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WHOLE-CELL PHYSIOLOGY AND GENE EXPRESSION PATTERNS IN THE CO-METABOLISM OF AND TOLERANCE TO PCBS BY *BURKHOLDERIA XENOVORANS* LB400.

presented by

John Jacob Parnell

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By

John Jacob Parnell

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ABSTRACT

WHOLE-CELL PHYSIOLOGY AND GENE EXPRESSION PATTERNS IN THE CO-METABOLISM OF AND TOLERANCE TO PCBS BY *BURKHOLDERIA XENOVORANS* LB400.

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Aerobic biodegradation of polychlorinated biphenyls (PCBs) occurs gratuitously via the biphenyl (bph) pathway. Although many PCB-degrading bacteria exhibit similar degradation profiles, I have found a wide range of sensitivity to PCBs and hypothesize involvement of genes outside the bph pathway in efficient degradation. To date, little information is available on mechanisms beyond the bph pathway that bestow PCB tolerance to microorganisms or increase degradation efficiency. This work combines physiological information on the reponse of *Burkholderia xenovorans* LB400 (LB400) with microarray techniques to investigate the transcriptomic response to growth substrate and growth phase on the degradation of PCBs. I report the induction of the bph and (chloro)catechol pathways during PCB degradation, however during growth on simple carbon sources, such as succinate, PCBs inhibit the bph pathway. Importantly, among the putative genes induced on degradation of PCBs, a putative chloroacetaldehyde dehydrogenase exibited 83% (amino acid) identity to a homolog responsible for rapid conversion of the highly toxic chlroroacetaldehyde intermediate in dichloroethane (DCE) degraders. The statistical analysis of genome-wide expression patterns of 11 different treatments reveals a close relationship in expression of the acetaldehyde dehydrogenase in the lower biphenyl pathway (BphJ) and chloroacetaldehyde dehydrogenase, suggesting a similar role. In addition, transcriptional profiling indicates very limited expression

response to PCB degradation compared with changes in growth rate and substrate and identifies several candidate genes, such as transporters and regulators that may be involved in the degradation of biphenyl. Resting-cell assays of LB400 show a dymanic change in degradation profiles as a result of culture conditions (carbon source and growth phase). Microarray analyses indicate several pathways associate with PCB degradation are influenced by sigma-54 (σ^{54}). Subsequent Q-RT-PCR analysis of both σ^{54} factors in the LB400 genome indicate the involvement of only one (located on the mini chromosome) associated with the degradation of biphenyl. Further Q-RT-PCR analyses of the response of the biphenyl pathway to carbon source competition and growth phase reveals inhibition of the biphenyl pathway by PCBs. Metabolic footprinting of knockout mutants of the C_1 pathway (LB400Δ*xoxF* and LB400Δ*flhAfdhAmtdB*) in LB400 indicates the involvement of this pathway in detoxification of formaldehyde that accumulates during growth on biphenyl and degradation of PCBs that has not been reported previously. Although I show that the affect of PCBs on gene expression profiles is minimal, several auxiliary mechanisms involved in PCB degradation have been identified. This work has outlined several factors, such as biotoxicity (detoxification), carbon source competition, and regulation and transport that are essential to the degradation of PCBs in an environmental context.

I dedicate the work I have done over the past five years to all of those that have allowed me to stand on their shoulders in order to keep my head above water. Primarily, I dedicate this to my wife, Kara and the kids. Their patience with me alone should earn them credit for much of the success of this work. Field trips have been cancelled and vacations postponed all in the name of science. Their endurance has been exemplary and without them, none of this would mean anything.

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Throughout the course of work for the fulfillment of the requirements for this degree, I have received help and support from many sources. Looking back on the progress I have had, I owe much to the faith I have in Heavenly Father. Following that source of guidance, I have to give thanks to my family and the support that they have offered, especially Kara, for being patient while I rolled out of bed in the middle of the night for growth curve readings or came in on weekends or holidays to finish experiments. My immediate family (including in-laws) was always there to bail me out and help when I needed it most. I especially thank Judy, Chuck, and Heidi Schurman for being our family in Michigan.

I gratefully acknowledge Ewa Daniellewicz and Alicia Pastor for their help with electron microscopy and A. Daniel Jones for his help with LC-MS analysis. Jeff Landgraff was instrumental in both analysis of gene expression patterns and Q-RT-PCR advice. I was greatly guided in experimental design and research strategies by Vincent Denef, Tamara Tsoi, Joon Park, Peter Bergholz, Stephen Callister, among others in the lab and of course Dr. Tiedje for always being available to meet with me and his input on the eventual manuscript products from this work. I also need to acknowledge Shannon Hinsa for her follow-up work with LB400 that will build on what I have accomplished. I never would have made it through all of the hurdles associated with forms and general requirements without the help of Rita House and Lisa Pline and have learned never to underestimate the value of good secretaries.

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CHAPTER 1

INTRODUCTION AND RATIONALE

Background

Polychlorinated biphenyls (PCBs) are among the most important environmental pollutants (Quensen, et al. 1998). PCBs are composed of a biphenyl nucleus with one to ten substituted chlorines; each different type of substitution is referred to as a congener. PCBs are generally found in industrial mixtures that contain 60 to 100 of the 209 different possible congeners and were produced commercially under the names Clophen (Bayer, Germany), Aroclor (Monsanto, USA), Kanechlor (Kanegafuchi, Japan), Santother (Mitsubishi, Japan), Phenoclor, and Pyralene (Prodolec, France). The degree of chlorination of each mixture is indicated by a number, the first indicating the number of carbon atoms and the latter denoting the percent chlorine by weight, thus Aroclor 1242 consists of 12 carbons and is 42% chlorine by weight. The persistence of PCBs in the environment increases with the extent of substitution as well as the position of chlorine atoms around the biphenyl nucleus and is generally associated with their aqueous solubility (Otto and Moon, 1996). Until their ban in 1977, more than 1.5 billion pounds of PCBs were manufactured in the United States alone (USEPA). Their industrial purposes varied greatly and included electric fluids in transformers and capacitors, wax and pesticide extenders, adhesives, de-dusting agents, cutting and compressor oils, flame retardants, heat transfer and dielectric fluids, hydraulic lubricants, electromagnets, fluorescent light ballasts, sealants, paints, inks, dyes and carbonless copy paper (Table 1.1)(Mondello, et al. 1997).

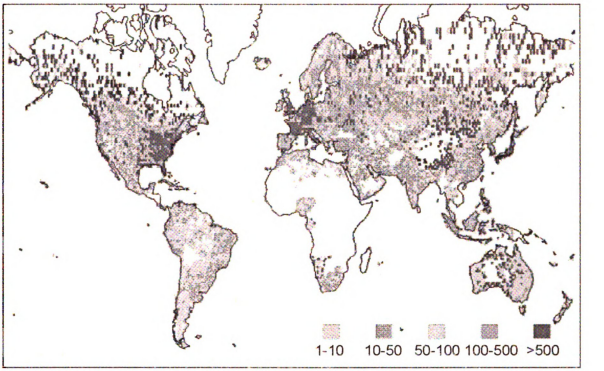


Figure 1.1. Estimated world-wide cumulative usage of PCBs in tons (from Brevik et al. 2002).

Table 1.1. Industrial usage in pounds and percent total of PCBs in the United States to 1977.

PCB Use	Pounds (millions)	Percentage of Total
Capacitors	755	50%
Transformers	400	26%
Placticizer uses	138	9%
Hydraulics and lubricants	100	6%
Carbonless copy paper	54	3%
Heat transfer fluids	24	1%
Petroleum additives	2	0.1%
Miscellaneous industrial uses	33	2%
TOTAL	1,506	97.1%

Ironically, the physicochemical characteristics that originally made PCBs so valuable—low vapor pressure and aqueous solubility, excellent dielectric properties, stability, inertness and persistence—are now the source of greatest environmental concern. Due to their low aqueous solubility, low volatility, and high octanol/water partitioning coefficients, PCBs readily become concentrated on sediment and soil surfaces (Chiou, 2002). Many of the physicochemical properties of PCBs also elicit negative effects in organisms. Because PCBs are very insoluble, they tend to accumulate in lipids of organisms (Chiou, 2002). The environmental dispersal of PCBs is pervasive due to the extent of commercial production, widespread industrial application, and persistence.

Significance

Health concerns with PCBs stem from their ability to bioaccumulate in the higher trophic levels (Kidd et al., 1998). The toxic effects of PCBs on eukaryotic organisms has been well documented and include oxidative stress and altered morphology of tissues that can lead to cell death (Stalker, et al. 1991; Livingstone, et al. 1991). In response to PCBs, aquatic organisms increase antioxidant defenses (Schlezinger, et al. 2000), such as superoxide dismutase, carotenoids, peroxidases, and glutathione (Leitao, et al. 2003). Because of the lipophilic properties of PCBs, they tend to partition into and concentrate in the lipid tissues of all organisms (Chiou, 2002), including humans (Pelletier et al., 2003), where they may alter immune functions (Grasman & Fox 2001; Smithwick et al., 2003) and cause neurological (Seegal 1996), developmental (Patandin et al., 1999),

respiratory (Swanson et al., 1995), and reproductive problems as well as some types of cancer due to estrogenic activity (Demers, et al. 2002). Numerous studies have focused on health concerns that demonstrate biotoxicity of elevated concentrations of PCBs over a wide variety of higher organisms. However, until now, the physiological response to PCB exposure has almost entirely focused on eukaryotic organisms. The characterization of the physiological response of bacteria to PCBs is essential as they play a major role in the food web where their exposure to PCBs is potentially greatest (soil/sediment surfaces) and they have the ability to degrade some congeners.

The most attractive solution to the problem of PCBs in the environment entails their complete *in situ* mineralization by microorganisms. While studies on the enzyme kinetics (Arnett, et al. 2000; Barriault, et al. 1998, 1999, 2004; Broadus and Haddock, 1998; Dai, et al. 2002; Furukawa 1982; Haddock, et al. 1993, 1995, 1997;) and characterization of PCB-degrading pathways (Asturias and Timmis, 1992; Barriault, et al. 2004; Bedard, et al. 1987; Brenner, et al. 1994; Furukawa 1982; Masai, et al. 1995) are extensive, predicting the fate of microbiologically mediated PCB destruction in the environment is hindered by complex environmental variables and limited knowledge of environmental rates or ecological performance.

Objectives of this study.

The main focus of this research is to gain insight into the ecology of PCB degradation by analyzing mechanisms of tolerance and co-metabolism of polychlorinated biphenyls by aerobic biodegradative microorganisms using *Burkholderia xenovorans*

LB400 as a model organism. To determine the extent of tolerance and breadth of co-metabolism of PCBs the following objectives were investigated:

Objective 1. Determine the sensitivity or tolerance of potential PCB-degrading strains to a commercial mixture of PCBs (Aroclor 1242).

Objective 2. Characterize the whole-cell physiological responses to LB400 following exposure to PCBs.

Objective 3. Analyze gene expression patterns involved in the response of LB400 following exposure to PCBs.

Objective 4. Compare transcriptional profiles of LB400 following growth on different carbon sources, growth phase and PCB exposure.

Objective 5. Examine the role of C1 compounds in PCB degradation by LB400.

REFERENCES

Abramowicz, D.A., M.J. Brennan, H.M. Van Dort, and F.L. Gallagher. 1993. Factors influencing the rate of polychlorinated biphenyl degradation in Hudson River sediments. Environ. Sci. Technol. **27:**1125–1131

Ahmed M., and D.D. Focht. 1973. Degradation of polychlorinated biphenyls by two species of *Achromobacter*. Can J Microbiol. **19:**47–52.

Arnett, C. M., J. V. Parales, and J. D. Haddock. 2000. Influence of chlorine substituents on rates of oxidation of chlorinated biphenyls by the BPDO of *Burkholderia* sp. strain LB400. Appl. Environ. Microbiol. **66:**2928-2933.

Asturias, J.A., and K.N. Timmis. 1993. Three different 2,3-dihydroxybiphenyl-1,2-dioxygenase genes in the gram-positive polychlorobiphenyl-degrading bacterium Rhodococcus globerulus P6. J Bacteriol. **175:**4631-40.

Barriault, D., J. Durand, H. Maaroufi, L.D. Eltis, and M. Sylvestre. 1998. Degradation of polychlorinated biphenyl metabolites by naphthalene-catabolizing enzymes. Appl. Environ. Microbiol. **64:**4637-4642.

Barriault, D., and M. Sylvestre. 1993. Factors affecting PCB degradation by an implanted bacterial strain in soil microcosms. Can J. Microbiol. **39:**594-602.

Barriault, D., M. Vedadi, J. Powlowski, and M. Sylvestre. 1999. *cis*-2,3-Dihydro-2,3-dihydroxybiphenyl dehydrogenase and *cis*-1,2-dihydro-1,2-dihydroxynaphthalene dehydrogenase catalyze dehydrogenation of the same range of substrates. Biochem. Biophys. Res. Commun. **260:**181-187.

Barriault, D., F. Lepine, M. Mohammadi, S. Milot, N. Leberre, and M. Sylvestre. 2004. Revisiting the regiospecificity of *Burkholderia xenovorans* LB400 biphenyl dioxygenase toward 2,2'-dichlorobiphenyl and 2,3,2',3'-tetrachlorobiphenyl. J. Biol. Chem. **279:**47489-47496.

Bedard, D. L., and J. F. Quensen, III. 1995. Microbial reductive dechlorination of polychlorinated biphenyls, p. 127-216. *In* L. Y. Young, and C. Cerniglia (ed.), Microbial transformation and degradation of toxic organic chemicals. Wiley-Liss Division, John Wiley & Sons, Inc., New York, N.Y.

Bedard, D.L., M.L. Haberl, R.J. May, and M.J. Brennan. 1987. Evidence for novel mechanisms of polychlorinated biphenyl metabolism in Alcaligenes eutrophus H850. Appl. Environ. Microbiol. **53:**1103–1112.

Bedard, D.L., R. Unterman, L.H. Bopp, M.J. Brennan, M.L. Haberl, and C. Johnson. 1986. Rapid assay for screening and characterizing microorganisms for the ability to degrade polychlorinated biphenyls. Appl. Environ. Microbiol. **51:**761–768.

Bedard, D.L., R.E. Wagner, M.J. Brennan, M.L. Haberl, and J.F. Brown Jr. 1987. Extensive degradation of Aroclors and environmentally transformed polychlorinated biphenyls by Alcaligenes eutrophus H850. Appl. Environ. Microbiol. **53:**1094–1102

Bopp L H. Degradation of highly chlorinated PCBs by *Pseudomonas* strain LB400. J Ind Microbiol 1986;**1**:23–29.

Brenner, V., J.J. Arensdorf, and D.D. Focht. 1994. Genetic construction of PCB degraders. Biodegradation **5:**359–377

Brevik, K., A. Sweetman, J. Pacyna, and K. Jones. 2002. Towards a global historical emission inventory for selected PCB congeners–A mass balance approach. The Science of the Total Environment. **290:**181-198.

Broadus R.M., and J.D. Haddock. 1998. Purification and characterization of the NADH:ferredoxin$_{BPH}$ oxidoreductase component of biphenyl 2,3-dioxygenase from *Pseudomonas* sp. strain LB400. Arch. Microbiol. **170:**106–112.

Butler C.S., and J.R. Mason. 1996. Structure-function analysis of the bacterial aromatic ring hydroxylating dioxygenases Adv. Microb. Physiol. **38:**47-84.

Chain, P.S.G., V.J. Denef, K. Konstantinidis, L.M. Vergez, L. Agulló, V.L. Reyes, L. Hauser, M. Córdova, L. Gómez, M. González, M. Land, V. Lao, F. Larimer, J.J. LiPuma, E. Mahenthiralingam, S.A. Malfatti, C.J. Marx, J.J. Parnell, A. Ramette, P. Richardson, M. Seeger, D. Smith, T. Spilker, W-J. Sul, T.V. Tsoi, L.E. Ulrich, I.B. Zhulin, J.M. Tiedje. 2006. *Burkholderia xenovorans* LB400 harbors a multi-replicon, 9.7 M bp genome shaped for versatility. Proc. Natl. Acad. Sci. (Submitted).

Chiou, C.T., 2002, Partition and adsorption of organic contaminants in environmental systems: New York, John Wiley & Sons

Dai, S., F.H.Vaillancourt, H. Maaroufi, N.M. Drouin, D.B. Neau, V. Snieckus, J.T. Bolin, and L.D. Eltis. 2002. Identification and analysis of a bottleneck in PCB biodegradation. Nat. Struct. Biol. **9:**934-939.

Demers, A. P. Ayotte, J. Brisson, S. Dodin, J. Robert, and E. Dewailly. 2002. Plasma Concentrations of Polychlorinated Biphenyls and the Risk of Breast Cancer: A Congener-specific Analysis. Am. J. Epidemiol., **155**(7):629 - 635.

Eltis, L. D., B. Hofmann, H. J. Hecht, H. Lunsdorf, and K. N. Timmis. 1993. Purification and crystallization of 2,3-dihydroxybiphenyl 1,2-dioxygenase. J. Biol. Chem. **268:**2727-2732.

Furukawa, K. 1982. Microbial degradation of polychlorinated biphenyls (PCBs). In: Chakrabarty A M. , editor; Chakrabarty A M. , editor. Biodegradation and detoxification of environmental pollutants. Boca Raton, Fla: CRC Press; pp. 33–56.

Furukawa K, and T. Miyazaki. 1986. Cloning of gene cluster encoding biphenyl and chlorobiphenyl degradation in *Pseudomonas pseudoalcaligenes.* J Bacteriol **166:**392-398.

Gibson, D. T., D. L. Cruden, J. D. Haddock, G. J. Zylstra, and J. M. Brand. 1993. Oxidation of polychlorinated biphenyls by *Pseudomonas* sp. strain LB400 and *Pseudomonas pseudoalcaligenes* KF707. J. Bacteriol. **175:**4561-4564.

Grasman K.A., and G.A. Fox. 2001. Associations between altered immune function and organochlorine contamination in young Caspian terns (*Sterna caspia*) from Lake Huron, 1997-1999. Ecotoxicology **10:**101-114.

Haddock J.D., and D.T. Gibson. 1995. Purification and characterization of the oxygenase component of biphenyl 2,3-dioxygenase from *Pseudomonas* sp. strain LB400. J Bacteriol. **177:**5834–5839.

Haddock J.D., J.R. Horton, and D.T. Gibson D.T. 1995. Dihydroxylation and dechlorination of chlorinated biphenyls by purified biphenyl 2,3-dioxygenase from *Pseudomonas* sp. strain LB400. J Bacteriol. **177:**20–26.

Haddock J.D., L.M. Nadim, and D.T. Gibson. 1993. Oxidation of biphenyl by a multicomponent enzyme system from *Pseudomonas* sp. strain LB400. J Bacteriol. **175:**395–400.

Haddock J.D., D.A. Pelletier, and D.T. Gibson. 1997. Purification and properties of ferredoxin$_{BPH}$, a component of biphenyl 2,3-dioxygenase of *Pseudomonas* sp. strain LB400. J Ind Microbiol Biotechnol. **19:**355–359.

Heaton S.N., S.J. Bursian, J.P. Giesy, D.E. Tillit, J.A. Render, P.D. Jones, D.A. Verbrugge, T.J. Kubiak, and R.J. Aulerich. 1995. Dietary exposure of mink to carp from Saginaw Bay, Michigan. 1. Effects on reproduction and survival, and the potential risks to wild mink populations. Arch Environ Contam Toxicol. **28:**334–343.

Hernandez B.S., J.J. Arensdorf, and D.D. Focht. 1995. Catabolic characteristics of biphenyl-utilizing isolates which cometabolize PCBs. Biodegradation. **6:**75–82.

Hirose, J., A. Suyama, S. Hayashida, and K. Furukawa. 1994. Construction of hybrid biphenyl *(bph)* and toluene *(tod)* genes for functional analysis of aromatic ring dioxygenase. *Gene* **138** 27–33.

Hooper S.W., C.A. Pettigrew, and G.S. Sayler. 1990. Ecological fate, effects and prospects for the elimination of environmental polychlorinated biphenyls (PCBs). Environ Toxicol Chem. **9:**655–667.

Hrywna, Y., T.V. Tsoi, O.V. Maltseva, J.F. Quensen, III, and J.M. Tiedje. 1999. Construction and Characterization of Two Recombinant Bacteria That Grow on *ortho-* and *para*-Substituted Chlorobiphenyls. Appl. Environ. Microbiol. **65**: 2163-2169.

Kidd, K.A., D.W. Schindler, R.A. Hesslein, and D.C.G. Muir. 1998. Effects of trophic position and lipid on organochlorine concentrations in fishes from subarctic lakes in Yukon Territory. Canadian Journal of Fisheries and Aquatic Sciences **55:**869-881.

Kimura, N., A. Nishi, M.Goto, and K. Furukawa. 1997. Functional analyses of a variety of chimeric dioxygenases constructed from two biphenyl dioxygenases that are similar structurally but different functionally. J. Bacteriol. **179**: 3936-3943.

Leitão, M.A.S., K.H.M. Cardozo, E. Pinto, and P. Colepicolo. 2003. PCB-Induced oxidative stress in the unicellular marine dinoflagellate Lingulodinium polyedrum Arch. Environ. Contam. Toxicol. **45:**59-65

Livingstone, D.R., J.K. Chipman, D.M. Lowe, C. Minier, R.K. Pipe . 2000. Development of biomarkers to detect the effects of organic pollution on aquatic invertebrates: recent molecular, genotoxic, cellular and immunological studies on the common mussel (Mytilus edulis L.) and other mytilids. Intl. J. Environ. Poll. **13:**56-91.

Masai, E., A. Yamada, J.M. Healy, T. Hatta, K. Kimbara, M. Fukuda, and K. Yano. 1995. Characterization of biphenyl catabolic genes of gram-positive polychlorinated biphenyl degrader Rhodococcus sp. strain RHA1. Appl Environ Microbiol. **61:**2079-85.

Massé R, F. Messier, L. Péloquin, C. Ayotte, and M. Sylvestre. 1984. Microbial biodegradation of 4-chlorobiphenyl, a model compound of chlorinated biphenyls. Appl Environ Microbiol. **47:**947–951.

McFarland, V.A., and J.U. Clarke. 1989. Environmental occurrence, abundance, and potential toxicity of polychlorinated biphenyl congeners: considerations for a congener-specific analysis. Environ. Health Perspect. **81:**225–239.

McKay, D. B., M. Seeger, M. Zielinski, B. Hofer, and K. N. Timmis. 1997. Heterologous expression of biphenyl dioxygenase-encoding genes from a gram-positive broad-spectrum polychlorinated biphenyl degrader and characterization of chlorobiphenyl oxidation by the gene products. J. Bacteriol. **179:**1924-1930.

Mondello, F.J. 1989. Cloning and expression in *Escherichia coli* of *Pseudomonas* strain LB400 genes encoding polychlorinated biphenyl degradation. J Bacteriol. **171:**1725–1732.

Otto, D.M.E., and T.W. Moon. 1996. Phase I and II enzymes and antioxidant responses in different tissues of brown bullheads from relatively polluted and non-polluted systems. Arch. Environ. Contam. Toxicol. **31:**141-147.

Patandin, S., P.C. Dagnelie, P.G.H. Mulder, E. Op de Coul, J.E. van der Veen, N. Weisglas-Kuperus, and P.J.J. Sauer. 1999. Dietary exposure to polychlorinated biphenyls and dioxins from infancy until adulthood: A comparison between breast-feeding, toddler, and long-term exposure. Environ. Health Persp. **107:**45-51.

Pelletier, C., P. Imbeault, and A. Tremblay. 2003. Energy balance and pollution by organochlorines and polychlorinated biphenyls. Obesity Rev. **4:**17-24

Schlezinger, J.J., and J.J. Stegeman. 2001. Induction and suppression of cytochrome P450 1A by 3,3',4,4',5- pentachlorobiphenyl and its relationship to oxidative stress in the marine fish scup (*Stenotomus chrysops*). Aquat Toxicol **52:**101-115.

Seegal, R,F,. and S. L. Schantz. 1994. Neurochemical and Behavioral Sequelae of Exposure to Dioxins and PCBs. In Dioxins and Health, A. Schecter, (ed.), Plenum Press, New York, pages 409-447.

Seeger, M., K.N. Timmis, and B. Hofer. 1995. Conversion of chlorobiphenyls into phenylhexadienoates and benzoates by the enzymes of the upper pathway for polychlorobiphenyl degradation encoded by the *bph* locus of *Pseudomonas* sp. strain LB400. Appl Environ Microbiol. **61:**2654–2658.

Smithwick, L.A., A. Smith, J.F. Quensen III, A. Stack, L. London, P.J. Morris. 2003. Inhibition of LPS-induced splenocyte proliferation by ortho-substituted polychlorinated biphenyl congeners. Toxicology **188:**319-333.

Stalker, M.J., G.B. Kirby, T.E. Kocal, I.R. Smith, and M.A.Hayes. 1991. Loss of glutathione S-transferases in pollution-associated liver neoplasms in white suckers (*Catostomus commersoni*) from Lake Ontario. Carcinogenesis **12:**2221–2226.

Swanson, G. M., H. E. Ratcliffe and L. J. Fischer. 1995. Human exposure to polychlorinated biphenyls (PCBs): A critical assessment of the evidence for adverse health effects. Regulatory Toxicology and Pharmacology **21**: 136-150.

Taira, K., J. Hirose, S. Hayashida, and K. Furukawa. 1992. Analysis of *bph* operon from the polychlorinated biphenyl-degrading strain of *Pseudomonas pseudoalcaligenes* KF707. J Biol Chem **267:**4844-4853.

van der Meer, J.R., W.M. DeVos, S. Harayama, and A.J.B. Zehnder. 1992. Molecular mechanisms of genetic adaptation of xenobiotic compounds. Microbiological Reviews, **56:**677-694.

CHAPTER 2

LITERATURE REVIEW

Microbial Biodegradation of PCBs.

The slow biodegradation of compounds in the natural environment may be caused by unfavorable physicochemical conditions (such as temperature, pH, redox potential, salinity, and oxygen concentration) or may be affected by the availablility of other nutrients, the accessibility of the substrates (solubility, dissociation from adsorbed materials), or predation. On the other hand, the low biodegradability may be due to the inability of microorganisms to effectively metabolize pollutants with uncommon man-made chemical structures or properties generally termed xenobiotics (van der Meer 1992). However, often the close structural resemblance of these compounds to nonxenobiotic analogs makes them very suitable for use in studies of metabolic adaptation. For instance, a general comparison of the major pathways for catabolism of aromatic compounds in bacteria has revealed that the initial conversion steps are carried out by different enzymes but that the compounds are transformed to a limited number of central intermediates such as protocatechuate and (substituted) catechols (van der Meer 1992; Chain et al. 2006). In the case of PCBs, microorganisms have extended their substrate range by developing peripheral enzymes, which are able to transform initial substrates into one of the central intermediates (Furukawa 1982; van der Meer 1995), making the microbial degradation of PCBs a realistic goal.

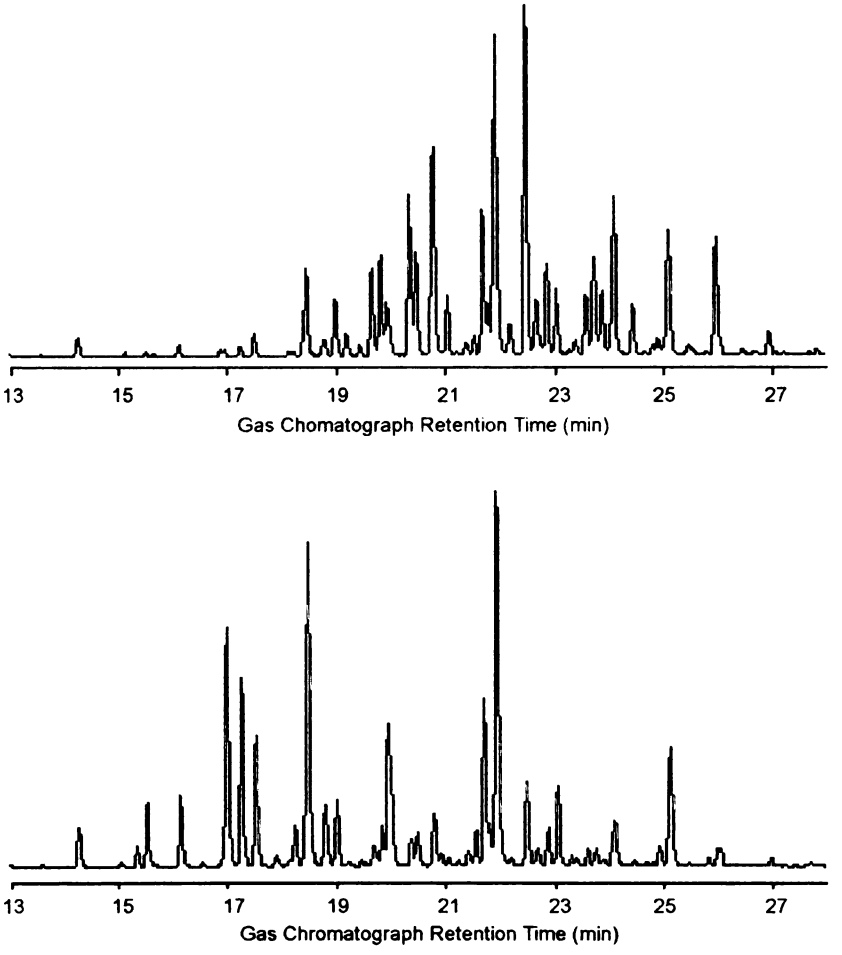


Figure 2.1. Gas chromatograms indicating reductive dechlorination of PCBs by anaerobic bacteria. Reductive dechorination removes chlorine from highly- chlorinated congeners resulting in lesser-chlorinated congeners, but does not eliminate PCBs. (from www.ct.ornl.gov/eds/rtg)

The scheme with most promise for remediation of PCB mixtures requires a sequential treatment of anaerobic reductive dechlorination followed by co-metabolism by aerobic bacteria (Abramowicz 1990). Microorganisms found in anaerobic sediments have been found to reductively dechlorinate higher chlorinated congeners (Alder, et al. 1993; Bedard, et al. 1995; Quensen, et al. 1998, 1990; Rhee et al. 1993a, 1993b, 1994; Sokol, et al. 1994; Ye, et al. 1995) accumulating congeners with fewer chlorines, usually found on *ortho* and *ortho+para* positions (Fish and Principe, 1994; Hrwyna et al. 1999). Unfortunately, reductive dechlorination does not reduce the amount of PCBs in sediments, and eventually reaches a plateau (Kim and Rhee, 1997). Anaerobic bacteria can reductively dechlorinate highly chlorinated congeners, but cannot complete the process and accumulate congeners with fewer chlorines (Figure 2.1) (Bedard, et al. 1995; Maltseva, et al. 1999; Rodrigues, et al. 2001).

The burden of PCB degradation (or destruction) lies with aerobic co-metabolism of the products of reductive dechlorination, by biphenyl- and chlorobenzoate-degrading organisms (Hrywna, et al 1999; Maltseva, et al 1999; Rodrigues, et al. 2001). Generally, aerobic PCB-degrading bacteria are only able to attack mono-, di-, tri-, and some tetrachlorobiphenyls using 2,3-dioxygenase and meta cleavage; the more chlorinated biphenyls have much lower rates of degradation, hence the importance of reductive dechlorination. Degradation of mono-, di-, and tri-chlorinated biphenyls is relatively common (Figure 2.1); only a few strains are capable of degrading PCBs with more than 4 chlorines (Bedard, et al, 1986) (Figure 2.2).

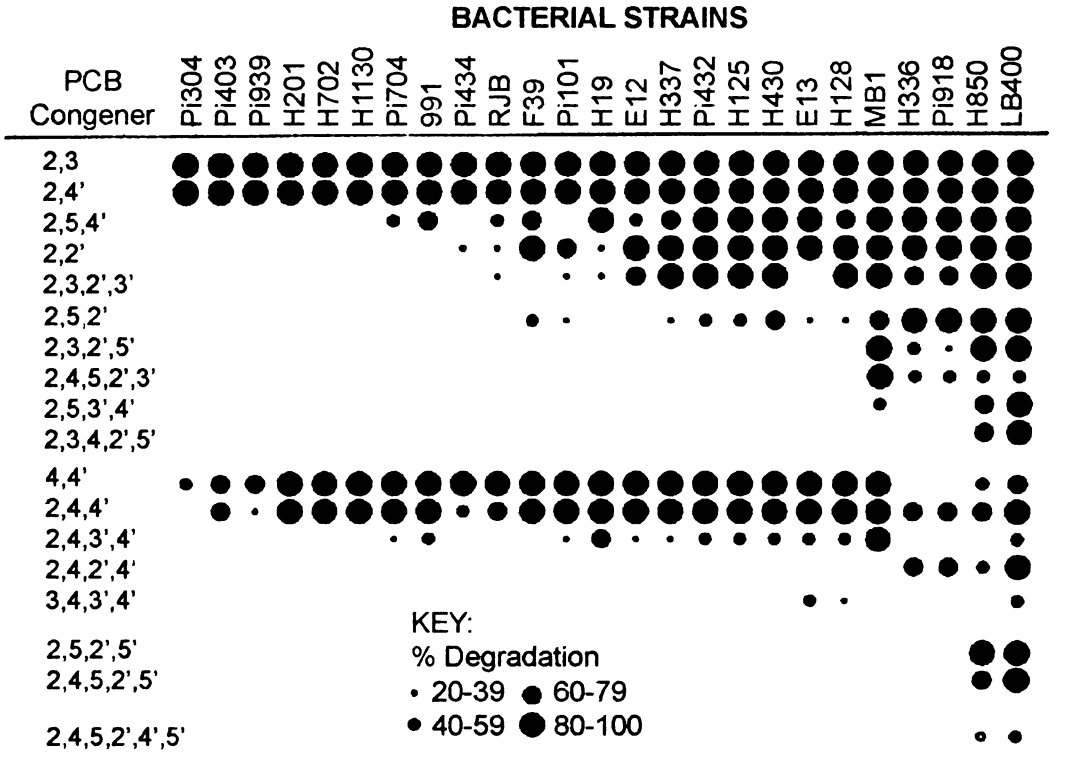


Figure 2.2. PCB-degradative competence of environmental isolates (from Bedard 1986).

