INVESTIGATION OF THE TOXICITY AND EFFLUX OF POLYCHLORINATED BIPHENYLS AND HYDROXYLATED POLYCHLORINATED BIPHENYLS IN ESCHERICHIA COLI

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ABSTRACT OF THESIS

INVESTIGATION OF THE TOXICITY AND EFFLUX OF POLYCHLORINATED BIPHENYLS AND HYDROXYLATED POLYCHLORINATED BIPHENYLS IN *ESCHERICHIA COLI*

Polychlorinated biphenyls (PCBs) are persistent organic pollutants. Due to their properties, PCBs accumulate in the food-chain and post a threat to the health of human beings and wildlife. Hydroxylated PCBs (OH-PCBs) are oxidative metabolites of PCBs and are more hydrophilic than their parent PCBs. One of the best approaches to break down these contaminants is through bioremediation, which is an environmental friendly process that uses microorganisms to restore natural environment.

Towards this goal, we have investigated the toxicity and accumulation of PCBs and OH-PCBs in a Gram-negative bacterium, *Escherichia coli*. We have also determined the role played by a primary multidrug efflux transporter AcrB on the accumulation of PCBs and OH-PCBs in bacterial cell. We found that none of the PCBs tested was toxic to *E. coli*, while different OH-PCBs have different levels of toxicity; the acrB knockout strain accumulated significantly more PCBs and OH-PCBs than the wild-type strain, suggesting that these compounds are substrates of the efflux pump; higher cytoplasmic concentrations of OH-PCBs were also observed in the acrB knockout strain using the biosensors. Based on these observations, we conclude that both PCBs and OH-PCBs are substrates of protein AcrB. Therefore the efflux activities of multidrug resistant pumps in Gram-negative bacteria should be considered while designing bioremediation approaches.

KEYWORDS: Polychlorinated biphenyls, hydroxylated polychlorinated biphenyls, *E. coli*, Multidrug Efflux Pump, Protein AcrB

Shen Geng

Jun 6, 2011
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THESIS

Shen Geng

The Graduate School
University of Kentucky
2011
INVESTIGATION OF THE TOXICITY AND EFFLUX OF POLYCHLORINATED BIPHENYLS AND HYDROXYLATED POLYCHLORINATED BIPHENYLS IN *ESCHERICHIA COLI*

THESIS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in the College of Arts and Sciences at the University of Kentucky

By

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Lexington, Kentucky

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Lexington, Kentucky

2011

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Chapter I: Introduction

1.1 Polychlorinated biphenyl (PCB), an environmental pollutant

PCBs are a group of synthetic compounds that are among the most notorious environmental contaminants. PCBs were widely used in industrial applications, particularly as electrical insulating and heat-exchange fluids in transformers and heat insulators since 1920s [Kucewicz, 2004]. Concerns over PCBs developed in 1965 after the detection of trace amount of these compounds in wildlife in Sweden [Jensen, 1969]. Later in 1979, upon the identification of PCBs in environmental samples, U.S. government banned the production of PCBs and began to strictly regulate the use of PCBs in industry. However, due to years of intensive use, improper disposal, and the intrinsic resistance of the compounds to degradation, large quantities of PCBs remain in the natural environment, corresponding to one-third of the 1.1 billion tons of PCBs ever produced [Weigel and Wu, 2000]. PCBs can be found in air, water, soils, and wildlife, posing a threat to all living creatures.

Because of their highly hydrophobic nature, PCBs preferentially adsorb on the surface of sediment at the bottoms of rivers. After being ingested by bottom feeders, PCBs accumulate along the food chain and circulate in the global ecosystem. PCBs have been found in about one-third of the Superfund National Priorities Sites [EPA, 2000]. Although considerable efforts have focused on cleaning up PCBs from the environment over the past couple of decades, large amount of PCBs still exist in the environment, threatening the well-being of both humans and wildlife [Ballschmitter, 1991].

1.1.1 Structure and property of PCBs

There are 209 PCB congeners. All PCB molecules have a biphenyl backbone as shown in Figure 1.1. Except for the two carbons that serve as the connecting sites,
the other ten carbons may attach either a hydrogen or a chlorine atom. According to the IUPAC nomenclature, the ring with more substitutions or substitutions that occur on carbons with smaller numbers in the biphenyl assembly is given unprimed numbers and the other ring with primed numbers. It has been found that the higher the degree of chlorine substitution, the harder the corresponding PCB to degrade. As a consequence, the most common PCBs that are detected from exposed animals are usually with high levels of chlorination, including 2, 2’, 3, 4, 4’, 5-hexachlorobiphenyl, 2, 2’, 4, 4’ 5, 5’-hexachlorobiphenyl, and 2, 2’, 3, 4, 4’, 5, 5’-heptachlorobiphenyl [McFarland and Clarke, 1989].

In terms of structures, PCBs fall into two categories: co-planar structures (dioxin-like PCBs) and non-planar structures (non-dioxin-like PCBs). The toxicity of co-planar PCBs is much higher than that of non-planar PCBs [Safe, 1993]. As for the synthesis of PCBs, two main methods have been adopted, including ferric ion and iron-catalyzed chlorination of biphenyls [Schaeffer, et al. 1984]. The chlorination is nevertheless non-region specific, which results in the formation of a complex mixture of isomers and congeners [Safe, 1993].

![Figure 1.1. The general structure of PCBs.](image)

PCBs are chemically inert to different types of chemical reactions including oxidation, reduction, addition, and substitution. They are also resistant to treatments
with acid, base, and heat. These qualities make PCBs very desirable in industrial applications, especially for the manufacturing of coolants and lubricants. However, these characteristics also make PCBs persistent environmental pollutants since they do not readily break down when exposed to chemical treatments or heat. Another characteristic that makes PCBs notorious pollutants lies in their hydrophobicity. They tend to accumulate in the fat tissues of oily fish (e.g. salmon, herring, sardines, tuna, and swordfish). PCBs in tissues from animals higher in the food chain that consume these fishes may reach a concentration that is many thousands of times higher than the PCB concentration in water. PCBs have been found all over the world including in the Arctic and Antarctic regions [Kucewicz, 2004]. Recently, it was reported that high levels of PCBs were found in polar bears [Skaare, et al. 2000]. Fish is also a food source for humans. Thus, it is not a surprise that detectable amounts of PCBs are widely found in human serum, adipose tissue, and breast milk, particularly from those who often consume contaminated fish [Buck, et al. 1997].

1.1.2 Adverse health effects of PCBs

Scientists have established connections between PCB exposure and adverse health effects. Laboratory animals have been shown to develop tumors under conditions of high-dose PCB exposure. U.S. Environmental Protection Agency, as well as other governmental or advisory agencies, classified PCBs as known animal carcinogens and possible human carcinogens. PCBs also attribute to eye irritations and skin disorders, such as chloracne in workers who were exposed to high levels of PCBs by skin contact and/or inhalation [James, 1993].

Excessive exposure to PCBs also affects the nervous system, immune system, reproductive system, and endocrine system. Studies with monkeys and humans reveal that exposure to PCBs leads to deficits in neurological development and changes in activity [Kimbrough, 2001]. PCBs have also been shown to suppress the immune system and therefore increase the risk of infections [EPA, 2008]. Reproductive defects
have been studied in a variety of animal species as well as humans who were exposed to PCB. Women who worked with PCBs in factories gave birth to children with decreased birth weights and gestational age [EPA, 2008]. PCBs have also been associated with type-2 diabetes through a study involving Swedish fishermen who consume contaminated fish from Baltic Sea [Lee, et al. 2006]. A positive correlation between levels of PCBs in their serum and prevalence of type-2 diabetes in the population has been established [Rylander, 2005].

1.1.3 Bioremediation of PCB contamination sites

Traditional methods to remedy PCB contamination sites include incineration, vitrification, solvent extraction and land filling [ATSDR, 1993]. However, these methods have the disadvantages of being time-consuming, expensive, and incomplete. In the last decade, many researchers began to focus on the development of an alternative approach to break down PCB wastes using microbes.

Microorganisms belonging to both Gram-negative and Gram-positive genera including *Pseudomonas, Alcaligenes, Achromobacter, Rhodococcus, Corynebacterium*, and *Bacillus* have been isolated from PCB contaminated sites [Unterman, et al. 1996]. These microbes are capable of growing on biphenyl as the sole carbon source. In 1973, Ahmed and Focht first reported that Achromobacter is capable of degrading several PCBs [Ahmed, et al. 1973]. Thereafter, the biodegradation of PCBs was explored from two aspects, dechlorination by anaerobic bacteria and oxidative degradation by aerobic ones [Furukawa and Fujihara, 2008].

