potential inducers used to test induction of beta-galactosidase activity in Cam-10 are indicated in parentheses. All treatments were supplemented with 1 mM pyruvate.

concentrations, 2-chlorobiphenyl, 4-chlorobiphenyl, salicylate and naphthalene induced beta-galactosidase activity above basal levels. Thus, these compounds appear to be inducers of *bphA* in Cam-1. However, none appears to be as strong an inducer as biphenyl, as none induced beta-galactosidase to the same level of activity in Cam-10 as did biphenyl. The induction of beta-galactosidase activity by biphenyl in Cam-10 was inhibited at 1 mM concentration of many of the potential inducers tested (see section 3.5). Moreover, at non-inhibitory concentrations (indicated in Fig. 16A), none of these compounds induced beta-galactosidase activity above basal levels in Cam-10. The level of beta-galactosidase activity after exposure to benzene, carvone, 3-chlorobiphenyl and pyruvate (Fig. 16A) was typical of that after exposure to other non-inducing aromatic compounds, terpenoids, chlorobiphenyls, sugars, alcohols and organic acids (data not shown, compounds tested listed in Table 12).

3.4. Inducers of beta-galactosidase activity in LB400-1.

In contrast to results with Cam-10, beta-galactosidase activity in cell suspensions of LB400-1 containing 1 mM biphenyl, carvone, cumene, cymene, pinene, limonene, fluorene, 3-chlorobiphenyl or toluene was similar to beta-galactosidase activity observed in cell suspensions containing only pyruvate (Fig. 16B). These results are consistent with S1 nuclease mapping studies of *bph* genes in LB400, which identified three transcriptional initiation sites (Erickson and Mondello, 1992). Activation from the promoter region furthest upstream of the BPDO translation start site (p3) is dependent on biphenyl. However, activation from the two proximal promoter regions (p2 and p1) is constitutive (Erickson and Mondello, 1992). Interestingly, 1 mM 2,3-dihydroxybiphenyl

had a slight inhibitory effect on induction of beta-galactosidase activity in LB400-1 (Fig. 16B).

3.5. Inhibitory effects of potential inducers.

At a concentration of 1 mM, most of the potential inducers tested actually inhibited induction by biphenyl of beta-galactosidase activity in Cam-10 (Table 12). Exceptions were compounds previously found to be inducers, naphthalene, salicylate, 2-chlorobiphenyl and 4-chlorobiphenyl, as well as naringenin, fructose, glucose and glycerol. With the exception of benzoate, acenaphthalene, fluorene, dioxin, anthracene, 3-chlorobiphenyl, 2-methylnaphthalene and dimethylnaphthalene, compounds inhibiting induction also inhibited cell growth. At concentrations less than 0.1 mM, none of the potential inducers were inhibitory to cell growth, yet at such concentrations, several of those compounds substantially inhibited induction of beta-galactosidase. In complex environments, inhibitory effects such as the ones found here can be expected to modulate expression of genes essential for PCB biodegradation. The inhibitory effect of soil extracts (Table 12) is consistent with this expectation.

3.6. Temperature dependence of *bphA* induction in Cam-1 and LB400

To investigate the role of *bphA* induction in the efficiency of PCB transformation at low temperature, I compared beta-galactosidase activity in cell suspensions of Cam-10 and LB400-1 incubated at 7°C with pyruvate or biphenyl plus pyruvate. Cell suspensions of Cam-10 were prepared using cells grown on pyruvate at 7°C. Since LB400 does not grow at 7°C (see chapter one), cell suspensions of LB400-1 were prepared using cells grown on pyruvate at 15°C. Samples from cell suspensions were obtained at several time points over 24 h, and transferred to 28°C to measure beta-galactosidase activity.

Table 12. Percent inhibition[a] of beta-galactosidase activity in Cam-10 by potential inducers at various concentrations.

Potential Inducer	% Inhibition at the following conc.:			
	1 mM	0.1 mM	0.01 mM	0.001 mM
Aromatic Compounds				
benzene	98	0	-	-
toluene	97	70	5	-
benzoate	51	0	-	-
catechol	100	64	15	0
2,3-dihydroxybiphenyl	100	70	52	2
acenaphthalene	50	19	0	-
fluorene	100	69	68	10
dioxin	100	100	85	43
2-methylnaphthalene	100	58	0	-
1,4-dimethylnaphthalene	100	67	0	-
anthracene (crystals)	41	0	-	-
phenanthrene	100	37	0	-
Terpenoids				
camphor	100	55	10	-
(s)-(+)-carvone	99	76	0	-
beta-citronellol	95	70	5	-
cumene	99	0	0	-
p-cymene	100	56	8	-
dehydroabietic acid	45	0		-
(+)-limonene	97	33	24	-
linoleic acid	81	0	-	-
pinene	88	49	0	-
Soil Extract and flavonoids				
Cambridge Bay (10%, 2%)	100	30	-	-
forest (10%)	52	0	-	-
Saglek (10%)	55	0	-	-
myricetin	90	90	-	-
Chlorinated Biphenyls				
3-chlorobiphenyl	100	100	32	0
2,2'-dichlorobiphenyl	-	20	-	-
4,4'-dichlorobiphenyl	-	0	-	-
Aroclor 1242 (100 ppm)	60	23	-	-

Table 12. continued.

[a] Inhibition of beta-galactosidase activity in Cam-10 by potential inducers was determined by incubating cells with 1 mM pyruvate, 1 mM biphenyl and the indicated concentration of potential inducer, then determining beta-galactosidase activity. Percent inhibition was measured by comparing beta-galactosidase activity in cell suspensions containing pyruvate and biphenyl without a potential inducer to activity in cell suspensions containing pyruvate and biphenyl plus the potential inducer.

(-) Not measured

Table 13. Effect of temperature on induction of beta-galactosidase activity in Cam-10 grown at 7°C or 30°C on pyruvate and LB400-1 grown at 15°C or 30°C on pyruvate.

Strain	Time	beta-galactosidase Activity (nmol o-nitrophenol/min/mg of cell protein)[a]	
		7°C[b]	30°C[c]
Cam-10	Initial[d]	18.0 ± 2.6	24.0 ± 0.6
	Final	74.0 ± 8.3[e]	118.6 ± 18.5[f]
LB400-1	Initial[d]	15.5 ± 4.0	76.3 ± 4.5
	Final	16.8 ± 1.0[e]	83.9 ± 0.7[f]

[a] Mean ± standard deviation (n=3)
[b] Cam-1 and LB400 cultures used were grown at 7°C and 15°C, respectively.
[c] Cam-1 and LB400 cultures used were grown at 30°C.
[d] beta-galactosidase activity at time 0
[e] beta-galactosidase activity at 24 h
[f] beta-galactosidase activity at 3 h

After 24 h, beta-galactosidase activity in Cam-10 incubated at 7°C with biphenyl was four times above that of cells incubated at 7°C with pyruvate (Table 13). Thus, *bphA* appears to be induced by biphenyl in Cam-1 at 7°C. Interestingly, initial beta-galactosidase activity was significantly less in LB400-1 grown at 15°C than in cells grown at 30°C (Table 13). As observed at 30°C, biphenyl did not induce beta-galactosidase activity in cell suspensions of LB400-1 at 7°C. These results further support the conclusion that *bphA* expression in LB400 is constitutive and indicate that the level of constitutive expression is temperature-dependent.

This research shows that regulation of the *bphA* is remarkably different between two different PCB-degrading bacteria. Induction of the *bphA* in Cam-1 is inducible at 7°C and at 30°C, and induction is greatest with biphenyl. In contrast, expression of *bphA* in LB400 is constitutive, and is reduced at low temperature. These results indicate that available chemical inducers, as well as physical environmental conditions can effect *bphA* expression in PCB-degrading bacteria. Consequently, knowledge of how physical and chemical environmental variables affect *bphA* induction in particular bacteria in a treatment system will be necessary to achieve optimal conditions for PCB bioremediation.

CHAPTER FOUR: Biphenyl Uptake by two PCB-degrading bacteria: mesophilic *Burkholderia* sp. Strain LB400 and psychrotolerant *Pseudomonas* sp. Strain Cam-1

4.0. Introduction.

Investigations described in chapters two and three determined whether intracellular processes (i.e. enzyme activities and gene regulation) account for the differences in PCB degradation activities of psychrotolerant and mesophilic bacteria that were illustrated in chapter one. However, prior to intracellular processing, PCBs must traverse the bacterial cell membrane, and this step might be rate limiting to PCB metabolism. In this chapter, biphenyl uptake by psychrotolerant *Pseudomonas* sp. strain Cam-1 and mesophilic *Burkholderia* sp. strain LB400 is compared by measuring ^{14}C-biphenyl accumulation in cells with time. To determine if biphenyl uptake is inducible, cultures used in uptake assays were prepared using different growth substrates. Metabolic inhibitors were used to determine if biphenyl transport across cell membranes requires energy. Finally, the kinetic parameters for biphenyl uptake by Cam-1 and LB400 were determined.