Through a combination of anaerobic/aerobic phases of degradation, reductive dechlorination provides lower-chlorinated PCBs that are more susceptible to aerobic attack and consequent degradation. Recently a sequential anaerobic-aerobic process was shown in laboratory scale to remove over 50% of Aroclor 1242 from river sediments (Rodrigues, et al. 2006).

(Polychlorinated) biphenyl degradation pathway

Aerobic degradation of PCBs occurs via oxidation by pathways normally responsible for metabolism of aromatic hydrocarbons such as naphthalene (Barriault, 1998; Fortin 2005) and biphenyl (Furukawa 1982; Gibson 1993). However, PCB degradation via the biphenyl pathway has thus far proven limited due to enzyme specificity of dioxygenases and other bph-pathway enzymes—such as hydrolases—and biotoxicity of PCBs and their metabolic intermediates. The pursuit of a PCB-degrading pathway has generally been narrowed to the biphenyl pathway because of its higher activity toward PCBs (Pellizari et al., 1996).

The DNA region encoding biphenyl dioxygenase (BphA), the first enzyme in the biphenyl-polychlorinated biphenyl degradation pathway, has been sequenced and well characterized (Erickson and Mondello 1992). BphA is a Resike-type, three-component enzyme, composed of a terminal dioxygenase and an electron transfer chain (Butler and Mason 1997; Mason and Cammack 1992). The first protein of the upper biphenyl pathway in LB400 is composed of six coding sequences for orf 0, bphA (or bphA1),

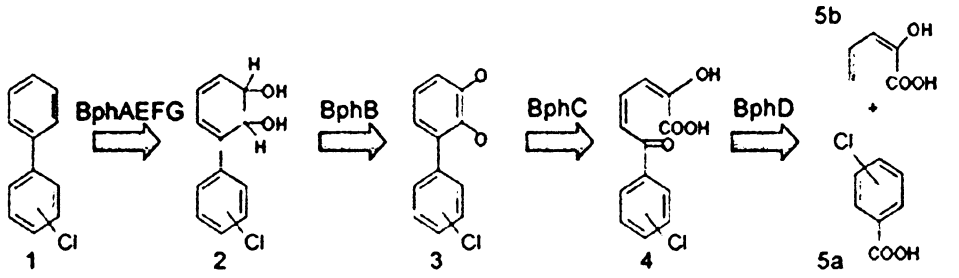


Figure 2.3. Biphenyl upper pathway encoded by the *bph* locus of *Burkholderia xenovorans* LB400. (Chloro)biphenyl (1) is converted to (chloro)biphenyl-2,3-dihydrodiol (2) by biphenyl dioxygenase (BphAEFG). (2) is converted to (chloro)2,3-dihydroxybiphenyl (3) by biphenyl-2,3-dihyddrodiol-2,3-dehydrogenase (BphB). (3) is converted to (chloro)2-hydroxy-6-oxo-6-phenylhexa-2,4-denoic acid (4) by 2,3-dihydroxybiphenyl-1,2-dioxygenase (BphC). (4) is cleaved to form 2-hydroxypenta-2,4-dienoic acid (5a) and (chloro)benzoic acid (5b) by 2-hydroxy-6-oxo-phenylhexa-2,4-dienoate hydrolase (BphD).

bphE (bphA2), orf 1, bphF (bphA3), and bphG (bphA4). The *bphA* and *bphE* genes encode encode a large and a small subunit of the terminal dioxygenase associating as an $\alpha_3\beta_3$ heterohexamer (Broadus and Haddock 1988; and Maeda, et al. 2001), *bphF* encodes ferredoxin, and *bphG* encodes ferredoxin reductase involved in electron transfer from NADH to reduce the terminal dioxygenase (Erickson, and Mondello 1992). BphA introduces molecular oxygen into the biphenyl molecule at the *ortho-* and *meta-* position to yield a 2,3-dihydro-2,3-diol (Furukawa and Miyazaki 1986; Masai, et al. 1995; Taira, et al. 1992). Following the introduction of oxygen into biphenyl by BphA, the 2,3-dihydro-2,3-diol is dehydrogenated to 2,3-dihydroxybiphenyl by dihydrodiol dehydrogenase (BphB). The second dioxygenase, 2,3-dihydroxybiphenyl dioxygenase (BphC), does not require any external reductant and cleaves the 2,3- dihydroxylated ring between carbon atoms 1 and 2 to produce 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (HOPDA, the ring *meta*-cleavage product)(Eltis 1993), which is then hydrolyzed to benzoic acid and 2-hydroxypenta-2,4-dienoate by a hydrolase (BphD).

Several studies have demonstrated limitation in the degradation of PCBs as BphA is a critical determinant in the substrate specificity for the degradation of PCB congeners (Barriault, et al. 2001; Erickson and Mondello 1993; Fortin, et al. 2005; Kimura, et al. 1997; McKay, et al. 1997, 2003; Seeger, et al. 1999; Zielinski, et al. 2002). The number of chlorines (Bedard, 1986, 1987a,b) as well as the substitution pattern on biphenyl (Arnett, 2000, Bedard 1990, Furukawa, et al. 1978) are the prevailing factors that influence the substrate specificity of BphA and consequent degradation of PCBs (Barriault, et al. 1993). The genetic difference in the biphenyl dioxygenase from environmental isolates accounts for much of the variation in their PCB degradation profiles.

The significance of substrate specificity of BphA has led to many studies targeting PCB-degrading strains for identification (Bedard 1986, Pellizari, 1997) and subsequent characterization of congeners degraded. (Bedard 1986, 1987; Bopp 1986). To date, two different modes of attack have been characterized for the BphA enzyme; a 2,3-dioxygenase attack on open 2,3 carbons via *meta*-cleavage, producing benzoate, and a 3,4-dioxygenase attack, forming a *cis*-dihydrodiol (Bedard, et al. 1987). The ability of the biphenyl dioxygenase to attack different positions results in a different degradation profile; 2,3-dioxygenase can degrade 4,4'-dichlorobiphenyl (*para*- substituted) via 2,3-dioxygenation (Masse, et al; Bedard 1986) and 3,4-dioxygenase allows degradation of 2,5,4'-trichlorobiphenyl and 2,5,2',5'-tetrachlorobiphenyl (*ortho*- and *meta*- substituted) via 3,4-dioxygenation (Bedard 1986, 1987). Several bacterial strains from the genera, *Pseudomonas, Vibrio, Aeromonas, Micrococcus, Acinetobacter, Bacillus, Rhodococcus* and *Streptomyces* can degrade mono-, di-, tri-, and some tetrachlorinated PCBs by *meta*-cleavage of unchlorinated 2,3-carbons (Bedard, et al 1986). Some strains, such as *Burkholderia xeovorans* LB400 (Bopp 1986), *Ralstonia eutropha* H850 (Bedard 1986), and *Rhodococcus globerulus* P6 (Asturias 1993) are exceptional PCB degraders capable of degrading up to hexa-chlorinated congeners (Bopp, 1986; Gibson, et al. 1993; Mondello, et al. 1997).

Recently the focus improving PCB degradation has turned to expanding the substrate specificity of BphA through genetic modification. In-depth studies of the protein structure of BphA and its interaction with substrate congeners have identified specific regions and amino acid residues key to degradation (Barriault 2004; Erickson 1993; Zielinski, 2002, 2003). With this in mind, research has shifted to exploit differences in the substrate specificity of different biphenyl dioxygenases via site-directed

mutagenesis (Erickson 1993) and directed evolution to create chimeras with improved PCB-degradation capabilities (Barriault 2001, 2002, 2004; Bruhlman 1999; Chebrou 1999; Furukawa 2004; Kimura 1997; Kumamaru 1998; Meyer 2002; Fuenaga 1999, 2001a,b).

Aside from the limitations of PCB degradation due to substrate speicificity, chlorinated metabolites produced on degradation of specific congeners can be problematic. The third critical step of PCB degradation involves a 2,3-dihydroxybiphenyl 1,2-dioxygenase that catalyzes ring cleavage (BphC) (Mondello 1989). *Ortho*-chlorinated PCB products can strongly inhibit BphC and promote its suicide inactivation effectively blocking the degradation of other compounds (Dai, et al. 2002; Vaillancourt, et al. 2002). Additionally, the following step, catalyzed by a serine hydrolase (BphD) to split the HOPDA molecule, is inhibited by a chlorine substitution on the dienoate moiety (Seah, et al. 2000, 2001). Consequently, co-metabolic oxidation of PCB often yields intermediates that can block the bph pathway and effect toxic stress to cells (Blasco et al. 1997; Maltseva et al. 1999; Dai, et al. 2002; Seah, et al. 2000; Vaillancourt, et al. 1998; Vaillancourt, et al. 2002; Hiraoka & Kimbara, 2002; Hiraoka, et al. 2002). Similar to BphA, genetic modification of downstream components of the biphenyl pathway have been attempted to alleviate toxicity posed by chlorinated metabolites of PCB degradation (Fortin, et al. 2005a,b).

Despite years of research on PCB degradation pathways, very little is known about the whole-cell physiology or genetic expression involved in PCB degradation, including stress responses induced by PCBs or their intermediates. Two of the most effective aerobic PCB degrading bacteria, *Burkholderia xenovorans* strain LB400 and *Rhodococcus* sp. RHA1, vary greatly in their PCB toxicity resistance (Seto, et al. 1996),

yet the sources of this difference are unknown. Indeed recent studies of PCB-tolerant LB400 (Denef, et al. 2004, 2005a, 2005b) suggest that cell systems other than the biphenyl pathway contribute to efficient degradation of biphenyl and of PCBs.

Biotoxicity of PCBs

Due to the lipophilic properties of PCBs, they tend to partition into the lipids of organisms, including biodegrading bacteria (Figure 2.4) (Chiou, 2002; Ramos, et al. 1997, 2002, 2004; Sikkemma, et al. 1995). The affinity of compounds such as PCBs with a high log K_{ow} value to membrane lipids causes them to accumulate in the lipid bilayer of bacteria altering fluidity and disrupting membrane function (Ramos, et al. 1997; Sikkema et al. 1995). Several studies have suggested that this membrane associated stress involved with PCBs can disrupt cellular functions including oxidative phosphorylation (Donato, et al. 1997; Kim, et al 2001; Sikkema, et al. 1995, Ramos, et al. 1997, 2004). The effects of membrane stress by PCBs could greatly influence the physiology and gene expression patterns of bacteria. Tolerance to aromatic hydrocarbons often results from different strategies employed by microorganisms including: (i) degradation of toxic compounds to benign materials; (ii) change in the composition of the membrane to prevent intrusion of toxic compounds; (iii) alteration of the extracellular surface characteristics to decrease cell permeability; (iv) active efflux of the toxic compound; and (v) removal of toxic compounds via vesicle formation (Ramos, et al. 2004).

An increase in the saturated fatty acid content is a well-documented response to environmental stresses (Hamamoto, et al. 1994; Loffeld, et al. 1996) and has been observed in *E. coli* in the presence of long-chain alcohols and aromatic compounds

(Ingram 1977). In adition, a rigidity in the cell membrane is often accomplished by rapid transformation of the fatty acid *cis*-9,10-methylene hexadecanoic acid (C17:cyclopropane) to unsaturated 9-*cis*-hexadecenoic acid (C16:1,9 *cis*) and subsequent transformation to the *trans* isomer (Ramos, et al. 2002). Kim, et al (2001) measured the effects of both a PCB congener (2,2',5,5'-tetrachlorobiphenyl) and biphenyl on the cytoplasmic membrane of *Ralstonia eutropha* H850 using fluorescence polarization. They suggest that the fatty acid profile shifts from unsaturated to saturated fatty acids on exposure to biphenyl and that the membrane modification during growth on biphenyl involves less of a fluidizing effect of PCBs on the membrane in those cells (Kim, et al. 2001). While the effect of membrane stress from compounds with properties similar to PCBs such as biphenyl (Kim, et al. 2001), chlorobenzoate (Sikkema, et al. 1995), and DDT (Donato, et al. 1997) have been investigated, there has yet been no direct analysis of the effects of PCBs themselves on membrane composition or adaptations in actively degrading cells.

Aside from the potential (local) toxicity of LB400 to PCB-degrading enzymes mentioned previously (Dai, et al. 2002; Seah, et al. 2000; Vaillancourt, et al. 1998, 2002) are global effects of potential intermediates of the biphenyl pathway. Blasco, et al (1997) found a toxic response to microorganisms growing with 4-chlorobiphenyl as a result of incomplete metabolism and accumulation of protoanemonin shown to be a toxic to microbes. Sondossi, et al (1992) also indicated the inhibition of PCB degradation due to the accumulation of dead-end chlorobenzoate metabolites, specifically 3-chlorobenzoate. In addition 2- and 4-chlorobenzoate inhibits the growth rate and induces global stress-response genes (Seeger, ND). Dihydrodiols and dihydroxybiphenyls are very toxic

metabolites that can accumulate and diminish cell viability. Camara et al (2004) demonstrated that the dihydrodiol or dihydroxybiphenyl derived from transformed 2-chlorobiphenyl damages the cell causing lysis. They also observed reduced viability from exposure to 2,3-dihydroxybiphenyl (Camara, et al. 2004). Similarly, Hiraoka et al (2002) found that the accumulation of 2- and 3- hydroxybiphenyl, produced through blocking *bphB*, inhibit bacterial cell separation during late-log growth. Additionally, hydroxylated metabolites like catechols formed within cells have shown toxic effects (Schweigert, et al. 2001). Finally, degradation of chlorinated compounds often produces a degradation-dependent stress caused by chlorinated aliphatic compounds which results in the reduction of cellular growth, viability, and respiratory activity (Alvarez-Cohen and McCarty 1991; Ensign, et al. 1992; Park, et al. 2002; Rasche, et al. 1991; van Hylckama Vlieg, et al. 1997; Yeager, et al. 2001). The build up of metabolic intermediates *in vivo* would resemble physiological conditions of bacteria in a bioremediation process.

Although some of the genes in the lower biphenyl pathway have shown degradative capability and in some cases dechlorinate the products of the upper biphenyl pathway (Hofer, et al. 1994; Kikuchi, et al. 1994), namely benzoic acid and pentadienoate, there is limited information regarding the downstream pathways responsible for degradation of chlorinated constituents from PCB degradation, while proven to be important (Seah, et al. 2000, 2001). In order to investigate the degradation intermediate-mediated toxicity, it is important to distinguish the effects membrane stress imposed by the accumulation of PCBs from co-metabolism.

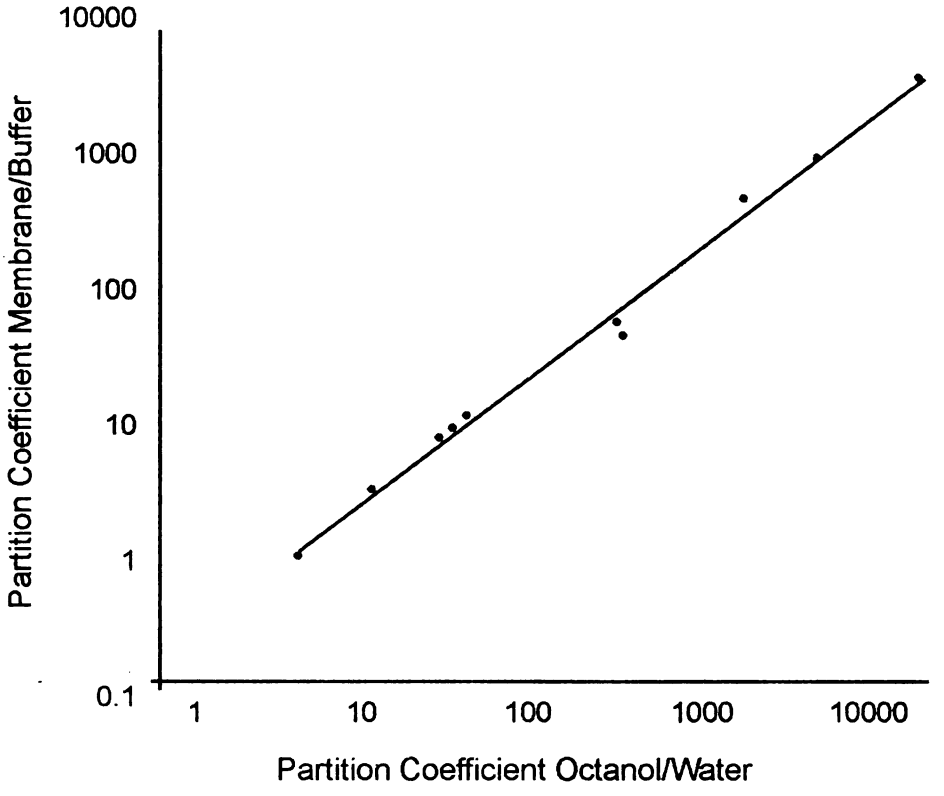


Figure 2.4. Correlation of the partition coefficient of hydrophobic compounds with their ability to partition into the cellular membrane (modified from Sikkema, et al. 1995).

Burkholderia xenovorans LB400

Thirty years ago *Burkholderia xenovorans* LB400 (Figure 2.5) was isolated from a PCB contaminated landfill in New York state (Bopp 1986; Goris, et al. 2004) and has since emerged as a model organism for aerobic degradation of PCBs. The success of LB400 as a PCB degrading microorganisms originate from the extremely wide range of congeners oxidized as well as its ability to grow on *ortho*+*meta*-congeners (Bopp 1986; Gibson, et al. 1993; Maltseva et al. 1999; Mondello et al. 1997).

Complete sequencing of the polychromosomal genome of the outstanding PCB dechlorinating and degrading *Burkholderia xenovorans* strain LB400 (http://genome.ornl.gov/microbial/bfun), and development of full-genome DNA microchips in collaboration with Xeotron (Invitrogen) Xeochip™ allow genomics-enabled investigation of the biology of chlorinated PAH degradation. The ~9.7 Mbp genome of LB400 contains 8989 predicted open reading frames (ORFs) in two chromosomes and one megaplasmid, and is the largest prokaryotic genome closed to date (Figure 2.6). The size of the LB400 genome, along with high catabolic versatility (Chain, et al 2006), makes this an ideal model for biodegradation studies.

Using genomic and proteomic techniques, Denef et al. (2005a, 2005b, 2006) investigated substrate- and phase- dependent expression of pathways involved in the degradation of PCBs in LB400. They found that genes directly involved in biphenyl degradation were up-regulated when grown on biphenyl in comparison to succinate-grown cells. In addition, three functional benzoate-catabolizing pathways: a catechol *ortho*-cleavage (*ben-cat*) pathway and two benzoyl~CoA pathways, were identified and shown to be dependent on growth substrate and growth phase. Subsequent deletion of

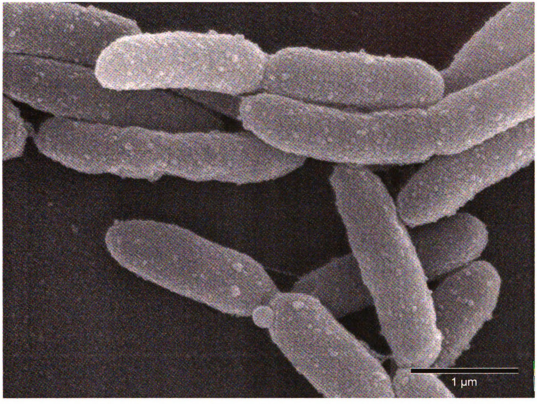


Figure 2.5. Electron micrograph of *Burkholderia xenovorans* LB400 grown on 1g/L of succinate in K1 mineral medium.

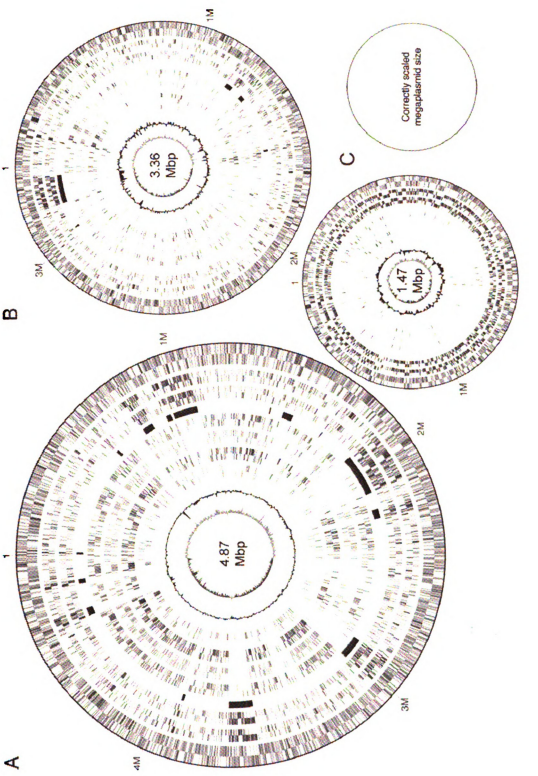


Figure 2.6. Genome of LB400 (from Chain, et al. 2006)

the ben-cat pathway demonstrated that the redundancy of the benzoate-degradation pathway does not directly contribute to PCB-degrading ability. Finally, they link the induction of the benzoyl~CoA pathway to reduced oxygen concentrations. Work on the gene expression of pathways involved in the degradation of biphenyl (and benzoate) sets the conceptual stage to study the effects of PCB degradation on LB400.

Microarray technology

Recent advances in high throughput genomic technologies have significantly expanded my abilities to explore transcriptional changes on a genomewide scale in response to natural or artificial stresses stimulus (Schena, et al. 1995, 1996; Lander, 1999; Lockhart, et al .1996; Fodor, et al. 1991; DeRisi, et al. 1997). After the completion of the genome sequence, predicted gene-coding sequences can be analyzed for the relative changes in mRNA of thousands of genes simultaneously (referred to as genome expression pattern) to environmental changes. The potential for this technology clearly extends to the degradation of xenobiotic compounds during bioremediation (Beliaev, et al. 2002; Denef, et al. 2004, 2005, 2006). To date, over a thousand studies have reported the expression patterns of dozens of whole genomes of various microorganisms (Wodicka, et al. 1997; Cho, et al. 1998; Hecker and Engelmann 2000; Hinchliffe, et al. 2003).

Each genome microarray is capable of producing thousands to tens of thousands of data points describing the transcriptional expression patterns due to variations in environmental conditions. The current challenge is to extract biologically relevant information from an abundance of gene expression data. Cluster analysis is commonly used since genes that have similar expression profiles over various conditions or

experiments group together reducing the complexity of data and indicating prominent patterns in the data (Autio, et al. 2003; Eisen, et al. 1998; Laws, et al. 2003; Sherlock 2000; Xia and Xie 2001; Zhao, et al. 2001). One of the most widely-used clustering techniques is the agglomerative, or bottom-up approach whereby single expression profiles are joined to form nodes, which are successively joined until all individual profiles and nodes have been joined to form a single hierarchical tree (Eisen, et al. 1998; Sherlock 2000). The advantage of this approach is that it is simple, and the end result can be easily visualized, from which coordinately regulated patterns can be relatively easily discerned by eye (Sherlock 2000).

Summary

The biodegradation of PCBs by aerobic bacteria is the keystone to *in situ* microbial remediation. To this point, the biphenyl pathway, responsible for the degradation of PCBs, has been extensively studied. However, a fundamental factor in successful PCB degradation, the tolerance and global changes involved, have been underrepresented. In order to achieve the highest leves of degradation possible, the biodegrading bacteria must be able to cope with conditions involved in PCB degradation including the effect of PCBs themselves on bacteria as well as the escalation of deleterious metabolites during degradation. By using the groundwork previously established by Chain, et al. (2006) and Denef, et al. (2004, 2005a, 2005b, 2006) involving sequencing of *Burkholderia xenovorans* LB400 genome and initial microarray optimization, I analyze the expression patterns involved in PCB degradation in conjunction with physiological changes.

REFERENCES

Abramowicz, D. A. 1990. Aerobic and anaerobic biodegradation of PCBs: a review. Crit. Rev. Biotechnol. **10:**241-248.

Abramowicz, D.A., M.J. Brennan, H.M. Van Dort, and F.L. Gallagher. 1993. Factors influencing the rate of polychlorinated biphenyl degradation in Hudson River sediments. Environ. Sci. Technol. **27:**1125–1131

Agullo, L., B. Camara, M. Hernandez, V. Latorre, and M. Seeger. 2006. Response to (chloro)biphenyls of the polychlorobiphenyl-degrader *Burkholderia xenovorans* LB400 involves stress proteins also induced by heat shock and oxidative stress.. FEMS Microbiol. Lett. Epub 2006 Dec. 8.

Ahmed M., and D.D. Focht. 1973. Degradation of polychlorinated biphenyls by two species of *Achromobacter*. Can J Microbiol. **19:**47–52.

Alder A.C., M.M. Haggblom, S.R. Oppenheimer, and L.Y. Young. 1993. Reductive dechlorination of polychlorinated biphenyls in anaerobic sediments. Envrion. Sci. and Technol. **27:**530-538

Alvarez-Cohen, L., and P. L. McCarty. 1991. Effects of toxicity, aeration, and reductant supply on trichloroethylene transformation by a mixed methanotrophic culture. Appl. Environ. Microbiol. **57:**228-235.

Arnett, C. M., J. V. Parales, and J. D. Haddock. 2000. Influence of chlorine substituents on rates of oxidation of chlorinated biphenyls by the BPDO of *Burkholderia* sp. strain LB400. Appl. Environ. Microbiol. **66:**2928-2933.

Asturias, J.A., and K.N. Timmis. 1993. Three different 2,3-dihydroxybiphenyl-1,2-dioxygenase genes in the gram-positive polychlorobiphenyl-degrading bacterium Rhodococcus globerulus P6. J Bacteriol. **175:**4631-40.

Barriault, D., J. Durand, H. Maaroufi, L.D. Eltis, and M. Sylvestre. 1998. Degradation of polychlorinated biphenyl metabolites by naphthalene-catabolizing enzymes. Appl. Environ. Microbiol. **64:**4637-4642.

Barriault, D., M-M. Plante, and M. Sylvestre. 2002. Family shuffling of a targeted *bphA* region to engineer biphenyl dioxygenase. J Bacteriol. **184:**3794-3800

Barriault, D., and M. Sylvestre. 1993. Factors affecting PCB degradation by an implanted bacterial strain in soil microcosms. Can J. Microbiol. **39:**594-602.

Barriault, D., C. Simard, H. Chatel, and, M. Sylvestre. 2001. Characterization of hybrid biphenyl dioxygenases obtained by recombining *Burkholderia* sp. strain

LB400 *bphA* with the homologous gene of *Comamonas testosteroni* strain B-356. Can. J. Microbiol. **47:**1025-1032.

Barriault, D., M. Vedadi, J. Powlowski, and M. Sylvestre. 1999. *cis*-2,3-Dihydro-2,3-dihydroxybiphenyl dehydrogenase and *cis*-1,2-dihydro-1,2-dihydroxynaphthalene dehydrogenase catalyze dehydrogenation of the same range of substrates. Biochem. Biophys. Res. Commun. **260:**181-187.

Barriault, D., F. Lepine, M. Mohammadi, S. Milot, N. Leberre, and M. Sylvestre. 2004. Revisiting the regiospecificity of *Burkholderia xenovorans* LB400 biphenyl dioxygenase toward 2,2'-dichlorobiphenyl and 2,3,2',3'-tetrachlorobiphenyl. J. Biol. Chem. **279:**47489-47496.

Barriault, D., and M. Sylvestre. 2004. Evolution of the biphenyl dioxygenase BphA from *Burkholderia xenovorans* LB400 by random mutagenesis of multiple sites in region III. J. Biol. Chem. **279:**47480-47488.

Beckering, C.L., L. Steil, M.H.W. Weber, U. Völker, and M.A. Marahiel. 2002. Genomewide Transcriptional Analysis of the Cold Shock Response in *Bacillus subtilis*. J Bacteriol. **184:**6395–6402.

Bedard, D. L., and J. F. Quensen, III. 1995. Microbial reductive dechlorination of polychlorinated biphenyls, p. 127-216. *In* L. Y. Young, and C. Cerniglia (ed.), Microbial transformation and degradation of toxic organic chemicals. Wiley-Liss Division, John Wiley & Sons, Inc., New York, N.Y.

Bedard, D.L., and M.L. Haberl. 1990. Influence of chlorine substitution pattern on the degradation of polychlorinated biphenyls by eight bacterial strains. Microb. Ecol. **20:**87–102.

Bedard, D.L., M.L. Haberl, R.J. May, and M.J. Brennan. 1987. Evidence for novel mechanisms of polychlorinated biphenyl metabolism in Alcaligenes eutrophus H850. Appl. Environ. Microbiol. **53:**1103–1112.

Bedard, D.L., R. Unterman, L.H. Bopp, M.J. Brennan, M.L. Haberl, and C. Johnson. 1986. Rapid assay for screening and characterizing microorganisms for the ability to degrade polychlorinated biphenyls. Appl. Environ. Microbiol. **51:**761–768.

Bedard, D.L., R.E. Wagner, M.J. Brennan, M.L. Haberl, and J.F. Brown Jr. 1987. Extensive degradation of Aroclors and environmentally transformed polychlorinated biphenyls by Alcaligenes eutrophus H850. Appl. Environ. Microbiol. **53:**1094–1102

Blasco, R., M. Mallavarapu, R-M. Wittich, K.N. Timmis, and D.H. Pieper. 1997. Evidence that formation of protoanemonin from metabolites of 4-

chlorobiphenyl degradation negatively affects the survival of 4-chlorobiphenyl-cometabolizing microorganisms. Appl. Environ. Microbio. **63**:427-434.

Bopp L H. Degradation of highly chlorinated PCBs by *Pseudomonas* strain LB400. J Ind Microbiol 1986;**1**:23–29.

Brenner, V., J.J. Arensdorf, and D.D. Focht. 1994. Genetic construction of PCB degraders. Biodegradation **5**:359–377

Brevik, K., A. Sweetman, J. Pacyna, and K. Jones. 2002. Towards a global historical emission inventory for selected PCB congeners–A mass balance approach. The Science of the Total Environment. **290:**181-198.

Broadus R.M., and J.D. Haddock. 1998. Purification and characterization of the NADH:ferredoxin$_{BPH}$ oxidoreductase component of biphenyl 2,3-dioxygenase from *Pseudomonas* sp. strain LB400. Arch. Microbiol. **170:**106–112.

Brown, J.F. Jr, D.L. Bedard, M.J. Brennan, J.C. Carnahan, H. Feng, and R.E.Wagner. 1987. Polychlorinated biphenyl dechlorination in aquatic sediments. Science **236:**709–712

Brown, J.F. Jr, R.E. Wagner, H. Feng, D.L. Bedard, M.J. Brennan, J.C. Carnahan, and R.J. May. 1987. Environmental dechlorination of PCBs. Environ. Toxicol. Chem. **5:**579–593

Brühlmann, F., and W. Chen. 1999. Tuning biphenyl dioxygenase for extended substrate specificity. Biotechnol. Bioeng. **63:**544-551.

Butler C.S., and J.R. Mason. 1996. Structure-function analysis of the bacterial aromatic ring hydroxylating dioxygenases Adv. Microb. Physiol. **38:**47-84.

Camara, B., C. Herrera, M. Gonzalez, E. Couve, B. Hofer, and M. Seeger. 2004. From PCBs to highly toxic metabolites by the biphenyl pathway. Environ. Microb. 6:842-850.

Chain, P.S.G., V.J. Denef, K. Konstantinidis, L.M. Vergez, L. Agulló, V.L. Reyes, L. Hauser, M. Córdova, L. Gómez, M. González, M. Land, V. Lao, F. Larimer, J.J. LiPuma, E. Mahenthiralingam, S.A. Malfatti, C.J. Marx, J.J. Parnell, A. Ramette, P. Richardson, M. Seeger, D. Smith, T. Spilker, W-J. Sul, T.V. Tsoi, L.E. Ulrich, I.B. Zhulin, J.M. Tiedje. 2006. *Burkholderia xenovorans* LB400 harbors a multi-replicon, 9.7 M bp genome shaped for versatility. Proc. Natl. Acad. Sci. (Submitted).

Chebrou, H., Y. Hurtubise, D. Barriault, and M. Sylvestre. 1999. Heterologous expression and characterization of the purified oxygenase component of *Rhodococcus globerulus* P6 biphenyl dioxygenase and of chimeras derived from it. J. Bacteriol. **181:**4805-4811.