The aerobic PCB degradation bacteria have a *bph* gene cluster which is present on their genomes or plasmids. These genes code for enzymes that are capable of catalyzing different steps of the reaction. Along the biphenyl degradative passway, one chlorinated benzoic acid and one dienoic acid are generated from the degradation of one chlorobiphenyl molecule as shown in Figure 1.2 [Gu, et al. 2004]. In terms of
dechlorination, microorganisms such as *Dehalococcoides* and *Thermotogales* are able to remove flanking chlorines on several PCBs. Dechlorination is expected to reduce the toxicity of PCBs as well as making them more susceptible to aerobic degradation [Liu, et al. 2010].

Figure 1.2. Biodegradation of PCBs catalyzed by Bhp proteins. One molecule of cis-2, 3-dihydro-2, 3-dihydroxychlorobiphenyls is generated from chlorobiphenyl in the presence of BphA. BphB catalyzes the next step to produce 2, 3-hydroxychlorobiphenyl. BphC and BphD catalyzes the next two steps of reactions and eventually generate one chlorobenzoic acid and one 2-hydroxy-penta-2, 3-dienoic acid [Gu, et al. 2004].

1.2 Hydroxylated polychlorinated biphenyls (OH-PCBs)

OH-PCBs are hydroxylated metabolites of polychlorinated biphenyls, with a common structure shown in Figure 1.3. The major metabolic pathway of PCBs is
biotransformation mediated by the cytochrome P450 isozymes, the terminal oxidases of the hepatic mixed-function oxidase system, to form hydroxylated PCBs [Lehmler, et al. 2010]. Even the most persistent PCB congeners can be transformed into a number of hydroxylated metabolites over time. Aerobic microorganisms degrade PCBs through another metabolic pathway to generate OH-PCBs with two hydroxyl substitutions on the parental PCBs, which are catalyzed by terminal dioxygenases. Furthermore, these OH-PCBs undergo degradation to generate chlorinated benzoic acids [Furukawa and Fujihara, 2008]. Abiotically, OH-PCBs may form via reactions of gaseous PCBs with OH radicals generated by UV degradation of ozone [Ueno, et al. 2007]. An inverse relationship between PCB concentration and OH radicals in the contaminated atmosphere has been reported, suggesting the hydroxylation of PCBs as the major removal pathway for PCBs in the atmosphere.

Figure 1.3. The general structure of monohydroxylated PCBs.

There are 837 possible monohydroxylated polychlorinated biphenyl congeners compared to 209 PCB congeners. The presence of hydroxyl groups renders these compounds more hydrophilic than their parent PCBs. Due to increased solubility, OH-PCBs are more readily absorbed from food source. The concentration of OH-PCBs in the environment is approximately 2.5 times higher than the concentration of PCBs [Turner, et al. 2007]. Higher concentrations of OH-PCBs were also detected in human and animal plasma than their parental PCBs [Park, et al 2009].

As discussed above, PCBs mainly accumulate in adipose tissues. However,
OH-PCBs are more water soluble than their parent PCBs, and therefore have a different distribution in the body. Most OH-PCB congeners are readily conjugated and excreted. They are able to circulate with blood, cross blood-placenta barriers and reach various organs [Dreiem, et al. 2009]. Due to structural similarities, OH-PCBs are antagonist of several hormones, especially thyroxin (Figure 1.4). Studies have revealed a significant reduction of thyroid hormone levels in plasma of animals exposed to OH-PCBs. It has been found that OH-PCBs can bind to human transthyretin (TTR), a thyroid hormone transport protein, with an affinity even higher than the natural ligand thyroxin [Brouwer and Van den Berg, 1986]. Some OH-PCB congeners show estrogenericity and antiestrogenicity in various mammalian cells, which can inhibit gap junctional intercellular communication, activate aryl hydrocarbon and estrogen receptors, and eventually play a role in tumor promotion [Machala, et al. 2004]. Furthermore, studies have shown that PCB metabolites including OH-PCBs and PCB quinones bind to nucleophiles, such as glutathione (GSH), and inhibit topoisomerase II \textit{in vitro}. As a consequence, cell susceptibility to oxidative damage increases due to the depletion of radical scavengers such as GSH [Srinivasan, et al. 2002]. In \textit{in vitro} studies, the oxygen species (ROS) generated by OH-PCBs have also been shown to attack cellular DNA and other nucleophiles, resulting in oxidative damage, such as DNA strand breakage [Srinivasan, et al. 2001].

![Figure 1.4. Structure of thyroxin shows similarity with OH-PCBs.](image)

1.3 Detection methods for PCBs and OH-PCBs
With increasing public concerns over environmental accumulation and toxic effects of PCBs and OH-PCBs, much effort has been focused on developing techniques and methods to monitor these contaminants. Traditional methods used to detect PCBs and OH-PCBs include gas chromatography (GC) or high performance liquid chromatography (HPLC) coupled with mass spectroscopy [Berger, et al. 2004]. These methods require pretreatments of samples through extraction, cleanup, and derivatization. GC in combination with a compound specific detector is considered as the primary detection technique for PCBs. It requires the compounds to be thermally stable at the temperature at which the analyzed samples are being vaporized. The detection of OH-PCBs by GC/MS on the other hand requires a derivatization step due to their low volatility. Recently, a two-dimensional gas chromatography (GC×GC) method has been developed to solve the problem of insufficient resolution of GC/MS. The GC×GC system has higher sensitivity, enhanced separation capacities, and simplified sample preparation process [Harynuk and Marriott, 2006]. LC/MS/MS system is usually utilized when GC/MS is not applicable due to thermolability, low volatility and high polarity of target analytes, such as in the detection of dihydroxylated PCBs. Overall, despite considerable cost and labor intensive sample preparation steps, traditional analytical methods involving gas or liquid chromatography and mass spectrometry are highly sensitive and reproducible in the detection of target pollutants in the nano-gram per liter range [Beyer and Biziuk, 2008].

On the other hand, biosensors are becoming more and more popular in the analyses of environmental and laboratory samples due to multiple advantages, including simplified sample preparation, rapid analysis, high selectivity, and low cost [Liu, et al. 2010]. Furthermore, these biosensing systems, especially whole-cell sensing systems, can provide information on bioavailability and toxic effects of the analytes to a “living” organism, which is not easily assessed by other cell-disrupting methods.
Biosensors are sensing systems or devices that involve the utilization of biological components, such as antibodies, enzymes, and receptors as the recognition or trapping elements [Daunert, et al. 2000]. In the presence of specific targets, the transducing elements that are connected to the recognition components, such as a binding protein, will produce measurable signals. The intensity of the signal is correlated with the amounts of the specific targets. The biological sensing element therefore confers selectivity while the transducing element determines the sensitivity in the biosensor. Although the selectivity of biosensors is comparable or even higher than traditional chromatography methods, the relatively low sensitivity, difficulties with immobilizing the recognition element and limited shelf-life are some challenges that limit wide applications of biosensors [Namiesnik, 2001].

1.4 Whole-cell biosensing system

Whole-cell sensing systems offer many benefits over other types of biosensors. First of all, other biosensors and traditional chromatography methods usually measure the total concentrations of analytes. However, living organisms are not always actually exposed to such a high amount of analytes, particularly when the target analytes have low bioavailability. These results thus might overestimate the actual effective amount of the analyte that may affect the living environment of humans and wildlife. Living cells, on the other hand, can reflect the bioavailability of the analytes and their toxic effects more accurately. Secondly, owing to the self-reproducing ability of microorganisms, whole-cell sensing systems have advantages such as low cost and lack of need for complex regeneration [Van der Meer, et al. 2004].

Microorganisms thriving in an environment with elevated concentration of chemicals have evolved different mechanisms to survive [Nikaido, 2009]. For example, many microbes living in hospitals have achieved increased resistance to multiple antimicrobials. The increased tolerance to alien chemicals could be a result
of an active efflux system that prevents the chemical from building up in their cytosol or special enzymes that degrade the compounds. The efflux pumps or enzymes involved in these mechanisms are usually encoded in the genome or plasmids of these microorganisms. However, the expression of these defense proteins is usually not constitutive. They are only produced in the presence of target compounds. The sensing and transcription regulation mechanism from different types of microorganisms have been exploited in the design of whole cell sensing systems for various toxic metals such as arsenic, antimony, mercury and lead, and organic aromatic compounds such as hydroxylated PCBs and chlorocatechols [Date, et al. 2007].