4.1. Biphenyl Uptake is inducible and requires energy.

Biphenyl uptake by *Pseudomonas* sp. strain Cam-1 and *Burkholderia* sp. strain LB400 was induced by growth on biphenyl (Fig. 17). Biphenyl uptake by Cam-1 and LB400 grown on pyruvate was indistinguishable from killed cell treatments or treatments without cells. A transcription termination site was previously inserted downstream of

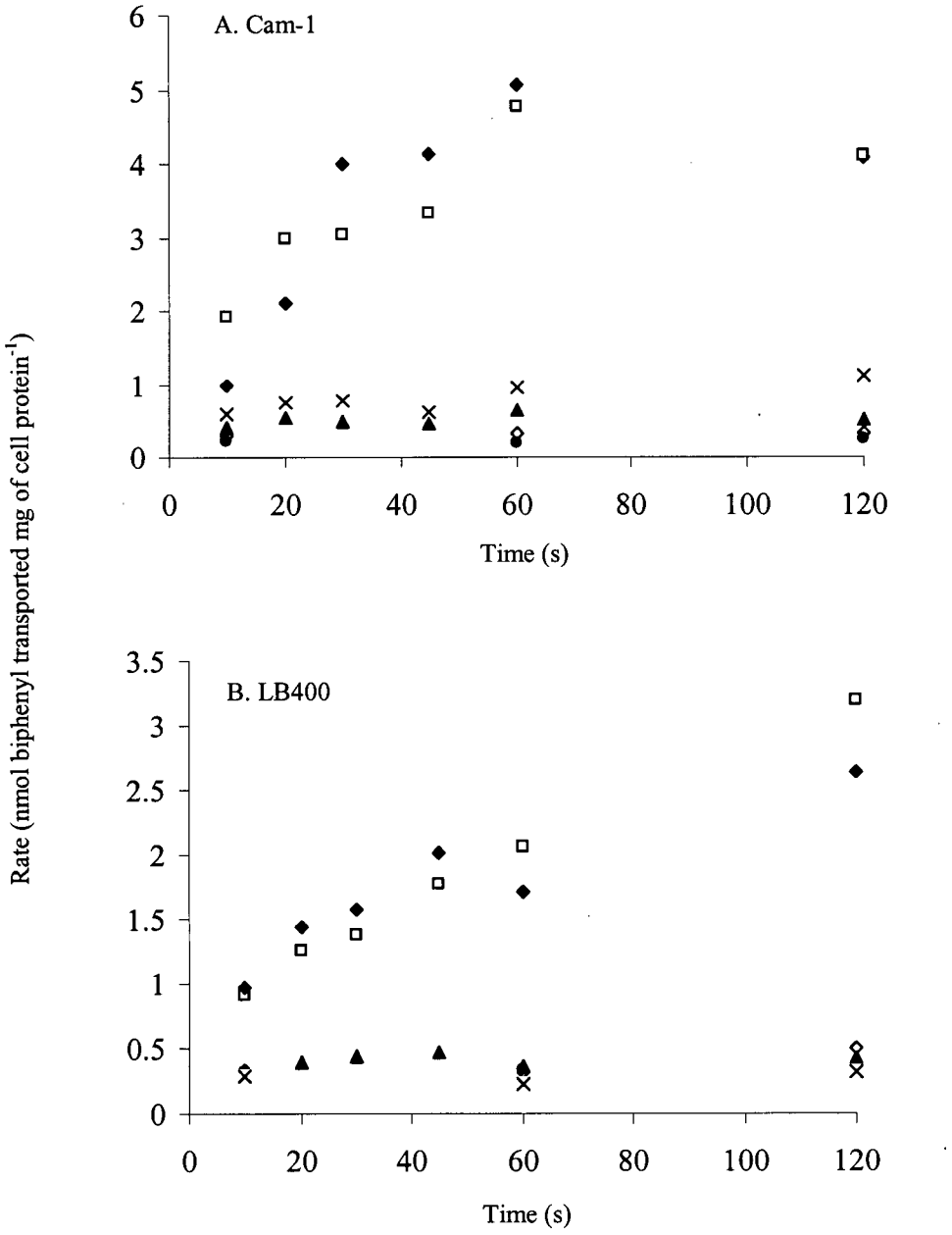


Fig. 17. The effect of growth substrate and metabolic inhibitors on biphenyl uptake at 22°C by *Pseudomonas* sp. strain Cam-1 (A) and *Burkholderia* sp. strain LB400 (B). Biphenyl uptake was tested using cells grown on biphenyl (◆), and treated with DCCD (□) or DNP (x), and using cells grown on pyruvate (▲). Controls were killed cells (◇) and no cells (•). Variations of 1.2 nmol/min/ mg of cell protein and 0.13 nmol/min/ mg of cell protein were observed in replicate treatments of Cam-1 and LB400 cultures, respectively.

bphA in Cam-1 to generate Cam-20 (see chapter 3). Interestingly, biphenyl uptake was not observed using Cam-20 grown on pyruvate with biphenyl. This result suggests that a metabolite of biphenyl degradation might be necessary for induction of biphenyl uptake, at least in Cam-1.

The energy dependence of biphenyl uptake was investigated by adding 2,4-dinitrophenol (DNP) or 1,3-dicyclohexylcarbodiimide (DCCD) to reaction mixtures to inhibit metabolic processes. DNP is a lipid-soluble, weak acid that dissipates the proton gradient by carrying protons across bacterial membranes, whereas DCCD disrupts ATP synthesis by binding to the F_o subunit of an ATPase complex (Bonting and de Pont, 1981). Biphenyl uptake by Cam-1 and LB400 was completely inhibited by DNP (Fig. 17). In contrast, the rates of biphenyl uptake by cells treated with DCCD were only slightly lower than non-inhibited cells. This result suggests that biphenyl uptake is driven by the proton motive force, but is not ATP dependent. Identifying genes that encode proteins for biphenyl transport across the cell membrane will enable more precise investigations of the energetics of the transport system.

4.2. Kinetics of biphenyl uptake.

The accumulation of biphenyl over 1 min (Fig. 17) showed saturable kinetics with respect to biphenyl concentration (Fig. 18). The initial rates of biphenyl uptake that were obtained over a range of biphenyl concentrations (10 μM – 200 μM) were fitted to the Michealis-Menten equation, and random trends in the residuals were observed. This observation is consistent with biphenyl entering the cell by a transport system (Cornish-Bowden, 1995). Steady-state kinetic parameters were obtained from the

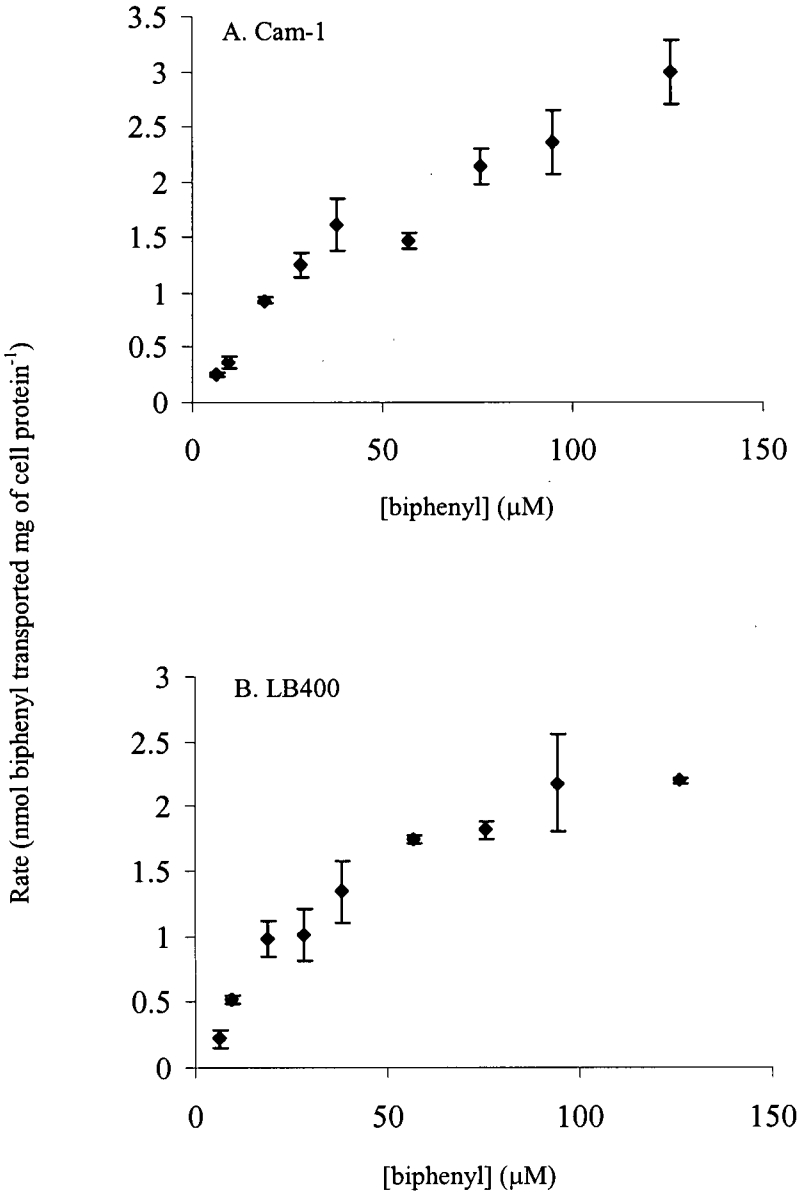


Fig. 18. Kinetics of biphenyl uptake at 22°C in *Pseudomonas* sp. strain Cam-1 (A) and *Burkholderia* sp. strain LB400 (B).

data fitted to the Michealis-Menten equation using the least-squares fitting and dynamic weighing options of LEONORA (Cornish-Bowden, 1995). For Cam-1, the K_m for biphenyl was 83.1 ± 15.9 μM with a V_{max} of 5.4 ± 1.7 nmol min^{-1} mg of cell $protein^{-1}$. For LB400, the K_m for biphenyl was 51.5 ± 9.6 μM with a V_{max} of 3.2 ± 0.3 nmol min^{-1} mg of cell $protein^{-1}$. These results suggest that the K_m and the V_{max} for biphenyl uptake by Cam-1 and LB400 are significantly different (Students *t*-test, $P < 0.05$).

4.3. Biphenyl Metabolism During Uptake Assays.

The initial step for biphenyl catalysis requires oxygen. Therefore, to verify that biphenyl metabolism does not account for observed rates of biphenyl uptake by Cam-1 and LB400, uptake assays were performed under anaerobic conditions. The production of $^{14}CO_2$ in reaction mixtures under anaerobic and aerobic conditions was also determined (Table 14). To enable comparisons with the no cells treatment, amounts (nmol) of biphenyl transported and CO_2 evolved were not standardized to cell protein.

Table 14. Amount (nmol) of biphenyl transported and CO_2 evolved after 1 min under aerobic and anaerobic conditions by Cam-1 and LB400[a].

Strain	nmol of biphenyl transported		% Difference	nmol CO_2 evolved		% Difference
	Aerobic	Anaerobic		Aerobic	Anaerobic	
Cam-1	0.14 ± 0.11	0.13 ± 0.11	7%	1.2 ± 0.010	0.99 ± 0.11	18%
LB400	0.13 ± 0.0014	0.14 ± 0.044	8%	1.8± 0.84	0.84 ± 0.071	53%
No cell				1.1		

[a]Values are averages of data obtained using different culture preparations (n = 2 or 3).

Importantly, biphenyl metabolism did not account for biphenyl uptake by Cam-1 and LB400, because the difference in biphenyl transported under aerobic and anaerobic conditions was significantly less than the difference in CO_2 evolved under these conditions. This is also consistent with the previous data suggesting that biphenyl uptake is not ATP-dependent (Fig. 17). Notably, the amount of radiolabel observed in anaerobic treatments was similar to that detected in treatments without cells (Table 14). Furthermore, biphenyl uptake was not detected using LB400 grown on pyruvate although the initial enzymes for biphenyl metabolism are constitutively expressed in LB400 (see chapter 3).