Chiou, C.T., 2002, Partition and adsorption of organic contaminants in environmental systems: New York, John Wiley & Sons

Dai, S., F.H.Vaillancourt, H. Maaroufi, N.M. Drouin, D.B. Neau, V. Snieckus, J.T. Bolin, and L.D. Eltis. 2002. Identification and analysis of a bottleneck in PCB biodegradation. Nat. Struct. Biol. **9:**934-939.

Demers, A. P. Ayotte, J. Brisson, S. Dodin, J. Robert, and E. Dewailly. 2002. Plasma Concentrations of Polychlorinated Biphenyls and the Risk of Breast Cancer: A Congener-specific Analysis. Am. J. Epidemiol., **155**(7):629 - 635.

Denef, V.J., J. Park, J.L. Rodrigues, T.V. Tsoi, S.A. Hashsham and J.M. Tiedje. 2003. Validation of a more sensitive method for using spotted oligonucleotide DNA microarrays for functional genomics studies on bacterial communities. Environ. Microbiol. **5**(10):933-43.

Denef, V.J., J. Park, T.V. Tsoi, J.-M. Rouillard, H. Zhang, J.A. Wibbenmeyer, W. Verstraete, E. Gulari, S.A. Hashsham, and J.M. Tiedje. 2004. Biphenyl and benzoate metabolism in a genomic context: Outlining genome-wide metabolic networks in *Burkholderia xenovorans* LB400. Appl. Environ. Microbiol. **70:**4961-4970.

Denef, V.J. M.A. Patrauchan, C. Florizone, J. Park, T.V. Tsoi, W. Verstraete, J.M.Tiedje, and L.D. Eltis. 2005. Growth substrate- and phase-specific expression of biphenyl, benzoate, and C_1 metabolic pathways in *Burkholderia xenovorans* LB400. J. Bacteriol. **187:**7996-8005.

Denef, V.J. J.A. Klappenbach, M.A. Patrauchan, C. Florizone, J.L.M. Rodrigues, T.V. Tsoi, W. Verstraete, L.D. Eltis, and J.M. Tiedje. 2006. Genetic and genomic insights into the role of benzoate-catabolic pathway redundancy in *Burkholderia xenovorans* LB400. Appl. Environ. Microbiol. **72:** 585-595.

DeRisi J.L., V.R. Iyer, and P.O. Brown. 1997 Exploring the metabolic and genetic control of gene expression on a genomic scale. Science. **278**:680–686.

Donato MM, Jurado AS, Antunes-Madeira MC, Madeira VM. 1997. Comparative study of the toxic actions of 2,2-bis(p-chlorophenyl)-1,1,1-trichloroethane and 2,2-bis(p-chlorophenyl)-1,1-dichloroethylene on the growth and respiratory activity of a microorganism used as a model. Appl. Environ. Microbiol. **63**:4948-51.

Eltis, L. D., B. Hofmann, H. J. Hecht, H. Lunsdorf, and K. N. Timmis. 1993. Purification and crystallization of 2,3-dihydroxybiphenyl 1,2-dioxygenase. J. Biol. Chem. **268:**2727-2732.

Ensign, S. A., M. R. Hyman, and D. J. Arp. 1992. Cometabolic degradation of chlorinated alkenes by alkene monooxygenase in a propylene-grown *Xanthobacter* strain. Appl. Environ. Microbiol. **58:**3038-3046.

Erickson, B. D., and F. J. Mondello. 1993. Enhanced biodegradation of polychlorinated biphenyls after site-directed mutagenesis of a biphenyl dioxygenase gene. Appl. Environ. Microbiol. **59:**3858-3862.

Fish, K.M., and J.M. Principe. 1994. Biotransformations of Aroclor 1242 in Hudson River test tube microcosms. Appl. Environ. Microbiol. **60:**4289-4296

Fodor, S.P.A., J.L. Read, M.C. Pirrung, L. Stryer, A.T. Lu, and D. Solas. 1991. Light-directed, spatially addressable parallel chemical synthesis. Science. **251**:767–773.

Fortin, P.D., I. MacPherson, D.B. Neau, J.T. Bolin, and L.D. Eltis. 2005. Directed evolution of a ring-cleaving dioxygenase for polychlorinated biphenyl degradation. J. Biol. Chem. **280:**42307-42314

Fortin, P.D., A.T.-F. Lo, M-A Haro, S. R. Kaschabek, W. Reineke, and L.D. Eltis. 2005. Evolutionarily divergent extradiol dioxygenases possess higher specificities for polychlorinated biphenyl metabolites. J. Bacteriol. **187:**415-421.

Furukawa, K., H. Suenaga, and M. Goto. 2004. Biphenyl dioxygenases: functional versatilities and directed evolution. J. Bacteriol. **186:**5189-5196.

Furukawa, K. 1982. Microbial degradation of polychlorinated biphenyls (PCBs). In: Chakrabarty A M. , editor; Chakrabarty A M. , editor. Biodegradation and detoxification of environmental pollutants. Boca Raton, Fla: CRC Press; pp. 33–56.

Furukawa K., K. Tonomura, and A. Kamibayashi. 1978. Effect of chlorine substitution on the biodegradability of polychlorinated biphenyls. Appl Environ Microbiol. **35:**223–227.

Furukawa K., and T. Miyazaki. 1986. Cloning of gene cluster encoding biphenyl and chlorobiphenyl degradation in *Pseudomonas pseudoalcaligenes.* J Bacteriol **166:**392-398.

Furukawa, K., and A.M. Chakrabarty. 1982. Involvement of plasmids in total degradation of chlorinated biphenyls. Appl. Environ. Microbiol. **44:**619-626

Gibson, D. T., D. L. Cruden, J. D. Haddock, G. J. Zylstra, and J. M. Brand. 1993. Oxidation of polychlorinated biphenyls by *Pseudomonas* sp. strain LB400 and *Pseudomonas pseudoalcaligenes* KF707. J. Bacteriol. **175:**4561-4564.

Grasman K.A., and G.A. Fox. 2001. Associations between altered immune function and organochlorine contamination in young Caspian terns (*Sterna caspia*) from Lake Huron, 1997-1999. Ecotoxicology **10:**101-114.

Haddock J.D., and D.T. Gibson. 1995. Purification and characterization of the oxygenase component of biphenyl 2,3-dioxygenase from *Pseudomonas* sp. strain LB400. J Bacteriol. **177:**5834–5839.

Haddock J.D., J.R. Horton, and D.T. Gibson D.T. 1995. Dihydroxylation and dechlorination of chlorinated biphenyls by purified biphenyl 2,3-dioxygenase from *Pseudomonas* sp. strain LB400. J Bacteriol. **177:**20–26.

Haddock J.D., L.M. Nadim, and D.T. Gibson. 1993. Oxidation of biphenyl by a multicomponent enzyme system from *Pseudomonas* sp. strain LB400. J Bacteriol. **175:**395–400.

Haddock J.D., D.A. Pelletier, and D.T. Gibson. 1997. Purification and properties of ferredoxin$_{BPH}$, a component of biphenyl 2,3-dioxygenase of *Pseudomonas* sp. strain LB400. J Ind Microbiol Biotechnol. **19:**355–359.

Hamamoto, T., N. Takata, T. Kudo, and K. Horikoshi K. 1994. Effect of temperature and growth phase on fatty acid composition of the psychrophilic Vibrio sp. strain no. 5710. FEMS Microbiol. Lett. **119:**77– 81.

Heaton S.N., S.J. Bursian, J.P. Giesy, D.E. Tillit, J.A. Render, P.D. Jones, D.A. Verbrugge, T.J. Kubiak, and R.J. Aulerich. 1995. Dietary exposure of mink to carp from Saginaw Bay, Michigan. 1. Effects on reproduction and survival, and the potential risks to wild mink populations. Arch Environ Contam Toxicol. **28:**334–343.

Hernandez B.S., J.J. Arensdorf, and D.D. Focht. 1995. Catabolic characteristics of biphenyl-utilizing isolates which cometabolize PCBs. Biodegradation. **6:**75–82.

Hiraoka, Y., Yamada, T., Tone, K., Futaesaku, Y., and Kimbara, K. 2002. Flow cytometry analysis of changes in the DNA content of the polychlorinated biphenyl degrader *Comamonas testosteroni* TK102: Effect of metabolites on cell-cell separation. Appl Environ Microbiol **68**: 5104-5112.

Hirose, J., A. Suyama, S. Hayashida, and K. Furukawa. 1994. Construction of hybrid biphenyl *(bph)* and toluene *(tod)* genes for functional analysis of aromatic ring dioxygenase. *Gene* **138** 27–33.

Hofer, B., S. Backhaus, and K. N. Timmis. 1994. The biphenyl/polychlorinated biphenyl-degradation locus (*bph*) of *Pseudomonas* sp. LB400 encodes four additional metabolic enzymes. Gene **144:**9-16.

Hooper S.W., C.A. Pettigrew, and G.S. Sayler. 1990. Ecological fate, effects and prospects for the elimination of environmental polychlorinated biphenyls (PCBs). Environ Toxicol Chem. **9:**655–667.

Hrywna, Y., T.V. Tsoi, O.V. Maltseva, J.F. Quensen, III, and J.M. Tiedje. 1999. Construction and Characterization of Two Recombinant Bacteria That Grow on *ortho-* and *para*-Substituted Chlorobiphenyls. Appl. Environ. Microbiol. **65**: 2163-2169.

Ingram, L.O. 1977. Changes in lipid composition of Escherichia coli resulting from growth with organic solvents and with food additives. Appl. Environ. Microbiol. **33:**1233– 36.

Kidd, K.A., D.W. Schindler, R.A. Hesslein, and D.C.G. Muir. 1998. Effects of trophic position and lipid on organochlorine concentrations in fishes from subarctic lakes in Yukon Territory. Canadian Journal of Fisheries and Aquatic Sciences **55:**869-881.

Kimura, N., A. Nishi, M.Goto, and K. Furukawa. 1997. Functional analyses of a variety of chimeric dioxygenases constructed from two biphenyl dioxygenases that are similar structurally but different functionally. J. Bacteriol. **179**: 3936-3943.

Kim, J., and G. Rhee. 1997 Population Dynamics of Polychlorinated Biphenyl-Dechlorinating Microorganisms in Contaminated Sediments. Appl. Environ. Microbiol. **63:**1771-1776.

Kikuchi, Y., Y. Yasukoshi, Y. Nagata, M. Fukuda, and M. Takagi. 1994. Nucleotide sequence and functional analysis of the meta-cleavage pathway involved in biphenyl and polychlorinated biphenyl degradation in *Psudomonas* sp. strain KKS102. J. Bacteriol. **176:**4269-4276.

Kitagawa, W., K. Miyauchi, E. Masai, and M. Fukuda. 2001. Cloning and Characterization of Benzoate Catabolic Genes in the Gram-Positive Polychlorinated Biphenyl Degrader *Rhodococcus* sp. Strain RHA1. J. Bacteriol. **183**: 6598-6606.

Kumamaru, T., H. Suenaga, M. Mitsuoka, T. Watanabe, and K. Furukawa. 1998. Enhanced degradation of polychlorinated biphenyls by directed evolution of biphenyl dioxygenase. Nat. Biotechnol. **16:**663-666.

Lander, E.S. 1999. Array of hope. Nat. Genet. Suppl. **21**:3–4.

Leitão, M.A.S., K.H.M. Cardozo, E. Pinto, and P. Colepicolo. 2003. PCB-Induced oxidative stress in the unicellular marine dinoflagellate Lingulodinium polyedrum Arch. Environ. Contam. Toxicol. **45:**59-65

Livingstone, D.R., J.K. Chipman, D.M. Lowe, C. Minier, R.K. Pipe . 2000. Development of biomarkers to detect the effects of organic pollution on aquatic invertebrates: recent molecular, genotoxic, cellular and immunological studies on the common mussel (Mytilus edulis L.) and other mytilids. Intl. J. Environ. Poll. **13:**56-91.

Loffeld, B., and H. Keweloh. 1996. Cis/trans isomerization of unsaturated fatty acids as possible control mechanism of membrane fluidity in *Pseudomonas putida* P8. Lipids **31:**811– 15

Maltseva, O.V., T.V. Tsoi, J.F. Quensen III, M. Fukuda, and J.M. Tiedje. 1999. Degradation of anaerobic reductive dechlorination products of Aroclor 1242 by four aerobic bacteria. Biodegradation. **10:**363-371.

Masai, E., A. Yamada, J.M. Healy, T. Hatta, K. Kimbara, M. Fukuda, and K. Yano. 1995. Characterization of biphenyl catabolic genes of gram-positive polychlorinated biphenyl degrader Rhodococcus sp. strain RHA1. Appl Environ Microbiol. **61:**2079-85.

Massé R, F. Messier, L. Péloquin, C. Ayotte, and M. Sylvestre. 1984. Microbial biodegradation of 4-chlorobiphenyl, a model compound of chlorinated biphenyls. Appl Environ Microbiol. **47:**947–951.

McFarland, V.A., and J.U. Clarke. 1989. Environmental occurrence, abundance, and potential toxicity of polychlorinated biphenyl congeners: considerations for a congener-specific analysis. Environ. Health Perspect. **81:**225–239.

McKay, D. B., M. Seeger, M. Zielinski, B. Hofer, and K. N. Timmis. 1997. Heterologous expression of biphenyl dioxygenase-encoding genes from a gram-positive broad-spectrum polychlorinated biphenyl degrader and characterization of chlorobiphenyl oxidation by the gene products. J. Bacteriol. **179:**1924-1930.

Meyer, A., A. Schmid, M. Held, A. H. Westphal, M. Rothlisberger, H. P. E. Kohler, W. J. H. van Berkel, and B. Witholt. 2002. Changing the substrate reactivity of 2-hydroxybiphenyl 3-monooxygenase from *Pseudomonas azelaica* HBP1 by directed evolution. J. Biol. Chem. **277:**5575-5582.

Mondello, F.J. 1989. Cloning and expression in *Escherichia coli* of *Pseudomonas* strain LB400 genes encoding polychlorinated biphenyl degradation. J Bacteriol. **171:**1725–1732.

Mondello, F.J., M.P. Turcich, J.H. Lobos, and B.D. Erickson. 1997. Identification and modification of biphenyl dioxygenase sequences that determine the specificity of polychlorinated biphenyl degradation. Appl. Environ. Microbiol. **63**: 3096-3103.

Oh, M.-K., L. Rohlin, K. C. Kao, and J. C. Liao. 2002. Global Expression Profiling of Acetate-grown Escherichia coli. J. Biol. Chem. **277:**13175 - 13183.

Otto, D.M.E., and T.W. Moon. 1996. Phase I and II enzymes and antioxidant responses in different tissues of brown bullheads from relatively polluted and non-polluted systems. Arch. Environ. Contam. Toxicol. **31:**141-147.

Park, J., Y.-M. Chen, J. J. Kukor, and L. M. Abriola. 2002. Influence of substrate exposure history on biodegradation in a porous medium. J. Contam. Hydrol. **51:**233-256.

Patandin, S., P.C. Dagnelie, P.G.H. Mulder, E. Op de Coul, J.E. van der Veen, N. Weisglas-Kuperus, and P.J.J. Sauer. 1999. Dietary exposure to polychlorinated biphenyls and dioxins from infancy until adulthood: A comparison between breast-feeding, toddler, and long-term exposure. Environ. Health Persp. **107:**45-51.

Pelletier, C., P. Imbeault, and A. Tremblay. 2003. Energy balance and pollution by organochlorines and polychlorinated biphenyls. Obesity Rev. **4:**17-24

Phadtare, S., Kato, I. and Inouye, M. 2002. DNA microarray analysis of the expression profile of *Escherichia coli* in response to treatment with 4,5-Dihydroxy-2-Cyclopenten-1-One. J. Bacteriol. **84:**6725-6729.

Polen, T., D. Rittmann, V. F. Wendisch, and H. Sahm. 2003. DNA microarray analyses of the long-term adaptive response of *Escherichia coli* to acetate and propionate. Appl. Environ. Microbiol. **69:**1759-1774.

Quackenbush J. 2001. Computational analysis of microarray data. Nat Rev Genet **2:**418–427.

Quensen, J.F., III, J.M. Tiedje and S.A. Boyd. 1988. Reductive dechlorination of polychlorinated biphenyls by anaerobic microorganisms from sediment. Science **242:**752-754.

Quensen, J.F., III, S.A. Boyd and J.M. Tiedje. 1990. Dechlorination of four commercial polychlorinated biphenyl mixtures (Aroclors) by anaerobic microorganisms from sediments. Appl. Environ. Microbiol. **56:**2360-2369.

Ramos, J. L., E. Duque, J-J. Rodriguez-Herva, P. Godoy, P., A. Haidour, F. Reyes, and A. Fernandez-Barrero. 1997. Mechanisms for solvent tolerance in bacteria. J. Biol. Chem. **272**:3887-3890.

Ramos, J.L., E. Duque, M-T Gallegos, P. Godoy, M.I. Ramos-Gonzalez, A. Rojas, W. Teran, and A. Segura. 2002. Mechanisms of solvent tolerance in Gram negative bacteria. Ann. Rev. Microbiol. **56**:743-768.

Rasche, M. E., M. R. Hyman, and D. J. Arp. 1991. Factors limiting aliphatic chlorocarbon degradation by *Nitrosomonas europaea*: cometabolic inactivation of ammonia monooxygenase and substrate specificity. Appl. Environ. Microbiol. **57:**2986-2994.

Rhee, G.Y., R.C. Sokol, B. Bush, and C.M. Bethoney. 1993. Long-term study of the anaerobic dechlorination of Aroclor 1254 with and without biphenyl enrichment. Environ. Sci. Technol. **27:**714-719.

Rhee, G.Y., R.C. Sokol, C.M. Bethoney, and B. Bush. 1993. A long-term study of anaerobic dechlorination of PCB congeners by sediment microorganisms: pathways and mass balance. Environ. toxicol. chem. **12:**1829-1834.

Rodrigues, J.L.M., O.V. Maltseva, T.V. Tsoi, R.R. Helton, J.F. Quensen, III, M. Fukuda and J.M. Tiedje. 2001. Development of a *Rhodococcus* recombinant strain for degradation of products from anaerobic dechlorination of PCBs. Environ Sci and Technol **35:**663-668.

Rodrigues, J.L.M., C.A. Kachel, M.R. Aiello, J.F. Quensen, O.V. Maltseva, T.V. Tsoi, and J.M. Tiedje. 2006. Degradation of Aroclor 1242 dechlorination products in sediments by *Burkholderia xenovorans* LB400(*ohb*) and *Rhodococcus* sp. strain RHA1(*fcb*). Appl Environ Microbiol **72:**2476-2482

Schena, M., D. Shalon, R.W. Davis, and P.O. Brown. 1995. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science. **270**:467–470.

Schena, M., D. Shalon, R. Heller, A. Chai, P.O. Brown, and R.W. Davis. 1996. Parallel human genome analysis: microarray-based expression monitoring of 1000 genes. Proc Natl Acad Sci **93**:10614–10619.

Schlezinger, J.J., and J.J. Stegeman. 2001. Induction and suppression of cytochrome P450 1A by 3,3′,4,4′,5- pentachlorobiphenyl and its relationship to oxidative stress in the marine fish scup (*Stenotomus chrysops*). Aquat Toxicol **52:**101-115.

Seah, S. Y. K., G. Labbe, S. R. Kaschabek, F. Reifenrath, W. Reineke, and L. D. Eltis. 2001. Comparative specificities of two evolutionarily divergent hydrolases involved in microbial degradation of polychlorinated biphenyls. J. Bacteriol. **183:**1511-1516

Seah, S. Y. K., G. Labbe, S. Nerdinger, M. R. Johnson, V. Snieckus, and L. D. Eltis. 2000. Identification of a serine hydrolase as a key determinant in the microbial degradation of polychlorinated biphenyls. J. Biol. Chem. **275:**15701-15708

Seegal, R,F,. and S. L. Schantz. 1994. Neurochemical and Behavioral Sequelae of Exposure to Dioxins and PCBs. In Dioxins and Health, A. Schecter, (ed.), Plenum Press, New York, pages 409-447.

Seeger, M., K.N. Timmis, and B. Hofer. 1995. Conversion of chlorobiphenyls into phenylhexadienoates and benzoates by the enzymes of the upper pathway for polychlorobiphenyl degradation encoded by the *bph* locus of *Pseudomonas* sp. strain LB400. Appl Environ Microbiol. **61:**2654–2658.

Seeger, M., K.N. Timmis, and B. Hofer. 1995. Degradation of chlorobiphenyls catalyzed by the *bph*-encoded biphenyl-2,3-dioxygenase and biphenyl-2,3-dihydrodiol-2,3-dehydrogenase of *Pseudomonas* sp. LB400. FEMS Microbiol Lett. **133:**259–264.

Seeger, M., M. Zielinski, K.N. Timmis, and B. Hofer. 1999. Regiospecificity of dioxygenation of di- to pentachlorobiphenyls and their degradation to chlorobenzoates by the *bph*-encoded catabolic pathway of *Burkholderia* sp. strain LB400. Appl Environ Microbiol. **65:**3614–3621.

Sherlock, G. 2000. Analysis of large-scale gene expression data. Curr Opin Immunol. **12**:201–205.

Sikkema, J., J. A. M. de Bont, and B. Poolman. 1995. Mechanisms of membrane toxicity of hydrocarbons. Microbiol. Rev. **59:**201-222.

Smithwick, L.A., A. Smith, J.F. Quensen III, A. Stack, L. London, P.J. Morris. 2003. Inhibition of LPS-induced splenocyte proliferation by ortho-substituted polychlorinated biphenyl congeners. Toxicology **188:**319-333.

Sondossi M, Sylvestre M, Ahmad D. 1992. Effects of chlorobenzoate transformation on the *Pseudomonas tesosteroni* biphenyl and chlorobiphenyl degradation pathway. Appl Environ Microbiol 1992;**58**:485–495.

Sokol, R.C., O-S Kwon, C.M. Bethoney, and G-Y. Rhee. 1994. Reductive dechlorination of polychlorinated biphenyls (PCBs) in St. Lawrence River sediments and variations in dechlorination characteristics. Environ. Sci. and Technol. **28:**2054-2064.

Stalker, M.J., G.B. Kirby, T.E. Kocal, I.R. Smith, and M.A.Hayes. 1991. Loss of glutathione S-transferases in pollution-associated liver neoplasms in white suckers (*Catostomus commersoni*) from Lake Ontario. Carcinogenesis **12:**2221–2226.

Suenaga, H., A. Nishi, T. Watanabe, M. Sakai, and K. Furukawa. 1999. Engineering a hybrid pseudomonad to acquire 3,4-dioxygenase activity for polychlorinated biphenyls. J. Biosci. Bioeng. **87:**430-435.

Suenaga, H., M. Mitsuoka, Y. Ura, T. Watanabe, and K. Furukawa. 2001. Directed evolution of biphenyl dioxygenase: emergence of enhanced degradation capacity for benzene, toluene, and alkylbenzenes. J. Bacteriol. **183:**5441-5444.

Suenaga, H., M. Goto, and K. Furukawa. 2001. Emergence of multifunctional oxygenase activities by random priming recombination. J. Biol. Chem. **276:**22500-22506.

Suutari, M., and S. Laakso. 1994. Microbial fatty-acids and thermal adaptation. Crit. Rev. Microbiol. **20:**129– 37.

Swanson, G. M., H. E. Ratcliffe and L. J. Fischer. 1995. Human exposure to polychlorinated biphenyls (PCBs): A critical assessment of the evidence for adverse health effects. Regulatory Toxicology and Pharmacology **21**: 136-150.

Taira, K., J. Hirose, S. Hayashida, and K. Furukawa. 1992. Analysis of *bph* operon from the polychlorinated biphenyl-degrading strain of *Pseudomonas pseudoalcaligenes* KF707. J Biol Chem **267:**4844-4853.

Vaillancourt, F.H., G. Labbe, N. M Drouin, P.D. Fortin, and L.D. Eltis. 2002. The mechanism-based inactivation of 2,3-dihydroxybiphenyl 1,2-dioxygenase by catecholic substrates. J. Biol. Chem. **277:**2019-2027.

van der Meer, J.R., W.M. DeVos, S. Harayama, and A.J.B. Zehnder. 1992. Molecular mechanisms of genetic adaptation of xenobiotic compounds. Microbiological Reviews, **56:**677-694.

van Hylckama Vlieg, J. E. T., W. de Koning, and D. B. Janssen. 1997. Effect of chlorinated ethene conversion on viability and activity of *Methylosinus trichosporium* OB3b. Appl. Environ. Microbiol. **63:**4961-4964.

Yeager, C. M., P. J. Bottomley, and D. J. Arp. 2001. Cytotoxicity associated with trichloroethylene oxidation in *Burkholderia cepacia* G4. Appl. Environ. Microbiol. **67:**2107-2115.

Ye, D., J.F. Quensen III, J.M. Tiedje, and S.A. Boyd. 1995. Evidence for para dechlorination of polychlorobiphenyls (PCBs) by methanogenic bacteria. Appl. Environ. Microbiol. **61:**2166-2171.

Zhao, L.P., R. Prentice, and L. Breeden. 2001. Statistical modeling of large microarray data sets to identify stimulus-response profiles. Proc Natl Acad Sci **98:**5631–5636.

Zielinski, M., S. Backhaus, and B. Hofer. 2002. The principal determinants for the structure of the substrate-binding pocket are located within a central core of a biphenyl dioxygenase {alpha} subunit. Microbiology **148:**2439-2448.

Zielinski, M., S. Kahl, H.-J. Hecht, and B. Hofer. 2003. Pinpointing biphenyl dioxygenase residues that are crucial for substrate interaction. J. Bacteriol. **185:**6976-6980.

Zielinski, M., S. Kahl, C. Standfuss-Gabisch, B. Camara, M. Seeger, and B. Hofer. 2006. Generation of novel-substrate-accepting biphenyl dioxygenases through segmental random mutagenesis and identification of residues involved in enzyme specificity. Appl. Environ. Microbiol. **72:**2191-2199.

CHAPTER 3

Parnell, J. Jacob, Joonhong Park, Vincent Denef, Tamara Tsoi, and James M. Tiedje. 2006. Coping with PCB toxicity: The physiological and genome-wide response of *Burkholderia xenovorans* LB400 to PCB (polychlorinated biphenyl)-mediated stress. Applied and Environmental Microbiology. **72:**6607-6614.

ABSTRACT

The biodegradation of polychlorinated biphenyls (PCBs) relies on the ability of aerobic microorganisms such as *Burkholderia xenovorans* sp. LB400 to tolerate two potential modes of toxicity presented by PCB degradation: passive toxicity as hydrophobic PCBs potentially disrupt membrane and protein function, and degradation-dependent toxicity from intermediates of incomplete degradation. I monitored physiological characteristics and genome-wide expression patterns of LB400 in response to the presence of Aroclor 1242 (500 ppm) under low expression of the structural biphenyl pathway (succinate and benzoate growth) and under induction by biphenyl. I found no inhibition of growth or change in fatty acid profile due to PCBs under non-degrading conditions. Moreover, I observed no differential gene expression due to PCBs themselves. However, PCBs did have a slight effect on the biosurface area of LB400 cells and caused slight membrane separation. On activation of the biphenyl pathway, I found growth inhibition from PCBs beginning after exponential phase growth suggestive of accumulation of toxic compounds. Genome-wide expression profiling revealed 47 (0.56% of all genes) differentially expressed genes under these conditions. The biphenyl and catechol pathways were induced as expected, but the quinoprotein methanol metabolic pathway and a putative chloroacetaldehyde dehydrogenase were also highly expressed. As the latter protein is essential to conversion of toxic metabolites in dichloroethane degradation, they may play a similar role in degradation of chlorinated aliphatic compounds resulting from PCB degradation.

INTRODUCTION

Polychlorinated biphenyls (PCBs) are organic chemicals belonging to a class of hydrocarbons that are among the most important environmental contaminants, as their physicochemical properties allow them to persist in the environment and bioaccumulate (34). PCBs accumulate in lipid-rich tissues of all organisms, including humans, where they alter immune functions (43), and cause neurological (40), developmental (31), respiratory (45), and reproductive problems as well as cancer due to estrogenic activity (12). These health concerns prioritize PCBs as major targets for environmental clean-up.

The bioremediation strategy with highest potential for destruction of PCB mixtures is a sequential anaerobic-aerobic process (1). Anaerobic bacteria can reductively dechlorinate highly chlorinated congeners, but cannot complete the process and accumulate congeners with fewer chlorines (4, 22, 35). The burden of PCB degradation lies with aerobic co-metabolism of the products of reductive dechlorination, by biphenyl- and chlorobenzoate-degrading organisms (22, 38). Recently a sequential anaerobic-aerobic process was shown in laboratory scale to remove over 50% of Aroclor 1242 from river sediments (39).

Two of the most effective aerobic PCB degrading bacteria, *Burkholderia xenovorans* strain LB400 and *Rhodococcus* sp. RHA1, vary greatly in their PCB toxicity resistance (41), yet the sources of this difference are unknown. Indeed recent studies of PCB-tolerant LB400 (13-15) suggest that cell systems other than the biphenyl pathway contribute to efficient degradation of biphenyl and PCBs. Although the biphenyl pathway has been extensively studied, the role of auxiliary mechanisms, which may provide tolerance to PCBs, has been lightly investigated and information on the

biological effects of PCBs (25) and their degradation products to bacteria is scarce (10, 21).

Complete sequencing of the LB400 genome (http://genome.ornl.gov/microbial/bfun; 11) led us to undertake genomics-enabled investigation of the biology of PCB degradation. The ~9.7 Mbp genome of LB400 contains 8958 predicted protein-coding genes (CDSs) in two chromosomes and one megaplasmid, and is among the largest prokaryotic genomes closed to date. Using genomic and proteomic approaches, an outline of genome-wide responses to a range of carbon and cell development conditions has been reported (13-15), establishing the groundwork for genomic analysis of PCB degradation.

As PCBs have been insinuated to impair microbial growth (25, 42), viability (10) and degradation (7, 44, 47, 48), successful remediation should also consider the physiological and genetic responses limiting or overcoming these toxic effects. In this study I investigate physiological and genome-wide cell responses and defenses against the toxicity posed by PCBs. To that end, I compare PCB tolerance levels of LB400 and other potential PCB-degrading bacteria and outline genome-wide gene expression patterns in response to different toxicity modes posed by PCBs. I further analyze LB400 differential gene expression patterns in response to PCBs with and without degradation. I find that LB400 is unusually tolerant to PCB degradation and suggest that some auxiliary pathways may remove toxic chlorinated intermediates.

MATERIALS AND METHODS

Bacteria, media and growth conditions. I determined the degree of sensitivity of 18 strains isolated on biphenyl and naphthalene from Brazil, Puerto Rico, Japan and USA (9, 28) to 100 ppm (low) and 500 ppm (high) concentrations of the commercial PCB mixture Aroclor 1242 (Table 1). Sixteen strains previously characterized by Pellizari, et al. (28) as potential PCB degraders and two well-studied PCB degraders, *Burkholderia xenovorans* LB400 (7) and *Rhodococcus* sp. RHA1 (26), were included in this study. To determine the effect of PCBs on growth, cultures of each strain were grown in triplicate on Luria-Bertani (LB) broth containing 0, 100, and 500 ppm Aroclor 1242 (Monsanto Co., St. Louis, Mo.) at 29 ± 1° C in 125 ml flasks agitated at 200 rpm. The percent growth, maximum optical density and estimated lag time was noted for PCB-containing cultures and compared with control medium that contained no PCBs. The maximum growth rate (μmax) was calculated using OD values during logarithmic growth. Maximum biomass was measured as cultures reached stationary-phase growth.

For studies in defined medium (13), batch cultures (25 mL) were prepared in 125-mL Wheaton® flasks and sealed with Teflon-lined lids. Succinate 1g/L (~10 mM) and benzoate 1g/L (~5 mM) were added to the medium prior to autoclaving. Sterile biphenyl 3g/L (~20 mM) was added directly to the sterilized medium in each flask. The PCB-containing medium was allowed to equilibrate for at least 24 h prior to addition of inoculum from a freshly-grown, biphenyl- adapted culture. Triplicates were prepared

Table 3.1. Tolerance of potential PCB-degrading strains to 500 ppm Aroclor 1242 in Luria Bertani medium. Tolerance (relative growth) was determined by comparing the maximum growth rate (μ max) and maximum biomass (Max OD at 600 nm) in the presence and absence of PCBs.