In term of detection mechanisms, there are mainly two types that can be employed in the whole-cell biosensors, the constitutive expression mechanism and the inducible expression mechanism [Gu, et al. 2004]. The constitutive expression system expresses the reporter protein at all times and yields a signal constantly. Toxic compounds, once present in the microorganism growing environment, will interfere with the growth of cells. The resultant decrease of cell density is reflected from the reduction of the signals. By generating a standard curve, the concentration of toxic targets can be measured. The constitutive expression system is not very selective, as it will detect the presence of any toxin that affects the growth of cell. Therefore, it is useful in the assessment of the overall contents of toxic compounds in a sample. A mammalian cell line harboring a plasmid PEGFP-N2, in which GFP gene is under the transcriptional control of the CMV promoter, has been constructed [Bi, 2002]. Using this cells line, the toxicity of many chemicals was characterized. Under harmful or toxic conditions, cells exhibited a lower growth rate and metabolism, which was reflected from a decreased fluorescence value.

In the inducible expression system, no signal can be detected in the absence of target compounds. The presence of the target compound triggers the expression of reporter proteins, which leads to detectable signals. The inducible expression system
is highly specific to analytes that induce the expression of the reporter protein. To construct a whole-cell biosensor of the inducible expression system, a special plasmid is usually created which includes a reporter gene under the control of a promoter as well as the gene coding for the corresponding regulator (Figure 1.5).

Figure 1.5. Plasmid designed for whole-cell biosensor. A regulator is placed upstream of a promoter in order to control the expression of the reporter gene.

The reporter gene/protein is the most universal component in a whole-cell biosensor. Reporters which are commonly used include \textit{LacZ/β}-galactosidase, green fluorescent proteins (GFP), and prokaryotic or eukaryotic luciferases. Owning to their unique properties, these reporters can either lead to a color change in the solution or generate light signals at certain wavelengths with or without the addition of substrates [Gu, et al. 2004].

\textit{LacZ/β}-galactosidase is an enzyme which catalyzes the cleavage of lactose
into galactose and glucose. It also cleave ortho-nitrophenyl-β-galactoside (ONPG) to generate galactose and ortho-nitrophenol (Figure 1.6). The yellow color of ortho-nitrophenol is utilized to quantify β-galactosidase. By measuring the time it takes for a reaction to change color, expression level of β-galactosidase can be evaluated. Cleavage of other substrates such as methylumbelliferyl-β-D-galactopyranoside (MUG) yields fluorescent product, which has also been utilized for the development of β-galactosidase quantification methods [Daunert, et al. 2000].

![β-galactosidase catalyzes the cleavage of o-nitrophenyl-β-galactoside to generate a galactose and a yellow product, o-nitrophenol.](image)

GFP was first isolated from the jellyfish, *Aequoria victoria*. GFP and its homologs isolated from other marine creatures have been utilized as fluorescent markers for labeling purposes [Chudakov, et al. 2010]. GFP absorbs light at ~480 nm and emits green fluorescence at ~510 nm. The excitation and emission wavelength may undergo blue- or red-shift by the influence of specific amino acids in the environment [Chudakov, et al. 2010]. By measuring the intensity of fluorescence emitted, the amount of expressed GFP could be quantified, which reflects the quantity of target analytes.

Luciferases were discovered and isolated from both prokaryotic cells such as
bacteria and eukaryotic cells such as firefly [Wilson and Hastings, 1998]. Their working mechanisms are slightly different. In bacteria, the continuous emission of bioluminescence requires five genes within the lux operon, luxCDABE. When substrates are added to the medium, only two genes, luxA and luxB, are required for light emission. Proteins LuxA and LuxB form a heterodimer which can catalyze the oxidation of their substrates, long-chain aldehydes, to fatty acids. This reaction involves the formation of an unstable high-energy bond and its breakage yields a blue light at around 490 nm. Proteins LuxC, LuxD and LuxE catalyze the formation of fatty aldehydes. These aldehydes will then serve as substrates for LuxA and LuxB to emit bioluminescence. In the presence of these three additional proteins (LuxC, LuxD and LuxE), there is no need for adding fatty aldehyde substrates. Firefly luciferase utilizes another type of working mechanism that is far more complicated than bacteria luciferase. It has been found that firefly luciferase is able to catalyze reactions to produce an unstable intermediate which soon degrades and leads to the emission of light (~550-570 nm) [Hastings, 1996]. The structure of substrates for firefly luciferase is also more complex than substrates for bacteria luciferase. In terms of application, both bacteria luciferases and firefly luciferase have been used in different biosensing systems [Taurianen, et al. 1998].

A typical inducible expression whole-cell sensing system for the detection of OH-PCBs is shown in Figure 1.7. A plasmid containing hbpR-P_{hbpC}-luxAB fusion as well as an ampicillin selectable marker was transformed into E. coli strain. hbpR gene codes for a regulatory protein, HbpR; P_{hbpC} is the promoter for hbpC gene while luxAB codes for bacteria luciferase. hbpC gene belongs to a group of genes called hbp genes. The hbp genes were found from Pseudomonas azelaica strain HPB1, coding for enzymes catalyzing OH-PCB degradation. With these genes, HPB1 strain can metabolize and live on OH-PCBs as its main carbon source. The hbp gene cluster consists of three open reading frames, hbpC, hbpA and hbpD. And the expression of these genes is under the control of two promoters, P_{hbpC} and P_{hbpA} and regulated by one protein, HbpR. On Pseudomonas azelaica chromosome, HbpR is located
upstream of $P_{hbpC}$ to control the expression of downstream genes. In the absence of OH-PCBs, HbpR binds with $P_{hbpC}$ and prevents the expression of $hbpC$. The binding of OH-PCBs leads to a conformational change in HbpR, which triggered the release of HbpR from $P_{hbpC}$. The release of $P_{hbpC}$ enables the production of HbpC to work on metabolizing OH-PCBs.

This sensing and response mechanism is employed in the design of a biosensing system. Instead of $hbpC$ gene, luxAB was fused downstream of $P_{hbpC}$. The release of $P_{hbpC}$ thus activates the transcription of luxAB, producing bacteria luciferase. With the addition of the substrate n-decanal, the intensity of bioluminescence generated indicates the expression level of luciferase, which in turn reflects the concentration of inducer target compounds. Therefore, the light intensity is directly correlated with OH-PCB concentration [Turner, et al. 2007].

![Figure 1. 7. Inducible expression whole-cell sensing system. A reporter gene is placed under the transcriptional promoter that is under the control of a regulator. (a) In the absence of analytes, the]
regulator binds the promoter and prevents expression of the reporter gene. (b) After the addition of analytes, the regulator will bind with analytes instead of promoter. The release of promoter leads to the expression of the reporter gene. And reporter protein will generate signals with the addition of specific substrates.

1.5 Efflux system of alien chemicals in Gram-negative bacteria

Bacteria have adapted to thrive at many harsh conditions including in the presence of high concentrations of toxic metalloids such as arsenic and antimony, toxic metals such as chromium, lead and mercury, as well as harmful organic compounds. To survive under these conditions, the resourceful bacteria have evolved intricate mechanisms to reduce the impact of the toxins on their own metabolism. A well-studied example of the adaptive evolution of microbes is the emerging and prevalence of antimicrobial resistance in microbial pathogens infecting human and domestic animals. Due to years of intensive usage of antibiotic in the treatments of infections, bacteria exposed to antibiotics have developed several types of defense mechanisms. Some bacteria produce specific enzymes such as β-lactamases to break down or deactivate antibiotics. Bacteria become resistant to one drug or one type of drugs through this mechanism. Some bacteria alter their cellular components targeted by antibiotics through mutations or enzymatic modifications. The affinities between antibiotics and their targets are therefore reduced. The third mechanism involves multidrug resistance efflux pumps. Bacteria can flush drugs out of the cell through the multidrug efflux pumps located on their membranes, and thus reduced the intra-cellular concentration of antibiotics to a sub-toxic level that is not lethal to the microbe.

Multidrug efflux pumps are encoded in the genomes of all bacteria. We speculate that these pumps might play a role in microorganisms used at the bioremediation site of PCBs. An analysis of *E. coli* genome reveals the existence of 37 open reading frames encoding putative drug transporters [Nashino, 2001]. Nishino
et al. have shown that 20 out of them are clearly involved in drug resistance, but not all 20 drug exporters are constitutively expressed. These identified drug efflux pumps were also found to be able to recognize novel drugs which are still under development by pharmaceutical companies [Nashino, et al. 2009].

