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An Ghníomhaireacht um Chaomhnú Comhshaoil

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Novel Approaches for the Detection of Polluting Chemicals in the Environment

STRIVE

Environmental Protection Agency Programme

2007-2013

SSTI

Comhshaol, Oidhreacht agus Rialtas Áitiúil
Environment, Heritage and Local Government

Environmental Protection Agency

The Environmental Protection Agency (EPA) is a statutory body responsible for protecting the environment in Ireland. We regulate and police activities that might otherwise cause pollution. We ensure there is solid information on environmental trends so that necessary actions are taken. Our priorities are protecting the Irish environment and ensuring that development is sustainable.

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EPA STRIVE Programme 2007-2013

Novel Approaches for the Detection of Polluting Chemicals in the Environment

Bacterial Detoxification Enzymes as Bioindicators and Biosensors of Environmental Pollution

(2007-FS-WRM-5-M5)

STRIVE Report

Prepared for the Environmental Protection Agency

by

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The EPA STRIVE Programme addresses the need for research in Ireland to inform policymakers and other stakeholders on a range of questions in relation to environmental protection. These reports are intended as contributions to the necessary debate on the protection of the environment.

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Executive Summary

Toxic synthetic organic compounds are ubiquitous in our modern environment and pose significant risks to human health. These compounds include polychlorinated biphenyls (PCBs) and many toxic chlorinated organic pesticides of concern as environmental pollutants. The glutathione transferases (GST) family of enzymes can play an important role in the detoxification of a wide range of these compounds. The aim of this research project was to investigate (known and novel) bacterial GSTs in environmental samples, and to study microbial diversity in these samples using molecular biological techniques. It was proposed that bacterial GSTs identified in soil/sediment contaminated with toxic chlorinated organic compounds could have the potential for bioremediation, and for development as bioindicators and biosensors of environmental pollutants, for example, toxic chlorinated organic PCBs and pesticides. A specific bacterial GST, $BphK^{LB400}$, was to be developed for use as a biosensor and for bioremediation.

A number of environmental soil/sediment samples that were of interest as potential sources of bacteria capable of degrading chlorinated organic compounds were sourced, both nationally and internationally, and used for genomic DNA isolation. A highly conserved gene (the 16S rRNA gene), which is used to identify microorganisms present in soil/sediment samples, was further investigated.

The DNA sequences of a number of bacterial GSTs capable of degrading organic compounds were analysed so as to identify highly conserved amino acids. These amino acids were then used in the design of experiments to identify (i) bacterial GST sequences (known and novel) in environmental samples and (ii) variants of a specific bacterial GST, $BphK^{LB400}$, with increased GST towards toxic chlorinated organic compounds found in the environment, which were to be used in the development of biosensors and for bioremediation. A number of bacterial GST sequences of interest (with increased activity towards pollutants) linked to a fluorescent tag were inserted into plant-associated bacteria. These modified bacteria were shown to be capable of colonising plants. However, more work needs to be done to further assess these laboratory-scale experiments. Although many environmental regulatory measures have been adopted in Ireland in recent decades, there has been little regulation regarding the protection of soil or the remediation of contaminated soil. With environmental technologies playing a key role in the Irish government's *Building Ireland's Smart Economy* paper (2008), and a global political shift towards sustainable and green bioremediation technologies, the development of plant-associated bacteria to degrade toxic synthetic organic compounds in environmental soil may provide an efficient, economic, and sustainable green remediation technology for the twenty-first century environment.

A relatively simple and inexpensive method (using gas chromotography-mass spectrometry [GC-MS]) was developed for the analysis of soil/sediment samples contaminated with chlorinated organic compounds. In 2006, the European Commission published the final Thematic Strategy for Soil Protection and a proposal for a directive that would establish a framework for the protection of soil involving the identification of the location and extent of soil contamination and the requirements for remediation. As an output of this project, it is recommended that this assay be further developed so that when the Soil Framework Directive is introduced and legally implemented in all member states of the European Union, a large number of Irish environmental soil/sediment samples can be analysed relatively quickly and inexpensively in Irish laboratories with standard GC-MS facilities.