Strain*	G+/G-[≡]	μ max (h^{-1})	Max OD	Rel growth†
Tolerant				
C. testosteroni VP44	G-	1.07±0.03	0.98±0.01	1.05
B. xenovorans LB400	G-	0.93±0.04	0.99±0.03	0.92
Moderately tolerant				
Unidentified strain PR4	G-	0.81±0.06	0.94±0.01	0.76
S. warnerii VP73	G+	0.87±0.22	0.83±0.06	0.73
A. xylosoxidans VP98	G-	0.75±0.06	0.95±0.01	0.71
X. maltophilia VP48	G-	0.73±0.02	0.94±0.01	0.69
P. gladioli VP86	G-	0.68±0.13	1.00±0.01	0.68
Unidentified strain PR2	G-	0.74±0.05	0.87±0.01	0.65
X. maltophilia VP90	G-	0.71±0.07	0.89±0.02	0.63
R. erythropolis NY05	G+	0.57±0.06	0.99±0.02	0.57
Moderately sensitive				
X. maltophilia VP11	G-	0.68±0.14	0.78±0.04	0.53
Unidentified strain VP69	G-	0.67±0.03	0.68±0.13	0.46
P. gladioli VP87	G-	0.58±0.12	0.79±0.03	0.46
P. gladioli VP49	G-	0.64±0.08	0.65±0.02	0.42
A. xylosoxidans PR5	G-	0.47±0.16	0.81±0.02	0.38
Rhodococcus sp. RHA1	G+	0.55±0.08	0.56±0.04	0.31
Sensitive				
P. gladioli VP71	G-	0.38±0.05	0.26±0.02	0.10
Unidentified strain VP103	G-	0.29±0.06	0.11±0.01	0.03

(±standard error = standard deviation/√n)
*Strains designated VP and PR are from Pellizari et al, (32).
[≡]Gram Positive/ Negative
†Relative growth is calculated by multiplying μ max and max OD (relative to PCB- controls).

for each growth condition. Microbial growth was monitored spectrophotometrically by measuring absorbance at 600 nm. To account for any effect of the (in)solubility of biphenyl or PCBs on absorbance, CFU counts were conducted by plating serial dilutions on plates with R2A agar.

Distribution of PCBs. Association of PCBs with bacteria was demonstrated by growing two strains incapable of PCB degradation (*Burkholderia xenovorans*, LMG 27120 and LMG 16229 (19)), in 25 ml of K1 with succinate and 500 ppm PCBs. Following growth to stationary phase (OD_{600} 1.2 and 1.0, respectively), the cultures were filtered using sterile glass wool and centrifuged at 4000 rpm for 10 min. A control of medium with PCBs but no cells was also filtered using glass wool, centrifuged, and analyzed as a reference for the distribution of PCBs without cells. The supernatant and cell fraction were separated and PCBs were extracted with octachloronaphthalene added as an internal standard.

PCB degradation by LB400 was measured in triplicate by disappearance of PCBs from cultures harvested at mid-logarithmic phase on each of the three carbon sources. Due to the difference in the growth rate on each carbon source, harvesting of LB400 varied from 12-24 h following inoculation, on reaching mid-logarithmic growth. Each PCB-containing culture was extracted three times using an equal volume of 1:1 hexane:acetone by shaking for 30 min and analyzed by gas chromatography as described previously (28). Percent degradation was calculated by determining the difference between the PCB concentration in experimental cultures and in non-inoculated controls.

Physiological adaptation to PCBs. To determine membrane adaptation of LB400 and RHA1 to PCBs, cells were grown in the presence or absence of PCBs and the fatty acid composition was analyzed as per the MIDI protocol (Microbial ID, Newark, DE). I also compared the morphology of LB400 grown in the presence and absence of PCBs by analyzing scanning electron micrograph preparations. SEM fixation was performed as previously described (26). Multiple fields (at least five) with multiple cells (>10) from two biological replicates were subsequently analyzed using the CMEIAS image analysis software (27, http://cme.msu.edu/cmeias). Due to the elongation of cells prior to cell division, I analyzed the smallest 25 percentile of the bacteria captured on electron micrographs for dimensional analysis. For transmission electron micrographs, cells were pelleted and the pellet resuspended in fixative (2.5% glutaraldehyde and 2.0% paraformaldehyde in 0.1M cacodylate buffer) overnight at 4°C. After fixation, cells were pelleted and the pellet was embedded in 2% agarose for post fixation with osmium tetroxide. Following postfixation, cells were dehydrated in acetone series and embedded in Spurr's resin. Sections were stained with uranyl acetate and lead citrate for examination on a JEOL 100CX transmission electron microscope.

Gene expression analysis. The genome-wide response to PCB stress was analyzed using a genomic microarray [Xeochip™ (13)] that contained 8,429 of the 8,989 total CDSs (93.8%) reported in the closed genome sequence of LB400 (http://genome.ornl.gov/microbial/bfun). LB400 cells were grown in succinate, benzoate, and biphenyl media in duplicate each with parallel cultures containing 500 ppm Aroclor 1242. After three serial transfers in the same medium, bacterial cells were harvested by centrifugation from the exponential phase (OD_{600} nm 0.3-0.4) at 4000 rpm for 10 min at

4°C. The culture was stored in RNAlater (Ambion) to protect against RNA degradation. Total RNA was extracted using an RNeasy mini kit (Qiagen), and the contaminant DNA was degraded by using DNase I (Roche). The RNA was quantified spectrophotometrically, and the quality was verified by gel analysis. RNA was labeled by amino-allyl dUTP (Sigma) and hybridized as before (13) using Cy5 and Cy3 fluorophores (Amersham). Two biological replicates were hybridized with at least 100 pmol of labeled material per Xeochip with dyes swapped. Hybridized chips were scanned with an Axon 4000B laser scanner and the data extracted using Genepix Pro 5.0 (Axon laboratories). Median expression data for each channel (Cy5 and Cy3) were imported into GeneSpring 7.2 (Silicon Genetics) and normalized using Lowess intensity-dependent normalization (13). Biological reproducibility was determined by calculating the coefficient of variation. Data were analyzed using GeneSpring software.

RESULTS

Tolerance to Aroclor 1242. I analyzed the degree of sensitivity or tolerance of potential PCB-degrading strains by determining the inhibition of growth due to addition of Aroclor 1242 (Table 3.1) to LB broth. Of the 18 potential PCB-degrading strains tested, only *Comamonas testosteroni* VP44 and *Burkholderia xenovorans* LB400 demonstrated tolerance to high concentrations of Aroclor. In contrast, both the growth rate and biomass production were decreased in *Rhodococcus* RHA1 with exposure to PCBs. Several strains, including *A. xylosoxidans,* exhibited extreme sensitivity to PCBs with diminished biomass, extremely slow growth rates and greater lag time. As expected, the ability to tolerate PCBs was better illustrated with 500 ppm than 100 ppm in the medium (data not shown). The length of lag-period prior growth in the presence of high

concentrations of PCBs appears to be consistent with the bacterial tolerance to PCBs. Due to its PCB tolerance and availability of genomics-enabled approaches, I selected LB400 for detailed elucidation of PCB toxicity and tolerance responses.

We monitored the effect of PCBs on growth when the biphenyl pathway expression was low (minimal to no degradation of PCBs) in succinate- and benzoate-grown cells. Regardless of the presence of PCBs, RHA1 was unable to grow on succinate in K1 medium. Growth of RHA1 on benzoate was not inhibited in the PCB-containing treatments (0.48 h^{-1} +/- .05) compared with controls (0.42 h^{-1} +/- .03). Likewise LB400 growth rates on both succinate (0.40 h^{-1} +/- .02) and benzoate (0.27 h^{-1} +/- .02) were not inhibited by PCBs (0.39 hr^{-1} +/- .02 and 0.28 hr^{-1} +/- .03 respectively). Plate counts confirmed this observation.

On expression of the biphenyl pathway during growth on biphenyl and subsequent PCB degradation, the growth of both RHA1 and LB400 was inhibited (Figure 3.1). No growth of RHA1 was observed in batch cultures containing biphenyl and PCBs (0.10 h^{-1} +/- .01 vs. 0.01 h^{-1} +/- .01). On the contrary, LB400 maintained growth rates similar to control cultures until mid-exponential phase before inhibition became apparent.

Cell-associated PCBs. Since PCBs have very low water solubility, I analyzed the amount of PCBs that partitioned to the biomass of two *Burkholderia xenovorans* strains incapable of PCB degradation after growth on succinate + PCBs in K1 medium. Virtually all of the PCBs recovered were found in the biomass fraction (Table 3.2). Based on previous measurements of membrane content of cells (30), this amount of PCBs corresponds to roughly 9.5 % of membrane weight if all cell- associated PCBs were localized in the membrane.

PCB fate under degrading and non-degrading conditions. Insignificant disappearance of PCBs by LB400 growing on either benzoate or succinate during exponential growth indicated that PCBs were not degraded on these carbon sources (Figure 3.2). However, during the transition to stationary phase, under carbon limited conditions, some degradation did occur (not shown). Although RHA1 did not grow on succinate, it grew well on benzoate and similar to LB400 showed no degradation of PCBs. When the bph-pathway was induced in LB400, I found significant disappearance of lesser- and *ortho*-chlorinated congeners such as 2-chlorobiphenyl and 2,3-dichlorobiphenyl, the more persistent congeners present in Aroclor 1242.

Morphological changes of LB400 due to PCBs. Morphological effects of PCBs on LB400 were analyzed using scanning electron micrographs of cells from exponential-phase growth of each of the treatments. Image analysis revealed a statistically significant reduction in the biosurface area caused by PCBs in succinate (14%) and benzoate (9%) grown cultures (Table 3.3). When LB400 was grown with biphenyl as the growth substrate, no significant reduction was detected in the biosurface area due to PCBs; however both biphenyl-grown treatments were significantly reduced (by 31%) compared with succinate and benzoate grown cells. Transmission electron micrographs from exponential phase LB400 grown on succinate with PCBs revealed pervasive separation of the inner and outer membrane (Figure 3.3A) compared to succinate alone (Figure 3.3B). Scanning electron micrographs of early stationary phase succinate + PCB grown cells show the formation of large membrane vesicles (Figure 3.3C) that are absent in control samples (Figure 3.3D). Nearly every micrograph collected from early stationary phase

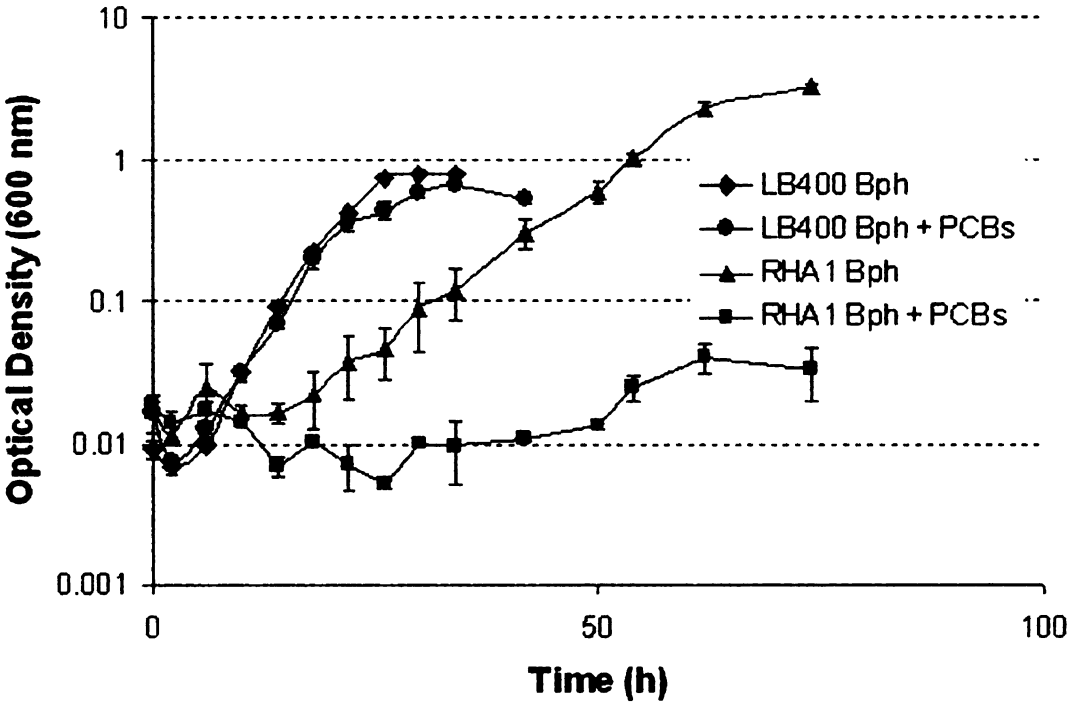


Figure 3.1. Growth curves of LB400 and RHA1 on biphenyl (3g/L) as carbon source with and without 500 ppm Aroclor 1242 (PCBs) in K1 medium.

Table 3.2. PCBs (ppm) extracted from two *Burkholderia xenovorans* strains incapable of PCB degradation after incubation with Aroclor 1242. Cultures and control were filtered using sterile glass wool to remove insoluble PCBs prior to centrifugation (± standard deviation).

Culture treatment	Supernatant (ppm)	Cell pellet (ppm)
Uninoculated medium*	4.0±0.1	5.1±0.1
LMG27120	2.9±0.1	75±5
LMG16229	3.3±0.3	57±5

*Control medium without bacterial inoculation.

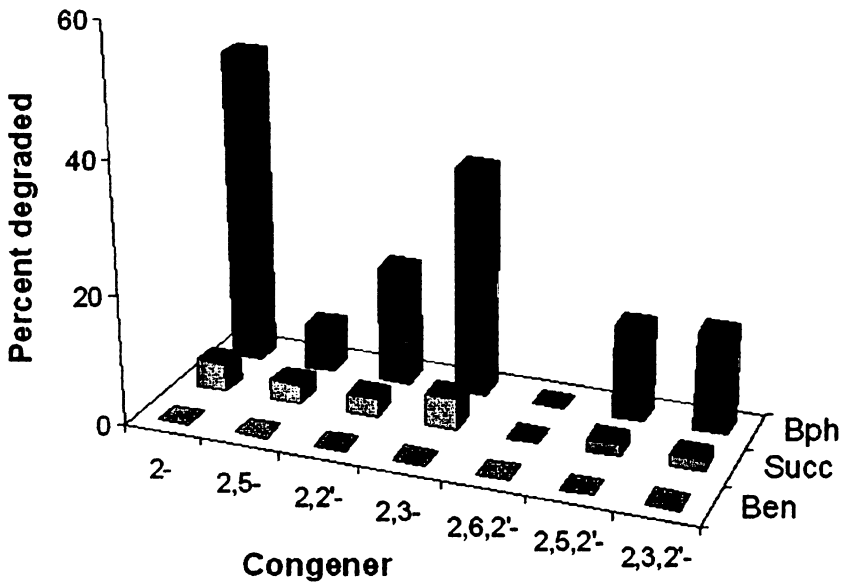


Figure 3.2. Percent degradation of lower-chlorinated PCB congeners (500 ppm Aroclor 1242) by LB400 harvested during exponential-phase growth on benzoate (Ben), succinate (Succ), and biphenyl (Bph) with PCBs.

cells with PCBs contained evidence of vesicles at a ratio of approximately 3:4 (vesicles:cell).

Fatty acid changes due to PCBs. The change in fatty acid composition of LB400 varied slightly for each carbon source. As general trends, unsaturated fatty acids, predominantly 18:1 and 16:1, decreased with increasing K_{ow} of C source (Figure 3.4). Succinate has the lowest K_{ow} (0.35) followed by benzoate (1.87) with biphenyl highest (3.90). Cyclic fatty acids (17:0 and 19:0) tended to increase with increasing K_{ow} of C source. On degradation of PCBs (induction by biphenyl), there was a significant shift from saturated fatty acids to cyclic fatty acids.

Gene expression in response to PCBs. No significant differential gene expression was found between benzoate or succinate control cultures and PCB-containing cultures (Figure 3.5A,B). The CVs for dye-swapped biological replicates were 9.5% for succinate + PCBs with succinate as reference and 5.4% for benzoate + PCBs with benzoate as reference.

Gene expression patterns measured from PCB-degrading conditions vs biphenyl growth indicate that relatively few genes, only 47 of 8429 CDSs are differentially expressed greater than 2-fold. Several of the structural genes in the upper biphenyl pathway were slightly more induced on degradation of PCBs as were several genes involved in (chloro)benzoate degradation via hydroxylation and CoA activation (Table 3.4). I also found genes induced that may play an ancillary role such as a cluster of genes involved in acetylacetone cleavage that contained a putative short chain alcohol

Table 3.3. Morphological measurements of the smallest quartile of LB400 cells grown on different carbon sources (succ = succinate, ben = benzoate, bph = biphenyl) on exposure to 500 ppm Aroclor 1242 as calculated by CMEIAS (± standard deviation). Statistically significant groups are indicated by different letters (determined by t-test $p<0.05$).

Growth conditions	Mean length (μm)	Mean width (μm)	Mean biosurface area (μm^2)	n	Grouping
Succ	1.86±0.19	0.47±0.04	3.2±0.4	27	A
Succ + PCBs	1.65±0.16	0.44±0.02	2.7±0.2	23	B
Ben	1.75±0.11	0.48±0.03	3.2±0.2	17	A
Ben + PCBs	1.61±0.15	0.49±0.04	2.9±0.2	23	B
Bph	1.41±0.14	0.41±0.06	2.1±0.4	17	C
Bph + PCBs	1.36±0.16	0.42±0.05	2.2±0.3	19	C

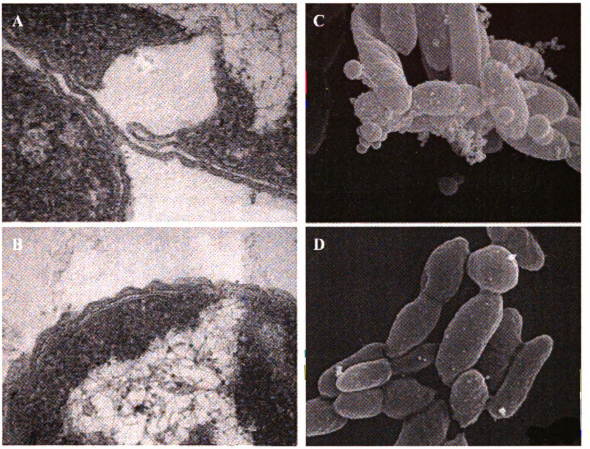


Figure 3.3. Electron micrographs of LB400 grown on succinate with and without PCBs: TEM image of exponential-phase cells with PCBs (A), and without PCBs (B), SEM of late exponential-phase cells with PCBs (C) and without PCBs (D).

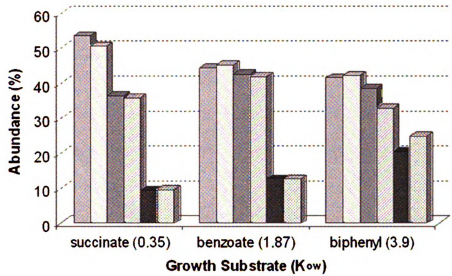


Figure 3.4. Fatty acid analysis of LB400 during growth on carbon sources with different octanol-water coefficients (Kow) in the presence (striped) and absence (solid) of PCBs. Fatty acids were grouped as unsaturated (light), saturated (gray) and cyclic (dark).

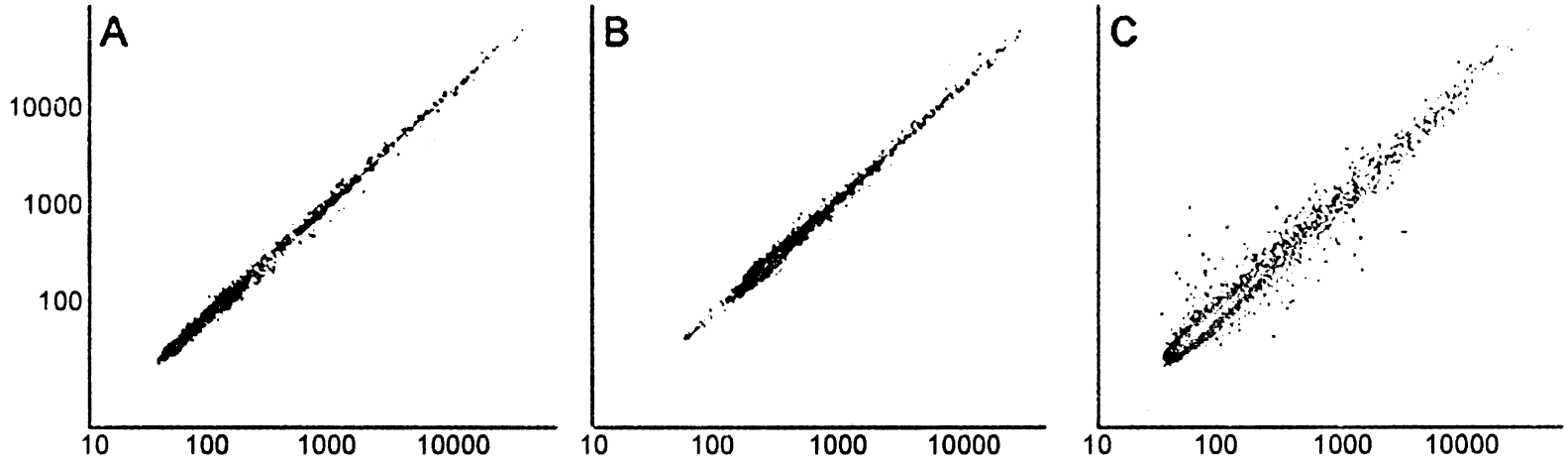


Figure 3.5. Logarithmic scale plots of spot intensities (arbitrary units). Transcriptional profiles from mid-log growth on succinate versus succinate with PCBs (A), benzoate versus benzoate with PCBs (B), and biphenyl versus biphenyl with PCBs (C). Each represents dye swap of biological replicates with Lowess normalization. Diagonal lines represent two-fold change in gene expression.

Table 3.4. Up-regulated genes of LB400 grown on biphenyl with PCBs with growth on biphenyl as the reference. Expression reported as the ratio.

Gene ID	Annotation	Expression
BxeA1129	Chloromuconate cycloisomerase	2.7
BxeA1130	Chlorocatechol-1,2-dioxygenase	3.2
BxeA1329	Sugar ABC transporter, periplasmic binding protein	2.7
BxeA1427	Amidase, hydantoinase/carbamoylase	2.1
BxeA2109	Catechol 1,2-dioxygenase	4.7
BxeA2876	Acetylacetone-cleaving enzyme	2.9
BxeA2877	Hypothetical protein	3.5
BxeA2878	Putative short-chain dehydrogenase/reductase	2.1
BxeA3675	ABC sugar transporter, ATPase subunit	2.5
BxeA4207	ABC nitrate/sulfonate/bicarbonate family transporter	2.1
BxeA4441	Chloroacetaldehyde dehydrogenase	4.9
BxeA4442	Hypothetical protein	2.4
BxeA4478	Putative exported protein precursor	3.1
BxeB1279	Glycogen synthase	1.9
BxeB1637	Cytochrome o ubiquinol oxidase subunit II precursor	1.9
BxeB2286	Unknown	1.7
BxeB2426	Cytochrome c-555 precursor[b]	5.4
BxeB2427	Methanol dehydrogenase large subunit-like protein[b]	9.3
BxeB2436	Formaldehyde activating enzyme[b]	16.0
BxeB2437	Hypothetical protein[b]	5.5
BxeB2469	Coenzyme PQQ synthesis D[b]	2.6
BxeB2473	TonB-dependent receptor[b]	3.0
BxeB2474	Hypothetical protein[b]	3.2
BxeC1082	Lipopolysaccharide biosynthesis protein	2.4
BxeC1187	4-hydroxy-2-oxovalerate aldolase[a]	1.4
BxeC1190	Glutathione S-transferase[a]	1.4
BxeC1191	2,3-Dihydroxybiphenyl dioxygenase[a]	1.5
BxeC1192	Cis-2,3-dihydrobiphenyl-2,3-diol dehydrogenase[a]	1.5
BxeC1196	Biphenyl dioxygenase small subunit[a]	1.7

[a] indicates CDSs associated with the biphenyl pathway.
[b] indicates CDSs associated with C1 metabolism.

dehydrogenase as well as a group of genes that contained a putative chloroacetaldehyde dehydrogenase. Additionally, on degradation of PCBs, gene expression patterns indicate an induction of genes potentially involved in methanol utilization including a methanol dehydrogenase-like protein, and formaldehyde activating enzyme as well as genes responsible for co-factors involved in C1 metabolism. Down-regulated genes (Table 3.5) include several membrane proteins, an oxidoreductase that is similar to formate dehydrogenase (BxeC0084), a cyclohexanone monooxygenase (BxeA3588), and a cluster of genes possibly involved in lysine biosynthesis (BxeB1798, BxeB1802 and BxeB1806). The CV for the biological replicates of PCB-degrading conditions (biphenyl + PCBs vs biphenyl) was 15.3% (Figure 3.5C).

DISCUSSION

The physiological and genome-wide response of *B. xenovorans* LB400 to PCBs enabled us to confine the source of toxicity to the byproducts of PCB degradation. Although both LB400 and RHA1 are considered model organisms for the study of PCB degradation, my data indicate that LB400 is much more tolerant to PCB-degradation dependent toxicity (Figure 3.1). In fact, LB400 is among the most tolerant PCB degraders identified in this study (Table 3.1). In both cases, PCBs *per se* did not affect the growth rate, viability or fatty acid profile and LB400 demonstrated no change in gene expression in the absence of degradation. Thus, toxicity of PCBs to RHA1 and LB400 was a direct result of the production of deleterious metabolites during co-metabolism. The difference in growth rates of LB400 and RHA1 during PCB degradation may indicate mechanisms beyond the biphenyl pathway that contribute to tolerance. LB400 gene expression data under biphenyl-grown, PCB-degrading conditions indicate that the biphenyl,

Table 3.5. Down-regulated genes of LB400 grown on biphenyl with PCBs with growth on biphenyl as the reference. Expression reported as the ratio.

Gene ID	Annotation	Expression
BxeA0676	Putative membrane protein	0.54
BxeA1007	16S rRNA processing protein	0.54
BxeA1178	Hypothetical protein	0.69
BxeA1741	RND efflux system, outer membrane lipoprotein	0.73
BxeA1939	Putative permease from ABC transporter	0.54
BxeA2187	Cytochrome c, class I precursor	0.58
BxeA3048	Putative bacteriophage protein GP46	0.35
BxeA3185	Putative enoyl-CoA hydratase	0.34
BxeA3588	Cyclohexanone monooxygenase	0.52
BxeA3722	Acetyl transferase	0.62
BxeB0560	Putative ADP-heptose--LPS heptosyltransferase	0.40
BxeB1798	TRAP dicarboxylate transporter-DctP subunit	0.49
BxeB1802	Dihydrodipicolinate synthetase	0.18
BxeB1806	MFS transporter, phthalate permease family	0.38
BxeB2010	Acriflavin resistance protein precursor	0.69
BxeB2146	Putative membrane protein precursor	0.15
BxeB2160	D-lactate dehydrogenase	0.38
BxeB2536	Putative LysR-family transcriptional regulator	0.30
BxeC0084	Formate dehydrogenase	0.55

3-chlorocatechol and catechol pathways are further induced on degradation of PCBs. The present data also indicate the induction of a putative chloroacetaldehyde dehydrogenase and components of the C_1 metabolism pathway that may be involved in PCB product degradation.

Microorganisms responsible for the biodegradation of hydrophobic compounds such as PCBs are subject to membrane effects such as fluidity disruption and interference with protein function (16, 42). Previous studies concerned with the adverse physiological responses to PCBs have attributed toxicity to PCBs themselves as well as to the products of their degradation (10, 21). While I confirmed that PCBs partition to the cell fraction of cultures (Table 3.2), this association did not correspond to a decrease in growth rate or viability in either the PCB-tolerant LB400 or -sensitive RHA1. Additionally, PCBs apparently do not interact with proteins that are responsible for cellular respiration as seen for other chlorinated aromatic compounds (16).

Recent studies suggest that PCB degradation does not occur when LB400 is grown with simple carbon sources such as glucose and succinate (6, 13). I found that PCBs were not degraded (Figure 3.2) and mechanisms involved in PCB degradation were not induced by PCBs during exponential-phase growth on succinate or benzoate (Figure 3.5A, B). Therefore, any physiological or genome-wide expression changes detected in succinate- or benzoate-grown LB400 cannot be attributed to degradation products of PCBs, but rather to interaction with PCBs themselves, effectively allowing us to decouple and evaluate the potential sources of PCB toxicity.

One physiological response to membrane stress caused by lipophilic compounds involves altered fatty acid chain length or composition (36, 37, 42). Although the fatty

acid composition of PCB-degrading *Ralstonia eutropha* HP850 shifts from saturated to unsaturated fatty acids during growth on biphenyl (25), the effect of PCBs themselves on membrane composition has not been documented. LB400 demonstrated no change in the fatty acid profile when grown on simple carbon sources (succinate or benzoate) with PCBs (Figure 3.4). However, LB400 may be similar to pseudomonads that do not alter their saturated-to-unsaturated fatty acid profile on exposure to lipophilic compounds (37). The only physiological changes I observed due to PCBs in non-degrading conditions appeared in electron micrographs of LB400 that indicate a reduction in the biosurface area of LB400 (Table 3.3). I also found frequent membrane aberrations caused by growth with PCBs (Figure 3.3B, D). Similar membrane separation caused by PCBs has been noted (10), and such membrane aberrations are often indicative of stressed conditions (5).

Although I found morphological evidence of PCB-membrane interactions in LB400, I found no differential gene expression (>2-fold) by microarray analysis in any of the 8429 CDSs tested in either succinate or benzoate treatments with PCBs relative to no-PCB treatments (Figure 3.5A & B). These analyses indicate that PCBs themselves do little to alter the composition of the membrane of LB400 and RHA1. Although PCBs do not affect growth rate or viability, the reduction in biosurface area and separation of inner and outer membrane do constitute a response to PCBs. However, dynamic morphological changes don't always elicit a change in gene expression (3). Either the responses reported here stem from gene expression changes below the sensitivity of microarray analysis or this is a direct physical effect of PCBs on membranes.

Past studies involved in the degradation of PCBs have focused exclusively on the biphenyl and chlorobenzoate pathways responsible for much of the degradation of simple

congeners (4, 17-19, 22, 28, 38), and as expected these pathways (biphenyl, catechol and chlorocatechol) were induced on degradation (Table 3.4). Although chlorinated aliphatic compounds derived from chlorinated pentadienoates may play an important role in PCB degradation, their fate and effect have been neglected. Potential metabolic derivatives of chlorinated pentadienoates have proven lethal to biodegrading microorganisms (2, 50, 51). Bacteria that are more capable of degrading or tolerating chlorinated aliphatic compounds would have a distinct advantage in environmental scenarios containing a mixture of PCB congeners. Among the putative genes induced on degradation of PCBs was an acetaldehyde dehydrogenase that is 83% (amino acid) identical to the chloroacetaldehyde dehydrogenase found in *Xanthobacter autotrophicus* GJ10, a known dichloroethane (DCE) degrader (24). Chloroacetaldehyde dehydrogenase is responsible for rapid conversion of the highly toxic chloroacetaldehyde intermediate, an essential step in DCE metabolism (24, 49). The alcohol dehydrogenase involved in DCE degradation is the same as the quinoprotein methanol dehydrogenase in methanol oxidation by Gram-negative methylotrophs (24, 33). The chloroacetaldehyde dehydrogenase genes and methanol oxidation pathway (and co-factors) were the most differentially expressed genes induced by PCB degradation.

Down-regulated genes demonstrate equally significant characteristics associated with the degradation of PCBs (Table 3.5). In agreement with the change in fatty acid profile on degradation of PCBs (Figure 3.4), a putative enoyl-CoA hydratase involved in fatty acid metabolism (29) is repressed. Although lysine biosynthesis has been shown to have an indirect association with fatty acid beta-oxidation in oxidative stress (8), the specific role of lysine repression in this case is unknown. Finally, the down-regulation of oxygen-dependent proteins may be indicative of oxygen limited conditions.

Although many of the mechanisms and components presented here may be involved in a general toxic response, such as oxygen limitation, some genes that are differentially expressed are almost certainly involved in minimizing the effects of toxic metabolites produced during degradation of PCBs. Unexpectedly, I found no growth inhibition or notable physiological response to PCBs under non-degrading conditions. Hence, factors beyond the upper biphenyl and benzoate degradation pathways contribute to successful PCB degradation. While RHA1 oxidizes a wide range of PCBs in resting cell assays (following growth on biphenyl) (27), it grew poorly on biphenyl in the presence of 500 ppm Aroclor 1242. Further comparison with other potential biodegrading bacteria, such as RHA1, shows that LB400 is extremely tolerant to the toxic metabolites that are created as a result of PCB degradation, possibly due to its superior ability to oxidize chlorinated aliphatic compounds. As LB400 is one of the bacteria most tolerant to PCB toxicity and has specificity to degrade a broad range of congeners, it becomes a model system for studying PCB degradation and its effects on bacteria, particularly for in situ biodegradation. Understanding the cell's responses to PCBs revealed in this study may provide information on the modes of toxicity and effective counter responses, eventually leading to development of more efficient PCB amelioration strategies.

REFERENCES

1. **Abramowicz, D. A.** 1990. Aerobic and anaerobic biodegradation of PCBs: a review. Crit. Rev. Biotechnol. **10:**241-248.

2. **Alvarez-Cohen, L., and P. L. McCarty.** 1991. Effects of toxicity, aeration, and reductant supply on trichloroethylene transformation by a mixed methanotrophic culture. Appl. Environ. Microbiol. **57:**228-235.

3. **Arends, S.J.R., and D. S. Weiss.** 2003. Inhibiting cell division in *Escherichia coli* has little if any effect on gene expression. J. Bacteriol. **186:**880-884.