Membrane transporters play an essential role in maintaining functionality of living cells. Other than protecting bacteria in a toxic environment, they keep ionic balances in between cytoplasm, periplasm and the outside environment by transporting ions such as Na$^+$ and K$^+$ across cell membranes. They also take up nutrients and exchange cellular communication molecules with the environment. There are more than 500 families of transporter systems belonging to several superfamilies, including the APT binding cassette (ABC) superfamily, the major facilitator superfamily (MFS), the resistance/nodulation/division (RND) superfamily, the amino acid-polyamine-organocation (APC) superfamily, and the voltage-gated ion channel (VIC) superfamily [Paulsen, 2003; Ren, et al. 2007]. Drug transporters in E. coli belong to five families, including MacAB from ABC superfamily, AcrAB from RND superfamily, EmrAB from MFS superfamily, Mdtk from multidrug and toxic compound exporters (MATE) family and EmrE from the small multidrug resistance (SMR) family. They work synergistically to build up the drug resistance of E. coli strains [Saier, et al. 2000; Pos, 2009].

Multidrug pump systems in E. coli have been studied extensively. Individual gene was knocked out separately in order to determine the contribution of each pump system in the drug resistant mechanism by Sulavid et al. It turned out that among all the pump systems, AcrA-AcrB-TolC system is the dominant and constitutively expressed one while others are weakly expressed under normal conditions [Sulavik, et al. 2001].

AcrA-AcrB-TolC is a tripartite protein complex with the structure shown in Figure 1.8. The AcrAB-TolC system has homologs in almost every gram-negative
bacterium strain. The major component AcrB is the multidrug efflux pump, belonging to the RND superfamily. Periplasmic fusion protein AcrA is the adaptor between AcrB and TolC and it contributes to the formation of a stable interaction between AcrB and TolC. TolC is a β-barrel protein located in the outer membrane. It functions as a channel to allow drugs passing through to the outside environment. TolC can also be employed by another pump system: EmrAB, to form another tripartite complex, EmrAB-TolC. The organization of AcrAB-TolC system provides effective protection for Gram-negative bacteria since once drugs are extruded to the outside environment, they have to traverse the outer membrane barrier again in order to reenter the cells [Nikaido and Takatsuka, 2009].
Figure 1.8. AcrAB-TolC transmembrane complex located in Gram-negative bacteria. TolC (pink) is the outer membrane channel. AcrB (green) locates at the interface of inner membrane and periplasm. AcrA (brown) works as an adapter between AcrB and TolC.
1.6 Protein AcrB

As the inner membrane component of the complex which provides a pathway for drug transport, AcrB became the focus of many studies and has been well characterized. AcrB exists as a trimer. In 2002, the crystal structure of AcrB was solved by Murakami et al., which reveals that AcrB is an asymmetric homotrimer. Each of the AcrB monomers is made up of 1049 amino acids. Its N-terminal half shows sequence homology and structural similarity with the C-terminal half [Seeger, et al. 2008]. The transmembrane domain of AcrB is made up of twelve α-helices. The periplasmic part of AcrB contains a TolC docking and a pore forming domain. A central cavity is formed in the center of AcrB trimer structure where substrates enter and be captured. The co-crystallization of AcrB and its substrates shows that these compounds are inside the central cavity. A loop structure located at the top of TolC docking domain from each monomer extends into the adjacent monomer and this interaction stabilizes the AcrB trimer structure. A vestibule area was found at the interface of transmembrane domain and periplasm domain and was suggested to be the first site where drugs enter to reach the central cavity (Figure 1.9).

AcrB employs a conformational cycling mechanism to export substrates, resembling that of a peristaltic pump. At any specific time, three monomers exhibit distinct conformations: loose (L), tight (T) and open (O). Each monomer rotates through these three conformations in turn (Figure 1.10). At the loose state, substrates enter the binding site. Then the monomer change conformation to the tight state in order to capture the substrate plus an H⁺ ion. Finally, it will change conformation again to the open state and pump substrates through the TolC channel while at the same time losing the H⁺. Through this mechanism, the transmembrane proton gradient is exploited to drive the conformational change that enabled the efflux of drugs [Seeger, et al. 2008; Pos, 2009].
Figure 1.9. AcrB monomer structure. AcrB monomer contains a transmembrane domain, a pore forming domain, and the TolC docking domain. A loop structure extends into another AcrB monomer to stabilize AcrB trimer.

Figure 1.10. Schematic representation of the AcrB conformational cycling transport mechanism. AcrB monomers are labeled different colors: orange, green and purple. The different geometric forms reflect conformational state loose (triangle), tight (rectangle), and open (circle). At Loose state, a substrate (indole) is fusing into AcrB. With a conformation change to tight state, AcrB exhibits high binding affinity to the substrate in the hydrophobic binding pocket. And finally, the substrate is released towards TolC following AcrB conformation changing to open state.
One characteristic of AcrB, similar with other multidrug efflux pumps, is that it has a wide range of substrates, including antibiotics (erythromycin, novobiocin, tetracycline, β-lactams, etc.), dyes (ethidium bromide, rhodamine 6G, crystal violet, Nile red, etc.), detergents (sodium dodecyl sulfate, Triton X-100, etc.), bile salts (cholate), and small organic molecules (hexane, cyclohexane, etc.) [Elkins and Nikaido, 2002; Takatsuka, et al. 2010]. These substrates can be charged or neutral, and differ dramatically in their molecular weights. However, these substrates show one thing in common: they all contain a large hydrophobic component. Accordingly, in terms of property, they are either hydrophobic or amphiphilic. Some examples of established AcrB substrates are shown in Figure 1.11. It was found that AcrB recognizes and picks up its substrates from the interface of inner membrane and periplasm where these lipophilic or amphiphilic molecules accumulate [Nikaido and Takatsuka, 2009]. This observation prompts us to examine the potential roles played by drug efflux pumps such as AcrB in the retention and efflux of PCBs and OH-PCBs, as such activities will affect the effectiveness of bioremediation of these compounds using microbes.
Figure 1.11. Structures of different substrates of AcrB
1.7 Summary

As discussed above, PCBs and its metabolites OH-PCBs are hazardous environmental pollutants that have an adverse effect on the health of both human beings and wildlife. In this thesis research, I investigated the ability of a major multidrug efflux transporter protein, AcrB, to efflux PCBs and OH-PCBs from the cell. A wild-type *E. coli* strain (BW25113) and its corresponding *acrB* gene knock-out strain (BW25113ΔacrB) were utilized to elucidate the potential role of AcrB in the uptake and retention of PCBs and OH-PCBs. PCB and OH-PCB toxicity assays were conducted using both strains to analyze the differences in their susceptibility. The accumulation of PCBs and OH-PCBs in the cells were first measured by GC/MS. Experimental results revealed that more of these compounds accumulated in the *acrB* gene knockout strain than the wild-type strain. I further confirmed this result using a genetically engineered whole-cell biosensing system to analyze OH-PCB concentrations in bacterial cytoplasm. A plasmid containing *HbpR*-P<sub>hbpC</sub>-lux<sub>AB</sub> fusion DNA was transformed into BW25113 and BW25113ΔacrB. In the presence of OH-PCBs, the regulatory protein HbpR binds to the analyte instead of the promoter and triggers the expression of luciferase coded by lux<sub>AB</sub> gene. By measuring the bioluminescence intensity, the intracellular concentrations of OH-PCB in the two strains were compared. When the same concentration of OH-PCBs were added in cell cultures, higher intensity of bioluminescence had been recorded in BW25113ΔacrB than in BW25113, indicating higher concentrations of OH-PCBs are present in the cytoplasm of BW25113ΔacrB. My results suggest that PCBs and OH-PCBs are likely substrates for AcrB. The efflux activities of membrane transporters should be considered while designing bioremediation experiments.
Chapter II: Materials and Methods

2.1 Reagents

Polychlorinated biphenyls (names and correlated structures shown in Figure 2.1) including PCB77, PCB104, PCB118, PCB126, PCB153, PCB166, PCB65 and PCB209, monohydroxylated polychlorinated biphenyls (names and correlated structures shown in Figure 2.2) including HPCB1001, HPCB2006, HPCB3001, HPCB4002 and HPCB5001, pyrene D-10 (C_{16}D_{10}) and N,O-Bis(trimethylsilyl)trifluoroacetamide with trimethylchlorosilane silylating reagent were purchased from AccuStandard Inc. (New Haven, CT). Dimethyl sulfoxide, sodium hydroxide, potassium hydroxide, ethylenediaminetetraacetic acid (EDTA), calcium dichloride dihydrate and kanamycin sulfate were purchased from BioWorld Inc. (Atlanta, GA). Hexane, isooctane, methylene chloride, hydrochloric acid and ferrous chloride were purchased from Fisher Scientific (Fair Lawn, NJ). Decanal, erythromycin, novobiocin, ampicillin, D-(+)-glucose, ammonium chloride, zinc sulfate heptahydrate, manganese sulfate monohydrate, copper sulfate pentahydrate, cobalt chloride, sodium borate, Luria-Bertani Broth, trypton, yeast extract, biotin, nicotinic acid and thiamine hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO). Nitritoltriacetic acid was purchased from Fluka (Switzerland). Sulfuric acid and magnesium sulfate were purchased from EMD Chemicals (Gibbstown, NJ). All chemicals were reagents grade or better and were used as received.
2.2 Bacterial strains and plasmids

Wild-type *Escherichia coli* strain BW25113 and the corresponding *acrB* gene
knockout strain BW25113ΔacrB (in which acrB gene was replaced by a kanamycin selectable marker) were obtained from Yale Coli Genetic Stock Center. E. coli strain DH10 harboring plasmid pHYBY109 was a kind gift from Dr. Sylvia Daunert. The plasmid pHYBY109 carries hbpR operon that confers resistance to OH-PCBs and LuxAB reporter gene coding for the bacteria luciferase as well as an ampicillin selectable marker. Plasmid pHYBY109 was transformed into BW25113 and BW25113ΔacrB. All bacteria strains were preserved as glycerol stocks (15% glycerol) and kept at -80 ºC.