Because of the ambitious nature of a number of these initial goals, not all the specific goals of this project were reached. However, data obtained from this project has the potential for the development of biosensors and for the bioremediation of chlorinated organic compounds in the environment, and for the development of a relatively simple and inexpensive method for detecting chlorinated organic compounds in environmental samples.

1 Introduction

Synthetic organic compounds are ubiquitous in the modern environment. They are found in homes, workplaces, public spaces and in agriculture. These organic compounds can enter soil, air and water through either local or diffuse contamination and can often be found far from their source of origin. Local or point-source pollution involves discrete locations of pollution – for example, industrial waste via factory or sewer pipes. Diffuse or nonpoint-source pollution involves pollution from multiple cumulative inputs over a large area, for example, agricultural waste (from farms) and municipal waste. While a large number of synthetic organic compounds are harmless, some are toxic and pose serious environmental and human health risks. The effects of contamination of environmental soil from toxic synthetic organic compounds include the poisoning of animals and plants, the altering of ecosystems, and risks to human health. International and national legislation attempts to address local sources of pollution by targeting industrial discharge. However, diffuse sources of pollution are more difficult to monitor and usually have a greater impact on the quality of the environment.

Because of their hydrophobic properties, many toxic synthetic organic compounds are persistent and are stored in fat tissue, resulting in bioaccumulation. Therefore, organisms at higher levels in food chains (e.g. humans) tend to have greater concentrations of these bioaccumulated toxins stored in their fat tissue than those at lower levels, resulting in the biomagnification of the physiological effects of the toxins in higher organisms. At the highest level in the food chain, that is, humans, these toxic organic compounds can be passed from mother to child either *in utero* via the placenta or post-natally via breast milk.

Synthetic organic compounds of concern as environmental contaminants include polychlorinated biphenyls (PCBs), pesticides, industrial solvents, petroleum products, dioxins and furans, explosives, and brominated flame retardants. Twelve organic compounds were listed as persistent organic pollutants (POPs) by the Stockholm Convention on Persistent Organic Pollutants, under the auspices of the United Nations Environment Programme (UNEP), an international agreement enforced in 2004 (Stockholm Convention Sectretariat, 2008, http://chm.pops.int/Convention/tabid/54/language/en-US/Default.aspx#convtext). The 12 POPs listed by the Stockholm Convention include PCBs, nine chlorinated organic pesticides (aldrin, chlordane, dichlorodiphenyltrichloroethane [DDT], dieldrin, endrin, mirex, heptachlor, hexachlorobenzene, and toxaphene), and dioxins and furans. Although the use of these POPs worldwide has been generally phased out because of their toxicity and persistence, they can still be found as contaminants in the natural environment due to their past use, and they continue to pose a threat to human health.

Traditional technologies routinely used for the remediation of contaminated environmental soil include excavation, transport to specialized landfills, incineration, stabilization and vitrification. Recently, however, there has been much interest in bioremediation technologies that can use plants and microorganisms (including bacteria) to degrade toxic contaminants in environmental soil into less-toxic and/or non-toxic substances. The United States Environmental Protection Agency (USEPA) defines bioremediation as a treatability technology which uses biological activity to reduce the concentration and/or toxicity of a pollutant. Bioremediation technologies offer many advantages over traditional remediation technologies as they can be applied *in situ* without the need for removing and transporting contaminated soil. They are usually less expensive and less labour intensive because they rely on solar energy, have a lower carbon footprint, and have a high level of public acceptance.

1.1 Polychlorinated Biphenyls and Synthetic Organic Pesticides

Widely used in industry in the twentieth century, polychlorinated biphenyls (PCBs) are toxic synthetic aromatic compounds that are notorious for their persistence and potential toxicity – a group of polychlorinated biphenyl compounds with 209 different congeners or related chemicals, some containing up to 10 chlorine atoms. Commercially available PCB