This research provides evidence for active transport of a non-ionic, aromatic substrate across bacterial cell membranes. More specifically, these are the first data suggesting that biphenyl uptake in at least some PCB-degrading bacteria is inducible and energy dependent. It is interesting to note that in LB400, biphenyl uptake was inducible while biphenyl metabolism was not. Thus, previous observations of the effects of co-substrates on biphenyl and PCB biodegradation may be due to induction of uptake, rather than of metabolism. Similar experiments using radiolabeled PCBs will reveal whether biphenyl uptake is equivalent to the transport of PCBs across bacterial membranes. If PCBs are actively transported into cells, then differences in the efficiency of PCB uptake by Cam-1 and LB400 may contribute to the differences in rates of PCB metabolism observed in Chapter 1. Moreover, the discovery of an active transport system for PCBs reveals a new target for improving the efficiency of PCB bioremediation.

CHAPTER FIVE: Sequential Anaerobic-Aerobic Treatment of Soil Contaminated with Weathered Aroclor 1260

5.0. Introduction.

The data from chapters two through four represent a reference to identify recombinant strains with improved PCB-degrading enzyme activities, optimal regulation of *bph* genes and effective PCB uptake ability. However, to verify that such recombinant bacteria improve the efficiency of PCB bioremediation, a quantitative analysis of PCB bioremediation using wild type bacteria is required. Sequential anaerobic-aerobic degradation of PCBs has been previously demonstrated (Anid *et al.*, 1993; Shannon *et al.*, 1994; Evans *et al.*, 1996). However, in this chapter it is shown for the first time a detailed quantitative analysis of sequential anaerobic-aerobic treatment of soil contaminated with a weathered mixture of Aroclor 1260. The anaerobic treatment was performed by B. Kuipers and V.W.-M. Lai from the department of Chemistry at the University of British Columbia. To summarize their work, following four months anaerobic treatment with an enrichment culture, all of the major components in Aroclor 1260 were completely or partially transformed to less chlorinated PCB congeners. The major products of reductive dechlorination were 2,4-,2',4'-tetrachlorobiphenyl and 2,4-,2',6'-tetrachlorobiphenyl, and the average chlorine substituents per PCB molecule decreased from 6.4 to 5.2. The subsequent aerobic treatment of the anaerobically treated soil was then conducted as part of my research. The effect of washing and aerating anaerobically treated soil, adding biphenyl to soil to stimulate PCB biodegradation, and bioaugmentation were determined. *Burkholderia* sp. strain LB400 was used to

bioaugment the aerobic treatment since this bacterium degrades a comparatively broad spectrum of PCB congeners. The details of the aerobic treatment of anaerobically treated PCB-contaminated soil are presented in this chapter.

5.1. Aerobic Treatment of PCB-contaminated Soil.

In PCB-contaminated Saglek soil that was not treated anaerobically, 90.2 ± 19.8 % (n=3) of Aroclor 1260 remained after the aerobic treatment. However, the amount of PCBs transformed during this treatment was not significant (Students *t*-test, $P < 0.1$). This result was not surprising since the majority of PCB congeners present in Saglek soil are substituted with 6 to 8 chlorine atoms. Consequently, most of these congeners are recalcitrant to aerobic biodegradation by LB400 (Bopp, 1986; Gibson *et al.*, 1993).

In aerobic treatments bioaugmented with LB400 and previously treated anaerobically, 80% to 92% of all major PCB congeners generated during the anaerobic stage were transformed (Fig. 19). In treatments bioaugmented with LB400, 67 % of total PCBs were transformed. Bioaugmentation with LB400 of anaerobically treated soil was necessary to achieve significant aerobic PCB-degradation (Fig. 20). Significantly, this result indicates that the products of anaerobic PCB dechlorination are available for aerobic microbial degradation and that the anaerobic treatment of the soil did not produce inhibitors of aerobic PCB transformation. PCB catabolism is also indicated by a decrease in the total moles of PCBs during the aerobic treatment (Table 15). The selectivity of the aerobic process for catabolism of less chlorinated PCB congeners is indicated by an increase in the average number of chlorine atoms per biphenyl molecule of PCBs (Table 15).

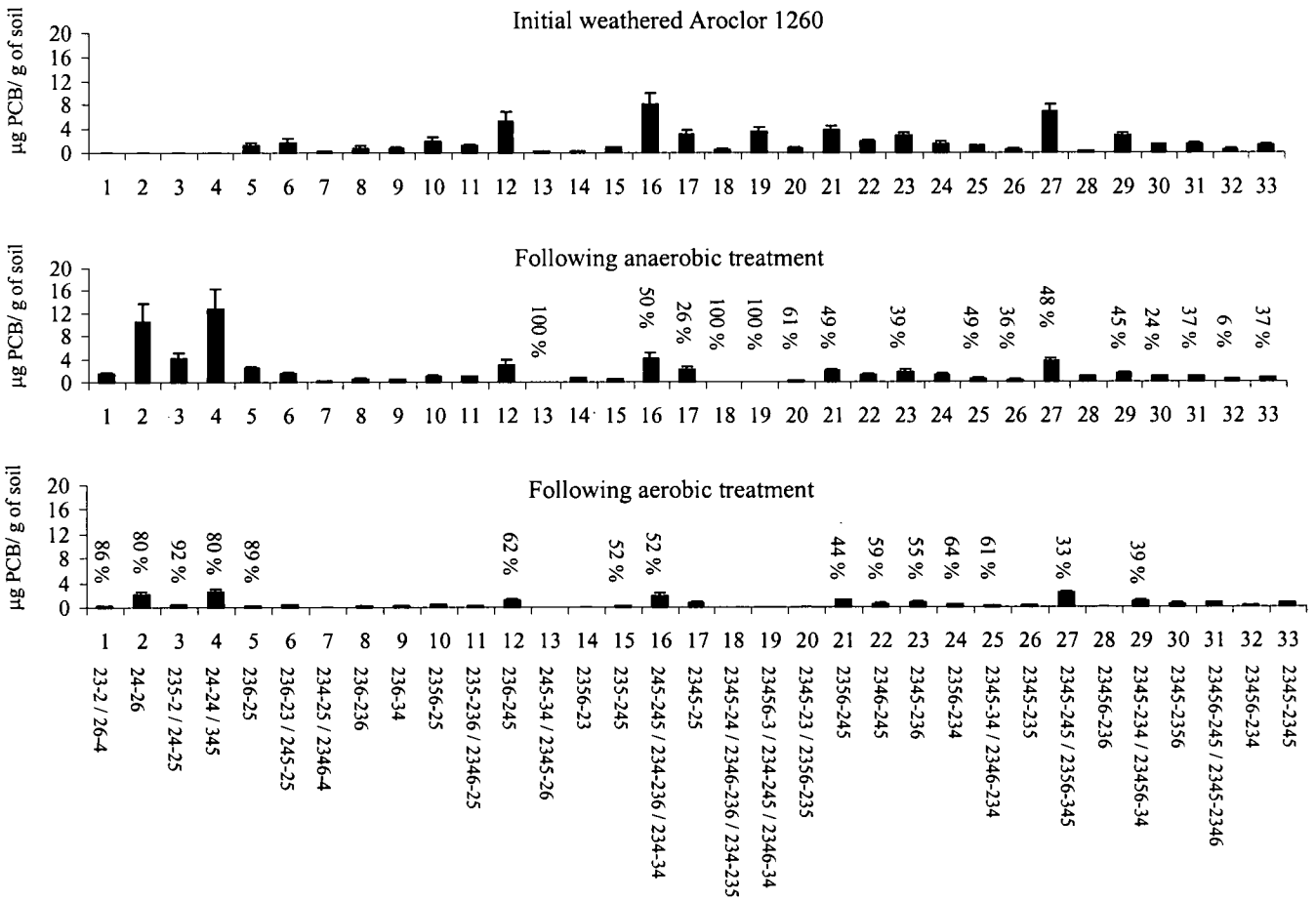


Fig. 19. Sequential treatment of PCB contaminated soil. Histograms show the major congeners of weathered Aroclor 1260 (> 0.5 µg/ g of soil) identified by positions of chlorine substitution. Bars indicate standard error; n = 3. Percent removal in each treatment phase is indicated for PCBs that were significantly degraded (Student's t-test, P < 0.1)

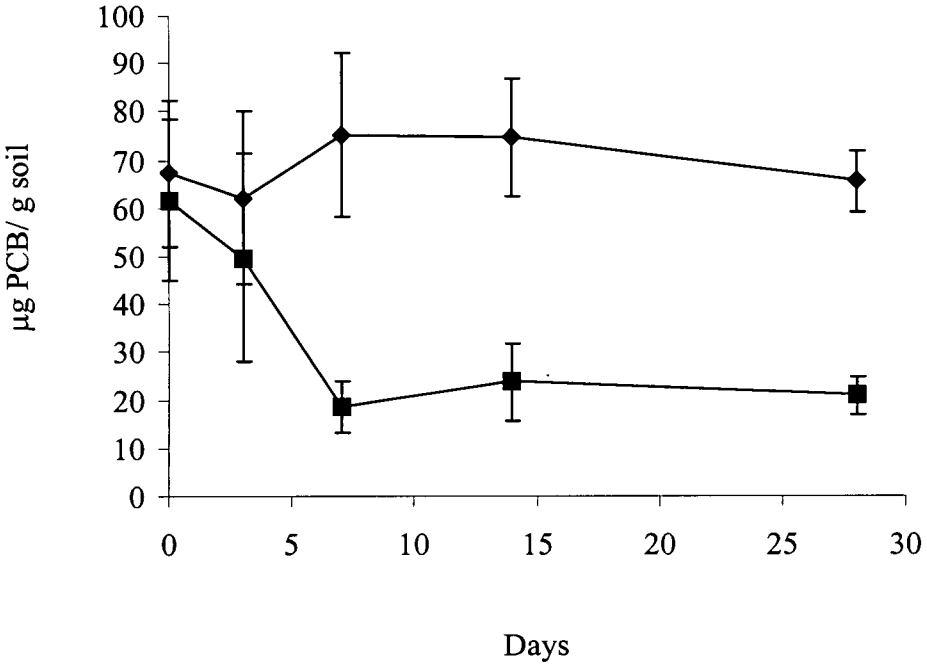


Fig. 20. Total PCBs during aerobic treatment of anaerobically treated soil (n = 3; bars indicate standard error). ◆, Aerobic treatment without inoculation; ■,Aerobic treatment with inoculation with *Burkholderia* sp. strain LB400.