2.3 Toxicity assay

PCB stock solutions were prepared by dissolving individual PCBs in hexane at the concentration of 100 μg/mL. OH-PCB stock solutions were prepared by dissolving individual OH-PCBs in DMSO at the concentration of 10⁻² M. Stock solutions were kept at 4 ºC. The stock solution was freshly diluted to the desired concentration right before each experiment.

3 mL of overnight culture in LB were prepared for all strains. For BW25113ΔacrB, 50 μg/mL kanamycin was added to the culture to prevent cross contamination. The cultures were incubated overnight at 37 ºC on an orbital shaker (New Brunswick Scientific Excella E24 Incubator Shaker, New Brunswick, Edison, NJ) at 250 rpm. The next morning, the overnight cultures were diluted 1000 times in fresh LB medium. In parallel, PCBs or OH-PCBs were added into wells of sterile 24-well cell culture plates (BioTek Inc., Winooski, VT). The solvent was allowed to evaporate. 800 μL of the diluted cell culture was then added into each well. The plates were shaken on a plate shaker at 37 ºC, 220 rpm for 6 hours. The optical density of cells at 600 nm in each well was then measured using the Epoch multi-volume spectrometer system (BioTek Inc., Winooski, VT).

2.4 Accumulation assay
2.4.1 Cell preparation

An overnight culture of each strain was prepared by adding 50 μL fresh culture to 50 mL LB medium. The culture for BW25113ΔacrB strain contained 50 μg/mL kanamycin. Flasks were shaken at 37 °C on an orbital shaker at 250 rpm overnight. PCB and OH-PCBs stock solutions were added to autoclaved 250 mL Pyrex flasks and solvents were allowed to evaporate. Next, 100 mL LB medium and 20 mL overnight culture were transferred into the flask containing either PCBs or OH-PCBs. The flasks were incubated at 37 °C in the orbital shaker at 220 rpm. 10 mL cultures were withdrawn after 1, 3, 5, 10, and 24 hours of incubation and processed as described below.

2.4.2 Extraction and treatment

10 mL cell culture was centrifuged using a Beckman Coulter Allegra 25R Centrifuge (Brea, CA) at 5000 rpm for 10 minutes. Cell pellets were resuspended in 1.5 mL PBS buffer (one liter PBS buffer containing: 1.42 g of Na₂HPO₄, 8.0 g of NaCl, 0.27 g of KH₂PO₄ and 0.20 g of KCl, with pH adjusted to 7.4). The solutions were then transferred into pre-weighed Eppendorf tubes and centrifuged at 5000 rpm for 5 min. The tubes were decanted to remove the supernatant. The residual liquid was absorbed by Kimwipe tissue paper. Cell weights were measured from the difference between the weight of Eppendorf tubes with cell pellets and the weight of the empty tubes.

Cells were then resuspended in 2 mL deionized water and immediately transferred to 5 mL VWR glass vials. After the addition of the recovery standard solution (100 μL PCB 166, 1.0 μg/mL for PCB accumulation assay or 300 μL HPCB1001, 2.0 μg/mL, for OH-PCB accumulation assay), cells were lysed using a Fisher Scientific sonicator (Hanover Park, IL). PCBs and OH-PCBs were then extracted by vortex mixing the cell lysate with 2 mL of hexane for 20 min and shaken
on an orbital shaker at room temperature, 100 rpm for another 10 min to achieve higher extraction efficiency. Organic phase was transferred to another 5 mL glass vial. 1 mL concentrated H\textsubscript{2}SO\textsubscript{4} was added to remove lipids from the organic phase. After vortexed for 1 min, the sample was left overnight to allow the separation of the two phases. The next morning, the organic phase was withdrawn and concentrated to 100 μL under a gentle stream of nitrogen.

For PCB accumulation assay, the concentrated samples were transferred using a glass syringe to 1.5 mL Agilent GC/MS glass vials (with glass insert). 10 μL of internal standard PCB209 (1 μg/mL) was spiked into each sample before it was injected into GC/MS. An internal standard (ISTD) is a compound added to a sample in known concentration to facilitate the qualitative identification and/or quantitative determination of the sample components [IUPAC, 1997].

For OH-PCB accumulation assay, after the concentration of the sample, 100 μL of silylating reagents (BSTFA: TMCS = 99:1, v/v) were added. The vials were then tightly capped and placed in a temperature controller (PolyScience Inc., Niles, IL) at 60 °C for 40 min to allow the derivatization of hydroxyl groups in OH-PCBs. After derivatization, silylating reagents were evaporated. The dried solute was re-dissolved in 100 μL hexane. The solution was transferred into 1.5 mL Agilent GC/MS glass vial (with glass insert). 10 μL ISTD pyrene-d\textsubscript{10} (1 μg/mL) was added as ISTD before the GC/MS analysis.

2.4.3 GC/MS operation

PCBs and OH-PCBs were analyzed using an Agilent 6890 Gas Chromatograph coupled with Agilent 5975 Inert Mass Selective Detector (Agilent, Santa Clara, CA). GC/MS conditions can be found in Table 2.1.
Capillary column: HP-5MS 5% Phenyl-Methyl Siloxane Column, Agilent
Length: 30m; Internal Diameter: 0.25 mm; Film Thickness: 0.25 μm

Carrier gas: Ultra-Pure Helium, 1.2 ml/min

Injection mode: Splitless (Purge on at 0.75 min)

Injection port temperature: 250 °C

Oven temperature program:
- 60 °C (hold 1 min, 40 °C /min) → 200 °C;
- 200 °C (4 °C /min) → 280 °C (hold for 5.5 min)

Run time: 30 min

* Inject hexane/methylene chloride between runs to reduce carry-over problem

Table 2.1 GC/MS conditions applied in this study

Calibration curves for different PCBs were established with seven concentration points for each species in the range of 0.05-3.5 μg/mL (0.05, 0.1, 0.2, 0.5, 1, 2, 3.5 μg/mL), each containing 0.1 μg/mL of ISTD (PCB209). Calibration curves for OH-PCBs were established with seven concentration points within the range of 0.1-10 μg/mL (0.2, 0.5, 1, 2, 5, 10 μg/mL), each containing 0.1 μg/mL of ISTD (pyrene-d10).

The ratio of peak for each compound to that of the ISTD was used for quantification.

2.5 Bioluminescence assay

2.5.1 Preparation of cells harboring pHYBY109

Plasmid pHYBY109 was transformed into BW25113 and BW25113ΔacrB using the heat shock method [Froger, et al. 2007]. Overnight cultures for both BW25113 and BW25113ΔacrB harboring plasmid pHYBY109 were prepared one
day before testing. For BW25113-pHYBY109, 50 mL LB medium containing ampicillin (100 μg/mL) was added in a 250 mL flask followed by the addition of 50 μL fresh cell culture. The culture for BW25113ΔacrB-pHYBY109 was made by adding 50 μL fresh cell culture to 50 ml LB medium containing kanamycin (50 μg/mL) and ampicillin (100 μg/mL). Flasks were kept at 37 °C on an orbital shaker at 250 rpm overnight.

For the luciferase induction assay, 3 mL overnight cultures was transferred into each flask containing 50 mL mineral medium (MM) [Jaspers, et al. 2001]. OH-PCB stock solutions were then added to the desired concentration. All tests were prepared in triplicate. These flasks were placed at 30 °C on an orbital shaker at 225 rpm for 5 h incubation period.