4. **Bedard, D. L., and J. F. Quensen, III.** 1995. Microbial reductive dechlorination of polychlorinated biphenyls, p. 127-216. *In* L. Y. Young, and C. Cerniglia (ed.), Microbial transformation and degradation of toxic organic chemicals. Wiley-Liss Division, John Wiley & Sons, Inc., New York, N.Y.

5. **Beveridge, T.J.** 1999. Structures of Gram-negative cell walls and their derived membrane vesicles. J. Bacteriol. **181**:4725-4733.

6. **Billingsley K A, Backus S M, Juneson C, Ward O P.** 1999. Comparison of the degradation patterns of polychlorinated biphenyl congeners in Aroclors by *Pseudomonas* strain LB400 after growth on various carbon sources. Can J Microbiol 1997;**43**:1172–1179.

7. **Blasco, R., M. Mallavarapu, R-M. Wittich, K.N. Timmis, and D.H. Pieper.** 1997. Evidence that formation of protoanemonin from metabolites of 4-chlorobiphenyl degradation negatively affects the survival of 4-chlorobiphenyl-cometabolizing microorganisms. Appl. Environ. Microbio. **63**:427-434.

8. **Bopp L H.** 1986. Degradation of highly chlorinated PCBs by *Pseudomonas* strain LB400. J Ind Microbiol. **1**:23–29.

9. **Breitling, R., O. Sharif, M.L. Hartman, and S.K. Krisans.** 2002. Loss of compartmentalization causes misregulation of lysine biosynthesis in peroxisome-deficient yeast cells. Eukaryot Cell. **6:**978-986.

10. **Camara, B., C. Herrera, M. Gonzalez, E. Couve, B. Hofer, and M. Seeger.** 2004. From PCBs to highly toxic metabolites by the biphenyl pathway. Environ. Microb. 6:842-850.

11. **Chain, P.S.G., V.J. Denef, K. Konstantinidis, L.M. Vergez, L. Agulló, V.L. Reyes, L. Hauser, M. Córdova, L. Gómez, M. González, M. Land, V. Lao, F. Larimer, J.J. LiPuma, E. Mahenthiralingam, S.A. Malfatti, C.J. Marx, J.J. Parnell, A. Ramette, P. Richardson, M. Seeger, D. Smith, T. Spilker, W-J. Sul, T.V. Tsoi, L.E. Ulrich, I.B. Zhulin, J.M. Tiedje.** 2006. *Burkholderia*

xenovorans LB400 harbors a multi-replicon, 9.7 M bp genome shaped for versatility. Proc. Natl. Acad. Sci. (Submitted).

12. **Demers, A. P. Ayotte, J. Brisson, S. Dodin, J. Robert, and E. Dewailly.** 2002. Plasma concentrations of polychlorinated biphenyls and the risk of breast cancer: A congener-specific analysis. Am. J. Epidemiol., **155**(7):629 - 635.

13. **Denef, V.J., J. Park, T.V. Tsoi, J.-M. Rouillard, H. Zhang, J.A. Wibbenmeyer, W. Verstraete, E. Gulari, S.A. Hashsham, and J.M. Tiedje.** 2004. Biphenyl and benzoate metabolism in a genomic context: Outlining genome-wide metabolic networks in *Burkholderia xenovorans* LB400. Appl. Environ. Microbiol. **70:**4961-4970.

14. **Denef, V.J. M.A. Patrauchan, C. Florizone, J. Park, T.V. Tsoi, W. Verstraete, J.M.Tiedje, and L.D. Eltis.** 2005. Growth substrate- and phase-specific expression of biphenyl, benzoate, and C_1 metabolic pathways in *Burkholderia xenovorans* LB400. J. Bacteriol. **187:**7996-8005.

15. **Denef, V.J. J.A. Klappenbach, M.A. Patrauchan, C. Florizone, J.L.M. Rodrigues, T.V. Tsoi, W. Verstraete, L.D. Eltis, and J.M. Tiedje.** 2006. Genetic and genomic insights into the role of benzoate-catabolic pathway redundancy in *Burkholderia xenovorans* LB400. Appl. Environ. Microbiol. **72:** 585-595.

16. **Donato MM, Jurado AS, Antunes-Madeira MC, Madeira VM.** 1997. Comparative study of the toxic actions of 2,2-bis(p-chlorophenyl)-1,1,1-trichloroethane and 2,2-bis(p-chlorophenyl)-1,1-dichloroethylene on the growth and respiratory activity of a microorganism used as a model. Appl. Environ. Microbiol. **63**:4948-51.

17. **Erickson, B. D., and F. J. Mondello.** 1993. Enhanced biodegradation of polychlorinated biphenyls after site-directed mutagenesis of a biphenyl dioxygenase gene. Appl. Environ. Microbiol. **59:**3858-3862.

18. **Furukawa, K. J.R. Simon, and A.M. Chakrabarty.** 1983. Common Induction and regulation of biphenyl, xylene/toluene, and salicylate catabolism in *Pseudomonas paucimobilis*. J. Bacteriol. **154**:1356-1362.

19. **Gibson, D. T., D. L. Cruden, J. D. Haddock, G. J. Zylstra, and J. M. Brand.** 1993. Oxidation of polychlorinated biphenyls by *Pseudomonas* sp. strain LB400 and *Pseudomonas pseudoalcaligenes* KF707. J. Bacteriol. **175:**4561-4564.

20. **Goris, J., P. De Vos, J. Caballero-Mellado, J. Park, E. Falsen, J.F. Quensen III, J.M. Tiedje, and P. Vandamme.** 2004. Classification of the PCB-and biphenyl degrading strain LB400 and relatives as *Burkholderia xenovorans* sp. nov. Int J Syst Evol Microbiol **54**:1677-1681.

21. **Hiraoka, Y., Yamada, T., Tone, K., Futaesaku, Y., and Kimbara, K.** 2002. Flow cytometry analysis of changes in the DNA content of the polychlorinated biphenyl degrader *Comamonas testosteroni* TK102: Effect of metabolites on cell-cell separation. Appl Environ Microbiol **68**: 5104-5112.

22. **Hrywna, Y., Tsoi, T.V., Maltseva, O.V., Quensen, III, J.F., and Tiedje, J.M.** 1999. Construction and characterization of two recombinant bacteria that grow on *ortho*- and *para*-substituted chlorobiphenyls. Appl Environ Microbiol **65**: 2163-2169.

23. **Imbeault, N.Y.R., J.B. Powlowski, C.L. Colbert, J.T. Bolin, and L.D. Eltis.** 2000. Steady-state kinetic characterization and crystallization of a PCB-transforming dioxygenase. J Biol Chem **275**:12430-12437.

24. **Janssen, D.B., A. Scheper, and B. Witholt.** 1984. Biodegradation of 2-chloroethanol and 1,2-dichloroethane by pure bacterial cultures, p 169-178. In E.H. Houwink and R.R. van der Meer (eds.), Innovations in biotechnology. Elsevier. Amsterdam

25. **Kim, I.S., H. Lee, and J.T. Trevors.** 2001. Effects of 2,2',5,5'-tetrachlorobiphenyl and biphenyl on cell membranes of *Ralstonia eutropha* H850. FEMS Microbiol Let **200**:17-24.

26. **Klomparens, K., Flegler, S.L., and Hooper, G.R.** 1986. Procedures for transmission and scanning electron microscopy for biological and medical science. Burlington, VT. Ladd Research Industries.

27. **Lui, J., F.B. Dazzo, O. Glagoleva, B. Yu, and A.K Jain.** 2001. CMEIAS: a computer aided system for the image analysis of bacterial morphotypes in microbial communities. Microb Ecol **41:**173-194.

28. **Maltseva, O.V., T.V. Tsoi, J.F. Quensen III, M. Fukuda, and J.M. Tiedje.** 1999. Degradation of anaerobic reductive dechlorination products of Aroclor 1242 by four aerobic bacteria. Biodegradation. **10:**363-371.

29. **Muller-Newen G, and W. Stoffel.** 1991. Mitochondrial 3-2trans-Enoyl-CoA isomerase. Purification, cloning, expression, and mitochondrial import of the key enzyme of unsaturated fatty acid beta-oxidation. Biol Chem Hoppe Seyler. **372:**613-24.

30 **Neidhardt, F.C., J.L. Ingraham, and M. Schaechter.** 1990. Physiology of the bacterial cell. Sinauer Associates, Inc., Sunderland, MA

31. **Patandin, S., P.C. Dagnelie, P.G.H. Mulder, E. Op de Coul, J.E. van der Veen, N. Weisglas-Kuperus, and P.J.J. Sauer.** 1999. Dietary exposure to polychlorinated biphenyls and dioxins from infancy until adulthood: A

comparison between breast-feeding, toddler, and long-term exposure. Environ. Health Persp. **107:**45-51.

32. **Pellizari, V. H., S. Bezborodnikov, J. F. Quensen III, and J. M. Tiedje.** 1996. Evaluation of strains isolated by growth on naphthalene and biphenyl for hybridization of genes to dioxygenase probes and polychlorinated biphenyl-degrading ability. Appl. Environ. Microbiol. **62:**2053-2058

33. **Poelarends, G.J.** 2001. Genetic adaptation of bacteria to halogenated aliphatic compounds : the recruitment and distribution of dehalogenase genes. Dissertation, University of Groningen.

34. **Quensen, J.F., III, J.M. Tiedje and S.A. Boyd.** 1988. Reductive dechlorination of polychlorinated biphenyls by anaerobic microorganisms from sediment. Science **242:**752-754.

35. **Quensen, J.F., III, S.A. Boyd and J.M. Tiedje.** 1990. Dechlorination of four commercial polychlorinated biphenyl mixtures (Aroclors) by anaerobic microorganisms from sediments. Appl. Environ. Microbiol. **56:**2360-2369.

36. **Ramos, J. L., E. Duque, J-J. Rodriguez-Herva, P. Godoy, P., A. Haidour, F. Reyes, and A. Fernandez-Barrero.** 1997. Mechanisms for solvent tolerance in bacteria. J. Biol. Chem. **272**:3887-3890.

37. **Ramos, J.L., E. Duque, M-T Gallegos, P. Godoy, M.I. Ramos-Gonzalez, A. Rojas, W. Teran, and A. Segura.** 2002. Mechanisms of solvent tolerance in Gram negative bacteria. Ann. Rev. Microbiol. **56**:743-768.

38. **Rodrigues, J.L.M., O.V. Maltseva, T.V. Tsoi, R.R. Helton, J.F. Quensen, III, M. Fukuda and J.M. Tiedje**. 2001. Development of a *Rhodococcus* recombinant strain for degradation of products from anaerobic dechlorination of PCBs. Environ Sci and Technol **35:**663-668.

39 **Rodrigues, J.L.M., C.A. Kachel, M.R. Aiello, J.F. Quensen, O.V. Maltseva, T.V. Tsoi, and J.M. Tiedje.** 2006. Degradation of Aroclor 1242 dechlorination products in sediments by *Burkholderia xenovorans* LB400(*ohb*) and *Rhodococcus* sp. strain RHA1(*fcb*). Appl Environ Microbiol **72:**2476-2482

40. **Seegal R,F,. and S. L. Schantz.** 1994. Neurochemical and behavioral sequelae of exposure to dioxins and PCBs. In Dioxins and Health, A. Schecter, (ed.), Plenum Press, New York, pages 409-447.

41. **Seto, M., N. Okita, K. Sugiyama, E. Masai, and M. Fukuda.** 1996. Growth inhibition of *Rhodococcus* sp. strain RHA1 in the course of PCB transformation. Biotechnol. Lett. **18:**1193-1198.

42. **Sikkema, J., J. A. M. de Bont, and B. Poolman.** 1995. Mechanisms of membrane toxicity of hydrocarbons. Microbiol. Rev. **59:**201-222.

43. **Smithwick, L.A., A. Smith, J.F. Quensen III, A. Stack, L. London, P.J. Morris.** 2003. Inhibition of LPS-induced splenocyte proliferation by ortho-substituted polychlorinated biphenyl congeners. Toxicology 188:319-333.

44. **Sondossi M, Sylvestre M, Ahmad D.** 1992. Effects of chlorobenzoate transformation on the *Pseudomonas tesosteroni* biphenyl and chlorobiphenyl degradation pathway. Appl Environ Microbiol 1992;**58**:485–495.

45. **Swanson, G. M., H. E. Ratcliffe and L. J. Fischer.** 1995. Human exposure to polychlorinated biphenyls (PCBs): A critical assessment of the evidence for adverse health effects. Regulatory Toxicology and Pharmacology **21**: 136-150.

46. **Tsoi, T. V., E.G. Plotnikova, J.R. Cole, W.F. Guerin, M. Bagdasarian, and J.M. Tiedje.** 1999. Cloning, expression and nucleotide sequence of the Pseudomonas aeruginosa strain 142 ohb genes coding for oxygenolytic ortho-dehalogenation of halobenzoates. Appl. and Environ. Microbiol. **65(5):**2151-2162.

47. **Vaillancourt, F.H., S. Han, P.D. Fortin, J.T. Bolin and L.D. Eltis.** 1998. Molecular basis for the stabilization and inhibition of 2,3-dihydroxybiphenyl 1,2-dioxygenase by *t*-butanol. J.Biol. Chem. **273:**34887-34895.

48. **Vaillancourt, F.H., G. Labbe, N. M Drouin, P.D. Fortin, and L.D. Eltis.** 2002. The mechanism-based inactivation of 2,3-dihydroxybiphenyl 1,2-dioxygenase by catecholic substrates. J. Biol. Chem. **277:**2019-2027.

49. **Van der Ploeg, J. M.P. Smidt, A.S. Landa, and D.B. Janssen.** 1994. Identification of chloroacetaldehyde dehydrogenase ivolved in 1,2-dichloroethane degradation. Appl. Environ. Microbiol. **60**:1599-1605.

50. **van Hylckama Vlieg, J. E. T., W. de Koning, and D. B. Janssen.** 1997. Effect of chlorinated ethene conversion on viability and activity of *Methylosinus trichosporium* OB3b. Appl. Environ. Microbiol. **63:**4961-4964.

51. **Yeager, C. M., P. J. Bottomley, and D. J. Arp.** 2001. Cytotoxicity associated with trichloroethylene oxidation in *Burkholderia cepacia* G4. Appl. Environ. Microbiol. **67:**2107-2115.

CHAPTER 4

SUPPORTING CAST OF PCB DEGRADATION: USING TRANSCRIPTIONAL PROFILING TO IDENTIFY GENES INVOLVED IN (POLYCHLORINATED) BIPHENYL DEGRADATION IN *Burkholderia xenovorans* LB400.

ABSTRACT

Over the past three decades, polychlorinated biphenyl (PCB) degradation by aerobic microorganisms has focused almost exclusively on the molecular structure and regulation of the biphenyl pathway. However, efficient degradation relies on more than the biphenyl pathway alone; detoxification of deleterious compounds, secondary regulation and transport of biphenyl are necessary components of optimal PCB degradation. The complete genome sequencing of *Burkholderia xenovorans* LB400 and subsequent transcriptional analyses have provided groundwork for more comprehensive investigation of PCB degradation. This study examines the transcriptional profile of 11 conditions involving three carbon sources, two growth phases and the presence of PCBs. Cluster analysis of transcriptional profiles successfully groups the upper and lower biphenyl pathway including ORF0, a regulator of the lower biphenyl pathway. Additionally, several transport-associated genes and regulatory elements cluster tightly with the biphenyl pathway implicating them in efficient biphenyl degradation. The acetaldehyde dehydrogenase BphJ, responsible for channeling acetaldehyde to pyruvate degradation clusters with a chloroacetaldehyde dehydrogenase, indicating similar function. This study expands (polychlorinated)biphenyl degradation beyond the biphenyl pathway identifying several candidate genes involved in improving efficiency of degradation.

INTRODUCTION

Polychlorinated biphenyls (PCBs) are xenobiotic compounds that have been widely used as industrial products. As a consequence of their physicochemical stability, PCBs have become one of the most important environmental contaminants. Since their ban by the Enrionmental Protection Agency three decades ago, several microorganisms capable of PCB degradation have been isolated and the biodegradation pathways involved have subsequently been characterized (Ahmed, et al. 1990; Furukawa, et al. 1982; Mondello 1989). Most studies have been directed toward organisms that degrade PCBs via the biphenyol pathway. One of the most studied PCB degraders is *Burkholderia xenovorans* LB400 (LB400) because of its ability to degrade highly chlorinated PCB congeners (Bopp 1986; Gibson, et al. 1993; Maltseva et al. 1999; Mondello et al. 1997) and its tolerance to high concentrations of PCBs (Parnell, et al. 2006).

Recent studies (Denef, et al. 2004, 2005, 2006; Parnell, et al. 2006) have shifted away from inspection of the biphenyl pathway in favor of a panoramic view of PCB degradation including auxiliary pathways and mechanisms involved in the improving efficiency of the degradation of biphenyl and PCBs by LB400. PCB degradation has consequently been found to be a dynamic process with variations due to carbon source (Billingsley, et al. 1997) as well as growth phase (Kohler, et al. 1988). While the contributions involving mechanisms and pathways outside the biphenyl pathway have improved my understanding of PCB degradation, conclusions from studies on global physiology, gene and protein expression continue to evolve.

Complete sequencing of the genome of the outstanding PCB dechlorinating and degrading LB400 (http://genome.ornl.gov/microbial/bfun) led us to undertake genomics-

enabled investigation of the biology of PCB degradation. The ~9.7 Mbp genome of LB400 contains 8989 predicted open reading frames (ORFs) in two chromosomes and one megaplasmid, and is among the largest prokaryotic genomes closed to date (Chain, et al. 2006). Using genomic and proteomic approaches, an outline of genome-wide responses to a range of carbon and cell development conditions has been reported (Denef, et al. 2004, 2005, 2006; Parnell, et al. 2006), establishing the groundwork for large-scale genomic analysis of PCB degradation.

As biodegrading microorganisms such as LB400 induce PCB degrading pathways on transition to early stationary phase (due to carbon limitation) expression of genes common to PCB degradation during growth (induced by biphenyl) can be examined. The most vigorous applications of expression analysis over a wide range of conditions is through transcriptional profiling, which entails the analysis of patterns of gene expression encompassing various experiments that examine a broad range of responses to different treatments (Quackenbush 2001). Statistical organization of gene expression patterns (Eisen, et al. 1998) has been used successfully to provide useful information outlining metabolism and global stress responses in other microorganisms (Oh, et al. 2001; Phadtare, et al. 2002; Polen, et al. 2003; Zheng, et al. 2001).

The aim of this study is to analyze gene expression patterns of different carbon sources (benzoate, biphenyl and succinate) and different growth phases [log-phase (ML) and transition phase (TP)] to provide a comprehensive overview of genes involved in PCB degradation and its regulation. By comparing these data with transcriptional data from the degradation of PCBs due to growth in the presence of biphenyl, I present a more comprehensive view of the degradation of PCBs.

MATERIALS AND METHODS

Bacterial strain and genome sequence. The annotated genome sequence of *Burkholderia xenovorans* LB400, originally isolated from a PCB-contaminated landfill (Bopp 1986; Goris, et al. 2004), was produced via a collaborative effort by the Department of Energy's Joint Genome Institute and Oak Ridge National Laboratory's Computational Genomics Group (Chain, et al. 2006). The final draft sequence is publicly available at http://genome.jgi-psf.org/finished_microbes/burfu/burfu.home.html.

Media and growth conditions. LB400 was grown in defined K1 medium (Denef, et al. 2004) in batch cultures supplemented with different carbon sources with and without 500 ppm (v:v) of Aroclor 1242. Batch cultures (25 mL) were prepared in acetone washed, sterilized 125-mL Wheaton® flasks and sealed with Teflon-lined lids. Succinate 1g/L (~10 mM) and benzoate 1g/L (~5 mM) were added to the medium as indicated previously (parnell; denef). Sterile biphenyl 3g/L (~20 mM) was added directly to the sterilized medium in each flask. The PCB-containing cultures were allowed to equilibrate for at least 24 h prior to addition of bacterial inoculum. Batch cultures were inoculated from freshly grown biphenyl adapted culture. Cultures were incubated at 30° C on a rotary shaker at 200 rpm. Growth was monitored spectrophotometrically at OD_{600} nm. Two biological replicates for each treatment (C source, +/- PCBs) were harvested during exponential-phase growth (ML) (at an OD of 0.3-0.4) and during transtition to stationary-phase growth (TP) (OD 0.8-1.0). The experimental setup for the effect of growth substrate and growth phase on PCB degradation is illustrated in Figure 4.1.

RNA extraction and labeling and hybridization. Bacterial cells were harvested by centrifugation at 5000 x g for 10 min at 4° C and resuspended in RNAlater (Ambion) to protect RNA against degradation.. RNA was extracted using RNeasy extraction kit (Qiagen), and remaining DNA was removed by 30 min incubation at ~25° C with 1.5 U DnaseI/μg nucleic acid (Roche). The quality of RNA and removal of DNA was verified by 1.2 % agarose gel electrophoresis. For direct incorporation (succinate vs. early stationary phase biphenyl), RNA was labeled as indicated previously (Denef, et al. 2004). All other hybridization experiments were performed using amino-allyl labeling (Denef, et al. 2004). All solutions were filtered (0.22 μm) for removal of particulate matter from microarray channels. All hybridizations were were performed as described previously (Denef, et al. 2004; Parnell, et al. 2006).

Data analysis. Median signals for each channel (Cy5 and Cy3) were imported into GeneSpring 7.2 (Silicon Genetics) and normalized using Lowess intensity-dependent normalization. Data from treatments benzoate + PCBs vs benzoate and biphenyl + PCBs vs biphenyl (Parnell, et al. 2006) were transformed using treatments benzoate vs succinate and biphenyl vs succinate (Denef, et al. 2004), respectively, in order to reference all treatments to succinate. Error propagation due to data transformation was calculated using the following equation:

$$\frac{\Delta z}{z} = \sqrt{\left(\frac{\Delta x}{x}\right)^2 + \left(\frac{\Delta y}{y}\right)^2 + \ldots}$$

P-values were calculated using GeneSpring's cross gene error model. Principal component analysis was performed on the ten treatments to determine sources of variance. Genes and treatments were clustered using Genespring's hierarchical clustering

algorithm with the similarity metric set for the standard error. Genes were further clustered according to treatments (ie growth phase, carbon source).

RESULTS

Transcriptome analysis of PCB degradation dependent on carbon source and growth phase. I have used whole-genome DNA microarrays with the goal of identifying the genes and pathways in *Burkholderia* xenovorans LB400 involved in PCB degradation. By this method, the reference treatment mRNA levels (cells harvested from succinate ML) and different carbon source, growth phase and PCB degradation were determined for each of the 8429 out of a total of 8989 (94%) genes in LB400. The results from my microarray analyses are expressed as ratios of treatment:reference mRNA levels. My microarray analyses include two components: first, to determine the effect of carbon source on the expression of the biphenyl pathway, and consequent PCB degradation, I included the transcriptional profile of succinate, benzoate and biphenyl (Denef, et al. 2004) for comparison with identical treatments with PCBs (Parnell, et al. 2006) and second, to determine the transition to PCB degradation on consumption of exogenous carbon sources, I analyzed the effect of growth phase (carbon limitation) on PCB degradation (Figure 4.1).

Principal component analysis indicated that nearly 72% of the variance encountered in the treatments was explained by four variables (component 1 = 44.23%, component 2 = 14.78% and component 3 = 12.86%). Subsequent plotting of treatments on a 3-dimensional grid indicates the strongest influence of growth phase, and carbon source on gene expression patterns (Figure 4.2). Hierarchical analysis also indicates that

treatments cluster by growth phase and carbon source rather than presence/absence of PCBs (Figure 4.3).

Clustering of the biphenyl pathway. Microarray analysis and degradation profiles of succinate-grown LB400 with PCBs shows no induction of the biphenyl pathway by PCBs during ML growth. Similarly, benzoate-grown LB400 with PCBs show no induction of the biphenyl pathway compared with benzoate without PCBs. However, on transition to stationary phase, the biphenyl pathway is induced by the PCBs present in the media and degradation does occur (Furukawa, et al. 1983).

The upper biphenyl pathway groups very tightly in hierarchical clustering analysis. BphA, E, F, G, B, C, and D all show similar expression profiles over the 10 treatments (Figure 4.4). Bph J and I, considered lower biphenyl pathway, do not cluster within this group. However, BphJ—an acetaldehyde dehydrogenase in the lower pathway—clusters with a putative chloroacetaldehyde dehydrogenase mentioned in the previous chapter.

DISCUSSION

DNA microarray technology is a useful tool in studying global expression patterns in response to a number of different growth conditions; here, I examine the response of *Burkholderia xenovorans* LB400 to growth substrate- and phase-dependent PCB degradation. This study provides the first information on global expression patterns associated with PCB degradation under growing and carbon-limited conditions (Figure 4.1). Degradation and transcriptional profiles indicate that the regulation of the biphenyl

pathway is controlled by growth substrate (Figures 4.2 and 4.3). In addition, transcriptional profiling across all treatments suggests differential regulation of the upper and lower biphenyl pathway (Figure 4.4). In this study, the combination of transcriptional profiling over a range of treatments has provided a productive approach to obtain a more complete holistic picture of the biodegradation process of PCBs.

Cluster analysis of gene expression patterns allowed us to group genes by function (Eisen, et al 1998). A good indication of the ability of genes to cluster according to function is the tight grouping of the upper biphenyl pathway. The upper biphenyl pathway clustered tightly together and was dissimilar to the expression of the lower biphenyl pathway across the conditions tested (Figure 4.4). While components of the lower biphenyl pathway (BphI and BphJ) are close to the upper biphenyl pathway cluster, the distance suggests differential regulation of the upper and lower pathways as noted previously (Denef, et al 2004). The gene product of *bphR1* (previously ORF0) clustered more closely with the lower biphenyl pathway than with the upper pathway (Figure 4.4). Watanabe, et al. (2003) demonstrate that the *bphR1* gene product in *Pseudomonas pseudoalcaligenes* KF707 is identified as a transcriptional regulator for itself as well as genes in the lower biphenyl pathway. Within the upper biphenyl expression profile cluster are several genes that may influence the biphenyl pathway including transcriptional regulators and several membrane transport proteins as well as genes with unknown function (Figure 4.5). A recent report has indicated that LB400 may utilize active transport in biphenyl catabolism (Master, et al. 2005). The highest expression of the lower biphenyl pathway is during ES growth on biphenyl and ES growth on succinate with PCBs. Close clustering of the acetaldehyde dehydrogenase (BphJ) involved in biphenyl degradation with a putative chloroacetaldehyde dehydrogenase identified

previously (Figure 4.6) (Parnell, et al. 2006) suggests involvement of chloroacetaldehyde dehydrogenase during biphenyl and PCB degradation. Furthermore, the transcriptional profiles of both dehydrogenases are similar to components involved in a PQQ containing alcohol dehydrogenase pathway.

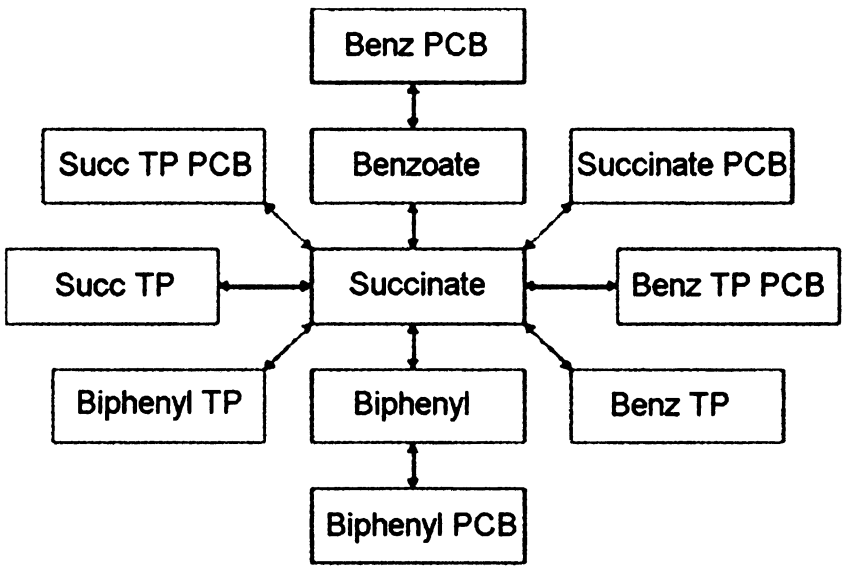


Figure 4.1. Schematic of treatments and comparisons used in this study. All were harvested from mid-logarithmic phase growth unless specified (T.P. indicates transition to early stationary phase).

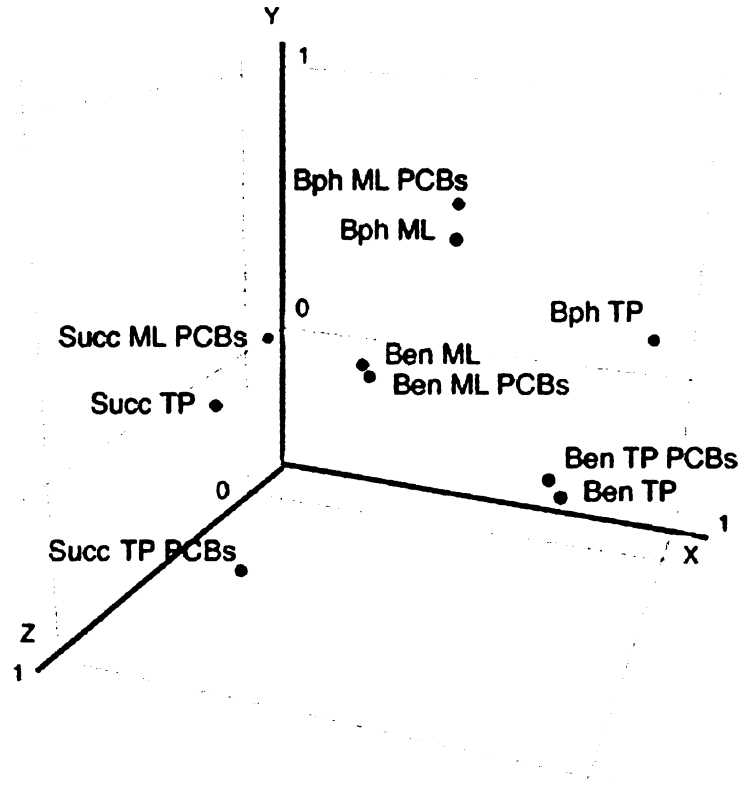


Figure 4.2. Pricipal component analysis of the genome-wide expression pattern of all treatments used in this study.

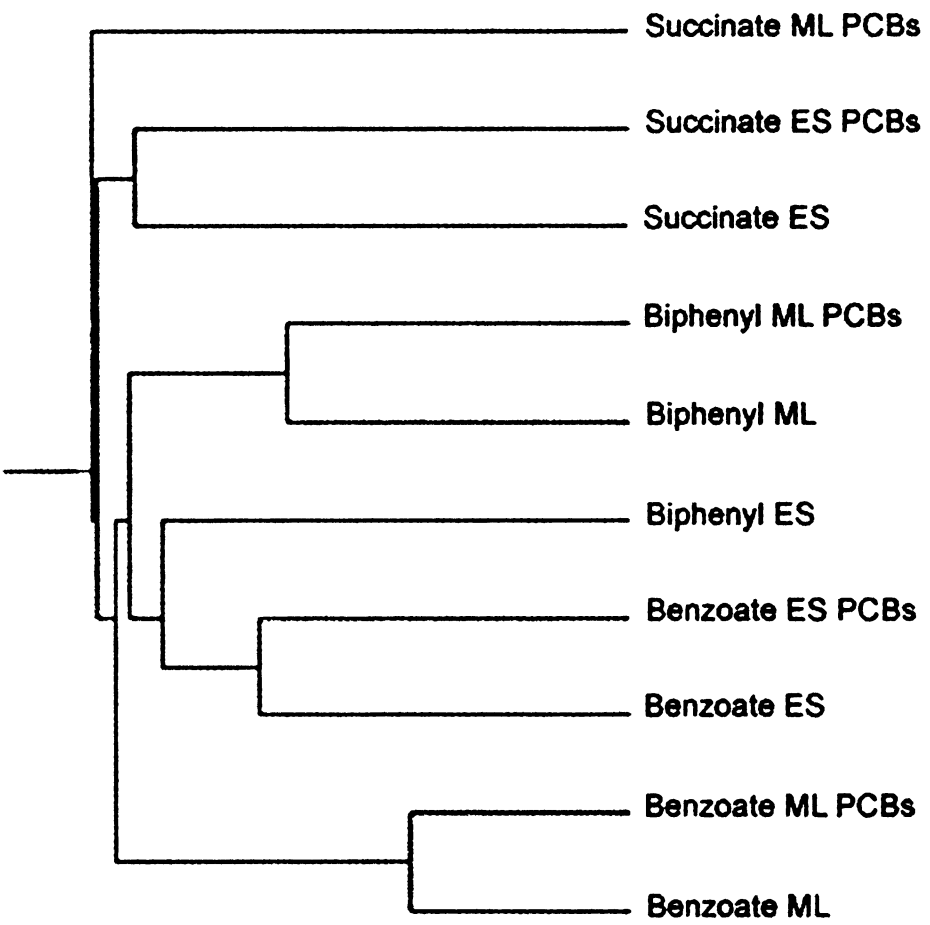


Figure 4.3. Cluster analysis of the total genome-wide expression patterns of all treatments used in this study.