mixtures (e.g. Arochlor), however, typically contain only 20–60 congeners. Because of their chemical stability, electronic-insulating properties, thermal stability and non-flammability, PCBs were used widely in flame retardants, dielectric fluids in capacitors, transformers, hydraulic fluids, surface coating, adhesives and dyes. The manufacture of PCBs was banned in the USA in 1978 because of their toxicity. Although banned, they remain a problem in the environment because of their persistence. Since PCBs were first synthesized in 1864, it is estimated that approximately 1 million tonnes have been manufactured worldwide (Hutzinger and Veerkamp, 1981) and that approximately 30% of all PCBs manufactured have been released into the natural environment, resulting in the contamination of soils and sediments (Holoubek, 2001). PCBs have been detected in polar bears in the Arctic, an environment far removed from industry, providing evidence of the dispersal of these toxic synthetic organic compounds in the natural environment (Skaare et al., 2000; 2002). In *The Management Plan for Polychlorinated Biphenyls (PCBs)* published by the EPA (2008a) in Ireland, it was estimated that there are 70,256 litres of PCBs (i.e. mixtures of substances with >0.005% or 50ppm PCBs by weight) in inventoried holdings in Ireland and 263,787 litres in non-inventoried holdings (EPA, 2008, www.epa.ie/downloads/pubs/waste/haz/pcb%20management%20plan.pdf).

Dichlorodiphenyltrichloroethane (DDT) is one of the best-known toxic chlorinated organic pesticides and although its use as an agricultural insecticide worldwide was banned by the Stockholm Convention, it is still used to control malaria in some parts of the world and remains controversial. DDT was used worldwide as an insecticide from the 1940s until the 1970s, when it was banned in the USA and other countries. In 1962, Rachel Carson in her popular book *Silent Spring* suggested that DTT and other pesticides were associated with cancer and that their agricultural use was a threat to wildlife, particularly birds (Carson, 1962). Along with the passing of the Endangered Species Act in 1973, the US ban on DTT is cited by scientists as a major factor in the comeback of the bald eagle in the USA (Stokstad, 2007). However, when a global ban on DDT was proposed in 2001, several countries in Africa claimed that DDT was still needed as an inexpensive and effective means for the control of the vector associated with malaria. Although DDT is generally not toxic to humans and was banned mainly for ecological reasons, subsequent research has shown that exposure to DDT at amounts that would be needed to control malaria might cause preterm birth and early weaning, eliminating the benefit of reducing infant mortality from malaria (Rogan and Chen, 2005; Guimares et al., 2007). Therefore, the use of DDT to help control malaria remains controversial because of its associated human health risks (Rogan and Chen, 2005; Guimares et al., 2007).

Two of the most commonly used pesticides in agriculture worldwide, the chlorinated organic compounds 2,4-dichlorophenoxyacetic acid (2,4-D) and atrazine, are not listed by the Stockholm Convention as POPs but have been listed by the USEPA as toxic and are associated with human health risks (Kiely et al., 2004). Both 2,4-D and atrazine are broad-leaf herbicides protecting many of the world's important crops, such as wheat, corn, and rice, which are cereal grains. Since its introduction in 1946, 2,4-D remains the most widely used herbicide worldwide. Although 2,4-D is biodegradable, it may persist in soil and water if microbes with the required capacity for biodegradation are not present in sufficient numbers. Atrazine has been banned by the European Union (EU) since 2007, but is still used in many parts of the world, and can remain in soil for more than one year after use, and leach into groundwater contaminating private and community wells (EXTOXNET, 1996 http://extoxnet.orst.edu/pips/atrazine.htm).

Other synthetic chlorinated organic pesticides of concern as contaminants of environmental soil include tetrachlorophenol (TCP), pentachlorophenol (PCP), and the tin-containing pesticide, tributylin (TBT). TCP is an insecticide and a bactericide and is widely used as a preservative for latex, wood, and leather. PCP is a disinfectant, a fungicide, and an extremely effective preservative for wood. In addition, PCP and its products are toxic to plants, facilitating their use as defoliants and general herbicides. TCP and PCP can be released into the environment as a result of their manufacture, storage, transport, or use as an industrial wood preservative. Their use at Finnish sawmills has led to extensive groundwater contamination (Kitunen et al., 1987). TCP and PCP are strong irritants and can produce skin and eye irritation upon contact. They are readily absorbed through the skin

and can produce systemic effects. Acute exposure to TCP and PCP in animals is associated with convulsant activity and inhibition of oxidative phosphorylation (Deichmann and Keplinger, 1981). The tin-containing biocide TBT is used to control a wide variety of organisms – in wood preservatives, as an anti-fouling pesticide in marine paints, and as an antifungal agent in industrial water systems. TBT compounds bioaccumulate as they move up the marine food chain and have been associated with toxicity in a number of marine organisms, such as molluscs, otters, dolphins and whales (Kannan et al., 1998; Murata et al., 2008). Chlorobenzoates, which are toxic metabolic intermediates produced from the biodegradation of a variety of compounds including PCBs and chlorinated aromatic pesticides, are also considered environmental contaminants.