Mineral medium (per liter) contains: 1.00 g of NH₄Cl, 3.49 g of Na₂HPO₄-2H₂O, 2.77 g of KH₂PO₄, 2.0 mL a vitamin solution, 20 mL Hunter’s vitamin-free mineral base, 0.01% trypton, 0.005% yeast extracts, and 10 mM D- (+)-glucose, adjusted to pH 6.8. The vitamin solution (per 100 mL) contained the following: 0.5 of mg biotin, 50 mg of nicotinic acid, and 25 mg of thiamine hydrochloride. Hunter’s vitamin-free mineral base (per liter) was prepared by mixing 10 g of nitrilotriacetic acid, 6.0 g of KOH, 14.5 g of MgSO₄•7H₂O, 3.33 g of CaCl₂•2H₂O, 9.74 mg of (NH₄)₆MoO₂₄•4H₂O, 99 mg of FeSO₄•7H₂O and 50 ml Metal 44 Solution. The Metal 44 Solution (per 100 ml) contains 387 mg of Na₄EDTA•4H₂O, 1.10 g of ZnSO₄•7H₂O, 914 mg of FeSO₄•7H₂O, 154 mg of MnSO₄•H₂O, 39.2 mg of CuSO₄•5H₂O, 24.8 mg of Co(NO₃)₂•6H₂O, 17.7 mg of NaB₄O₇•19H₂O and it was neutralized with H₂SO₄. MM was sterilized and stored at 4 °C before use.

2.5.2 Bioluminescence detection

After the 5 hours incubation, 50 mL cell culture was centrifuged at 5000 rpm
for 10 min. Cell pellet was resuspended in 3 mL PBS buffer. For the final assay, 0.3 mL of cell was transferred into a far UV quartz cuvette. Following the addition of 30 μL n-decanal (50 mM in 1:1 ethanol/H₂O), bioluminescence was immediately measured using a PerkinElmer LS55 Fluorescence Spectrometer (Waltham, MA) set at bioluminescence measurements. Light output was integrated from 5 s to 30 s and the total light signal was expressed as relative light unit (RLU).
3.1 PCBs and OH-PCBs used in this study

Five PCBs were selected as model compounds in this study, including PCB77, PCB104, PCB118, PCB126, and PCB153. Different levels of chlorination are present in the chosen PCBs, from four chlorine substitutions to six chlorine substitutions. Three of the PCBs (PCB77, PCB118, and PCB126) are co-planar congeners while the remaining two (PCB104 and PCB153) are non-planar congeners. PCBs were kept at 4 °C upon received and dissolved in hexane before performing tests.

Although there are 837 possible monohydroxylated-PCB congeners, only limited numbers of these compounds are commercially available. Four monohydroxylated-PCBs were selected as target compounds in this study, including HPCB2006, HPCB3001, HPCB4002, and HPCB5001. All selected OH-PCBs contain one hydroxyl group substitution on carbon position 2 of the biphenyl structure. Different levels of chlorination are present in chosen OH-PCBs, from two chlorine substitutions in HPCB2006 to five chlorine substitutions in HPCB5003. The chosen OH-PCBs have been included in the study of a whole-cell biosensing system (Turner et al., 2007). OH-PCBs were kept at 4 °C and dissolved in DMSO before performing tests.

3.2 PCB toxicity on E. coli

Toxicity of chosen PCBs were determined in both wild-type E. coli strain BW25113 and an acrB gene knockout strain BW25113ΔacrB. PCBs were applied as a mixture of all five PCBs and the total PCB concentration in cell cultures ranged from 0.8 to 12.8 μg/mL. Optical densities of cells at 600 nm were measured after 6 hours of incubation (Figure 3.1).
PCBs showed no toxicity at levels examined in both BW25113 and BW25113ΔacrB, presumably due to the low solubility and bioavailability of these hydrophobic compounds.

3.3 OH-PCB toxicity on *E. coli*

Toxicity of chosen OH-PCBs was investigated in both BW25113 and BW25113ΔacrB. Individual OH-PCB was tested separately in order to reveal the differences in their toxic effects. Different concentrations of OH-PCBs in the range from 0.4 to 6.4 μg/mL were present in cell cultures. Optical densities of cells at 600 nm were measured after 6 hours incubation. Testing results for each OH-PCB along with the negative control are shown in Figure 3.2. to Figure 3.5. Data are normalized to the optical densities of the cell culture in the absence of OH-PCBs.
Figure 3. 2. Toxicity assay for HPCB2006 in BW25113 (orange) and BW25113ΔacrB (blue). AU, arbitrary unit.

Figure 3. 3. Toxicity assay for HPCB3001 in BW25113 (orange) and BW25113ΔacrB (blue). AU, arbitrary unit.
Figure 3.4. Toxicity assay for HPCB4002 in BW25113 (orange) and BW25113ΔacrB (blue). AU, arbitrary unit.

Figure 3.5. Toxicity assay for HPCB5001 in BW25113 (orange) and BW25113ΔacrB (blue). AU, arbitrary unit.
Different levels of toxicity were observed for different OH-PCBs as well as for the two *E. coli* strains. Among the four OH-PCBs tested, the levels of toxicity decreases from HPCB5001 to HPCB2006, and then to HPCB3001 and HPCB4002. BW25113 exhibits higher tolerance to OH-PCBs than BW25113ΔacrB, indicating the significance of protein AcrB in conferring bacteria resistance toward OH-PCBs. Knocking out *acrB* gene directly leads to the lack of protein AcrB in the cell, through which BW25113ΔacrB strain loses its major multidrug efflux pump. As a result, OH-PCBs accumulate in cells and cause lethal effects to the cells.

3.4 PCB and OH-PCB accumulation assay in *E. coli*

3.4.1 Establishment of GC/MS method

All seven PCBs could be completely separated on the GC/MS chromatogram within 30 min under applied conditions. Each species displayed a single peak with a nice peak shape. A chromatogram of a sample containing mixed PCBs with each peak identified is shown in Figure 3.6. Target PCB identifications were confirmed by mass spectra as shown in Figure 3.7. The molecular ions [M+] of eight PCBs were 498, 292, 292, 326, 326, 326, 360, and 360, for PCB209, PCB77, PCB65, PCB104, PCB118, PCB126, PCB153, and PCB166, respectively. The highest abundant fragment ion was utilized for quantification and analysis.
Figure 3.6 Gas chromatogram for model PCBs. AU, arbitrary unit.

Peak identity: 1. PCB65; 2. PCB104; 3. PCB77; 4. PCB118; 5. PCB153; 6. PCB126; 7. PCB166; 8. PCB209 (ISTD).
A.

PCB77

B.

PCB104
Figure 3.7 Mass spectra for five target PCBs: PCB77 (A), PCB104 (B), PCB118 (C), PCB126 (D), and PCB153 (E). The molecular ion [M⁺] for quantification is marked as bold black numbers on each plot.

In this study, PCB166 were used as PCB recovery standards. Extraction recoveries for all PCBs at different incubation times ranged from 14.6% to 62.7%. Standard calibration curves for each PCB were shown in Figure 3.8.
A. Integrated Peak Area, AU

Response = 6.14e+005 * Amt - 5.54e+004
Coef of Det (r^2) = 0.999  Curve Fit: Linear

B. Integrated Peak Area, AU

Response = 6.20e+005 * Amt - 1.54e+004
Coef of Det (r^2) = 0.999  Curve Fit: Linear
C.

**Integratd Peak Area, AU**

<table>
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<th>[PCB118], µg/mL</th>
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<td>Coef of Det (r^2)</td>
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D.

**Integratd Peak Area, AU**

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<td>Curve Fit: Linear</td>
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Figure 3.8. Standard calibration curves and coefficient for all PCBs: PCB77 (A), PCB104 (B), PCB118 (C), PCB126 (D), PCB153 (E), and PCB166 (F). AU, arbitrary unit.

3.4.2 Accumulation of PCBs in wild-type and acrB knockout E. coli

As described in Chapter II, BW25113 and BW25113ΔacrB cell cultures were
incubated in the presence of PCBs. At specified time, small portions of the cell culture were taken and processed to determine the PCB contents in the cell pellet. The quantity of PCB was normalized to the weight of the cell pellet. The accumulation data determined over time are shown in Figure 3.9.

A.
Figure 3.9 PCB accumulation assay in BW25113 (orange) and BW25113ΔacrB (blue). The concentrations of PCB in cell pellets collected at 1, 3, 5, 10, and 24 h were determined and normalized over the cell weight.

Two observations were made through the accumulation studies: First, the
accumulation of PCB in cells did not increase over time. For BW25113, the level of PCBs remained stable within the error range over the time period collected. For BW25113ΔacrB, the accumulation level reached the maximum value within one hour, and then decreased slightly over time. Second, comparing the one hour accumulation data, higher concentration of PCBs accumulated in BW25113ΔacrB than in BW25113. This observation is consistent with our speculation that PCBs are substrates of AcrB. BW25113ΔacrB lacked functional AcrB, and therefore was less effective in pumping out PCBs.