Table 15. Summary of Treatment of PCB Contaminated Soil. Values are means (standard errors)

Treatment	nmol of PCBs/g of soil	μg of PCB/g soil	average Cl/PCB molecule
Initial soil[a]	157.4 (15.7)	58.7 (5.7)	6.4 (0.031)
After Anaerobic Step[b]	162.1 (45.4)	58.3 (14.4)	5.2 (0.068)
After Aerobic Step[b]	55.5 (10.5)	19.6 (3.6)	5.5 (0.12)

a. n = 4
b. n = 3

Notably, not all congeners shown to be degraded by LB400 were transformed during the aerobic treatment (Bedard and Haberl, 1990; Bopp, 1986; Haddock *et al.*, 1995). Also, transformation of certain PCB congeners with 7 chlorine substituents was detected. The range of PCBs degraded by pure cultures of LB400 might differ from that of LB400 in soil. Also, detectable loss of particular heptachlorobiphenyls may be due to indigenous microbial activity, or sampling variation. Certain PCB congeners that were present in the original soil sample were aerobically degraded only after the anaerobic treatment. This result suggests that highly chlorinated PCBs may inhibit biodegradation of less chlorinated PCBs. Alternatively, the anaerobic treatment might increase the bioavailability of weathered PCBs. Interestingly, the extent of PCB degradation in soil bioaugmented with strain LB400 was similar at 7 days and 28 days (Fig. 20), and none of the congeners were completely removed by LB400 (Fig. 19). The remaining PCBs might be strongly sorbed to the soil matrix, and poorly available for microbial degradation.

Also, metabolites that are inhibitory to PCB degradation by LB400 may have accumulated during the aerobic treatment (Brenner *et al.*, 1994). Thus, it would be interesting to determine if co-inoculation of LB400 and certain chlorobenzoic acid degraders would increase total aerobic PCB transformation (Focht, 1995). Finally, the biphenyl added to each treatment was completely consumed after 7 days. LB400 removes highly chlorinated PCBs less efficiently in the absence of biphenyl, than in the presence of biphenyl (Mondello, 1989). Thus, it is possible that adding more biphenyl to the soil treatments during incubation, would increase the extent of PCB transformation (Barriault and Sylvestre, 1993).

In conclusion, the sequential anaerobic-aerobic treatment of soil contaminated with highly chlorinated and weathered PCB congeners results in initial decrease in major components of Aroclor 1260, followed by aerobic degradation of the resulting less chlorinated PCBs. Overall, the concentration of PCBs was decreased by 67 % (from 59 μg/g of soil to 20 μg/g of soil), the number of moles PCBs was decreased by 65 %, and the average number of chlorine atoms per PCB molecule decreased from 6.4 to 5.5. As a result of this treatment, the concentration of PCBs in soil was reduced below the 50 μg/g cleanup limit. Thus, this laboratory-scale treatment of weathered PCBs demonstrates the potential for PCB bioremediation. The methods used are currently being applied to design simple, soil slurry bioreactors that can be used on-site. Future experiments will attempt to establish if total aerobic PCB transformation is enhanced by co-inoculation of additional PCB-degrading bacteria and chlorobenzoic acid degrading bacteria, or repeated addition of biphenyl during the aerobic treatment.

DISCUSSION

This thesis is the first report of isolation and characterization of PCB-degrading psychrotolerant bacteria. Bioremediation of PCB-contaminated Arctic soil using indigenous bacteria, as opposed to using mesophilic PCB-degrading bacteria, could be advantageous since Arctic bacteria might be adapted for PCB degradation at low temperature. As a result, bioaugmentation of contaminated Arctic soil using Arctic bacteria could reduce costs for on-site bioremediation, particularly heating costs.

To evaluate the potential of bacteria indigenous to Arctic soil for bioremediation of PCBs, the effect of temperature on PCB transformation by Arctic bacteria and the mesophile *Burkholderia* sp. strain LB400 was compared. LB400 was used because this strain transforms a comparatively broad range of PCB congeners (Bedard *et al.*, 1990). Unlike LB400, which transforms PCBs with up to 6 chlorine substituents, the Arctic bacteria transformed PCBs that have up to 4 chlorine substituents (Fig. 8, p.57). However, at low temperature the psychrotolerant bacteria transformed many PCB congeners at higher initial rates than LB400 (Table 7, p.64). Furthermore, PCB transformation by the Arctic bacteria was diminished at 50°C, whereas PCB transformation by LB400 was higher at 50°C than at 37°C (Fig. 11, p.65). These results are consistent with the expression of cold-adapted PCB-degrading enzymes in the Arctic soil isolates. Alternatively, the expression of *bph* genes in the Arctic bacteria might be higher than in LB400, or PCB uptake might occur at higher rates in the Arctic bacteria than in LB400. To determine the basis for higher rates of PCB transformation at low temperature by Arctic bacteria compared to LB400, *Pseudomonas* sp. strain Cam-1 and LB400 were compared in terms of PCB-degrading enzyme activity, *bph* gene expression

and biphenyl uptake. The Arctic bacterium Cam-1 was chosen for further analyses because at 7°C, Cam-1 transforms more individual congeners at higher rates than the other Arctic soil isolates.

1.0 The effect of temperature on activities of $BPDO_{Cam1}$ and $BPDO_{LB400}$.

As the initial enzyme in the biphenyl catabolic pathway, BPDO plays an important role in determining rates of PCB biotransformation. Consequently, to determine if the differences in rates of PCB transformation by Cam-1 and LB400 result from the cold-adaptation of PCB-degrading enzymes in Cam-1, BPDO from Cam-1 ($BPDO_{Cam1}$) and LB400 ($BPDO_{LB400}$) were compared in vitro. The terminal oxygenase components (ISPs) of each BPDO were overexpressed in *E. coli*; purified ISPs were complemented by his-tagged ferredoxin and reductase subunits for activity assays. Importantly, the overexpression of ISP_{Cam1} and ISP_{LB400} in *E.coli* gave higher yields of active enzyme from less starting culture and fewer purification steps than previously reported procedures for purification of ISPs (Arnett *et al.*, 2000; Haddock and Gibson, 1995; Hurtubise *et al.*, 1998; Imbeault *et al.*, 2000). The specific activity of ISP_{LB400} was greater than that reported for aerobically purified preparations of the enzyme (Arnett *et al.*, 2000; Haddock and Gibson. 1995) and the purities of ISP_{Cam1} and ISP_{LB400} were similar to that reported for anaerobically purified preparations of ISP from *C. testosteroni* B-356 (ISP_{B356}) (Imbeault *et al.*, 2000). In addition, ISP_{Cam1} and ISP_{LB400} contained a full complement of Rieske-type [2Fe-2S] cluster and mononuclear Fe^{2+} centres. Thus, the procedure described in this thesis for the purification of ISP_{Cam1} and ISP_{LB400} improves the procedure for anaerobic purification of ISPs that was described by

Imbeault *et al.* (2000), because in my research less time was required to obtain enzyme preparations of comparable quality.

Consistent with observations using whole cells, $BPDO_{Cam1}$ displayed high catalytic activity with biphenyl (k_{cat}^{app}) at low temperature and low thermal stability compared to $BPDO_{LB400}$ (Fig. 13, p.72; Table 9, 73). To ascertain whether Cam-1 expresses a cold-adapted BPDO, the relative activities of $BPDO_{Cam1}$ and $BPDO_{LB400}$ were compared with previous reports of cold-adapted enzymes. In general, cold-adapted enzymes exhibit high specific activity at low temperature and limited thermal stability compared to mesophilic homologues (Feller *et al.*, 1997; Feller *et al.*, 1996; Feller *et al.*, 1995; Gerday *et al.*, 1997). Furthermore, characteristic effects of temperature on cold-adapted and mesophilic enzymes can often be predicted from differences in respective thermodynamic parameters. The activation energy (ΔG^*) and enthalpies of activation (ΔH^*) are often lower in reactions catalyzed by cold-adapted enzymes than in reactions catalyzed by homologous mesophilic enzymes (reviewed in Lonhienne *et al.*, 2000). For instance, Hoyoux *et al.* (2001) recently reported a cold-adapted beta-galactosidase from the Antarctic psychrophilic bacterium, *Pseudoalteromonas haloplanktis*. At temperatures below 20°C, the specific activity of beta-galactosidase from *P. haloplanktis* was approximately 2 times higher than that of beta-galactosidase from *E. coli*. In addition, beta-galactosidase from *P. haloplanktis* was less thermostable than the *E. coli* enzyme (Hoyoux *et al.*, 2001). Finally, the thermodynamic activation parameters ΔG^* and ΔH^* were lower in reactions catalyzed by beta-galactosidase from *P. haloplanktis* than in reactions catalyzed by beta-galactosidase from *E. coli* (Hoyoux *et al.*, 2001). Likewise, the kinetic parameters of $BPDO_{Cam1}$ were determined at 4°C, although $BPDO_{LB400}$

activity was not detectable at this temperature (Table 9, p.73). Moreover, at 55°C the half-life of ISP_{LB400} was more than two times longer than ISP_{Cam1} (Results 2.4, p.74). Finally, the differences in the thermodynamic activation parameters of $BPDO_{Cam1}$ and $BPDO_{LB400}$ were consistent with the relative activities of these enzymes (Table 10, p.73).