Glyphosate is an organophosphate broad-spectrum herbicide originally sold in the 1970s under the tradename Roundup™. Today, glyphosate is the most widely used herbicide in the USA. Although less toxic than chlorinated organic pesticides, it is a suspected endocrine disruptor. A review of at least 58 studies on the effects of Roundup™ suggests that non-target organisms were exposed to only minimal acute and chronic risk (Giesy et al., 2000). However, more recent research reports that glyphosate induces a variety of functional abnormalities in the specific activity of the enzymes in the liver, heart and brain, in pregnant rats and their foetuses (Daruich et al., 2001). Glyphosate was also reported to interfere with an enzyme involved in testosterone production in mouse cell culture (Walsh et al., 2000) and to interfere with an oestrogen biosynthesis enzyme in cultures of human placental cells (Richard et al., 2005).

A number of synthetic organic compounds, for example, PCBs and many pesticides, are suspected endocrine disruptors and have been associated with the feminization of males. A number of scientists attribute the striking drop in sperm counts among men worldwide to these endocrine disruptors. In a landmark study, Danish researchers reviewed 61 studies and reported in 1992 that the number and motility of sperm in men's semen had declined by 50% since 1938 (Carlsen et al., 1992). Other studies have linked endocrine disruptors and rising rates of testicular cancer (Adami et al., 1994) and breast cancer (Falck et al., 1992; Wolff et al., 1993). The exposure of humans to PCBs and a number of chlorinated organic pesticides has been associated with an increased risk of developing cancer (Flower et al., 2004) and with developmental disabilities (Faroon et al., 2001) in children. A recent study reported an association between exposure to pesticides and an increased incidence of Parkinson's disease (Ascherio et al., 2006).

As the effects of these chemicals on human heath become more widely understood, reducing the levels of PCBs and toxic synthetic chlorinated organic pesticides in the environment would appear to be an issue of growing urgency.

1.2 Remediation Technologies

1.2.1 Traditional Technologies for the Remediation of Contaminated Soil

It is estimated that traditional global remediation costs are in the range of $US25–50 billion annually (Glass, 1999; Tsao, 2003). Unfortunately, this high cost of remediation contributes to the abandonment worldwide of a large number of polluted commercial sites or brownfields. For example, in the USA, the USEPA Office of Underground Storage Tanks reported that 34% of known contaminated sites chose to be in non-compliance in 2008 (US EPA, 2008, http://www.epa.gov/OUST/pubs/OUST_FY08_Annual_Report-_Final_3-19-09.pdf). Some of the reasons for non-compliance are typically (i) non-compliance has a lower immediate cash cost than compliance using traditional remediation technologies and (ii) the demand for green remediation is not yet powerful enough to drive action and does not have an impact on sales and revenue. However, with (i) an increasing public awareness of the need to move towards a low-carbon economy, (ii) the introduction of regulations with an increased emphasis on a low-carbon economy, and (iii) the development of sustainable bioremediation metrics, there is an increased interest in moving away from traditional technologies for the remediation of contaminated soil (e.g. excavation, transport to specialized landfills, incineration, stabilization and vitrification) towards bioremediation technologies by regulators, consultants and representatives from industry.