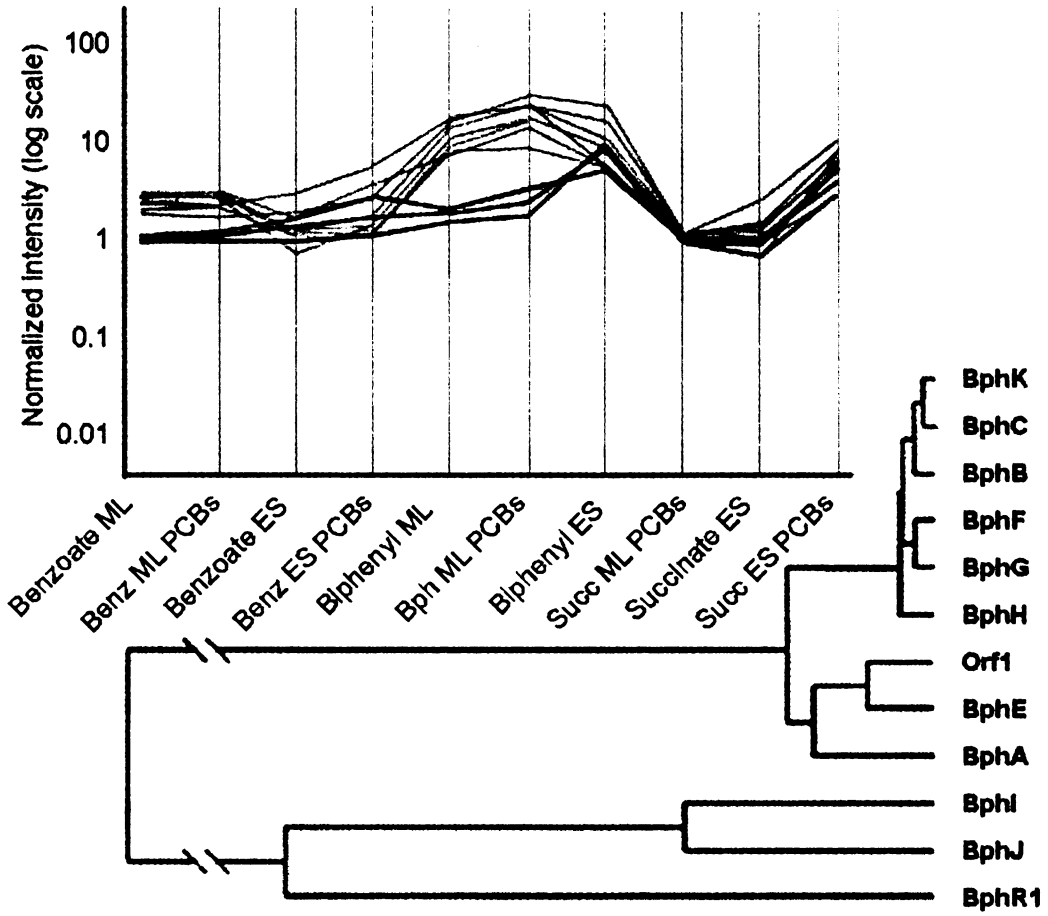
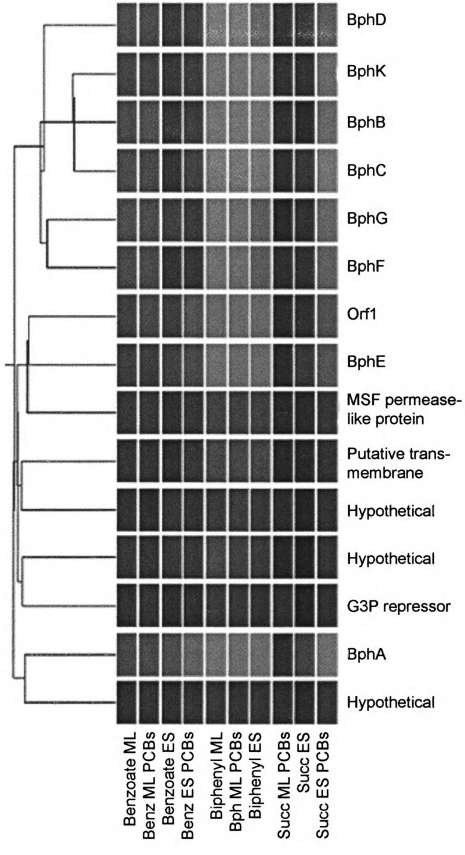


Figure 4.4. Expression profile (y axis) of the biphenyl pathway across all treatments (x axis) (top) and clustered according to their expression profile (bottom).

Figure 4.5. Clade containing the expression profiles for the genes in the upper biphenyl pathway along with other genes that may be involved in (polychlorinated) biphenyl degradation by cluster analysis. Expression of genes in each treatment (x axis) relative to succinate ML. Light = up-regulated, Black = No change in expression.


BphD
BphK
BphB
BphC
BphG
BphF
Orf1
BphE
MSF permease-like protein
Putative trans-membrane
Hypothetical
Hypothetical
G3P repressor
BphA
Hypothetical
Benzoate ML
Benz ML PCBs
Benzoate ES
Benz ES PCBs
Biphenyl ML
Bph ML PCBs
Biphenyl ES
Succ ML PCBs
Succ ES
Succ ES PCBs

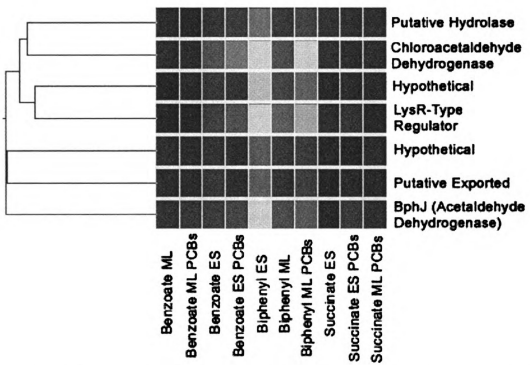


Figure 4.6. The Expression profile for the acetaldehyde dehydrogenase (BphJ) gene in the lower biphenyl pathway clusters with other genes that may be involved in (polychlorinated) biphenyl degradation. Expression of genes in each treatment (x axis) relative to succinate ML. Light = up-regulated, Black = No change in expression.

REFERENCES

Ahmed M., and D.D. Focht. 1973. Degradation of polychlorinated biphenyls by two species of *Achromobacter*. Can J Microbiol. **19:**47–52.

Billingsley K A, Backus S M, Juneson C, Ward O P. 1997. Comparison of the degradation patterns of polychlorinated biphenyl congeners in Aroclors by *Pseudomonas* strain LB400 after growth on various carbon sources. Can. J. Microbiol. **43**:1172–1179.

Bopp L H. Degradation of highly chlorinated PCBs by *Pseudomonas* strain LB400. J Ind Microbiol 1986;**1**:23–29.

Chain, P.S.G., V.J. Denef, K. Konstantinidis, L.M. Vergez, L. Agulló, V.L. Reyes, L. Hauser, M. Córdova, L. Gómez, M. González, M. Land, V. Lao, F. Larimer, J.J. LiPuma, E. Mahenthiralingam, S.A. Malfatti, C.J. Marx, J.J. Parnell, A. Ramette, P. Richardson, M. Seeger, D. Smith, T. Spilker, W-J. Sul, T.V. Tsoi, L.E. Ulrich, I.B. Zhulin, J.M. Tiedje. 2006. *Burkholderia xenovorans* LB400 harbors a multi-replicon, 9.7 M bp genome shaped for versatility. Proc. Natl. Acad. Sci. (Submitted).

Denef, V.J., J. Park, J.L. Rodrigues, T.V. Tsoi, S.A. Hashsham and J.M. Tiedje. 2003. Validation of a more sensitive method for using spotted oligonucleotide DNA microarrays for functional genomics studies on bacterial communities. Environ. Microbiol. **5**(10):933-43.

Denef, V.J., J. Park, T.V. Tsoi, J.-M. Rouillard, H. Zhang, J.A. Wibbenmeyer, W. Verstraete, E. Gulari, S.A. Hashsham, and J.M. Tiedje. 2004. Biphenyl and benzoate metabolism in a genomic context: Outlining genome-wide metabolic networks in *Burkholderia xenovorans* LB400. Appl. Environ. Microbiol. **70:**4961-4970.

Denef, V.J. M.A. Patrauchan, C. Florizone, J. Park, T.V. Tsoi, W. Verstraete, J.M.Tiedje, and L.D. Eltis. 2005. Growth substrate- and phase-specific expression of biphenyl, benzoate, and C_1 metabolic pathways in *Burkholderia xenovorans* LB400. J. Bacteriol. **187:**7996-8005.

Denef, V.J. J.A. Klappenbach, M.A. Patrauchan, C. Florizone, J.L.M. Rodrigues, T.V. Tsoi, W. Verstraete, L.D. Eltis, and J.M. Tiedje. 2006. Genetic and genomic insights into the role of benzoate-catabolic pathway redundancy in *Burkholderia xenovorans* LB400. Appl. Environ. Microbiol. **72:** 585-595.

Eisen, M.B., P. T. Spellman, P. O. Brown and D. Botstein. 1998. Cluster analysis and display of genome-wide expression patterns. Proc. Natl. Acad. Sci. **95:**14863-14868.

Furukawa, K. J.R. Simon, and A.M. Chakrabarty. 1983. Common Induction and regulation of biphenyl, xylene/toluene, and salicylate catabolism in *Pseudomonas paucimobilis*. J. Bacteriol. **154**:1356-1362.

Furukawa, K. 1982. Microbial degradation of polychlorinated biphenyls (PCBs). In: Chakrabarty A M. , editor; Chakrabarty A M. , editor. Biodegradation and detoxification of environmental pollutants. Boca Raton, Fla: CRC Press; pp. 33–56.

Gibson, D. T., D. L. Cruden, J. D. Haddock, G. J. Zylstra, and J. M. Brand. 1993. Oxidation of polychlorinated biphenyls by *Pseudomonas* sp. strain LB400 and *Pseudomonas pseudoalcaligenes* KF707. J. Bacteriol. **175:**4561-4564.

Goris, J., P. De Vos, J. Caballero-Mellado, J. Park, E. Falsen, J.F. Quensen III, J.M. Tiedje, and P. Vandamme. 2004. Classification of the PCB-and biphenyl degrading strain LB400 and relatives as *Burkholderia xenovorans* sp. nov. Int J Syst Evol Microbiol **54**:1677-1681.

Kohler, H.-P.E., D. Kohler-Staub, and D.D. Focht. 1988. Cometabolism of polychlorinated biphenyls: Enhanced transformation of Aroclor 1254 by growing bacterial cells. Appl. Environ. Microbiol. **54:**1940-1945.

Maltseva, O.V., T.V. Tsoi, J.F. Quensen III, M. Fukuda, and J.M. Tiedje. 1999. Degradation of anaerobic reductive dechlorination products of Aroclor 1242 by four aerobic bacteria. Biodegradation. **10:**363-371.

Mondello, F.J. 1989. Cloning and expression in *Escherichia coli* of *Pseudomonas* strain LB400 genes encoding polychlorinated biphenyl degradation. J Bacteriol. **171:**1725–1732.

Mondello, F.J., M.P. Turcich, J.H. Lobos, and B.D. Erickson. 1997. Identification and modification of biphenyl dioxygenase sequences that determine the specificity of polychlorinated biphenyl degradation. Appl. Environ. Microbiol. **63**: 3096-3103.

Oh, M.-K., L. Rohlin, K. C. Kao, and J. C. Liao. 2002. Global Expression Profiling of Acetate-grown Escherichia coli. J. Biol. Chem. **277:**13175 - 13183.

Phadtare, S., Kato, I. and Inouye, M. 2002. DNA microarray analysis of the expression profile of *Escherichia coli* in response to treatment with 4,5-Dihydroxy-2-Cyclopenten-1-One. J. Bacteriol. **84:**6725-6729.

Polen, T., D. Rittmann, V. F. Wendisch, and H. Sahm. 2003. DNA microarray analyses of the long-term adaptive response of *Escherichia coli* to acetate and propionate. Appl. Environ. Microbiol. **69:**1759-1774.

Quackenbush J. 2001. Computational analysis of microarray data. Nat Rev Genet **2:**418–427.

Watanabe, T., H. Fujihara, and K. Furukawa. 2003. Characterization of the second LysRtype regulator in the biphenyl-catabolic gene cluster of *Pseudomonas pseudoalcaligenes* KF707. J. Bacteriol. **185**: 3575-3582.

Zheng, M., X. Wang, L.J. Templeton, D.R. Smulski, R.A. LaRossa, and G. Storz. 2001. DNA microarray-mediated transcriptional profiling of the *Escherichia coli* response to hydrogen peroxide. J. Bacteriol. **183**: 4562-4570.

CHAPTER 5

ENVIRONMENTALLY RELEVANT PARAMETERS AFFECTING PCB DEGRADATION: INVESTIGATION OF THE EXPRESSION OF THE BIPHENYL PATHWAY AND ASSOCIATED GENES IN *Burkholderia xenovorans* LB400

ABSTRACT

The principal means for microbial degradation of polychlorinated biphenyls (PCBs) is through the biphenyl pathway. Although molecular aspects of the regulation of the biphenyl pathway have been studied, information on environmental facets such as the effect of alternative carbon sources on (polychlorinated) biphenyl degradation is limited. The variation in resting cell PCB degradation profiles of *Burkholderia xenovorans* LB400 in relation to conditions prior to PCB exposure (carbon source and growth phase) led us to examine these factors. Genome-wide expression patterns reveals 25 genes commonly up-regulated during PCB degradation and growth on biphenyl in transition to stationary phase including detoxification pathways and nutrient-scavenging systems potentially regulated by sigma factor 54 (σ^{54}). While previous studies have revealed inactivation of the biphenyl pathway on exposure to simple carbon sources and association with σ^{54}, this effect has not been studied in detail until now. Q-RT-PCR analysis of the response of genes in the upper biphenyl pathway (*bphA*, *bphD*, and *bphR1*), σ^{54} and detoxification genes in the LB400 genome indicate similarities between the biphenyl pathway, σ^{54} and chloroacetaldehyde dehydrogenase. The response of genes in the upper biphenyl pathway to carbon source competition and growth phase reveals inhibition of the biphenyl pathway by PCBs. Expression data suggest induction of the biphenyl pathway during growth on succinate without degradation, indicating involvement of post-transcriptional regulation or transport of biphenyl. This information is crucial to understanding PCB degradation in an environmental context as biodegrading bacteria experience a range of carbon sources and growth phases.

INTRODUCTION

Over the past three decades, microbial biodegradation of polychlorinated biphenyls (PCBs) has focused primarily on the molecular regulation and gene structure of the biphenyl pathway fortuitously responsible for the co-metabolism of lower-chlorinated congeners (Ahmed and Focht, 1973). In order for successful *in situ* microbial biodegradation of PCBs to occur; PCBs must be available to biodegrading microorganisms, and PCB-degrading pathways in aerobic bacteria need to be induced (reviewed by Ohtsubo, et al. 2004). The biphenyl degradation pathway responsible for the degradation of PCBs has been analyzed and several studies have identified molecular components key to the regulation of the biphenyl pathway. BphS is a GntR-type repressor (Muoz, et al. 1999) shown in *Pseudomonas* sp. KKS102 to negatively affect the biphenyl pathway (Ohtsubo, et al. 2000). ORF0 is another regulator belonging to the GntR family and was identified as a positive regulator for itself and the biphenyl pathway in *Pseudomonas pseudoalcaligenes* KF707 (Watanabe, et al. 2003) and as a regulator for *bphA1* in *Burkholderia xenovorans* LB400 (Beltrametti, et al. 2001; Denef, et al. 2004, Erickson and Mondello, 1993). In addition to local regulation of the biphenyl pathway by BphS and ORF0, sigma factor 54 (σ^{54}), associated with global regulation in response to various physiological states such as carbon and nitrogen limitation (Kim, et al 1995; Merrick and Stewart, 1985) as well as degradative pathways (Cases and deLorenzo 2000; Cases, et al. 1996; Ehrt, et al. 1994; Sze, et al. 1996), has been indirectly linked to transposon-associated biphenyl degradation in *Ralstonia metallidurans* CH34 (Muoz, et al. 2001).

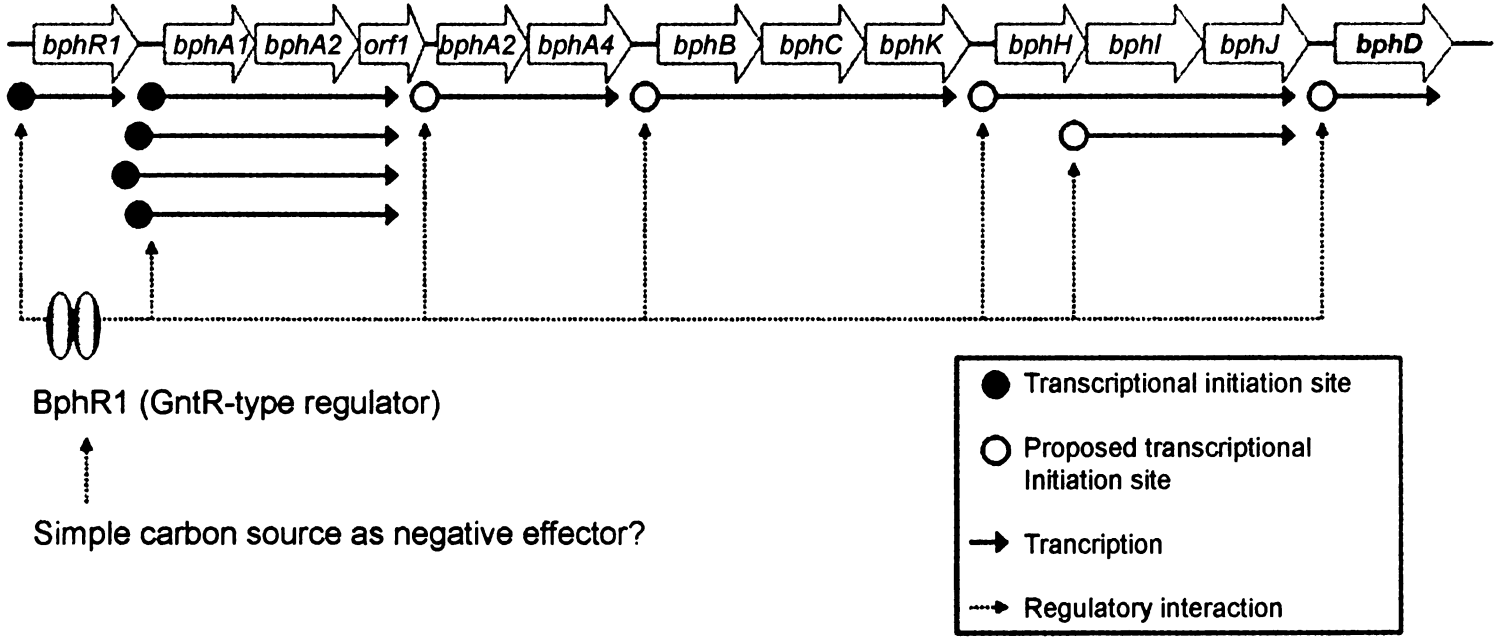
Burkholderia xenovorans LB400 (LB400) (Chain, et al. 2006; Goris, et al. 2004) has become a model organism to study the aerobic biodegradation of PCBs because of

the extremely wide range of congeners oxidized as well as its ability to grow on *ortho+meta*-congeners (Bopp, 1986; Gibson, et al. 1993; Maltseva, et al. 1999; Mondello, et al. 1997). While LB400 has no homolog for BphS, regulation of the biphenyl pathway in LB400 involves BphR1 (formerly ORF0) (Beltrametti, et al. 2001, Denef, et al. 2004; Watanabe, et al. 2003). BphR1 is a positive regulator of the biphenyl pathway in LB400 through a promoter localized upstream of *bphA* and has been identified as a biphenyl-inducible regulator of *bphA* (Beltrametti, et al. 2001, Denef, et al. 2004; Erickson and Mondello, 1993), and possibly *bphD* (Denef, et al. 2004) (Figure 1).

Although molecular mechanisms involved in the regulation of the biphenyl pathway have been described, information on environmental factors affecting PCB degradation is limited. Previous results indicate that the degradation of PCBs does not occur during exponential-phase growth with simple carbon compounds such as glucose, succinate and benzoate (Billingsley, et al. 1997; Furukawa, et al 1983; Parnell, et al. 2006). However, following removal of simple carbon sources (either artificially, as in resting cell conditions, or by natural consumption), PCBs are capable of inducing the biphenyl pathway (Abramowicz, et al. 1993; Ahmed and Focht 1973; Barriault, et al. 1998; Bedard and Quensen 1995; Bedard, et al. 1986, 1987; Gibson, et al. 1993; Seeger, et al. 1995). Although the effect of carbon source on PCB degradation has been studied (Billingsley, et al. 1997), details have been limited to overall degradation profiles.

Most environmental settings where *in situ* bioremediation of PCBs by microorganisms would be most advantageous contain a wide range of naturally-occurring carbon sources at different concentrations and a varied physiological state of the bacteria. This investigation focuses on the expression of the biphenyl pathway and several genes suggested to be co-regulated based on transcriptomic studies from an ecological context

Figure 5.1. Regulatory model for the biphenyl pathway in *Burkholderia xenovorans* LB400. Adapted from Denef, V.J., 2005.


bphR1
bphA1
bphA2
orf1
bphA2
bphA4
bphB
bphC
bphK
bphH
bphI
bphJ
bphD
BphR1 (GntR-type regulator)
Simple carbon source as negative effector?
Transcriptional initiation site
Proposed transcriptional Initiation site
Trancription
Regulatory interaction

of competing carbon sources (succinate, biphenyl and PCBs). Furthermore, we examine the effect of different physiological states (growth phase) on the biphenyl pathway of LB400 and resting cell degradation profiles.

MATERIALS AND METHODS

Media and growth conditions. Growth curve experiments allowed us to determine the effects of simple carbon sources and growth phase (carbon starvation) on the biphenyl pathway in *Burkholderia xenovorans* LB400. We grew triplicate cultures of LB400 in 25 ml mineral medium (K1) with combinations of biphenyl (3 g/L), succinate (1 g/L) and Aroclor 1242 (500 ppm) as described previously (Denef, et al. 2004, Parnell, et al. 2006). Cultures were grown in 125 ml Wheaton® flasks and incubated on an orbital shaker (200 rpm) at 30±2°C. A matrix of all conditions tested is displayed in Table 5.1. The growth rate of LB400 was determined by measuring the maximum slope during logarithmic growth. Cell cultures were harvested at different growth phases—either mid-logarithmic (ML) or transition to stationary-phase (TP) growth—for further analysis (Figure 5.2).

For carbon source utilization, LB400 was grown on biphenyl until early logarithmic growth (OD 0.35) and amended with succinate. Following the addition of succinate, samples were harvested at 5min, 30 min, and 1h for further study.

Degradation of PCBs. Growing cell assays of PCB degradation by LB400 were measured as described previously (Parnell, et al. 2006) in triplicate by disappearance of PCBs from succinate cultures harvested at mid-log (ML), late log, and transition to

Table 5.1. Treatment matrix of carbon competition (row) and growth phase (column) involved in this study. Check marks indicate samples harvested for Q-RT-PCR analysis.

Carbon Sources	ML[†]	ML+5min	ML+30min	ML+1h	TP[°]
Biphenyl	✓	✓	✓	✓	✓
Biphenyl with Succinate[*]		✓	✓	✓	✓
Biphenyl with PCBs	✓				✓
Succinate	✓				✓
Succinate with PCBs	✓				✓
Succinate with Biphenyl	✓				✓

* Succinate added to culture grown on biphenyl to mid-log phase.

[†]ML= Mid-log phase growth (OD 600 nm 0.3-0.4)

[°]TP= Transition to stationary phase growth (OD 600 nm 0.8-1.0)

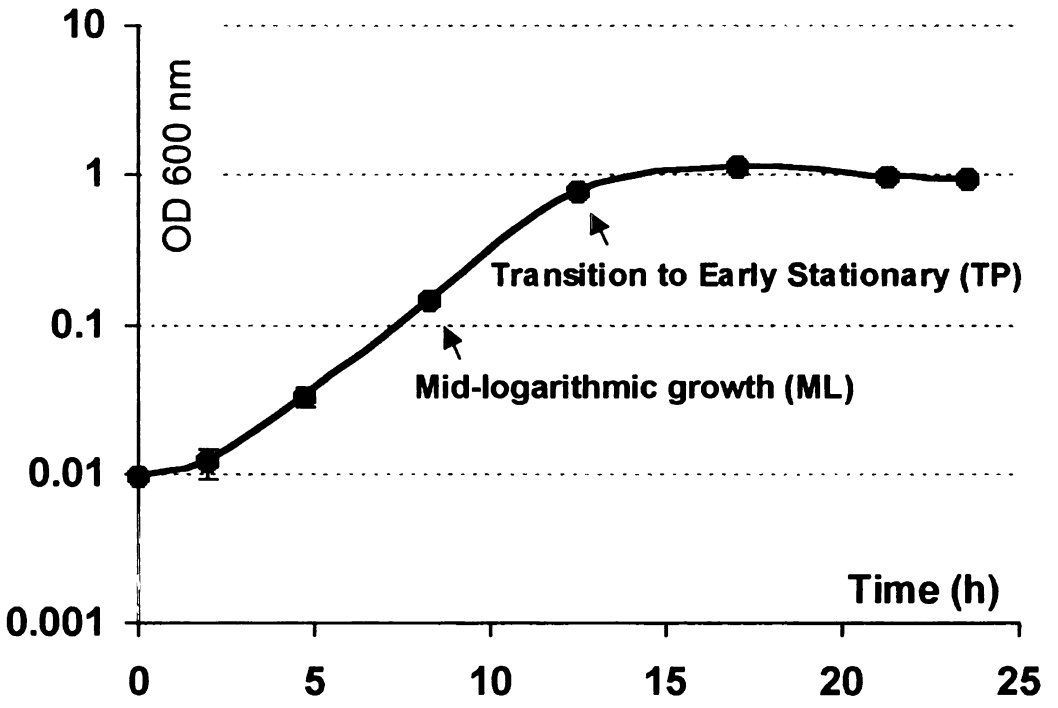


Figure 5.2. Diagram demonstrating sample collection points for growth-phase studies. Mid-logarithmic phase samples (ML) were collected at 0.3-0.4 OD_{600} nm. Early stationary phase samples (TP) were collected at 0.8-1.0 OD_{600} nm.

stationary phase (TP) (0.4, 0.7 and 1.0 OD_{600}, respectively). Each PCB-containing treatment was extracted three times using an equal volume of 1:1 hexane:acetone solution by shaking for 30 min. and analyzed by gas chromatography as described previously (Quensen, et al. 1990). Controls were performed by extraction from uninoculated media. Percent degradation was calculated as described earlier (Parnell, et al. 2006).

Resting cell PCB degradation profiles by LB400 were determined from cultures grown on succinate and biphenyl harvested from ML and from TP by centrifugation and rinsed to remove exogenous carbon. Resting cell assays were measured in triplicate using traditional methods described previously (Barriault, et al. 1998), except for growth phase. Each chlorobiphenyl congener used in resting cell assays was at a final concentration of 2 ppm and a control consisting of heat-inactivated cells was run in parallel.

cDNA preparation. RNAlater (Ambion) was added in a 1:1 ratio to the culture to protect RNA against degradation. Bacterial cells were harvested by centrifugation at 5000 x g for 10 min at 4 °C. RNA was extracted using RNeasy RNA extraction kit (Qiagen), and remaining DNA was removed by 30 min incubation at room temperature with 1.5 U DnaseI/µg nucleic acid (Roche, 10 U/µl). Integrity of RNA and absence of DNA was verified by 1.2 % agarose gel electrophoresis. 2 µg of total RNA was incubated overnight at 45 °C using 6 µg random primers (Invitrogen), and Superscript II reverse transcriptase (Invitrogen). Purification was performed using the QiaQuick PCR purification kit (Qiagen). Total cDNA concentration was determined spectrophotometrically (Nanodrop® Wilmington, DE).

Q-RT-PCR analysis. Primers for *bphA*, *bphD*, *bphR1* and two potential σ^{54} transcripts were designed using Primer Express software (Applied Biosystems, Foster City, CA). Triplicate Q-RT-PCR runs were performed for *bphA*, *bphD*, *bphR1*, and each σ^{54}. One ng of cDNA from each condition was utilized in triplicate for 40 cycle, two step PCR in an ABI 7900HT (Applied Biosystems, Foster City, CA) using SYBR Green Master Mix (Applied Biosystems, Foster City, CA) and 125 nM of each primer (Table 5.2). Amplicon size (80-100 bps) and reaction specificity were confirmed by agarose gel electrophoresis and product dissociation curves. The number of target copies in each sample was interpolated from its detection threshold (C_T) value using a purified PCR product standard curve. 16S rRNA expression was measured as internal control, and the measured internal control signal was used to normalize variations due to different reverse transcription efficiency (Denef, et al. 2004). In addition, Q-RT-PCR of the σ^{70} transcript was used for relative comparison to σ^{54}. All conditions were normalized to succinate-grown cells harvested during ML growth.

RESULTS

Effect of growth phase on PCB degradation. Growing cell assays for PCB degradation of LB400 with succinate demonstrate a growth phase-dependent shift to PCB degradation (Figure 5.3). Early growth (0.4 OD) indicates little degradation of

Table 5.2. Primers used for Q-RT-PCR analysis in this study including biphenyl pathway elements detoxification pathway genes sigma-70 and both sigma-54 factors (sc = small chromosome BxeB1172, c = large chromosome BxeA4122).

Primer	Sequence
bphA-f	ggctacgtgggtacaaggc
bphA-r	tagccgacgttgccagg
bphD-f	cgcactcaccgaaagttctac
bphD-r	ttacccgcctcgttgtagtg
bphR1-f	gtcagttcgtatcaccggc
bphR1-r	ccactgattgaacaagtgaacc
σ^{70}-f	gccagatccaacaggaagc
σ^{70}-r	ttccgaacaccgttggagg
σ^{54}-sc-f	tcgcgaaatatcgtgaagc
σ^{54}-sc-r	cttgcgcaggttgactgcc
σ^{54}-c-f	tcgcggacagtgtcgatctc
σ^{54}-c-r	accagcaactcgtcttcggtc
ClAc-f	atcgccaagattgcgtttac
ClAc-r	gtacacacctcgccctgatt
xoxF-f	aaggcaggtgaaaccaacac
xoxF-r	catacgtcgtcgtcttgtcg
Fae-f	aacaaggtcaccatcaaggg
Fae-r	ggaccgaacatctgcacc

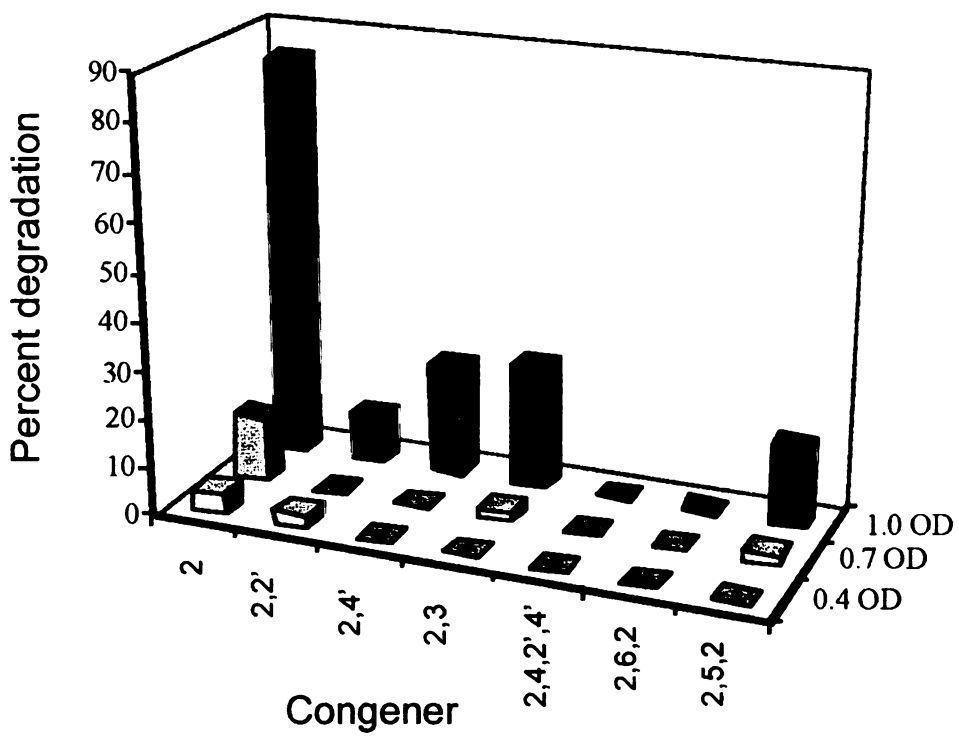


Figure 5.3. Percent degradation of PCBs during growth on Succinate (y axis). Specific PCB congeners degraded (x axis) throughout different growth phases in the growth curve (z axis).