Although $BPDO_{Cam1}$ is more active than $BPDO_{LB400}$ at low temperature, and is less thermostable than $BPDO_{LB400}$, several considerations indicate that these properties do not result from cold-adaptation of $BPDO_{Cam1}$. First, the k_{cat}^{app} of $BPDO_{LB400}$ at 4°C did not increase with increasing concentrations of biphenyl. This result might indicate that the activation of oxygen by $BPDO_{LB400}$ is poorly coupled to biphenyl hydroxylation at 4°C or that the activity of $BPDO_{LB400}$ at 4°C is below the detection limit of the oxygen electrode. By determining the kinetic parameters of other mesophilic BPDOs at 4°C, the significance of $BPDO_{Cam1}$ activity at 4°C will be revealed. Second, catalytic efficiency ($k_{cat}^{app} / K_m^{app}$) is a better indicator of thermal adaptation than k_{cat}^{app} since substrate binding may be affected by temperature (Feller and Gerday, 1997). Notably, between 0 and 30°C, the $k_{cat}^{app} / K_m^{app}$ of most cold-adapted enzymes is higher than that of mesophilic homologues (Feller and Gerday, 1997). In contrast, at 10 and 25°C the values of $k_{cat}^{app} / K_m^{app}$ for $BPDO_{Cam1}$ and $BPDO_{LB400}$ were not significantly different. Third, the steady-state kinetic parameters of $BPDO_{Cam1}$ for biphenyl at 25°C were similar to those reported for $BPDO_{B356}$. Therefore, under certain conditions $BPDO_{Cam1}$ displays similar characteristics to those of a mesophilic enzyme. Finally, the catalytic subunit (BphA) of ISP_{Cam1} and of the ISP from the mesophile *P. pseudoalcaligenes* KF707 (ISP_{KF707}) differ by only amino acid 178, which is alanine in $BphA_{Cam1}$ and valine in $BphA_{KF707}$

(Mondello *et al.*, 1997). Single amino acid substitutions are responsible for the cold-adaptation of certain enzymes (reviewed in Feller and Gerday, 1997). However, structural comparisons of most homologous cold-adapted and mesophilic enzymes suggest that the modification of many weak interactions is the prevailing mechanism of enzyme adaptation to temperature (Feller and Gerday, 1997). Thus, although the difference in activities of $BPDO_{Cam1}$ and $BPDO_{LB400}$ is consistent with data obtained using whole cells, in vitro kinetic data suggest that $BPDO_{Cam1}$ is not significantly cold-adapted. Nevertheless, because the activity of $BPDO_{LB400}$ was not detectable at 4°C, at low temperature and high substrate concentration as in PCB-contaminated Arctic soil, it might be advantageous for bacteria to express $BPDO_{Cam1}$ as opposed to $BPDO_{LB400}$. Accordingly, it will be interesting to study the kinetics of PCB transformation by $BPDO_{Cam1}$ and $BPDO_{LB400}$.

The difference in k_{cat}^{app} of $BPDO_{LB400}$ and $BPDO_{B356}$ at 25°C for biphenyl (Table 10, p.73) was not expected because previous comparisons concluded that their activities were similar under these conditions (Hurtubise *et al.*, 1998). Importantly, the activity of $BPDO_{LB400}$ did not differ in separate enzyme preparations. Moreover, each preparation of ISP_{LB400} contained a full complement of Rieske-type [2Fe-2S] clusters and mononuclear Fe^{2+} centres, and reached similar purity as preparations of ISP_{Cam1} and ISP_{B356} (Imbeault *et al.*, 2000). Previous comparisons of $BPDO_{LB400}$ and $BPDO_{B356}$ used aerobically purified oxygenases and a discontinuous enzyme assay to measure activity (Hurtubise *et al.*, 1998). Thus, procedures first described by Imbeault *et al.* (2000) for the anaerobic purification of ISPs and continuous measurement of BPDO activity may enable the detection of differences between BPDOs that would otherwise be missed.

Four regions were previously identified in alignments of BphA amino acid sequences, which were consistently associated with oxygenases that have broad substrate specificity but weak activity towards di-*para*-substituted biphenyl (LB400-type BphAs), or narrow substrate range and high activity towards di-*para*-substituted biphenyl (KF707-type BphAs) (Mondello *et al.*, 1997). For example, the range of PCB congeners transformed by BPDO was correlated to region III of BphA, which includes amino acids 336 through 376. Moreover, site-directed mutagenesis of Thr-376 (found in KF707-type BphAs) to Asn-376 (found in LB400-type BphAs) increases the range of PCB congeners that are transformed by $BPDO_{KF707}$ (Kimura *et al.*, 1997; Mondello *et al.*, 1997). Region I of BphA includes amino acids 1 through 276, and contains residues that are expected to form the active site of the oxygenase (Fig.14, p.76). Interestingly, the alignment of deduced amino acid sequences of ISP_{Cam1} and ISP_{LB400} revealed that 8 of the 21 amino acid differences between $BphA_{Cam1}$ and $BphA_{LB400}$ occur within region I of the subunit (Fig.14A, p.76).

To identify the determinants of the activities and thermostability of $BPDO_{Cam1}$ and $BPDO_{LB400}$ more accurately, the quaternary structure of ISP_{Cam1} was modeled using the solved structure of ISP_{B356}. Amino acids 237 and 238 are positioned near the coordinates of a Rieske-type [2Fe-2S] cluster and a mononuclear Fe^{2+} centre. Consequently, the differences in amino acids encoded at these positions may affect the properties of the catalytic groups (e.g., the reduction potential of the mononuclear iron), and thereby affect the rate of biphenyl transformation. Interestingly, the activities of $BPDO_{B356}$ and $BPDO_{Cam1}$ with biphenyl at 25°C are similar (Imbeault *et al.*, 2000), and like $BphA_{Cam1}$, $BphA_{B356}$ has methionine and serine at positions 237 and 238 respectively. In contrast,

Ralstonia eutrophus H850 degrades a similar range of PCBs as LB400, and like $BphA_{LB400}$, $BphA_{H850}$ contains threonine residues at positions 237 and 238 (Bedard and Haberl. 1990; Mondello *et al.*, 1997). Investigating the kinetics of $BPDO_{H850}$ may reveal if there exists a basis for adaptational compromise between broad substrate specificity and high catalytic activity in BPDOs. Furthermore, engineering BPDOs by exchanging region I of $BphA_{LB400}$ for region I from $BphA_{Cam1}$ may confirm the identity of amino acids that affect the kinetics of BPDOs.

The structural model of ISP_{Cam1} also revealed amino acids that might affect the thermostability of this enzyme. Twelve of the 21 amino acid differences between $BphA_{Cam1}$ and $BphA_{LB400}$ appear to be positioned within the core of this subunit. Nine of these 12 amino acids (amino acids 237, 277, 283, 285, 324, 325, 335, 337 and 340) are smaller in $BphA_{Cam1}$ than are the corresponding amino acids in $BphA_{LB400}$. The occurrence of smaller amino acids within the core of $BphA_{Cam1}$ might decrease the ability of these residues to participate in molecular interactions that stabilize $BphA_{LB400}$. Ile340Thr represents a decrease in the size of the amino acid and an increase in the polarity of the core of $BphA_{Cam1}$. Importantly, increasing the polarity of an enzyme core is correlated with decreased enzyme stability (see Introduction, section 4). Five amino acid differences occur between β-subunits (BphE) of ISP_{Cam1} and ISP_{LB400}. In particular, Arg41Ser appears to occur at the interface of two BphEs, and corresponds to an increase in the polarity of this residue in $BphE_{Cam1}$. Hydrophobic interactions are stabilized by heat; thus, decreasing the hydrophobicity of interactions between $BphE_{Cam1}$ subunits might reduce the thermostability of $BPDO_{Cam1}$ (Gerday *et al.*, 1997).

As mentioned above, $BphA_{Cam1}$ and $BphA_{KF707}$ differ by only one amino acid. However, $BphE_{KF707}$ is identical to $BphE_{LB400}$. A single amino acid at a subunit contact site accounted for the unique effect of temperature on the activities of closely related lactate dehydrogenases (Feller and Gerday, 1997). Thus, it will be interesting to compare the activity of $BPDO_{KF707}$ with $BPDO_{Cam1}$ and $BPDO_{LB400}$. If $BPDO_{KF707}$ has similar activities and thermostability as $BPDO_{Cam1}$, then determining the kinetic parameters of recombinant BPDOs previously generated by shuffling *bphA* genes from LB400 and KF707 may reveal if broad substrate range and low specific activity are necessarily linked. Otherwise, if $BPDO_{KF707}$ has similar activities and thermostability as $BPDO_{LB400}$, then the relation between subunit interaction and BPDO activity should be further investigated.

Others have demonstrated that only a few amino acid substitutions significantly affect the specificity of BPDOs (Mondello *et al.*, 1997). Likewise, my data suggest that few amino acid substitutions affect the kinetics and thermostability of BPDOs. As a result, studying the kinetics of PCB transformation by $BPDO_{Cam1}$ and $BPDO_{LB400}$ with respect to structural information of BPDOs may reveal rational strategies for engineering BPDOs with high catalytic activity, broad substrate specificity and high activities at low temperatures.

2.0. The regulation of *bph* genes in *Pseudomonas* sp. strain Cam-1 and *Burkholderia* sp. strain LB400

In addition to differences in BPDO activities, the regulation of *bph* genes in Cam-1 and LB400 is remarkably different. The regulation of *bph* genes in Cam-1 and LB400 was investigated by inserting a *lacZ* reporter gene between *bphA* and *bphE* to generate

Cam-10 and LB400-1 respectively. The assay conditions used to study the effect of temperature and various compounds on *bph* gene expression in Cam-1 and LB400 were determined by performing beta-galactosidase assays using Cam-10 and LB400-1 after incubation with biphenyl for various time points (Fig.15, p.81). Induction of beta-galactosidase activity in LB400-1 did not depend on the presence of biphenyl, suggesting that *bphA* is constitutively expressed in LB400. Notably, a gradual increase in beta-galactosidase activity in LB400-1 over time was consistently observed (Fig.15B, p.81). This result may reflect recovery from a decrease in beta-galactosidase activity during harvesting and preparation of cell suspensions of LB400-1. In contrast, beta-galactosidase activity in Cam-10 was stimulated by the presence of biphenyl, and was maximal after 3 h of incubation (Fig.15A, p.81). The observed decrease of beta-galactosidase activity in Cam-10 upon biphenyl depletion is consistent with observations by others suggesting that repeated addition of biphenyl to soil microcosms is necessary for optimal PCB biodegradation by certain bacteria (Barriault and Sylvestre, 1993). Decreased induction of *bphA* upon biphenyl depletion may also explain why pure cultures of certain PCB-degrading bacteria remove more PCBs when cells are growing on biphenyl rather then when resting cells are used (Kohler *et al.*, 1988). Interestingly, although the solubility of biphenyl is approximately 0.044 mM (Foreman and Bidleman, 1985), higher induction of beta-galactosidase activity was consistently observed in cell suspensions of Cam-10 containing 1 mM biphenyl than in cell suspensions containing 0.33 mM biphenyl (Fig. 15A, p.81). It is possible that larger amounts of biphenyl lead to more consistent high concentration of soluble biphenyl, and therefore higher beta-galactosidase activity. Alternatively, this result may suggest that bacteria use biphenyl

via direct contact with the crystals instead of, or in addition to, uptake of dissolved biphenyl.