1.2.2 Bioremediation Technologies for the Remediation of Contaminated Soil

In the remediation of the environment, bioremediation is a treatment process that uses microorganisms (including bacteria) and plants to degrade toxic contaminants into less toxic or non-toxic substances. According to the United States Sustainable Remediation Forum (US SURF), sustainable remediation is broadly defined as a remedy or combination of remedies whose net benefit on human health and the environment is maximized through the judicious use of limited resources (US SURF, 2009). Because bioremediation technologies can be applied *in situ* without the need for the removal and transport of contaminated soil, are usually less expensive and less labour intensive (relying on solar energy), have a lower carbon footprint, and have a high level of public acceptance than traditional remediation technologies, they potentially offer a sustainable solution to the problem of contaminated environmental soil. However, conditions in the contaminated environmental soil need to be optimized for effective biodegradation of the target contaminants – moisture levels, pH and temperature in the soil will dictate survival ranges for microorganisms and plants used for bioremediation; abundant oxygen will facilitate the mineralization of soil contaminants; concentrations of nutrient and hydrocarbons in the soil will need to be balanced for efficient bioremediation; and suitable microorganisms and plants will be required to degrade and/or mineralize target contaminants.

Although it appears that the advantages associated with the use of bioremediation technologies clearly outweigh the disadvantages, when compared to traditional remediation technologies, other factors to consider when using bioremediation technologies include the length of time required (months or years), geographic limitations on the use of specific plants, and the seasonal limitations associated with the use of specific plants. Choosing a technology for the sustainable remediation of contaminated environmental soil requires detailed analyses of the environmental impact. Bioremediation technologies compare favourably with traditional remediation technologies when analysed using sustainability remediation metrics. Sustainable remediation metrics include economic, societal and environmental metrics for comparing and selecting remedies and monitoring success, and include important elements such as water use, worker safety, community impact, and the net environmental benefit (US SURF, 2009).

1.2.2.1 Biodegradation of toxic organic compounds in environmental soil

A number of bacterial strains have been identified in a wide variety of contaminated environments with enzymes capable of degrading toxic organic compounds. Anaerobic bacteria can convert highly chlorinated PCB congeners into less chlorinated biphenyls by reductive dechlorination (Bedard and Quensen, 1995; Brown et al., 1984). Aerobic bacteria, for example, *Burkholderia xenovorans* LB400 (Hofer et al., 1993) and *Rhodococcus* sp. strain RHA1 (Masai et al., 1997), can then cleave lesser chlorinated biphenyl rings to yield chlorinated benzoates and pentanoic acid derivatives which are often degradable by other bacteria. The dechlorinating bacteria *Dehalococcoides ethenogenes*, *Dehalobacter restrictus*, *Desulfitobacterium dehalogenans*, *Dehalospirillum multivorans*, *Desulfuromonas chloroethenica*, and *Desulfomonile tiedjei* are capable of dehalogenating PCE (Damborsky, 1999) and other chlorinated aromatic compounds (Mohn and Tiedje, 1992). *Dehalococcoides ethenogenes* strain 195, which is the only bacterial strain that dechlorinates PCE completely to yield ethylene, is of interest because of its potential use in the bioremediation of TCE- and PCE-contaminated sites (Maymo-Gatell et al., 1997). Mannisto et al. (2001) identified bacterial strains *Herbaspirullum* sp K1, *Sphingomonas* strains K74 and MT1, *Nocardioides* sp K44, that could degrade TCP faster at lower than room temperature. *Sphingobium chlorophenolicum* strain ATCC 39723 can completely mineralize PCP (Cai and Xun, 2002). Bacteria involved in the biodegradation of petroleum products in a number of different environmental soil types have also been identified (Saadoun, 2002; Wang et al., 2008).

1.2.2.2 Enhancement of bacterial degradation of toxic organic compounds

Endophytic bacteria (non-pathogenic bacteria that occur naturally in plants) and rhizospheric bacteria (bacteria that live near the roots of plants) have been shown to contribute to the biodegradation of toxic organic compounds in contaminated soil and could have the potential for phytoremediation. Endophytic and rhizospheric bacterial degradation of toxic organic compounds (either naturally occurring or genetically enhanced) in contaminated soil in the environment

could have positive implications for human health worldwide. Using biotechnology, bacteria (rhizospheric and/or endophytic) can be engineered, via natural gene transfer or recombinant DNA technology, to produce specific enzymes capable of degrading toxic organic pollutants found in the environment. Genetic engineering of endophytic and rhizospheric bacteria for use in plant-associated degradation of toxic compounds in soil is considered one of the most promising new technologies for remediation of contaminated environmental sites.