PCBs; primarily degradation consists of congeners with a chlorine-free ring. Although some degradation of higher chlorinated PCBs are evident in later logarithmic phase (0.7 OD), the greatest amount of PCB degradation occurs as the initial growth substrate is consumed on transition to stationary phase (1.0 OD) where cells are still growing, but likely in a state of carbon limitation.

Based on resting cell PCB degradation assays, the most significant change in degradation profile was revealed in LB400 harvested from TP biphenyl-grown cells. The increased degradation was generally observed for di-para substituted PCBs (Table 5.3). Ortho substituted congeners appear readily degraded on removal of exogenous carbon consistent with growing cell data while highly chlorinated para-substituted congeners such as 2,4,6,2'4'6'-hexachlorobiphenyl show no indication of degradation.

Transcriptional profile comparison. Using genome-wide expression data from earlier studies (Denef, et al. 2004; Parnell, et al. 2006), a comparison of the gene expression patterns from biphenyl transition-phase and degradation of PCBs during growth on biphenyl indicate the induction of several common genes and pathways (Figure 5.4). Of the 114 genes up-regulated during PCB degradation (ML growth on biphenyl with PCBs) with reference to succinate ML, 49 (43%) were also induced during TP growth on biphenyl. Of the 49 genes commonly induced during PCB degradation and TP growth on biphenyl, 25 were unique relative to biphenyl ML growth (Table 5.4), including several genes involved in C_1 degradation and a putative chloroacetaldehyde dehydrogenase. Several of the genes induced with

Table 5.3. Percent degradation of PCB congeners from resting cell assays of LB400 harvested during exponential-phase (ML) growth and transition into stationary-phase (TP).

PCB Congener	Succinate ML	Succinate TP	Biphenyl ML	Biphenyl TP
23-	100	95	100	100
2-4-	100	100	100	100
2-2-	100	98	100	100
4-4-	11	1	ND	44
24-4-	24	3	13	85
25-2-	100	97	100	100
25-4-	100	95	100	100
25-25-	100	100	100	99
23-25-	100	99	98	99
23-23-	98	97	99	96
24-24-	2	ND	24	89
25-34-	91	85	96	100
24-34-	11	3	ND	55
34-34-	ND	ND	ND	8
245-23-	21	5	1	61
245-245-	ND	ND	ND	29
246-246-	ND	ND	ND	ND
245-25-	85	80	91	98
234-25-	46	23	45	95
245-245-	7	3	4	33

ND- No degradation detected

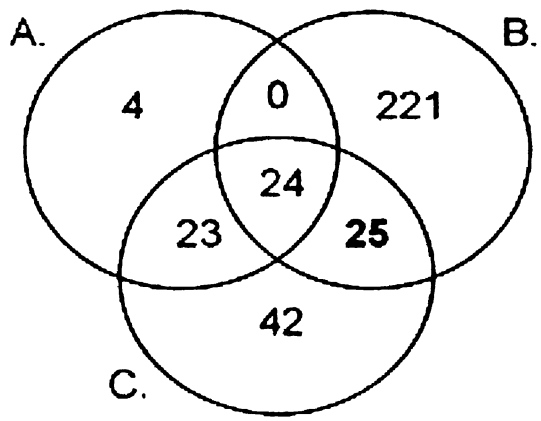


Figure 5.4. Venn diagram of up-regulated genes from *Burkholderia xenovorans* LB400 grown on biphenyl (A) harvested during ML growth, (B) harvested during TP growth, and (C) grown with biphenyl and 500 ppm Aroclor 1242 and harvested during ML growth (all relative to succinate ML growth). Bold indicates genes induced during conditions most favorable for PCB degradation.

Table 5.4. Genes identified through genome-wide microarray analyses commonly induced during growth on biphenyl transition to stationary-phase (TP) and exposure to PCBs during mid-log (ML PCBs) divergent from genes induced during growth on biphenyl alone (ML). Highlighted genes indicate previously identified detoxification pathways that may be involved in PCB degradation.

[a] Expression ratio is reported relative to LB400 grown on succinate and harvested during ML growth.

[b] Data from Parnell, et al. 2006.

[c] Data from Denef, et al. 2004.

[e] Formaldehyde detoxification pathway characterized by Marx, et al. 2004.

Gene ID	Annotation	Associated Function	Expression Ratio[a] ML[c]	ML PCBs[b]	TP[c]
BxeC0962	Hemerythrin-like metal binding protein	Unknown	1.6	2.5	5.3
BxeC1188	(BphJ) Semialdehyde dehydrogenase	Biphenyl Pathway	1.8	2.2	6
BxeB1679	Putative transport-associated protein	Unknown	1.5	2.8	12.9
BxeB2425	**MxaJ-like solute binding protein**	**Formaldenyde Detoxification[d]**	1.4	2.3	12
BxeB2426	**Cytochrome c-555 precursor**	**Formaldenyde Detoxification**	1	4.8	42.3
BxeB2427	**(XoxF) Methanol dehydrogenase-like protein**	**Formaldenyde Detoxification**	0.9	8	47.1
BxeB2436	**(Fae) Formaldehyde activating enzyme**	**Formaldenyde Detoxification**	1.3	20.7	160.3
BxeB2437	**Conserved hypothetical protein**	**Formaldenyde Detoxification**	1.1	5.8	66.8
BxeB2470	**Coenzyme PQQ synthesis protein E**	**Formaldenyde Detoxification**	1.2	2.2	7
BxeB2473	**TonB-dependent receptor precursor**	**Formaldenyde Detoxification**	0.9	2.8	14.8
BxeB2474	**Conserved hypothetical protein**	**Formaldenyde Detoxification**	0.9	3	22
BxeB2469	**Coenzyme PQQ synthesis protein D**	**Formaldenyde Detoxification**	1.1	2.6	9.7
BxeA0355	Conserved hypothetical protein	Unknown	1.1	2.1	5.9
BxeA1028	Putative exported protein	Unknown	2	2.7	4
BxeA1129	Chloromuconate cycloisomerase	Chlorobenzoate Degradation	1	2.6	2.2
BxeA1130	Chlorocatechol 1,2-dioxygenase	Chlorobenzoate Degradation	1.1	3.1	4.6
BxeA1629	Putative sugar ABC transport, substrate-binding protein	Unknown	1.8	6.7	2.3
BxeA1994	Putative Cyd operon protein YbgT	Unknown	1.3	2.2	2.3
BxeA2773	Regulatory protein, TetR	Unknown	1.6	2.6	4.3
BxeA2801	Putative peptidase A24A, prepilin type IV precursor	Unknown	1.7	2.1	3.8
BxeA2944	Conserved hypothetical protein	Unknown	1.4	2.6	2.9
BxeA3322	Putative membrane protein	Unknown	1.8	2.2	2.7
BxeA4441	**(Chloro)actetaldehyde dehydrogenase**	**Chloroacetaldehyde Detoxification[b]**	1.8	8.1	34.3
BxeA4442	**Conserved hypothetical protein**	**Chloroacetaldehyde Detoxification**	1	2.2	7.3
BxeA4478	Conserved hypothetical protein	Unknown	1.2	3.5	39.1

PCB degradation and TP biphenyl-grown conditions were hypothesized regulated by σ^{54} (Denef, et al. 2004).

Q-RT-PCR expression analysis. Samples harvested from various points along the growth curve give a representation of the induction of the biphenyl pathway according to carbon source competition as well as growth stage. During growth on succinate with PCBs, the *bphA* and *bphD* are down-regulated compared to growth on succinate alone (0.6±0.2 and 0.3±0.2, respectively). LB400 grown with succinate and biphenyl concurrently demonstrate an increased expression in the biphenyl pathway as compared to succinate alone during both ML- and TP-growth as *bphA* expression is 4.3±1.8- and 9.7±1.8-fold increased and *bphD* is 2.8±1.7- and 4.0±2.1-fold increased (Table 5.5).

Regression analysis of the expression patterns gives an indication of the association of different genes with carbon source and growth phase. As expected, the biphenyl pathway elements (*bphA*, *bphD* and *bphR1*) are all statistically correlated. In addition, σ^{70}, the σ^{54} located on the chromosome (σ^{54}c) and the putative chloroacetaldehyde dehydrogenase gene (ClAc) are correlated with the biphenyl pathway (Table 5.6).

Carbon source competition. Growth rates of LB400 on a combination of carbon sources indicate primary substrate utilization. Maximum growth rates were calculated for growth on succinate (0.42±0.03 cell divisions/h), succinate and biphenyl (0.41±0.02 cell divisions/h), succinate and PCBs (0.42±0.05 cell

Table 5.5. Expression values from quantitative RT-PCR analysis demonstrating the effect of different carbon sources [succinate (Succ), biphenyl (Bph), and polychlorinated biphenyls (PCB)] and different growth phases [Mid-Logarithmic (ML) and Transition to stationary-phase (TP)] on biphenyl pathway components, regulatory elements, and detoxification genes potentially involved in biphenyl degradation. Expression values are reported relative to succinate-grown *Burkholderia xenovorans* LB400 harvested from mid-logarithmic growth (Succ ML).

Treatment	*bphA*	*bphD*	*bphR1*	σ^{70}	σ^{54}sc	σ^{54}c	Fae	*xoxF*	ClAc
Succ TP	1.3±1.0	1.9±1.4	0.8±0.1	1.5±1.0	1.3±0.4	1.0±0.5	1.2±0.3	1.2±0.3	0.4±0.4
Succ PCB ML	0.6±0.2	0.3±0.2	1.4±0.1	1.6±0.1	1.2±0.4	0.5±0.2	0.8±0.1	1.2±0.3	1.3±0.5
Succ PCB TP	2.1±1.0	1.1±0.6	1.0±0.2	1.2±0.6	0.5±0.3	0.9±0.3	7.2±2.3	2.0±1.6	6.9±2.4
Succ Bph ML	4.3±1.8	2.8±1.7	4.4±1.7	1.7±0.3	1.6±0.9	0.6±0.3	1.1±1.0	3.6±1.4	10.8±5.5
Succ Bph TP	9.7±1.8	4.0±2.1	6.6±1.4	1.5±0.2	1.0±0.6	2.4±0.1	10.0±3.3	6.0±2.4	15.7±6.2
Bph Succ ML+5min	19.7±6.1	20.0±4.2	8.9±1.6	3.2±0.3	1.2±0.2	0.6±0.4	2.5±1.0	0.8±0.4	410±130
Bph Succ ML+30min	5.1±0.2	17.3±6.7	6.5±0.9	1.8±0.8	0.7±0.2	0.6±0.8	2.0±0.6	0.8±0.6	59.0±18.4
Bph Succ ML+1h	6.0±1.2	8.2±2.0	6.7±0.7	1.8±0.3	0.4±0.1	1.4±0.7	2.3±2.1	0.7±0.2	21.6±11.4
Bph ML+5min	24.2±2.7	32.2±2.5	14.5±0.3	3.2±0.6	6.3±4.6	2.7±0.8	2.4±1.7	6.0±2.1	391±181
Bph ML+30min	12.5±5.6	22.5±6.7	15.6±0.9	3.1±0.8	2.6±1.6	1.7±0.8	17.5±5.2	8.2±1.6	435±140
Bph ML+1h	13.6±7.7	26.4±2.0	11.9±0.7	2.9±0.7	1.1±0.3	2.1±0.2	63.2±15.8	12.4±3.7	306±118
Bph PCB ML	26.2±4.9	27.0±3.2	17.5±2.1	3.1±0.8	3.5±1.8	1.4±0.4	9.5±2.0	5.4±0.5	356±123
Bph PCB TP	14.4±3.1	34.5±7.2	8.0±2.3	1.4±0.5	2.2±1.7	2.2±0.7	70.1±21.2	9.1±2.0	251±75.8

Table 5.6. Correlation matrix of R^2 values from regression analysis of the expression of components of the biphenyl pathway, its potential regulators, and detoxification pathway genes possibly associated with biphenyl degradation in *Burkholderia xenovorans* LB400. Quantitative RT-PCR expression data were converted to Log_2 values prior to regression analysis. Significant p-values (<0.05) are bold.

	bphA	*bphD*	*bphR1*	σ^{70}	σ^{54}sc	σ^{54}c	Fae	*xoxF*	ClAc
bphA	-	**0.84**	**0.84**	**0.48**	0.25	**0.36**	0.30	0.27	**0.85**
bphD		-	**0.77**	**0.44**	0.20	**0.30**	**0.31**	0.19	**0.82**
bphR1			-	**0.58**	0.25	0.27	0.23	0.26	**0.84**
σ^{70}				-	**0.33**	0.06	0.02	0.08	**0.55**
σ^{54}sc					-	0.16	0.02	**0.36**	0.22
σ^{54}c						-	**0.46**	**0.54**	0.21
Fae							-	**0.57**	**0.60**
xoxF								-	0.19

divisions/h), biphenyl (0.22±0.01 cell divisions/h), and biphenyl with PCBs (0.20±0.02 cell divisions/h). In addition, the growth rate of LB400 grown on biphenyl to ML following the addition of succinate was calculated (0.37±0.02 cell divisions/h) (Figure 5.5).

Within 5min of addition of succinate to biphenyl-grown culture during ML phase, the expression of *bphD* decreases significantly (from 32.3±2.5- to 20.0±4.2-fold induction relative to succinate ML). Similarly, *bphR1* expression decreases significantly (from 14.5±0.5- to 8.9±1.6-fold induction) within 5 min of succinate addition. The expression level of *bphA* does not show significant decrease until within 30 min of succinate addition (12.5±5.5- to 5.6±0.2-fold induction) (Figure 5.6).

DISCUSSION

This study places (polychlorinated) biphenyl degradation in a context relevant to environmental scenarios by studying factors such as carbon source competition and growth phase effects on expression of the biphenyl pathway in LB400. Billingsley, et al. (1997) reported that LB400 conditioned to biphenyl growth results in more efficient PCB degradation than LB400 grown on other sources (Billingsley, et al. 1997). These results are somewhat expected as the physiological state of LB400 grown on biphenyl is more comparable to PCB-degrading conditions. As co-metabolism of PCBs relies on removal of all exogenous carbon, energy becomes a limiting factor and dynamic changes in gene expression patterns (such as a switch from a dissimilar growth substrate like succinate) require energy that could otherwise be allocated to PCB degradation.

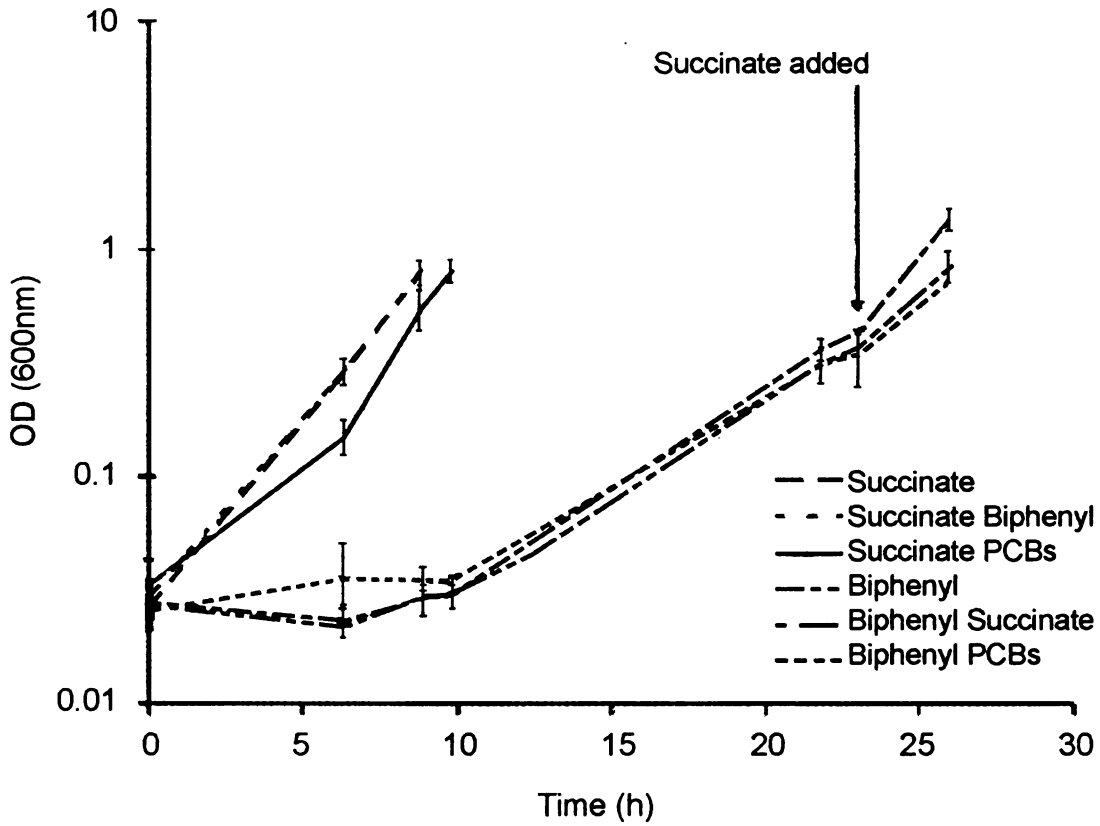
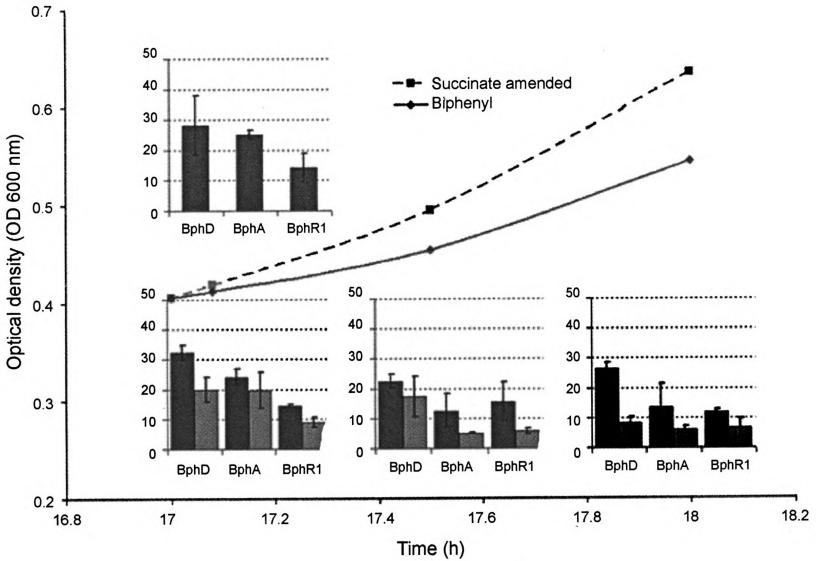


Figure 5.5. Growth curves of *Burkholderia xenovorans* LB400 grown with succinate (1g/L), biphenyl (3g/L) and PCBs (500 ppm v:v Aroclor 1242) in K1 mineral medium. Succinate was added to biphenyl-grown culture during mid-logarithmic growth.

Figure 5.6. Expression of genes (*bphA* and *bphD*) and the regulator (*bphR1*) involved in the biphenyl degradation pathway relative to succinate mid-log growth (columns) and growth curves (OD_{600}nm) of LB400 grown on biphenyl to mid-log growth (blue) and amended with succinate (green) (lines). Data collected at 0 min (A), 5 min (B), 30 min (C) and 1h (D) following addition of succinate.


Optical density (OD 600 nm)
Time (h)
Succinate amended
Biphenyl
BphD
BphA
BphR1

The effect of energy availability on PCB degradation was demonstrated by Kohler, et al. (1988) by comparing growing cell assays with resting cell assays (both grown on biphenyl). Not only did they find an increase in the magnitude of PCB degradation, but the range of congeners degraded also increased in growing cells (Kohler, et al. 1988). They concluded that PCB degradation during growth on biphenyl affords more physiological stability leading to more active cells (Kohler, et al. 1988). Similarly, resting cell assays presented here of cells harvested from different carbon sources (succinate and biphenyl) and growth phases (ML and TP) demonstrate a physiologically-mediated range in PCB degradation (Table 5.2). These findings in conjunction with recent genomic (Denef, et al. 2004, Parnell, et al. 2006) and proteomic analyses (Denef, et al. 2005) suggest the activation of mechanisms beyond the biphenyl pathway that provide stability to LB400 and improve PCB degradation during TP growth on biphenyl.

Transcriptional analysis of active PCB degradation (during growth on biphenyl) and of TP biphenyl cells (condition harvested for resting cells that included greatest PCB degradation) identified 25 potential genes involved in efficient degradation of PCBs (Table 5.3). Many of those genes such as motility-associated genes (Jagannathan, et al. 2001) oxygen scavenging and C_1 pathway genes are potentially subject to regulation by σ^{54} (Denef, et al. 2004). In addition, two pathways induced during PCB degradation and biphenyl TP growth have potential for detoxification of damaging compounds. Marx, et al (2004) identified the C_1 pathway in LB400 as responsible for removal of formaldehyde (27). Additionally, chloroacetaldehyde dehydrogenase may eliminate toxic products of incomplete PCB degradation (Parnell, et al. 2006). Induction of these pathways prior to exposure to increased levels of formaldehyde- or chloroacetaldehyde-like compounds

would impart an enormous advantage toward physiological stability of resting cell conditions and improve PCB degradation.

In order to determine the association of both of the σ^{54} factor homologs in LB400 and the chloroacetaldehyde and formaldehyde degradation pathways with biphenyl degradation, we examined expression of these pathways across a range of carbon sources and growth phases. Of the two σ^{54} homologs in LB400, one (BxeB1172) is located on the small chromosome (σ^{54}sc) while the other (BxeA4122) is found on the large chromosome (σ^{54}c); only the σ^{54}c was found in an operon structure similar to previous documentation (Powell, et al. 1995). Regression analysis of the expression patterns indicate that the expression of σ^{70}, the σ^{54}c, and the chloroacetaldehyde dehydrogenase are all significantly correlated to expression of the biphenyl pathway. Conversely, the expression of elements of the C_1 pathway (XoxF and Fae) is not strongly correlated to the induction of biphenyl pathway genes, although Fae and *bphD* expression is significant (Table 5.6).

Despite previous studies indicating that the biphenyl pathway (degradation of PCBs) is not induced (and possibly repressed) during growth on simple carbon compounds (Billinglsley, et al. 1997, Denef, et al. 2004, 2005; Furukawa, et al. 1983; Parnell, et al. 2006), information on the effect of simple carbon sources on the induction of the biphenyl pathway is cursory. Carbon source utilization analysis indicates that despite an extended lag phase for adaptation, the eventual maximum growth rate for LB400 grown on both succinate and biphenyl is identical to growth on succinate alone. Addition of succinate to biphenyl-grown ML cultures changes the growth rate of LB400 from biphenyl- to succinate-like growth rates, suggesting a switch to succinate as carbon

source and possible catabolite repression (Figure 5.6). In addition, compared to biphenyl treatments, the expression of *bphD* and *bphR1* in succinate amended treatments declines significantly (within 5 min). By 30 minutes, expression of *bphA* has decreased significantly in succinate amended treatments (Figure 5.6). By 1h (Bph Succ ML+1h), the expression level of all three components of the biphenyl pathway examined have diminished to levels similar to succinate with biphenyl (Succ Bph ES) (Table 5.6). This information indicates that succinate does play a role in repression or inactivation of the biphenyl pathway. Additional evidence presented in this study, in effect suggests that PCB degradation does not occur during growth on succinate until transition to stationary-phase growth where succinate becomes limited (Figure 5.3).

On the surface, this evidence appears to contradict previous work by Master and Mohn (2001) indicating constitutive expression of the biphenyl pathway in LB400 during growth on simple carbon sources (Master and Mohn, 2001). However, this study clarifies this paradox by evaluating the expression of the biphenyl pathway during growth on multiple carbon compounds and during different physiological states using Q-RT-PCR analysis. Q-RT-PCR analyses indicate that genes in the biphenyl pathway (*bphA*, *bphD* and *bphR1*) are induced during growth on succinate with biphenyl (Table 5.5) as compared to succinate only. In addition, during growth on succinate with PCBs, the biphenyl pathway is down-regulated indicating some level of expression of the biphenyl pathway during growth on succinate and repression of the biphenyl pathway by PCBs. Although the biphenyl pathway is expressed during growth on simpler carbon compounds such as succinate, little degradation occurs. This data suggests additional elements possibly involved post-transcriptional regulation or that the active transport of

biphenyl in LB400 as reported by Master, et al. (2005) play a more crucial role in biphenyl degradation than previously thought (Master, et al. 2005).

This work has shown for the first time a repression of the biphenyl pathway in LB400 by PCBs during growth with a simple carbon source such as succinate. As many environmental sites contaminated with PCBs contain a range of carbon sources, this becomes an important concern for *in situ* PCB degradation. Furthermore, although biphenyl appears not to be utilized during growth on succinate with biphenyl, the biphenyl pathway is expressed, suggesting post-transcriptional regulation or active transport involved in biphenyl degradation (Master, et al. 2005). While LB400 has two σ^{54} factor homologs (σ^{54}sc and σ^{54}c), only the σ^{54}c is associated with expression of the biphenyl pathway. Additionally, the expression of the chloroacetaldehyde dehydrogenase correlates to expression of the biphenyl pathway in LB400, suggesting peripheral involvement of the chloroacetaldehyde dehydrogenase in biphenyl (and PCB) degradation. This work identifies genes associated with the biphenyl pathway in imparting stability to LB400 during PCB degradation and fills a gap in the knowledge of the activation of the biphenyl pathway under competing carbon sources and the effect PCBs may have on the biphenyl pathway in environmental scenarios.

REFERENCES

Abramowicz, D.A., M.J. Brennan, H.M. Van Dort, and F.L. Gallagher. 1993. Factors influencing the rate of polychlorinated biphenyl degradation in Hudson River sediments. Environ. Sci. Technol. **27:**1125–1131

Ahmed M., and D.D. Focht. 1973. Degradation of polychlorinated biphenyls by two species of *Achromobacter*. Can. J. Microbiol. **19:**47–52.

Barriault, D., J. Durand, H. Maaroufi, L.D. Eltis, and M. Sylvestre. 1998. Degradation of polychlorinated biphenyl metabolites by naphthalene-catabolizing enzymes. Appl. Environ. Microbiol. **64:**4637-4642.

Bedard, D. L., and J. F. Quensen, III. 1995. Microbial reductive dechlorination of polychlorinated biphenyls, p. 127-216. *In* L. Y. Young, and C. Cerniglia (ed.), Microbial transformation and degradation of toxic organic chemicals. Wiley-Liss Division, John Wiley & Sons, Inc., New York, N.Y.

Bedard, D.L., M.L. Haberl, R.J. May, and M.J. Brennan. 1987. Evidence for novel mechanisms of polychlorinated biphenyl metabolism in Alcaligenes eutrophus H850. Appl. Environ. Microbiol. **53:**1103–1112.

Bedard, D.L., R. Unterman, L.H. Bopp, M.J. Brennan, M.L. Haberl, and C. Johnson. 1986. Rapid assay for screening and characterizing microorganisms for the ability to degrade polychlorinated biphenyls. Appl. Environ. Microbiol. **51:**761–768.

Beltrametti, F., D. Reniero, S. Backhaus, and B. Hofer. 2001. Analysis of transcription of the *bph* locus of *Burkholderia* sp. strain LB400 and evidence that the ORF0 gene product acts as a regulator of the *bphA1* promotor. Microbiology **147**: 2169-2182.

Billingsley K A, Backus S M, Juneson C, Ward O P. 1997. Comparison of the degradation patterns of polychlorinated biphenyl congeners in Aroclors by *Pseudomonas* strain LB400 after growth on various carbon sources. Can. J. Microbiol. **43**:1172–1179.

Bopp L H. Degradation of highly chlorinated PCBs by *Pseudomonas* strain LB400. J. Ind. Microbiol. 1986;**1**:23–29.

Cases, I., and V. de Lorenzo. 2000. Genetic evidence of distinct physiological regulation mechanisms in the σ^{54} *Pu* promoter of *Pseudomonas putida*. J. Bacteriol. **182:**956-960.

Cases, I., J. Perez-Martin, and V. de Lorenzo. 1996. Involvement of σ^{54} in exponential silencing of the *Pseudomonas putida* TOL plasmid *Pu* promoter. Mol. Microbiol. **19:**7-17.

Chain, P.S.G., V.J. Denef, K. Konstantinidis, L.M. Vergez, L. Agulló, V.L. Reyes, L. Hauser, M. Córdova, L. Gómez, M. González, M. Land, V. Lao, F. Larimer, J.J. LiPuma, E. Mahenthiralingam, S.A. Malfatti, C.J. Marx, J.J. Parnell, A. Ramette, P. Richardson, M. Seeger, D. Smith, T. Spilker, W-J. Sul, T.V. Tsoi, L.E. Ulrich, I.B. Zhulin, J.M. Tiedje. 2006. *Burkholderia xenovorans* LB400 harbors a multi-replicon, 9.7 M bp genome shaped for versatility. Proc. Natl. Acad. Sci. **103:**15280-15287.

Denef, V.J., J. Park, T.V. Tsoi, J.-M. Rouillard, H. Zhang, J.A. Wibbenmeyer, W. Verstraete, E. Gulari, S.A. Hashsham, and J.M. Tiedje. 2004. Biphenyl and benzoate metabolism in a genomic context: Outlining genome-wide metabolic networks in *Burkholderia xenovorans* LB400. Appl. Environ. Microbiol. **70:**4961-4970.

Denef, V.J. M.A. Patrauchan, C. Florizone, J. Park, T.V. Tsoi, W. Verstraete, J.M.Tiedje, and L.D. Eltis. 2005. Growth substrate- and phase-specific expression of biphenyl, benzoate, and C_1 metabolic pathways in *Burkholderia xenovorans* LB400. J. Bacteriol. **187:**7996-8005.

Denef, V.J. 2005. A functional genomics approach to elucidate the biphenyl and benzoate metabolic network integration in *Burkholderia xenovorans* LB400. Dissertation in Applied and Biological Sciences. Faculty of bioscience engineering, Universiteit Gent.

Ehrt, S., L. N. Ornston, and W. Hillen. 1994. RpoN (σ^{54}) is required for conversion of phenol to catechol in *Acinetobacter calcoaceticus*. J. Bacteriol. **176:**3493-3499.

Erickson, B. D., and F. J. Mondello. 1993. Enhanced biodegradation of polychlorinated biphenyls after site-directed mutagenesis of a biphenyl dioxygenase gene. Appl. Environ. Microbiol. **59:**3858-3862.

Furukawa, K. J.R. Simon, and A.M. Chakrabarty. 1983. Common Induction and regulation of biphenyl, xylene/toluene, and salicylate catabolism in *Pseudomonas paucimobilis*. J. Bacteriol. **154**:1356-1362.

Gibson, D. T., D. L. Cruden, J. D. Haddock, G. J. Zylstra, and J. M. Brand. 1993. Oxidation of polychlorinated biphenyls by *Pseudomonas* sp. strain LB400 and *Pseudomonas pseudoalcaligenes* KF707. J. Bacteriol. **175:**4561-4564.

Goris, J., P. De Vos, J. Caballero-Mellado, J. Park, E. Falsen, J.F. Quensen III, J.M. Tiedje, and P. Vandamme. 2004. Classification of the PCB-and

biphenyl degrading strain LB400 and relatives as *Burkholderia xenovorans* sp. nov. Int J Syst Evol Microbiol **54**:1677-1681.

Hernandez, B. S., J. J. Arensdorf, and D. D. Focht. 1995. Catabolic characteristics of biphenyl-utilizing isolates which cometabolize PCBs. Biodegradation **6:**75-82.

Hugouvieux-Cotte-Pattat, N., T. Kohler, M. Rekik, and S. Harayama. 1990. Growth-phase-dependent expression of the *Pseudomonas putida* TOL plasmid pWW0 catabolic genes. J. Bacteriol. **172:**6651-6660.

Jagannathan, A., C. Coinstantinidou, and C. W. Penn. 2001. Roles of *rpoN*, *fliA*, and *flgR* in expression of flagella in *Campylobacter jejuni*. J. Bacteriol. **183:**2937-2942.

Kim, Y., L.S. Watrud, and A. Matin. 1995. A carbon starvation survival gene of *Pseudomonas putida* is regulated by σ^{54}. J. Bacteriol. **177:**1850-1859.

Kohler, H.-P.E., D. Kohler-Staub, and D.D. Focht. 1988. Cometabolism of polychlorinated biphenyls: Enhanced transformation of Aroclor 1254 by growing bacterial cells. Appl. Environ. Microbiol. **54:**1940-1945.

Maltseva, O.V., T.V. Tsoi, J.F. Quensen III, M. Fukuda, and J.M. Tiedje. 1999. Degradation of anaerobic reductive dechlorination products of Aroclor 1242 by four aerobic bacteria. Biodegradation. **10:**363-371.

Marx, C.J., J.A. Miller, L. Chistoserdova, and M.E. Lidstrom. 2004. Multiple formaldehyde oxidation/detoxification pathways in *Burkholderia fungorum* LB400. J. Bacteriol. **186:**2173-2178.

Master, E.R., and W.W. Mohn. 2001. Induction of *bphA*, encoding biphenyl dioxygenase, in two polychlorinated biphenyl-degrading bacteria, psychrotolerant *Pseudomonas*strain Cam-1 and mesophilic *Burkholderia* strain LB400. Appl. Environ. Microbiol. **67**: 2669-2676.