These results have implications for bioremediation strategies that involve bioaugmentation. The preparation of PCB-degrading bacteria for bioaugmentation of PCB-contaminated soil is generally performed by growing the bacteria on biphenyl. The rate of growth and final cell densities of bacteria are often lower when growing on biphenyl than on certain alternative substrates such as pyruvate. My results demonstrate that a PCB-degrading bacterium can be grown on pyruvate (or a cheaper substrate) quickly and to high optical densities, then induced within hours to remove biphenyl. It is possible then, that this method can be applied for preparing bacterial inocula for bioremediation of PCB-contaminated soil, particularly in cases where the bacterial inoculum is defined and where catabolic genes are chromosomally located, rather than on plasmids which can be lost during growth on substrates other than biphenyl (Furukawa and Chakrabarty, 1983; Higson, 1992). However, it may be important to determine the effect of biphenyl on other physiological parameters such as membrane composition, and how these parameters effect PCB biodegradation.

The *bphA* gene in Cam-1 was inducible at 7°C and 30°C and induction was highest when using biphenyl as the inducer (Fig. 16, p.83; Table 13, p.87). Notably, at 7°C the maximum induction of the *bphA* gene in Cam-1 occurred after longer incubation time with biphenyl than at 30°C. At low temperature, the solubility of biphenyl is decreased, which may result in decreased rates of biphenyl transport across bacterial membranes, causing lower initial intracellular concentration of biphenyl and slower induction of *bphA*. The expression of *bphA* in LB400 is constitutive and is lower at

lower temperature (Fig. 15B, p.81; Table 13, p.87). The synthesis of constitutively expressed proteins in mesophilic bacteria may be repressed at low temperature, which has been correlated with increased synthesis of cold acclimation proteins (reviewed in Panoff *et al.*, 1998). Thus, decreased induction of *bphA* at 7°C in LB400 may result from a general decrease in the synthesis of constitutively expressed proteins. My comparisons of PCB transformation by whole cells at 7°C used Cam-1 and LB400 grown at 7°C and 15°C respectively. Therefore, the concentration of PCB-degrading enzymes might have been higher in Cam-1 than in LB400. If intracellular enzyme concentrations are rate-limiting to PCB degradation, then relatively high *bph* gene expression in Cam-1 at low temperature may contribute to higher rates of PCB transformation by this bacterium under these conditions.

Despite constitutive expression of the *bph* genes in LB400 from p1 and p2 (Fig.4, p.9) (Mondello, 1989; Brazil *et al.*, 1995), LB400 grown on biphenyl is able to degrade di-*para*-substituted PCBs and tetra- and penta-chlorobiphenyls more effectively than LB400 grown on succinate or on biphenyl plus succinate (Mondello, 1989). Mondello (1989) used transcriptional maps to show that transcription products from p3 contain *bphA* gene sequences. Moreover, biphenyl increases the expression of *lacZ* inserted downstream of a cloned DNA fragment that contains p3, *orf0*, p2 and p1 (de Lorenzo, V., personal communication). Thus improved PCB degradation by cells grown on biphenyl may result from increased transcription of *bphA*. However, Beltrametti *et al.* (2001) showed that transcription of *orf0* in LB400 terminates before transcription of *bphA* begins. To examine the effect of pyruvate plus biphenyl on *bphA* gene induction in LB400, beta-galactosidase activity in cell suspensions of LB400-1 containing pyruvate

alone, pyruvate and biphenyl, or biphenyl alone were compared. Similar levels of beta-galactosidase activity were detected in all treatments (Fig.16B, p.83). These results are consistent with constitutive expression of p1 and p2, and transcription termination between *orf0* and *bphA*. The discrepancy between these results and those described by de Lorenzo, (personal communication) may result from differences in the sensitivity of beta-galactosidase assays that were performed, or differences between the promoter sequences in the LB400 strains used. Notably, in LB400-1 the *lacZ* reporter gene is inserted between *bphA* and *bphE*, whereas other investigators constructed transposons to insert a chromosomal copy of the *lacZ* reporter gene, under the control of *bph* promoters, into LB400. Thus, it is possible that the chromosomal context of *bph* gene promoter sequences may affect the activity of these promoters. Despite this possibility, the promoter regions that have been used to study *bph* gene regulation in LB400 should be sequenced to verify that they are identical.

Although it is not clear if *bphA* is transcribed from p3 in LB400, constitutive expression of p1 and p2 has been reported by several independent research groups. Thus, constitutive expression of genes encoding the initial enzymes for biphenyl degradation by LB400 may explain why LB400 grown on glucose, glycerol (Billingsley *et al.*, 1997), or terpenoid compounds (Donnelly *et al.* 1994) removes PCBs. It would be interesting to determine if the regulation of *bph* genes in other organisms, which were induced for PCB transformation after growth on substrates other than biphenyl (Billingsley *et al.*, 1997; Donnelly *et al.* 1994; Gilbert and Crowley, 1997) is constitutive.

Transcriptional activation from p3 results in transcription of *orf0* (Fig. 4, p.9; Erickson and Mondello, 1992). The presence of *orf0* in our strain of LB400 was verified

using PCR. The *orf0* gene from LB400 is 86% similar to *orf0* from *Pseudomonas pseudoalcaligenes* KF707. In KF707, Orf0 is autoregulated and is necessary for the expression of genes encoding enzymes in the biphenyl degradation pathway downstream of *bphC* (Watanabe *et al.*, 2000). Likewise, *bph* genes downstream of *bphC* in LB400 may be positively regulated by Orf0. Induction of genes downstream of *bphC* allows cells to grow with biphenyl and minimizes the accumulation of metabolites resulting from biphenyl and PCB catabolism. Thus, as opposed to increased expression of *bphA*, decreased accumulation of metabolites from PCB transformation may explain why LB400 grown on biphenyl degrades di-*para* substituted PCBs and tetra- and penta-chlorobiphenyls more effectively than LB400 grown on other substrates (Mondello, 1989). Also, other physiological effects of growth on biphenyl, such as changes to membrane composition, may be necessary for transformation of certain PCB congeners.

Adding biphenyl to soil to stimulate PCB biodegradation is problematic because biphenyl is poorly soluble in water and is toxic. To identify compounds other than biphenyl that induce the expression of *bph* genes, beta-galactosidase activity in Cam-10 following incubation with various compounds was determined. Induction of *bphA* was highest with biphenyl, however, naphthalene, salicylate, 4-chlorobiphenyl and 2-chlorobiphenyl induced the expression of *bphA* to levels higher than background (Fig.16, p.83). Because Cam-10 grew with naphthalene, and since salicylate is a metabolite of naphthalene degradation, it is possible that the observed induction by naphthalene of beta-galactosidase activity in Cam-10 is due to salicylate, or its catabolites. Induction of beta-galactosidase activity in Cam-10 by salicylate is consistent with the observation that certain *Pseudomonas* species grown on salicylate readily oxidize biphenyl, and readily

oxidize salicylate when grown on biphenyl (Furukawa *et al.*, 1983). Others have proposed the use of naphthalene as a growth substrate for PCB-degrading bacteria (Pellizari *et al.*, 1996). Naphthalene is a natural component of soil, and has been used in solvents and motor oils. Consequently, naphthalene frequently occurs as a co-contaminant at PCB-contaminated sites (Jones *et al.*, 1989). Pellizari *et al.* (1996) found that bacteria isolated on biphenyl remove PCBs more extensively than bacteria isolated on naphthalene. In those experiments, PCB transformation was assayed using resting cells of bacteria grown on the substrate of isolation. My results are consistent with the findings of Pellizari *et al.* (2000) because naphthalene induced *bphA* in Cam-1, although to lower levels than did biphenyl. As has been proposed (Pellizari *et al.*, 1996), the stimulatory effect of naphthalene on PCB-degradation may be sufficient for PCB bioremediation in cases where extensive initial dechlorination has occurred.

Metabolites of several potential inducers may have inhibited beta-galactosidase induction in Cam-10. Transformation of 3-chlorobiphenyl by Cam-10 to 3-chlorocatechol was apparent from the formation of black-coloured catecholic polymers in cell suspensions (Brenner *et al.*, 1994). Since 3-chlorocatechol is a potent inhibitor of 2,3-dihydroxybiphenyl 1,2-dioxygenase (Focht, 1995; Sondossi *et al.*, 1992), inhibition of beta-galactosidase induction in Cam-10 by 3-chlorobiphenyl may result from negative regulation by accumulated metabolites. Cam-10 rapidly transformed 2,3-dihydroxybiphenyl to the *meta*-cleavage product, indicated by the production of a bright yellow metabolite. Thus, inhibition of beta-galactosidase induction in Cam-10 by 2,3-dihydroxybiphenyl may also result from negative regulation by accumulated metabolites. Fluorene, catechol, dioxin and 2-methylnaphthalene were also transformed by Cam-10,

as indicated by the production of coloured metabolites. Interestingly, these compounds that were transformed by Cam-10 include the most potent inhibitors of beta-galactosidase induction (Table 12, p.86).

Detailed biochemical studies will be necessary to identify the regulators of *bph* gene expression in Cam-1 and thereby determine if inhibition of *bphA* induction by particular compounds involves negative genetic regulation. The genetic organization and DNA sequences of *bph* genes in Cam-1, LB400 and *Pseudomonas pseudoalcaligenes* KF707 are very similar if not identical. Thus, it is likely that a gene that encodes a regulator with homology to Orf0 is positioned upstream of *bphA* in Cam-1. However, since the regulation of *bph* genes in Cam-1 is remarkably different from the regulation of *bph* genes in LB400 and KF707, the promoter sequence upstream of *bphA* in Cam-1 might contain the binding sequence for Orf0 or *bphA* might be cotranscribed with *orf0* in Cam-1. Notably, BphS from *Pseudomonas* sp. KKS102 and *Ralstonia eutropha* A5 is approximately 40% identical to Orf0 from KF707 and LB400, however, BphS negatively regulates *bph* genes (Mouz *et al.*, 1999; Ohstubo *et al.*, 2001). The repression of *bph* genes by BphS is eliminated in the presence of biphenyl metabolites (Ohstubo *et al.*, 2001). Similarly, the induction *bph* genes in Cam-1 requires biphenyl. Thus, in contrast to KF707 and LB400, *bph* genes in Cam-1 might be negatively regulated.

As mentioned, the induction of *bph* genes downstream of *bphC* in KF707 and probably LB400 requires biphenyl. More specifically, the biphenyl metabolite 2,3-dihydroxybiphenyl and 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid was shown to enhance the binding of Orf0 to the operator regions within the *bph* gene cluster in KF707 (Watanabe *et al.*, 2000). Since naphthalene, 2-chlorobiphenyl and 4-chlorobiphenyl

induced *bphA* expression in Cam-1, albeit to lower levels than biphenyl, it would be interesting to determine if metabolites from naphthalene or monochlorobiphenyl degradation also induce *bph* genes downstream of *bphC* in KF707 and LB400. From an applied point of view, stimulating PCB bioremediation by adding naphthalene or certain chlorinated biphenyls to contaminated soil is not a viable alternative to using biphenyl. However, if naphthalene or monochlorinated biphenyls are present in contaminated soil, then adding biphenyl to stimulate PCB bioremediation may not be necessary.