Studies using two genetically modified strains of the rhizospheric bacteria *Pseudomonas fluorescens* F113, i.e. *Pseudomonas fluorescens* F113*rifbph* (with a single chromosomal insertion of the *bph* operon) (Brazil et al., 1995) and *Pseudomonas fluorescens* F113: 1180 (with a single chromosomal insertion of the *bph* operon under the control of the *Sinorhizobium meliloti* nod regulatory system) (Villacieros et al., 2005) reported that (i) the modified rhizospheric bacteria colonized roots as effectively as the wildtype rhizospheric bacteria, (ii) *bph* genes were expressed *in situ* in soil, and (iii) the modified rhizospheric bacteria could degrade PCBs more efficiently than the wildtype rhizospheric bacteria, indicating considerable potential for the manipulation of the rhizosphere as a useful strategy for bioremediation. *Pseudomonas fluorescens* F113: 1180 does not contain antibiotic resistance genes from the vector making this strain more suitable for *in situ* applications. Since the *bph* element in *Pseudomonas fluorescens* F113: 1180 is stable, lateral transfer of the *bph* element to a homologous recipient would not be expected to occur at detectable frequencies in the rhizosphere (Ramos et al., 1994).

Dzantor (2007) recently reviewed the use of biotechnology to enhance rhizospheric microbial degradation of POPs. However, because toxic organic compounds can enter the root xylem from the soil before they are degraded, and these contaminants can remain in the xylem for up to two days (McCrady et al., 1987), plant-associated endophytes that have been genetically enhanced so as to degrade toxic organic compounds appear to offer more potential than rhizospheric bacteria for reducing phytotoxicity. Endophytic bacteria can be isolated from host plants of interest (e.g. plants native to a geographical region) and enhanced genetically to contain degradation pathways or genes to degrade target contaminants before being reinoculated back into the host plant for bioremediation purposes.

In the project team's laboratory, Germaine et al. (2009) reported that a genetically enhanced endophytic strain of the poplar endophyte *Pseudomonas putida* VM1441, i.e. *Pseudomonas putida* VM1441 (pNAH7), could protect inoculated pea plants from the toxic effects of naphthalene. They also showed that inoculation of plants with this strain facilitated higher (40%) naphthalene degradation rates compared with uninoculated plants in artificially contaminated soil (Germaine et al., 2009). Barac et al. (2004) reported that a genetically enhanced endophytic strain of the soil bacterium *Burkholderia cepacia* G4 could increase inoculated yellow lupine plant tolerance to toluene, and decrease phytovolatilization of toluene from the plant into the atmosphere by 50–70% in laboratory-scale experiments. In this study, the plasmid, pTOM, which encodes a pathway for the degradation of toluene, was transferred via conjugation to the natural endophyte, providing the genes for toluene degradation. Later, Taghavi et al. (2005) extended this work to poplar trees and showed that this degradative plasmid, pTOM, could transfer naturally, via horizontal gene transfer, to a number of different endophytes *in planta*, promoting more efficient degradation of toluene in poplar plants. Horizontal gene transfer results in the natural endophyte population having the capacity to degrade environmental pollutants without the need to establish the inoculants strain long term. Endophytes that have been engineered by horizontal gene transfer have the distinct advantage that they may not be considered to be genetically modified microorganisms (GMMs) and could, therefore, be exempt from current international and national GM legislation, thus facilitating the testing of these microorganisms in the field at an accelerated pace.

1.3 Bacterial Glutathione S-transferases

One family of proteins/enzymes that plays an important role in detoxification is glutathione S-transferases (GSTs: EC 2.5.1.18), the ubiquitous multifunctional prokaryotic and eukaryotic proteins involved in the cellular detoxification and excretion of a large variety of electrophilic compounds of both endobiotic and xenobiotic origin. GSTs have been purified from humans, animals, plants, fish, insects, fungi, yeast,

and bacteria. Understanding of the role of bacterial GSTs in metabolism, however, is still in its infancy. The association of bacterial GST DNA with other genes involved in the degradation of toxic pollutants, including PCBs, indirectly suggests a role for bacterial GSTs in biodegradation.