While the same initial concentrations were used, different PCBs displayed different accumulation levels. The difference in accumulation could result from two factors: first, the differences in the AcrB efflux efficiency of various PCBs; second, the difference in the intrinsic solubility and bioavailability of the PCBs. The differences in the AcrB efflux efficiency are reflected from the differences in the PCB accumulation amounts in BW25113 and BW25113ΔacrB. Through comparing, after 1 hour of incubation, the accumulation levels of all five PCBs in BW25113 are approximately 44-50% of the level in BW25113ΔacrB, indicating AcrB efflux the five PCBs with a similar efficiency (Table 3.1).

To compare the intrinsic solubility and bioavailability of the PCBs, we compared the accumulation in the BW25113ΔacrB strain (Figure 3.10). While the same concentration of PCBs existed in the cell culture, distinct accumulation levels were observed. Among all five PCBs, PCB126 has the highest while PCB153 has the lowest bioavailability. The bioavailability of PCBs is correlated with their structures. In this study, PCB126 and PCB77 have co-planar structures which lead to their relatively high water solubility and bioavailability, while PCB104, PCB118, and PCB153 have non-planar structures and thus low solubility and bioavailability. Therefore, distinguished accumulation levels of PCBs are likely due to differences in bioavailability of target PCBs.
Table 3.1 PCB accumulation in BW25113 and BW25113ΔacrB with one hour incubation.

<table>
<thead>
<tr>
<th></th>
<th>BW25113 (PCB/Cell Weight, μg/g)</th>
<th>BW25113ΔacrB (PCB/Cell Weight, μg/g)</th>
<th>WT / ΔacrB (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCB77</td>
<td>99.96</td>
<td>222.32</td>
<td>44.90</td>
</tr>
<tr>
<td>PCB104</td>
<td>64.73</td>
<td>131.41</td>
<td>49.26</td>
</tr>
<tr>
<td>PCB118</td>
<td>69.98</td>
<td>142.36</td>
<td>49.17</td>
</tr>
<tr>
<td>PCB126</td>
<td>106.90</td>
<td>236.80</td>
<td>45.14</td>
</tr>
<tr>
<td>PCB153</td>
<td>49.81</td>
<td>110.17</td>
<td>45.24</td>
</tr>
</tbody>
</table>

Figure 3.10. Accumulation of five PCBs in BW25113ΔacrB with one hour incubation. PCB amounts are normalized to cell weights.

One question remains to be answered, why the PCB level in BW25113ΔacrB decreased over time. After 24 hours of incubation, the concentration of PCB in BW25113ΔacrB decreased to a level comparable to the level in wild-type E. coli. On the contrary, the PCB level in the wild-type strain BW25113 remained at a constant level. We speculate that the presence of PCBs might have triggered the producing of
other efflux pumps which are coded in the bacteria genome.

3.4.3 Accumulation of OH-PCBs in wild-type and acrB knockout E. coli

The presence of the -OH group in monohydroxylated PCBs increases the difficulty in using GC for analysis, since it drastically reduced the volatility of the compound. Therefore, derivatization is necessary to overcome the low volatility of OH-PCBs. I tested two derivatization approaches, methylation and silylation. I found silylation was superior, because the reaction occurs in milder conditions than methylation, with shorter incubation time and relatively low temperature. Silylation procedure with BSTFA/TMCS reagent (at 60°C for 40 min) for derivatization [Shi et al, 2009] is applied in this study. Silylating reaction of HPCB2006 is shown in Figure 3.11.

GC chromatograms were collected using silylated OH-PCBs. All four compounds were completely resolved as shown in Figure 3.12. Peaks were identified with standards as well as known spectra from the compound library. Molecular ions [M+] detected in each of OH-PCB silylation products were at 275, 310, 346, 380, and 414, for HPCB1001, HPCB2006, HPCB3001, HPCB4002, and HPCB5001, respectively. An ion with the mass to charge ratio of 93 was selected for quantifying OH-PCBs. As shown in Figure 3.13, mass spectra for OH-PCB silylation products were obtained as well.

In this study, pyrene-d_{10} was chosen as ISTD. HPCB1001 was used as recovery standard for OH-PCBs. The extraction recovery for all four OH-PCBs is in the range from 13.4% to 95.6%. The standard calibration curves for the OH-PCBs used in this study are shown in Figure 3.14.
Figure 3.11. HPCB2006 silylating reaction equation.
Figure 3.12. Gas chromatogram of a sample containing a mixture of silylated OH-PCBs. AU, arbitrary unit. Peak identity: 1. HPCB1001-Si(CH$_3$)$_3$; 2. HPCB3001-Si(CH$_3$)$_3$; 3. HPCB2006-Si(CH$_3$)$_3$; 4. HPCB4002-Si(CH$_3$)$_3$; 5. pyrene-d$_{10}$ (ISTD); 6. HPCB5001-Si(CH$_3$)$_3$. 
Figure 3.13. Mass spectra of four OH-PCB silylation products: HPCB2006 (A), HPCB3001 (B), HPCB4002 (C), and HPCB5001 (D). Chemical structures of targets are shown in the plots. Mass ion used for quantification is marked with a bold black number on each plot.
A.

Integrated Peak Area, AU

\[ \text{Resp Ratio} = 7.36\text{e-002} \times \text{Amt} \times 1.78\text{e-001} \]

Coef of Det \((r^2)\) = 0.998  Curve Fit: Linear

B.

Integrated Peak Area, AU

\[ \text{Resp Ratio} = 5.62\text{e-002} \times \text{Amt} \times 7.91\text{e-002} \]

Coef of Det \((r^2)\) = 0.999  Curve Fit: Linear
C.

Integrated Peak Area, AU

HPCB3001

Resp Ratio = 7.05e-002 \* Amt \* 9.70e-002

Coef of Det (r^2) = 0.999 Curve Fit: Linear

D.

Integrated Peak Area, AU

HPCB4002

Resp Ratio = 4.62e-002 \* Amt \* 1.18e-001

Coef of Det (r^2) = 0.998 Curve Fit: Linear

55
A mixture of four OH-PCBs was added to BW25113 and BW25113ΔacrB to mimic the real samples in which PCB metabolites are present in a mixture. Total OH-PCB concentration in cell cultures reached 0.4 μg/mL (0.1 μg/mL of each OH-PCB, respectively) and was controlled at equal to or less than 0.4 μg/mL in the following tests. According to previous toxicity assays, OH-PCBs at these concentrations did not have a significant toxic effect. Accumulation of individual OH-PCB in cell strains was shown in Figure 3.15. Several observations were made.

First, there is a drastic difference in OH-PCB accumulation levels between BW25113 and BW25113ΔacrB. This result is consistent with previous observations with PCBs. Again, it supports the hypothesis that OH-PCBs are AcrB substrate. Second, in the wild-type strain BW25113, OH-PCB accumulation level maintained relatively constant up to 24 hours of incubation. While in the gene knockout strain BW25113ΔacrB, the amount of PCB reduced with time. This observation is again similar to previous experimental results obtained for the accumulation experiment of
PCBs. Third, the efflux efficiencies of AcrB for different OH-PCBs were clearly different, as revealed by the difference between the accumulation levels of each OH-PCB in BW25113 and BW25113ΔacrB (Table 3.2). Among four OH-PCBs, HPCB4002 accumulated in BW25113ΔacrB 12 fold more than that in BW25113, suggesting high efflux efficiency of protein AcrB to transport HPCB4002. HPCB5001 accumulation amount in BW25113ΔacrB was only 1.5 fold more than the level in BW25113, which indicated that AcrB has relatively low efflux efficiency towards HPCB5001. Bioavailability of different OH-PCBs is reflected from the accumulation amount in BW25113ΔacrB as shown in Figure 3.16. The accumulation level of HPCB2006 which has a co-planar structure is 3 fold the amount of HPCB3001 and HPCB4002, and 1.3 fold the amount of HPCB5001, suggesting its high bioavailability, while the other three OH-PCBs have non-planar structure which directly leads to their low bioavailability.

A.
Figure 3.15. Accumulations of HPCB2006 (A), HPCB3001 (B), HPCB4002 (C), and HPCB5001 (D) in BW25113 (orange) and BW25113ΔacrB (blue). OH-PCB accumulations in *E. coli* were measured after 1, 3, 5, 10, and 24 h of incubation.