3.0. The effect of biphenyl uptake on biphenyl and PCB metabolism

The analyses of BPDO activities and of *bph* gene induction in Cam-1 and LB400 suggest that differences in enzyme activities as well as *bph* gene regulation may contribute to the differences in PCB transformation activities initially observed using whole cells. PCBs must traverse the bacterial membrane for catalysis, thus differences in rates of PCB uptake by Cam-1 and LB400 might also contribute to differences in rates of PCB transformation by these bacteria. Since PCBs are metabolized via the biphenyl catabolic pathway, the mechanism used for biphenyl transport in bacteria might also be used to transport certain PCBs. As a result, the uptake of radiolabeled biphenyl was used as a preliminary model for PCB transport into cells. Importantly, investigating PCB uptake directly will be necessary to confirm the mode of transport of these compounds.

Biphenyl uptake by Cam-1 and LB400 was inducible and showed saturable kinetics with respect to biphenyl concentration, which suggests that a transport system is involved in biphenyl uptake by these bacteria (Fig. 17, p.90; Fig. 18, p.92). In addition, biphenyl uptake was inhibited by the ionophore 2,4-dinitrophenol (DNP), which suggests that a transport system for biphenyl might be energized by the proton motive force.

Analysis of the kinetic data indicate that the apparent V_{max} for biphenyl transport by Cam-1and LB400 at 22°C (5.4 ± 1.7 nmol min^{-1} and 3.2 ± 0.3 nmol min^{-1}, respectively) were within the same order of magnitude as the rates of uptake of other aromatic compounds. For instance, the rate of uptake of 4-hydroxybenzoate by *Pseudomonas putida* was 25 nmol min^{-1}mg of cell $protein^{-1}$ (Nichols and Harwood, 1997); the rate of uptake of 4-toluene sulfonate by *Comamonas testosteroni* was 26.5 nmol min^{-1}mg of cell $protein^{-1}$ (Locher *et al.*, 1993); the rate of uptake of 4-chlorobenzoate by Coryneform bacterium NTB-1 was 5.1 nmol min^{-1}mg of cell $protein^{-1}$ (Groenewegen *et al.*, 1990) and the rate of uptake of naphthalene by *Pseudomonas fluorescens* Uper-1 was 0.3 nmol min^{-1}mg of cell $protein^{-1}$ (Whitman *et al.*, 1998). The values of apparent K_m for biphenyl uptake by Cam-1and LB400 (83.1± 15.9 μM and 51.5 ± 9.6 μM, respectively) were above the solubility of biphenyl in water (44 μM). It is possible that the solubility of biphenyl in natural environments is higher than in water, in which case the maximum rate of biphenyl uptake by Cam-1 and LB400 might be achieved. However, high rates of biphenyl transport to the cytosol may not be advantageous if the rate of biphenyl catalysis is not significantly higher than the rate of biphenyl uptake. Moreover, lower rates of biphenyl transport than biphenyl transformation may minimize the accumulation of inhibitory metabolites within the cell.

These data are the first evidence for active transport of biphenyl across bacterial membranes. Notably, passive transport of 2,4,2',4'-tetrachlorobiphenyl by *Ralstonia eutrophus* H850 was reported, however biphenyl uptake by this bacterium was not investigated (Kim *et al.*, 2001). Depending on the selectivity of the possible biphenyl transport system, different PCB congeners might be transported to the cytosol via

different mechanisms. As a result, determining the substrate selectivity of the biphenyl transport system will be necessary to determine if differences in rates of PCB uptake by Cam-1 and LB400 contribute to the difference in rates of PCB transformation by these bacteria. Notably, benzoate inhibits the utilization of 4-hydroxybenzoate by *Pseudomonas putida* by inhibiting the expression of *pcaK*, which encodes a permease for 4-hydroxybenzoate (Cowles *et al.*, 2000). As a result, in the presence of benzoate and 4-hydroxybenzoate, *P. putida* initially uses benzoate, which supports a higher growth rate for this microorganism (Cowles *et al.*, 2000). Likewise, certain inhibitors of *bph* gene induction in Cam-1 (see Chapter 3) might function by inhibiting biphenyl transport in Cam-1. However, most of the compounds that inhibited *bph* gene expression in Cam-1 did not support growth of this microorganism (Appendix II). Identifying the genes that are necessary for biphenyl uptake will confirm that biphenyl is transported across cell membranes via a protein transport system, and will enable more accurate examination of how this transport system is energized and regulated. Finally, the discovery of an active transport system for biphenyl and PCBs will identify a new target for improving PCB biodegradation.

This investigation of PCB metabolism by psychrotolerant and mesophilic bacteria has fundamental significance. The enzyme kinetic analyses showed that, despite the distinct substrate specificities of BPDOs, very minor amino acid differences significantly affect the catalytic activities and thermostability of these enzymes. The induction studies showed that the regulation of gene clusters that have very similar if not identical DNA sequences and organization can be remarkably different, which supports the hypothesis that regulatory and functional genes might evolve independently (de Lorenzo and Perez-

Martin, 1996). Finally, although aromatic compounds are generally thought to traverse cell membranes via passive diffusion, evidence for the active uptake of biphenyl was obtained. In addition to the fundamental significance of these observations, these results can be applied to optimize PCB bioremediation by wild type bacteria and improve designs for recombinant PCB-degrading bacteria. However, to identify improved strategies for PCB bioremediation, or detect recombinant bacteria with optimal PCB-degradation activity, a careful analysis of PCB bioremediation using wild type strains and few manipulations is required. To this end, a detailed quantitative analysis of sequential anaerobic-aerobic treatment of soil contaminated with weathered Aroclor 1260 was performed.

4.0. Bioremediation of PCB-contaminated soil.

Following prolonged presence in soil, PCBs that can be degraded will be removed and remaining congeners will sorb to soil particles. As a result, weathered Aroclor 1260 can have a higher proportion of recalcitrant, highly-chlorinated PCB congeners than when initially synthesized. Consequently, demonstrating remediation of weathered Aroclor 1260 is important for assessing the application of bioremedaition to treat soil with a history of contamination with PCBs. The anaerobic treatment of PCB-contaminated soil was performed by B. Kuipers and V.W.-M. Lai from the Department of Chemistry at the University of British Columbia, and the subsequent aerobic treatment was performed as part of my research. After four months, the anaerobic treatment had primarly dechlorinated *meta*-substituted chlorines. The number of moles of PCBs before and after the anaerobic treatment did not change significantly, which confirms that the anaerobic treatment results in PCB dechlorination, as opposed to degradation of the

biphenyl molecule. Notably, a significant reduction in PCB concentration by weight as a result of dechlorination was not observed (Table 15, p.99), which is likely due to high variability during soil sampling.

Aerobic treatments, which were bioaugmented with *Burkholderia* sp. strain LB400, significantly degraded the major PCB congeners that were produced during the anaerobic treatment. However, none of the PCB congeners were completely transformed and the extent of PCB transformation did not increase after 7 days. Several explanations might account for this observation. First, the biphenyl content in the aerobic treatments was depleted after 7 days. Since biphenyl is probably required to induce *bph* genes downstream of *bphC*, then incomplete PCB transformation in the aerobic treatment might result from the accumulation of the *meta*-cleavage product (*i.e.* chlorinated 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid) in LB400, thereby reducing the flux through the PCB-degradation pathway. Second, additional metabolites that inhibit PCB degradation by LB400 might accumulate due to the specificities of the various enzymes in the biphenyl pathway. For instance, 3,4-dihydrodihydroxybiphenyls and chloro-substituted *cis*-dihydrodiols are relatively poor substrates for BphB (Barriault *et al.*, 1999; Hulsmeyer *et al.*, 1998; Sylvestre *et al.*, 1996a); catalysis by BphC is slowed by 3,4-dihydroxybiphenyls and chlorine substituents located on the dihydroxylated ring (Hirose *et al.*, 1994; Khan *et al.*, 1997), and catalysis by BphD is slowed by chlorine substituents adjacent to the hydrolysis position (Seah *et al.*, 2000). Finally, chlorobenzoates generated by the hydrolysis of *meta*-cleavage products, might accumulate and inhibit PCB-biodegradation. More specifically, certain chlorobenzoates are transformed to chlorocatecols via benzoate dioxygenases or toluate dioxygenases (Reineke, 1998).

Chlorocatechols are then transformed via the modified *ortho*-cleavage pathway to TCA cycle intermediates (Harayama and Timmis, 1989). However, 2,3-dihydroxylbiphenyl dioxygenases are often inhibited by chlorocatechols, and in particular by 3-chlorocatechol (Adams *et al.*, 1992; Arensdorf and Focht, 1994; Brenner *et al.*, 1994; Sondossi *et al.*, 1992). In addition, 3-phenylcatechol inhibits *ortho* fission of 3-chlorocatechol (Adams *et al.*, 1992; Brenner *et al.*, 1994). Probably due to the incompatibility of the *meta*-cleavage and *ortho*-cleavage pathways, PCB-degrading bacteria that express the modified *ortho*-cleavage pathway have not been isolated. Notably, chloroaliphatic acids are also generated by the hydrolysis of *meta*-cleavage products. Most chloroaliphatic acids are metabolized by PCB-degrading bacteria. However, few bacteria have been isolated that degrade chloroacetate (Focht, 1995; Hernandez *et al.*, 1995). Therefore chloroacetate might also accumulate in certain PCB-degrading bacteria and inhibit PCB biodegradation.

The sequential anaerobic-aerobic treatment of PCB-contaminated soil, which is described in this thesis, did not completely remove the PCBs that were present in the soil sample. However, this treatment reduced the concentration of PCBs in the soil to below regulatory requirements (*i.e.* below 50 mg/ kg of soil). PCB bioremediation might be improved by maintaining a sufficient concentration of the inducer molecule; co-inoculating contaminated soil with bacteria that degrade PCBs, chlorobenzoate and chloroaliphatic acid; using recombinant strains that express enzymes that degrade PCBs, chlorobenzoates and chloroaliphatic acids and broadening the substrate specificities of the biphenyl pathway enzymes.