Burkholderia sp. LB400 is a well-characterized aerobic PCB-degrading microorganism. The genes in the *bph* operon are responsible for the ability of this bacterium to degrade PCB (Bopp, 1986; Hofer et al., 1993; Hofer et al., 1994). A gene, *bphK*, encoding a protein with significant sequence similarity to prokaryotic and eukaryotic GSTs, was found in a central location within this gene cluster, and GST activity of the protein product, BphK^{LB400}, was demonstrated (Hofer et al., 1994). All the genes in the *bph* operon of *Burkholderia* sp. LB400 have been assigned a function except for *bphK*. The GST enzyme activity of BphK^{LB400} was studied in greater detail by Bartels et al. (1999). BphK^{LB400} was found to have a broad pH optimum ranging from 6.0 to 8.0 and still had maximal activity at a temperature of 55^{o}C, showing that the enzyme remains in its active form over a wide range of conditions (Bartels et al., 1999). Unlike the majority of GSTs which have a broad substrate specificity, BphK^{LB400} showed only activity towards the model substrate used in GST activity assays, 1-chloro 2,4-dinitrobenzene (CDNB), when assayed using a variety of widely used electrophilic substrates (Bartels et al., 1999). It has been reported that *bphK* is not essential for the utilization of biphenyl as a carbon source, although approximately the same induction factor was observed for 2,3-dihydroxybiphenyl 1,2-dioxygenase activity, which is encoded by the 5'-adjacent *bphC* gene, suggesting that the expression of *bphK* is co-regulated with the expression of genes responsible for the catabolism of biphenyls (Bartels et al., 1999). In the laboratory, the *bphK* gene from *Burkholderia* LB400 was cloned independently of the *bph* operon into an over-expression vector in *E. coli* to allow further studies and genetic analysis of this gene (Gilmartin et al., 2003). Bacterial cellular extraction and GST activity assay of BphK^{LB400} were optimized to identify potential substrates for this enzyme (Gilmartin et al., 2003). High performance liquid chromotography (HPLC) studies suggested an additional dechlorination function for BphK^{LB400} in relation to the substrate 4-chlorobenzoate (4-CBA) (Gilmartin et al., 2003).

More recently, the project team reported enzymatic activity of BphK^{LB400} towards a number of chlorinated organic compounds, including chlorobenzoates ([CBA], PCB degradation products) and commonly used toxic pesticides, functioning as endocrine disruptors and associated with cancer. BphK^{LB400} substrates include 3-CBA and 4-CBA; and the pesticides, 2,4-dichlorphenoxyacetate (2,4-D), atrazine, pentanochlor, Clean-up®, chloromequat chloride and triphenyltin chloride (McGuinness et al., 2006; 2007). These compounds are associated with varying levels of ecotoxicity. Pentanochlor is a selective herbicide applied on wheat, barley and oats. The US EPA has classified pentanochlor as a toxic compound with ecological effects on birds, aquatic organisms and animals (Pesticide Action Network, 2011, http://www.pesticideinfo.org/). Clean-up$^{(R)}$, a commercial herbicide, prevents the recurrence of weeds on kerbs, paths, patios, driveways and yards, and contains 60% atrazine and 40% aminotrizole. Chloromequat chloride (CMC) is a plant growth regulator and a suspected human health hazard, and triphenyltin chloride is used as a biocidal intermediate, rodent repellent, fungicide and insecticide worldwide and is ranked as one of the most hazardous compounds (worst 10%) to ecosystems (Pesticide Action Network, 2011, http://www.pesticideinfo.org/). These findings suggest a potential role for BphK^{LB400} in bioremediation of the environment.