Master, E.R., J.J. McKinlay, G.R. Stewart, and W.W. Mohn. 2005. Biphenyl uptake by psychrotolerant *Pseudomonas* sp. strain Cam-1 and mesophilic *Burkholderia* sp. strain LB400. Can. J. Microbiol. **51:**399–404.

Merrick, M.J., and W.D.P. Stewart. 1985. Studies on the regulation and function of the *Klebsiella pneumoniae ntrA* gene. Gene. **35:**297-303.

Mondello, F.J., M.P. Turcich, J.H. Lobos, and B.D. Erickson. 1997. Identification and modification of biphenyl dioxygenase sequences that determine the specificity of polychlorinated biphenyl degradation. Appl. Environ. Microbiol. **63**: 3096-3103.

Mouz, S., C. Merlin, D. Springael, and A. Toussaint. 1999. A GntR-like negative regulator of the biphenyl degradation genes of the transposon Tn4371. Mol. Gen. Genet. **262:**790-799.

Mouz, S., E. Coursange, and A. Toussaint. 2001. *Ralstonia metallidurans* CH34 RpoN sigma factor and the control of nitrogen metabolism and biphenyl utilization. Microbiology **147:**1947-1954.

Ohtsubo, Y., T. Kudo, M. Tsuda, and Y. Nagata. 2004. Strategies for bioremediation of polychlorinated biphenyls. Appl. Microbiol. Biotechnol. **65:**250-258.

Ohtsubo, Y., Y. Nagata, K. Kimbara, M. Takagi, M., and A. Ohta. 2000. Expression of the *bph* genes involved in biphenyl/PCB degradation in *Pseudomonas* sp KKS102 induced by the biphenyl degradation intermediate, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid. Gene **256:** 223-228.

Parnell, J.J., J. Park, V. Denef, T. Tsoi, S. Hashsham, J. Quensen III, and J.M. Tiedje. 2006. Coping with PCB toxicity: The physiological and genome-wide response of *Burkholderia xenovorans* LB400 to PCB (polychlorinated biphenyl)-mediated stress. Appl. Environ. Microbiol. **72:**6607-6614.

Powell, B.S., D.L. Court, T. Inada, Y. Nakamura, V. Michotey, X. Cui, A. Reizer, M.H. Saier Jr., and J Reizer. 1995. Novel proteins of the phosphotransferase system encoded within the *rpoN* operon of *E. coli*. Enzyme IIA^{Ntr} affects growth on organic nitrogen and the conditional lethality of an era^{ts} mutant. J. Biol. Chem. **270:**4822-4839

Quensen, J.F., III, S.A. Boyd and J.M. Tiedje. 1990. Dechlorination of four commercial polychlorinated biphenyl mixtures (Aroclors) by anaerobic microorganisms from sediments. Appl. Environ. Microbiol. **56:**2360-2369.

Seeger, M., K.N. Timmis, and B. Hofer. 1995. Conversion of chlorobiphenyls into phenylhexadienoates and benzoates by the enzymes of the upper pathway for polychlorobiphenyl degradation encoded by the *bph* locus of *Pseudomonas* sp. strain LB400. Appl. Environ. Microbiol. **61:**2654–2658.

Sze, C.C., T. Moore, and V. Shingler. 1996. Growth-phase dependent transcription of the σ^{54}-dependent Po promoter controlling the *Pseudomonas*-derived (methyl)phenol *dmp* operon of pVI150. J. Bacteriol. **178:**3727-3735.

Watanabe, T., H. Fujihara, and K. Furukawa. 2003. Characterization of the second LysRtype regulator in the biphenyl-catabolic gene cluster of *Pseudomonas pseudoalcaligenes* KF707. J. Bacteriol. **185**: 3575-3582.

CHAPTER 6.

ACCUMULATION AND DETOXIFICATION OF FORMALDEHYDE IN *Burkolderia xenovorans* LB400 DURING (POLYCHLORINATED) BIPHENYL DEGRADATION.

ABSTRACT

The biphenyl pathway responsible for the degradation of polychlorinated biphenyls has been well-documented over the past few decades including enzymes and metabolic products. Induction of C_1 pathway genes in *Burkholderia xenovorans* LB400 during growth on biphenyl and PCB degradation prompted this study to examine the metabolic footprint of biphenyl and PCB degradation. I analyzed low molecular-weight aldehydes that accumulate during growth on biphenyl and degradation of PCBs for LB400 wild type and two knockout mutations of the C_1 pathway (LB400Δ*xoxF* and LB400Δ*flhA fdhA mtdB*). I found an accumulation of formaldehyde on degradation of biphenyl and PCBs that is diminished with expression of the C_1 pathway. In addition, I found that XoxF is responsible for the accumulation of formaldehyde. This work is the first to associate formaldehyde accumulation with the degradation of biphenyl. Although the source of formaldehyde (substrate for XoxF) is unknown, the role of the C_1 pathway in LB400 is clear.

INTRODUCTION

Over the past thirty years, *Burkholderia xenovorans* LB400 has been extensively studied due to the wide range of polychlorinated biphenyl (PCB) congeners degraded via the biphenyl pathway (Bopp 1986, Ericson and Mondello 1992). Nonetheless, most of the studies of LB400 had been reductionist in nature, focused primarily on the biphenyl pathway. Completion of the genome sequence reveals a diverse network of often redundant metabolic pathways that provide clues to ecological niches and function (Chain, et al. 2006). Recently, several studies have expanded beyond the scope of the biphenyl pathway describing functional redundancy of benzoate (Denef 2004, 2005, 2006) and C_1 degradation pathways (Marx 2004). However the explicit role of many of these pathways in an ecological context and in PCB degradation remains unclear.

Earlier studies focusing on the ecology of PCB degradation indicate a relationship between methylotrophy and PCB degraders, however details with regards to this relationship are lacking. Recent whole genome expression studies by Denef, et al. (2004) indicate a significant induction of the C_1 pathway during growth on biphenyl as LB400 transitions into stationary phase. Follow-up studies indicate that some of the C_1 genes may be induced due to the formation of formate on degradation of benzoate via CoA oxidation (Denef, et al. 2005; Zaar, et al. 2001, 2004), though they also point out that formate detoxification genes are only a subset of the C_1 genes induced in transition phase (Denef, et al. 2004). Additionally, the C_1 pathway is induced during degradation of PCBs with no evidence of up-regulation of CoA oxidation genes (Parnell, et al. 2006); the C_1 genes are independent of benzoate CoA during degradation of PCBs.

Although the C_1 utilization pathway in LB400 is homologous to that of the well-studied methanol-degrading *Methylobacterium extorquens* AM-1 (Amaratunga, et al. 1997), LB400 is incapable of growth on methanol or other C_1 compounds (Marx, et al. 2004), possibly a result of an incomplete methanol dehydrogenase. While Marx et al. (2004) establish the C_1 pathway in LB400 as formaldehyde detoxification involved in choline degradation (Figure 1), other work has demonstrated the formation of formaldehyde in other Burkholderia during growth on methylated aromatic compounds (Singer, et al. 2003); however, the presence of these compounds, and hence induction of the C_1 pathway is not expected in either biphenyl or PCB degradation.

In this study I use metabolic footprinting (metabolomics) to place the C_1 pathway of LB400 in an ecological context in PCB degradation (Kell, et al. 2005). I examine the metabolic profile of simple carbon compounds on deletion of key genes involved in the C_1 pathway (Marx, et al. 2004).

MATERIALS AND METHODS

Bacterial strains and growth conditions. Throughout this study I used three strains of *Burkholderia xenovorans* LB400: LB400 wild type (wt), LB400Δ*xoxF*, and LB400Δ*flhA fdhA mtdB* (Marx, et al. 2004). Growth curve experiments allowed us to determine the effects of the C_1 pathway on the degradation of biphenyl and PCBs. I grew triplicate cultures of LB400 in 25 ml mineral medium (K1) and biphenyl (3 g/L) with and without Aroclor 1242 (500 ppm) as described previously (Denef, et al. 2004; Parnell, et al. 2006). Cultures were grown in 125 ml Wheaton® flasks and incubated on an orbital shaker (200 rpm) at 30±2°C.

Metabolic fingerprinting. Aliquots (5 ml) were harvested in triplicate from biphenyl mid-logarithmic phase (OD 600 nm 0.4) and early stationary phase growth (OD 600 nm 0.8) for treatments with and without PCBs. Each aliquot was filtered through a 0.22 micron filter (Microcon) preparatory for LC-MS analysis. In order to detect aldehydes, samples were derivitized prior to LC-MS injection.

RESULTS

Growth effects of C_1 pathway knockouts. In order to determine the effects of the C_1 pathway knockouts on growth of LB400 on biphenyl with and without PCBs, I analyzed the growth curves and compared with LB400wt. I found no difference in growth rate, indicating no fatal phenotypes and that the C_1 pathway is not essential to LB400 during growth on biphenyl or PCBs.

Metabolic fingerprint of LB400wt, LB400Δ*xoxF*, and LB400Δ*flhA fdhA mtdB*. The metabolic profile of all three LB400 strains was analyzed with particular focus on low molecular weight aldehydes that accumulate during biphenyl and PCB degradation. The retention time for modified formaldehyde was calculated at approximately 1.34 min. LB400wt harvested from mid-logarithmic (ML) phase growth exhibited increased levels of formaldehyde. In addition, biphenyl grown ML LB400wt with PCBs shows slight elevation in formaldehyde, as does LB400wt early stationary-phase (TP) growth (Figure 6.2). The triple knockout of all three redundant formaldehyde detoxification pathways (LB400Δ*flhA fdhA mtdB*) harvested from transition to stationary phase (TP) growth indicated little, if any accumulation of formaldehyde. However,

LB400Δ*flhA fdhA mtdB* harvested from ML phase with PCBs indicates an accumulation of formaldehyde (Figure 6.3). Finally, LB400Δ*xoxF* revealed no accumulation of formaldehyde harvested from transition to stationary phase (TP) growth or from ML phase with PCBs.

DISCUSSION

The role of the C_1 pathway proven enigmatic in LB400 as it is unable to grow on simple carbon compounds like homolog-bearing *Methylobacterium extorquens* AM-1 (Amaratunga, et al. 1997; Marx, et al. 2004). The role has become increasingly baffling as components are highly expressed in both biphenyl (Denef, et al. 2004) and PCB degradation (Parnell, et al. 2006). Metabolic fingerprinting of selected knockout mutations of LB400 (LB400Δ*xoxF* and LB400Δ*flhA fdhA mtdB*) in this study have helped to clarify the role of the C_1 pathway in LB400, but not in (polychlorinated) biphenyl degradation.

Formaldehyde is formed by the methanol dehydrogenase-like XoxF in LB400. The formaldehyde can then be routed through any one of three redundant pathways, a glutathione-S transferase, NAD-linked pathway (FlhA), an NAD-linked, glutathione-S transferase independent pathway (FdhA), and and NAD(P)-linked methylene H_4MPT pathway (see Figure 1).

During logarithmic-phase growth of LB400wt on biphenyl, regardless of the presence of PCBs, I found an accumulation of formaldehyde. The highest accumulation was in biphenyl during mid-log growth (Figure 2). Previous studies (Denef, et al. 2004; Parnell, et al. 2006) noted an induction in the C_1 pathway that may explain a lower

accumulation in both transition to stationary phase, grown on biphenyl, and PCB degradation.

The elimination of all three modes of formaldehyde detoxification (LB400Δ*flhA fdhA mtdB*) should lead to a marked accumulation of formaldehyde. During PCB degradation (growth on biphenyl and harvested in logarithmic growth), I found a high accumulation of formaldehyde (higher than the same treatment for LB400wt). However, formaldehyde accumulation was much lower in transition to stationary phase growth on biphenyl (Figure 6.3).

As XoxF is similar to methanol dehydrogenase (MxaF), but shows no affinity for methanol or other C_1 compounds (Marx, et al. 2004). In the homologous pathway in AM-1, the methanol dehydrogenase is responsible for the production of formaldehyde. In LB400 I would expect to see no accumulation of formaldehyde in any of the treatments. Indeed, I found no accumulation of formaldehyde in either of the treatments (Figure 6.4), indicating that XoxF is responsible for the formation of formaldehyde.

This study demonstrates a previously undocumented accumulation of formaldehyde during (polychlorinated) biphenyl degradation caused by XoxF in LB400. While this explains the role of the C_1 pathway in LB400 (formaldehyde detoxification), the role of the C_1 pathway in biphenyl and PCB degradation remains a mystery. In addition, though I have found the product of XoxF to be formaldehyde, the substrate is still unclear.

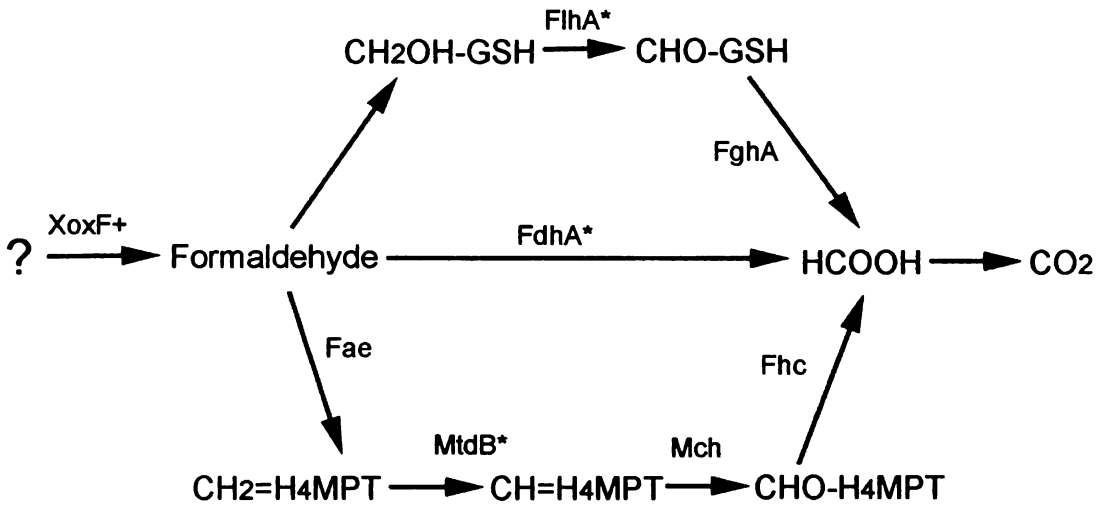


Figure 6.1. Functional redundance of three formaldehyde detoxification pathways in *Burkholderia xenovorans* LB400 (from Marx, et al. 2004). Formaldehyde is produced by XoxF and channeled into three pathways. Knockout mutations used in this study are designated LB400Δ*xoxF* (+) and LB400Δ*flhAfdhAmtdB* (*).

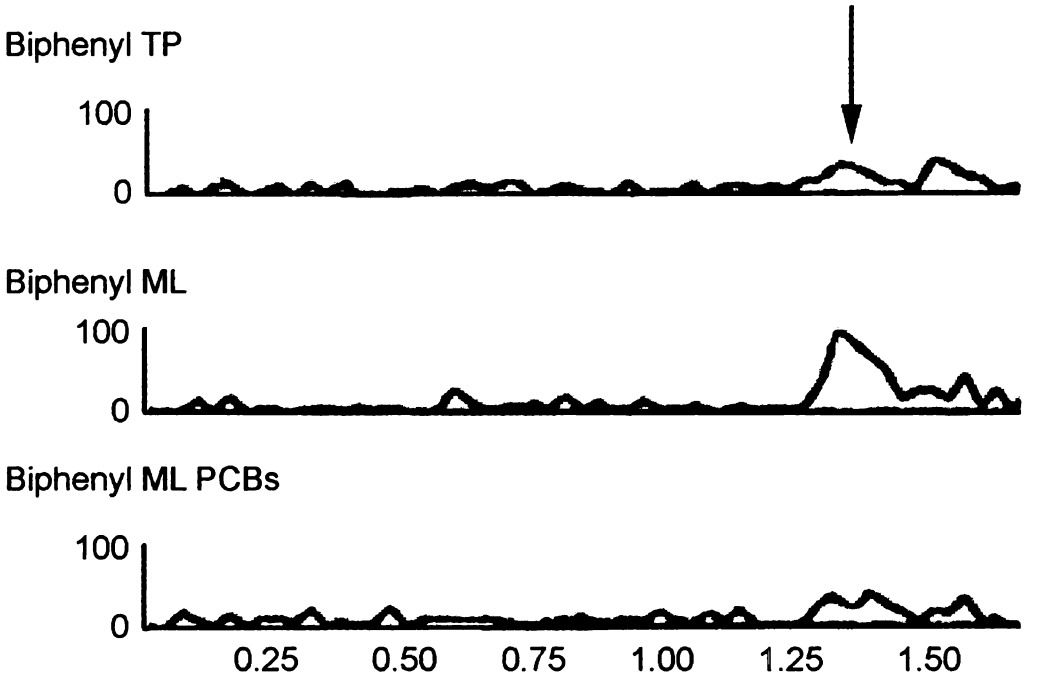


Figure 6.2. LC-MS profile of LB400wt grown on biphenyl with and without PCBs harvested from mid-logarithmic (ML) and early stationary phase growth (ES). The retention time for modified formaldehyde is highlighted.

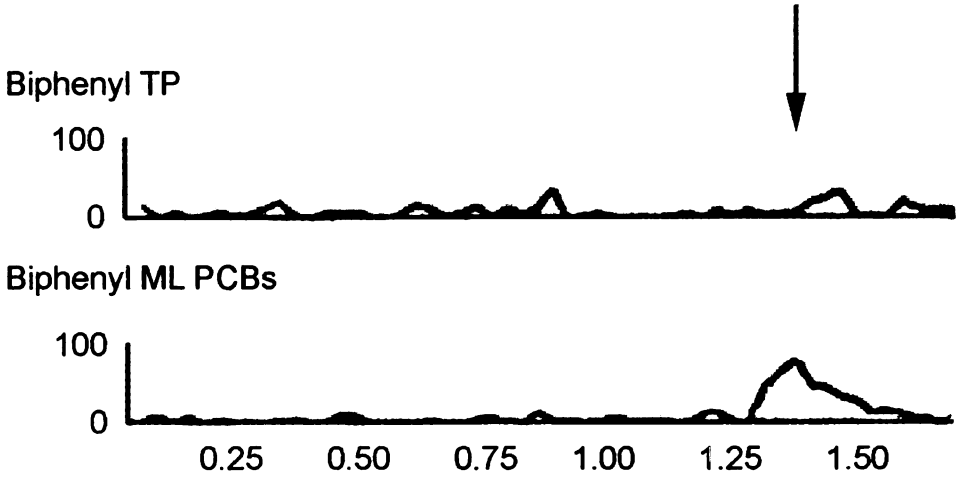


Figure 6.3. LC-MS profile of LB400Δ*flhA fdhA mtdB* grown on biphenyl and harvested during early stationary phase (ES) and mid logarithmic phase (ML) with PCBs. The retention time for modified formaldehyde is highlighted.

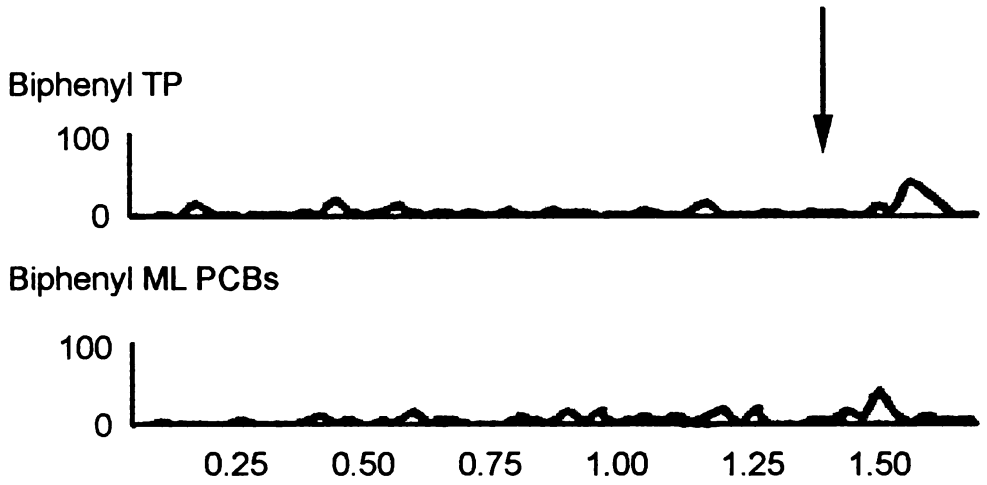


Figure 6.4. LC-MS profile of LB400Δ*xoxF* grown on biphenyl and harvested during early stationary phase (ES) and mid logarithmic phase (ML) with PCBs. The retention time for modified formaldehyde is highlighted.

REFERENCES

Amaratunga, K. P.M. Goodwin, C.D. O'Connor, and C. Anthony. 1997. The methanol oxidation genes *mxaFJGIR(S)ACKLD* in *Methylobacterium extorquens*. FEMS Microb. Lett. **146:**31-38.

Bopp L H. Degradation of highly chlorinated PCBs by *Pseudomonas* strain LB400. J Ind Microbiol 1986;**1**:23–29.

Chain, P.S.G., V.J. Denef, K. Konstantinidis, L.M. Vergez, L. Agulló, V.L. Reyes, L. Hauser, M. Córdova, L. Gómez, M. González, M. Land, V. Lao, F. Larimer, J.J. LiPuma, E. Mahenthiralingam, S.A. Malfatti, C.J. Marx, J.J. Parnell, A. Ramette, P. Richardson, M. Seeger, D. Smith, T. Spilker, W-J. Sul, T.V. Tsoi, L.E. Ulrich, I.B. Zhulin, J.M. Tiedje. 2006. *Burkholderia xenovorans* LB400 harbors a multi-replicon, 9.7 M bp genome shaped for versatility. Proc. Natl. Acad. Sci. **103:**15280-15287.

Denef, V.J., J. Park, T.V. Tsoi, J.-M. Rouillard, H. Zhang, J.A. Wibbenmeyer, W. Verstraete, E. Gulari, S.A. Hashsham, and J.M. Tiedje. 2004. Biphenyl and benzoate metabolism in a genomic context: Outlining genome-wide metabolic networks in *Burkholderia xenovorans* LB400. Appl. Environ. Microbiol. **70:**4961-4970.

Denef, V.J. M.A. Patrauchan, C. Florizone, J. Park, T.V. Tsoi, W. Verstraete, J.M.Tiedje, and L.D. Eltis. 2005. Growth substrate- and phase-specific expression of biphenyl, benzoate, and C_1 metabolic pathways in *Burkholderia xenovorans* LB400. J. Bacteriol. **187:**7996-8005.

Denef, V.J. J.A. Klappenbach, M.A. Patrauchan, C. Florizone, J.L.M. Rodrigues, T.V. Tsoi, W. Verstraete, L.D. Eltis, and J.M. Tiedje. 2006. Genetic and genomic insights into the role of benzoate-catabolic pathway redundancy in *Burkholderia xenovorans* LB400. Appl. Environ. Microbiol. **72:** 585-595.

Erickson, B. D., and F. J. Mondello. 1993. Enhanced biodegradation of polychlorinated biphenyls after site-directed mutagenesis of a biphenyl dioxygenase gene. Appl. Environ. Microbiol. **59:**3858-3862.

Kell, D.B., M. Brown, H.M. Davey, W.B. Dunn, I. Spasic, and S.G. Oliver. 2005. Metabolic footprinting and systems biology: the medium is the message. Nat. Rev. Microbiol. **3:**557-565

Marx, C.J., J.A. Miller, L. Chistoserdova, and M.E. Lidstrom. 2004. Multiple formaldehyde oxidation/detoxification pathways in *Burkholderia fungorum* LB400. J. Bacteriol. **186:**2173-2178.

Parnell, J.J., J. Park, V. Denef, T. Tsoi, S. Hashsham, J. Quensen III, and J.M. Tiedje. 2006. Coping with PCB toxicity: The physiological and genome-wide response of *Burkholderia xenovorans* LB400 to PCB (polychlorinated biphenyl)-mediated stress. Appl. Environ. Microbiol. **72:**6607-6614.

Singer, A. C., D. E. Crowley, and I. P. Thompson. 2003. Secondary plant metabolites in phytoremediation and biotransformation. Trends Biotechnol. **21:**123-130.

Zaar, A., W. Eisenreich, A. Bacher, and G. Fuchs. 2001. A novel pathway of aerobic benzoate catabolism in the bacteria *Azoarcus evansii* and *Bacillus stearothermophilus*. J. Biol. Chem. **276**: 24997-25004.

Zaar, A., J. Gescher, W. Eisenreich, A. Bacher, and G. Fuchs. 2004. New enzymes involved in aerobic benzoate metabolism in *Azoarcus evansii*. Mol. Microbiol. **54**: 223-238.

CHAPTER 7

SUMMARY AND FUTURE PERSPECTIVES.

MAJOR CONCLUSIONS

The main focus of this research is to gain insight into the mechanisms of tolerance and co-metabolism of polychlorinated biphenyls by aerobic biodegradative microorganisms using *Burkholderia xenovorans* LB400 as a model organism. To determine the extent of tolerance and breadth of co-metabolism of PCBs, I first determined the sensitivity or tolerance of potential PCB-degrading strains to a commercial mixture of PCBs (Aroclor 1242).

In comparing 18 different bacterial strains with PCB-degrading potential, I found a wide range in sensitivity to PCBs. *Burkholderia xenovorans* LB400 is one of the most tolerant to high concentrations of PCBs and because of the completion of the genome sequence and the availability of microarray techniques, this became a model organism for the study of tolerance as well as investigations into additional mechanisms involved in co-metabolism of PCBs and efficient biphenyl degradation. Moreover, the range of physiological characteristics associated with the bacteria selected for this study allow further focus of the source of PCB tolerance. In order to design a successful *in-situ* PCB biodegradation scheme, the physiological and environmental context under which PCBs are degraded by aerobic microorganisms must be determined.

One such context is the biotoxicity of PCBs to biodegrading microorganisms. In Chapter 3, I characterize the whole-cell physiological responses to LB400 following exposure to PCBs. As PCBs become associated with lipids of organisms, they have the

tendency to accumulate in the membrane of biodegrading bacteria such as LB400. The physiological changes induced by the presence of PCB can provide information regarding environmental conditions present in the medium. This knowledge can eventually lead to better understanding of mechanisms responsible for tolerance and their exploitation in environmental settings. I found that LB400 does not degrade PCBs during mid-logarithmic phase growth on simple carbon sources such as succinate and benzoate. These treatments served to demonstrate the innate toxicity of PCBs to bacteria in an environemtal context. This work was the first to demonstrate that PCBs themselves have no significant physiological effect on biodegrading bacteria.

Aside from PCBs themselves, biodegrading bacteria are faced with the potential toxic effects of degradation products. A characterization of the whole-cell physiological response to PCBs, due to both PCBs and the products of their degradation (biphenyl pathway induced by growth on biphenyl), clarify the effect of PCB degradation on microorganisms. In order to determine the mechanisms involved in tolerance to PCB degradation, I analyzed gene expression patterns involved in the response of LB400 following exposure to PCBs. The advent of bacterial genome sequencing has ushered an era of large-scale discovery involving the response of bacteria to a broad range of conditions. Until now, the majority of studies on PCB degradation have focused primarily on the PCB-degrading biphenyl pathway; little consideration has been given to peripheral pathways or mechanisms involved in whole-cell responses to PCBs and the products of their degradation. In order to design a successful *in-situ* PCB biodegradation scheme, the environmental and ecological context under which PCBs are degraded by aerobic microorganisms must be determined, including global gene expression responses. While other genes are certainly involved in PCB degradation, no information has been

reported on the global expression patterns involved in PCB degradation. Degradation of PCBs by LB400 (biphenyl with PCBs vs biphenyl) induces pathways involved in PCB degradation (biphenyl, and (chloro)catechol degradation pathways) as well as several other pathways that may be associated with detoxification of deleterious metabolites from incomplete PCB degradation. The C1 pathway is up-regulated during degradation of PCBs, although the basis of its induction is unknown. The chloroacetaldehyde dehydrogenase that is induced is very similar to the chloroacetaldehyde involved in the detoxification of chlorinated aldehydes produced on degradation of DCE. BphI is responsible for cleaving 4-hydroxy-2-oxovalerate into pyruvate and acetaldehyde. However a chlorinated substrate may lead to a chloroacetaldehyde that would be toxic for biodegrading bacteria. Further evidence for the formation of chloroacetaldehyde is the similar clustering of BphJ, which funnels acetaldehyde into pyruvate metabolism and the chloroacetaldehyde dehydrogenase in transcriptional profiling (Chapter 4).

The most vigorous applications of transcriptional profiling entail the analysis of patterns of gene expression encompassing various experiments that examine a broad range of responses to different treatments. I analyzed the gene expression patterns 11 different conditions involving different carbon sources (benzoate, biphenyl and succinate) different growth phases (log-phase and early stationary phase) and presence/absence of PCBs to provide a comprehensive overview of genes involved in PCB degradation and its regulation. Statistical analysis of gene expression patterns clustered the upper biphenyl tightly separate from the lower biphenyl pathway, indicating the value of transcriptional profiling. Many of the genes grouped in the same cluster of the upper biphenyl pathway have no known association with biphenyl degradation and serve as viable candidates for

future study. In addition, BphJ clustered tightly with the chloroacetaldehyde dehydrogenase mentioned previously, suggesting similar function.

PCB degradation has traditionally been reported using resting cell assays that induce the biphenyl pathway following removal of other available carbon sources. While this technique has obvious advantages in detecting PCB degradation and characterizing congener specificity, LB400 harvested from different growth phases leads to different PCB degradation patterns. The most profound difference in PCB degradation profile was in early stationary phase LB400 grown on biphenyl. Transcriptional profiles of PCB degradation and early stationary phase LB400 grown on biphenyl identify 25 genes that are induced in both conditions that may impart stability during PCB degradation. Among the genes induced is the pathway responsible for formaldehyde detoxification (C1), BphJ, chloroacetaldehyde dehydrogenase, and fimbrial-associated proteins. Many of the genes are potentially regulated by sigma-54 (formaldehyde detoxification and fimbrial-associated genes). LB400 contains two potential sigma-54 genes that were examined via Q-RT-PCR during different conditions leading to a range in PCB degradation. I found that the expression of one of the sigma-54 genes (located on the chromosome) correlates with expression of the biphenyl pathway, as does sigma-70, and the chloroacetaldehyde dehydrogenase.

The effect of carbon source on biphenyl degradation has long been known. PCB degradation does not occur in the presence of simple carbon sources. While the molecular mechanisms for the regulation of the biphenyl pathway have been studied, the environmental aspects, such as the role of competing carbon sources have not been documented. I tested the effect of a simple carbon source (succinate) on the expression of the biphenyl pathway using Q-RT-PCR. This is the first indication that the biphenyl

pathway is induced by biphenyl when LB400 is grown with succinate. However, I found no evidence of degradation of biphenyl, suggesting either post-transcriptional factors or transport limited availability of biphenyl. Furthermore, I found that PCBs in the presence of simple carbon sources represses the biphenyl pathway. These are crucial factors involved in environmental scenarios as PCB-contaminated sediments often contain additional carbon sources.

I found induction of the C1 metabolic pathway in LB400 in response to the degradation of PCBs. Using knockout mutations of the C1 pathway, I performed metabolic footprinting focusing primarily on low molecular-weight acetaldehydes. I found for the first time that formaldehyde is produced during degradation of biphenyl and PCBs. I also report that XoxF, a methanol dehydrogenase-like protein is involved in the production of formaldehyde and the downstream C1 elements are responsible for detoxification of formaldehyde.

This work has focused on (polychlorinated) biphenyl external to the biphenyl pathway such as toxicity, regulation, and other mechanisms possibly involved in increasing efficiency of degradation. Understanding these factors and treating PCB degradation as a whole-cell process is crucial to placing biphenyl and PCB degradation in an ecological context.

FUTURE PERSPECTIVES

This work has revealed the importance of factors external to the biphenyl pathway involved in efficient and successful degradation of PCBs and biphenyl. Some of these factors impart tolerance to LB400 to high concentrations of PCBs where other potential

PCB degraders are sensitive. Although I implicate the chloroacetaldehyde dehydrogenase as a key component to tolerance, more detailed study of the specific role of chloroacetaldehyde dehydrogenase in the degradation of PCBs. In addition, information on the occurrence of membrane vesicles on exposure to PCBs (mentioned in chapter 3) should be pursued as this may be a defense mechanism to minimize PCB-membrane interaction or a general response to stress. Finally, physiological information on the toxicity of PCBs to LB400 indicates some expression changes that are below the detection for traditional microarray studies and require more sensitive methods to determine effect.

Transcriptional profiling has identified several genes that may be involved in biphenyl degradation including regulators and membrane-associated proteins. Further characterization of these proteins via knockout mutation could determine the function and role of these genes in biphenyl degradation.

In addition, LB400 has two sigma-54 factors; the first, chromosomally localized factor appears to be associated with PCB degradation. While In addition, sigma general nutrient starvation, and the second seems to be associated with the degradation of biphenyl. Future studies on the specific involvement of the latter sigma factor, such as knockout mutation, could indicate how much association is involved between the biphenyl pathway and sigma-54 activity.