Barriault and Sylvestre (1993) found that the repeated addition of biphenyl to PCB-contaminated soil increased the extent of PCB-biotransformation compared to adding surfactant or repeatedly bioaugmenting the soil with *Comamonas testosteroni* B-356. Likewise, other researchers demonstrated that cells growing on biphenyl transformed more PCBs than resting cells (Kohler *et al.*, 1988). These experiments emphasize the importance of maintaining sufficient quantities of the inducer molecule for effective bioremediation. However, these experiments did not demonstrate PCB mineralization or determine if metabolites that inhibited PCB biodegradation were accumulating in the treatments.

Several researchers have demonstrated that co-cultures of PCB-degrading bacteria and chlorobenzoate-degrading bacteria increase total PCB transformation and PCB mineralization (Adriaens *et al.*, 1989; Adriaens and Focht, 1990; Brunner *et al.*, 1985; Fava *et al.*, 1994; Furukawa and Chakrabarty, 1982; Hickey *et al.*, 1993; Kikuchi *et al.*, 1995). However, chlorobenzoate-degrading bacteria do not transform all chlorobenzoates that are produced during PCB biotransformation, and chlorobenzoate degradation can be inhibited by metabolites of PCB biotransformation (Adriaens and Focht, 1991; Hernandez *et al.*, 1991; Stratford *et al.*, 1996). The inhibition of chlorobenzoate degradation might be overcome by using co-cultures of chlorobenzoate-degrading bacteria that have different substrate specificities (Reineke, 1998; Fava *et al.*, 1994).

Regulating the activity of a co-culture of bacteria is more difficult than regulating the activity of a singe bacterial strain. Thus, an alternative to using co-cultures of bacteria to bioremediate PCBs is to use recombinant strains that express both PCB-

degrading enzymes and chlorobenzoate-degrading enzymes (Adams *et al.*, 1992; Focht *et al.*, 1996; Hrywna *et al.*, 1999; McCullar *et al.*, 1994; Rodrigues *et al.*, 2000). PCB mineralization by recombinant bacteria may be improved by expressing chlorobenzoate-degrading enzymes that have broader specificity than wild type enzymes (Brenner *et al.*, 1993). However, as discussed above, designing ring cleavage dioxygenases (BphC) that are not inhibited by chlorocatechols, as well as dihydrodiol dehydrogenases (BphB) and *meta*-cleavage product hydrolases (BphD) that have increased substrate specificities, is also essential to the success of these recombinant strains (Barriault et al, 1999; Seah *et al.*, 2000; Vaillancourt *et al.*, 1998).

Finally, data presented in this thesis demonstrate that the induction of PCB-degrading enzymes and the transport of substrate across the cell membrane for catalysis might also limit the potential of PCB bioremediation. As a result, optimizing the flux through the PCB metabolic pathway, which could be distributed in a microbial consortium or assembled in a recombinant bacterium, might require differential regulation of enzymes to minimize the accumulation of inhibitory PCB metabolites and minimize the metabolic burden on the host strains. The effect of PCB-uptake on PCB mineralization can be better investigated if genes that encode a transport system for PCBs or biphenyl are identified.

CONCLUSIONS

Comparisons of PCB transformation by psychrotolerant bacteria isolated from Arctic soil and the mesophile *Burkholderia* sp. strain LB400 suggested that the psychrotolerant bacteria are adapted to degrade PCBs at low temperature. LB400 and the Arctic bacterium *Pseudomonas* sp. strain Cam-1 were further analysed to determine the basis for superior PCB transformation at low temperature in Cam-1.

Although the kinetic parameters of BPDO from Cam-1 ($BPDO_{Cam1}$) were determined at 4°C, BPDO from LB400 ($BPDO_{LB400}$) had no detectable activity at this temperature. Furthermore, $BPDO_{Cam1}$ was less thermostable than $BPDO_{LB400}$. However, at 10°C and 25°C the apparent catalytic efficiencies ($k_{cat}^{app} / K_m^{app}$) of $BDPO_{Cam1}$ and $BPDO_{LB400}$ were not significantly different. As a result, I conclude that the differences in activities of $BPDO_{Cam1}$ and $BPDO_{LB400}$ may not result from cold-adaptation of $BPDO_{Cam1}$. The kinetic parameters of other mesophilic BPDOs should be determined at 4°C to evaluate the significance of $BPDO_{Cam1}$ activity at this temperature. Interestingly, amino acid sequence comparisons indicate that only a few residues near the active site might determine the specific activities of $BPDO_{Cam1}$ and $BPDO_{LB400}$. Thus, it might be possible to generate BPDOs with broad substrate specificity and high catalytic activity by changing only a few amino acids in $BPDO_{LB400}$.

Despite the high sequence similarity and identical organization of *bph* genes in Cam-1 and LB400, the regulation of *bphA* in these strains is remarkably different. Although *bphA* in LB400 is constitutively expressed, *bphA* expression in Cam-1 required biphenyl. I was unable to identify compounds that induce *bphA* in Cam-1 more than biphenyl, however naphthalene, salicylate, 2-monochlorobiphenyl and 4-

monochlorobiphenyl induced the expression of *bphA* above background levels. Interestingly, at 7°C, the constitutive expression of *bphA* in LB400 was reduced whereas *bphA* expression in Cam-1 was induced, albeit after longer incubation with biphenyl than at 30°C. Thus, higher rates of PCB transformation at low temperature in Cam-1 than in LB400 may result from higher levels of expression of enzymes that degrade PCBs in Cam-1.

The rate of biphenyl uptake at 22°C by Cam-1 and LB400 was not significantly different. These data suggest that differences in PCB transformation activity in Cam-1 and LB400 may not result from differences in intracellular concentrations of these compounds. PCBs may enter cells differently than biphenyl. Furthermore, the rate of biphenyl and PCB uptake by Cam-1 and LB400 may differ at low temperature. Thus, additional studies are required to determine the contribution of substrate uptake on differences in PCB biotransformation activities in Cam-1 and LB400.

FUTURE EXPERIMENTS

Several new research objectives result from observations made in this thesis. First, the kinetics of PCB transformation by $BPDO_{Cam1}$ and $BPDO_{LB400}$ should be determined. If congeners that are transformed by $BPDO_{Cam1}$ and $BPDO_{LB400}$ were transformed at higher rates by $BPDO_{Cam1}$, then it would be interesting to identify amino acid residues that significantly affect the catalytic activity of BPDOs. Subsequent analyses of recombinant enzymes might reveal whether broad substrate specificity and low catalytic activity are necessarily linked.

Second, regulators of *bph* genes in *Pseudomonas* sp. strain Cam-1 should be identified. Accordingly, DNA sequences upstream of *bphA* and downstream of *bphC* in Cam-1 should be determined. In addition, genes in Cam-1 that are homologous to *orf0* from *Pseudomonas pseudoalcaligenes* KF707 and *Burkholderia* sp. strain LB400 could be identified using Southern hybridization. It would then be interesting to determine if regulators of *bph* genes can be modified to respond to different environmental signals, such as different carbon sources or temperature.

Finally, considerably more research is required to characterize biphenyl and PCB uptake by bacteria. The effect of temperature on biphenyl uptake by Cam-1 and LB400 should be determined. In addition, PCB uptake by Cam-1 and LB400 should be investigated. In this way, the importance of substrate uptake on rates of PCB metabolism may be revealed. Eventually, the biphenyl uptake system should be identified to enable more careful characterizations of the proteins involved. A biphenyl transport system could be identified by screening mutants that grow slower on biphenyl than wild type strains, or by using reverse genetics or microarrays.

APPENDIX I

The rate of removal of selected single PCB congeners by *Pseudomonas* sp. strain Cam-1 was determined. Cam-1 was grown on biphenyl at 7°C, and cell suspensions were prepared as described in the materials and methods (3.1). Selected PCB congeners (10 μg/ ml of a PCB congener and 2 μg/ ml of the internal standard, 2,2'4,4'6,6'-hexachlorobiphenyl) were added to each tube, and then the reaction tubes were incubated at 7°C. Sulfuric acid was used to stop PCB removal activity in cell suspensions at regular time intervals over 24 h. Remaining PCBs were extracted from the cell suspensions and analyzed as described in the materials and methods (3.2).

Table 16. Removal of Single PCB Congeners.

PCB Congener	Rate of Removal[a] ($\mu g\ h^{-1}$ g of cell $protein^{-1}$)	% Removed [b]
2,2'-dichlorobiphenyl	0	0
4,4'-dichlorobiphenyl	1279	56
2,3,2'-trichlorobiphenyl	0	0
2,6,2'-trichlorobiphenyl	0	0
2',3,4-trichlorobiphenyl	ND	43
2,4,4'-trichlorobiphenyl	1517	23
2,5,4'-trichlorobiphenyl	3354	29
2,3,2',3'-tetrachlorobiphenyl	0	0
2,4,6,4'-tetrachlorobiphenyl	0	0

[a] These data are also presented in the 448 project summary written by Daryl J. Smith (1999).

[b] Determined at 24 h. ND. Not determined.

APPENDIX II

The compounds used as potential inducers of *bph* genes in Cam-1 were tested as growth substrates for Cam-1 and Cam-10. These experiments were performed in screw capped, teflon lined tubes, and test substrates were added to mineral medium (PAS). Cultures were incubated at 22°C on a tube roller.

Table 17. Use of test inducers by Cam-1 and Cam-10.

Substrate	Substrate Concentration	Results[a] for	
		Cam-1	Cam-10
Benzene	100 mg/L	NG	NG
Toluene	100 mg/L	NG	NG
Benzoate	100 mg/L	G	G
Acenaphthalene	200 mg/L	NG	NG
Fluorene	200 mg/L	NG	NG
Naphthalene	200 mg/L	NG	NG
Carvone	100 mg/L	NG	NG
Citronellol	100 mg/L	NG	NG
Cumene	100 mg/L	NG	NG
Cymene	100 mg/L	NG	NG
Limonene	100 mg/L	NG	NG
Linoleic acid	1 g/L	G	G
Pinene	100 mg/L	NG	NG
Forest soil extract	20 %	NG	NG
Saglek soil extract	20 %	NG	NG

Cambridge Bay soil extract	20 %	NG	NG
Biphenyl	200 mg/L	G	G
4-chlorobiphenyl	200 mg/L	G	G
Acetone	200 mg/L	NG	NG
Glucose	1 g/L	G	G
Glycerol	1 g/L	G	G
Methanol	200 mg/L	NG	NG

[a]G, supports growth as sole organic substrate; NG, does not support growth. Readings were obtained after 3 days and 1 month.

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