<table>
<thead>
<tr>
<th></th>
<th>BW25113</th>
<th>BW25113ΔacrB</th>
</tr>
</thead>
<tbody>
<tr>
<td>(PCB/Cell Weight, μg/g)</td>
<td>(PCB/Cell Weight, μg/g)</td>
<td>(%)</td>
</tr>
<tr>
<td>HPCB2006</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.441</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPCB3001</td>
<td>0.911</td>
<td></td>
</tr>
<tr>
<td>HPCB4002</td>
<td>0.286</td>
<td></td>
</tr>
<tr>
<td>HPCB5001</td>
<td>4.671</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.16. Accumulation of four OH-PCBs in BW25113ΔacrB with one hour incubation. OH-PCB amounts are normalized to cell weights.

To evaluate the effect of initial OH-PCB concentration on cellular accumulations, we measured HPCB2006 accumulation after 1 hour of incubation at three different concentrations, 0.1, 0.2, and 0.4 μg/mL. HPCB2006 accumulation in BW25113 and BW25113ΔacrB after 1, 3, 5, 10, and 24 h of incubation were measured by GC/MS. As shown in Figure 3.17 and Figure 3.18, HPCB2006 accumulation level correlates positively with initial concentrations. When HPCB2006 concentration increases from 0.1 to 0.4 μg/mL, the accumulation in BW25113ΔacrB increases from 1.5 fold to 4 fold of that in BW25113 strain. The results further confirm that protein AcrB works on extruding OH-PCBs. Additionally, the results suggest that the AcrB pump efflux efficiency is dependent on the amount of toxic compounds present. Moreover, OH-PCB accumulation decreases rapidly with time in BW25113ΔacrB as comparing Figure 3.17 and Figure 3.18. The decrease ratio is dependent on the concentration of OH-PCBs. This observation is in agreement with previous results on PCBs and again we speculate that the presence of toxic
compounds such as OH-PCBs triggers the self-defense system in bacteria: other efflux pumps are therefore being expressed. The response, however, takes certain amount of time to be reflected from the compound accumulation results.

Figure 3.17. HPCB2006 accumulation in BW25113 (Orange) and BW25113ΔacrB (Blue) with 1 hour of incubation in response to initial concentration applied.
Figure 3.18. HPCB2006 accumulation in BW25113 (Orange) and BW25113ΔacrB (Blue) with 10 hours of incubation in response to initial concentration applied.

3.5 Bioluminescence assay

The hbp genes enable P. azelaica to degrade hydroxylated biphenyls and use them as sole carbon sources. The mechanism involved has been well characterized [Kohler, et al. 1988]. A group of plasmids containing different hbp genes as well as promoters were constructed by Jaspers et al to further explore the characteristics of the promoter genes [Jaspers, 2000]. They found a transcription activator hbpR protein and two promoters that control the expression of hbp genes. Later, a plasmid (pHYBY109) containing hbpR-P_{hbpC-luxAB} fusion was created and transformed into E. coli for the studying of P_{hbpC} promoter activity [Jaspers, 2001]. Upon the addition of OH-PCBs, increase of bioluminescent signals was detected, suggesting the binding of OH-PCBs to HbpR leads to direct activation of the promoter P_{hbpC}.

In our study, the plasmid pHYBY109 was transformed to both wild-type E. coli strain BW25113 and acrB gene knockout strain BW25113ΔacrB in order to
investigate the intracellular concentration of OH-PCBs. Based on our previous studies, more OH-PCBs accumulated in BW25113ΔacrB than in BW25113. However, the above experiment could not differentiate the difference between OH-PCBs attached to the cell membrane and those entered into the cell. By using this bioluminescent whole-cell sensing system, we aimed at evaluating the differences of OH-PCB concentrations in the cytoplasm of the two strains.

As shown in Figure 3.19, in the plasmid used in this study, \textit{hbpR}, coding for the regulator HbpR protein, is placed upstream of the promoter \(P_{\text{hbpC}}\) and the reporter \textit{luxAB}, which codes for bacteria luciferase. In this way, the expression of \textit{luxAB} is activated by the promoter \(P_{\text{hbpC}}\), which is directly regulated by HbpR. In the absence of OH-PCBs, HbpR binds with \(P_{\text{hbpC}}\), and prevents the expression of luciferase, while the addition of OH-PCBs triggers a conformational change in HbpR to release the promoter. As a result, expression of \textit{luxAB} is induced. Bioluminescence is then measured immediately after the addition of the substrate.

The mechanism involved in generating bioluminescence is shown in the following reaction:

\[
\text{FMNH}_2 + RCHO + O_2 \xrightarrow{\text{luciferase}} \text{FMN} + \text{RCPOOH} + \text{hv}(500nm)
\]

Bacteria luciferase catalyzes the monooxygenation of a long-chain aldehyde to the corresponding carboxylic acid with production of light due to the decay of an exited state intermediate. Here we used decanal as the substrate.
Important assay parameters were optimized in order to improve the performance of the sensing system. Different incubation temperatures including 25 °C, 30 °C, and 37 °C, incubation length including 3, 5, and 7 hours as well as cell densities for the final bioluminescent assay were evaluated. Based on the sensitivity of the instrument, the optimum conditions under which the strongest bioluminescent signals can be measured, which are used in this study, are the following: the incubation temperature and time were set at 30 °C for 5 hours; optical densities of cells in the final solution were at 3.0. The maximum bioluminescence emission wavelength was determined to be 500 nm. Immediately after addition of the substrate solution, the bioluminescent output was recorded. A bioluminescence emission curve can be observed. A representative emission spectrum is shown in Figure 3.20.
We chose to work in the OH-PCB concentration range from 0.1 to 3.2 or 6.4 μg/ml, as higher OH-PCB concentration slowed down the growth of the knockout strain, which is more sensitive to the toxicity of OH-PCBs. An increase of the bioluminescent signals was detected with the increasing concentration of OH-PCBs, consistent with results reported in literature [Turner, et al. 2007]. Bioluminescent signals in both BW25113 and BW25113ΔacrB were detected in the presence of four concentrations of each OH-PCB (Figure 3.21).

Consistent with earlier toxicity and accumulation studies, different OH-PCBs have different efficiency in inducing the bioluminescent signal (Figure 3.21). However, all four OH-PCBs tested induced significantly higher signals in BW25113ΔacrB compared to BW25113. The stronger signals in BW25113ΔacrB suggested a higher cytoplasmic OH-PCB concentration in the acrB knockout strain.

For three OH-PCBs tested, HPCB2006, HPCB3001, and HPCB5001, the
bioluminescent levels in the wild-type strain BW25113 were constant at different OH-PCB level (Figure 3.21). For HPCB4002, an increase of OH-PCB concentration in the cell culture did result in an increase of the bioluminescent signal. On the contrary, all OH-PCBs induced the emission of bioluminescent signal in a concentration dependent manner in BW25113ΔacrB.

This result indicates that protein AcrB does interact with OH-PCBs and functions on efflux these toxic compounds out of cells. Therefore, with absence of protein AcrB, OH-PCBs are more likely to stay inside the cells and causing damage to the cell.

A.
Figure 3.21. Bioluminescent signal of BW25113 (orange) and BW25113ΔacrB (blue) in the presence of OH-PCBs: HPCB2006 (A), HPCB3001 (B), HPCB4002 (C), and HPCB5001 (D). Data are the integration of bioluminescent signals from 5 s to 30 s divided by optical densities of cells at 600 nm in the final testing solutions. RLU, relative light unit.
Chapter IV: Conclusions

In this project, the interaction between a major multi-drug efflux transporter protein, AcrB, and PCBs as well as their metabolites, OH-PCBs, was investigated through the use of a wild-type *E. coli* strain BW25113 and its corresponding *acrB* gene knockout strain BW25113ΔacrB strain. I have found that PCBs have little toxic effects to both *E. coli* strains, presumably due to low bioavailability. Different OH-PCBs have distinguished levels of toxic effects. By measuring PCB and OH-PCB accumulation in both cell strains using GC/MS, apparent differences were observed between BW25113 and BW25113ΔacrB. PCBs and OH-PCBs accumulate more in BW25113ΔacrB than in BW25113, suggesting protein AcrB functions in extruding these compounds out of cells. The results are in agreement with the bioluminescent assays using a whole-cell sensing system. With the same concentration of OH-PCBs in the cell culture, a higher level of OH-PCBs enters the cytoplasm of BW25113ΔacrB than BW25113. My results indicate that PCBs and OH-PCBs are likely to be substrates of protein AcrB. Therefore, AcrB can recognize and transport PCBs and OH-PCBs out of cells. This finding suggests that the potential role of efflux pumps should be considered while using microbes in bioremediation applications involving PCBs and OH-PCBs.
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