In the project laboratory, bacteria expressing a specific bacterial GST isolated from *Burkholderia xenovorans* LB400, BphK^{LB400} (wildtype and mutant [Ala180Pro]), capable of dehalogenating toxic chlorinated organic pesticides was shown to protect a legume, the pea plant (*Pisum sativum*), from the effects of a chlorinated organic pesticide, CMC (McGuinness et al., 2007). When pea plants were grown under stressful conditions, inoculation of peas with *E. coli* expressing BphK ^{LB400} (wildtype and mutant) before treatment with CMC resulted in an increase in biomass and root length of plants. This suggested that BphK^{LB400} could protect the pea plants from the effects of CMC. Previously, it had been shown that mutating the conserved amino acid at position 180 in BphK^{LB400} from Ala to Pro resulted in an approximate 2-fold increase in GST activity towards a number of chlorinated organic substrates tested, including other commonly used pesticides (McGuinness et al., 2006; 2007). Therefore, BphK^{LB400}

and other bacterial GSTs could have the potential in the phytoremediation of toxic chlorinated organic pesticides in environmental soil if expressed by rhizospheric or endophytic bacteria.

1.4 Objectives

The National Development Plan, 2007–2013 (The Stationery Office, 2007), states that 'the development of a knowledge-based economy is one of the key challenges and opportunities facing Ireland' and emphasizes the continued development of a world-class research system. With Europe emerging as a world leader in environmental technologies and engineering, the current research project is at this leading edge of research. At EU level, the current emphasis in the field of environmental technologies is on developing eco-efficient system solutions to mitigate effects on the environment (http://ec.europa.eu/environment/etap/). The EU Commission (CEC) adopted a Soil Thematic Strategy (CEC, 2006a) and a proposal for a Soil Framework Directive (CEC, 2006b) on 22 September 2006 with the objective of protecting soils across the EU. While this EU Soil Framework Directive is a work in progress and will not be legally implemented for a number of years, the aims of this current research project are in line with the remediation goals of this Directive. Having identified contaminated sites, EU member states will be obliged to ensure that these are then remediated so that they no longer pose any significant risk to human health and the environment.

The Pesticide Control Service of the Department of Agriculture, Fisheries, and Food has responsibility for regulating the use of pesticides in Ireland. While it is unlikely that pesticide usage represents a major threat to Irish soil because of the relatively small area of land devoted to crops, in the future soil remediation will be much more to the forefront with the introduction of EU legislation. It is envisaged that in the future the EU Soil Framework Directive will provide the guidelines for Ireland's approach to developing regulations for the protection of soil (i.e. regulation of contamination levels and remediation of contaminated soil).

Two of four major areas in environmental health research defined by the EPA in Ireland as current research challenges, are: (i) persistent organic pollutants (POPs) in soil and water and (ii) emerging diseases, for example, due to increased use of the chlorinated organic pesticide DTT in developing countries to control human disease vectors, such as mosquitoes that transmit malaria (EPA STRIVE Meeting, Kilkenny, May 2007). Reducing the levels of PCBs and toxic synthetic chlorinated organic pesticides in the environment would appear to be an issue of growing urgency as the effects of these chemicals on human health become understood more widely.

In a more recent EPA report *State of the Environment* (EPA, 2008b), four major environmental challenges were identified. They were:

1 Limiting and adapting to climate change;

2 Reversing environmental degradation;

3 Mainstreaming environmental considerations; and

4 Complying with environmental legislation and agreements.

The main issues relating to 'reversing environmental degradation' were identified as:

- Preventing eutrophication and other water pollution;
- Protecting natural habitats and species populations;
- Remediating of contaminated soil.

Contaminated sites include disused landfills, abandoned mines, and sites of old industrial activities, such as steel or gas works. The EPA has estimated that there are approximately 2000 sites in Ireland where there is potential for soil and/or groundwater contamination but the actual number is unknown as there is currently no national inventory. Unlike many other European countries, Ireland lacks specific legislation for dealing with contaminated sites (McIntyre, 2006) and has yet to develop environmental quality standards for soil.

The aim of this research project was to identify bacterial GSTs (known and novel) in environmental samples and, using molecular biological techniques, to study microbial diversity in these samples. Bacterial GSTs identified in soil contaminated with toxic chlorinated organic compounds could have the potential for bioremediation, and for development as bioindicators and biosensors of environmental pollutants, for example toxic chlorinated organic PCBs and pesticides. A specific bacterial GST, $BphK^{LB400}$, was to be developed for use as a biosensor and for bioremediation.

2 Methodology and Results

2.1 Collection of Environmental Samples and Isolation of Genomic DNA

Genomic DNA was isolated from environmental soil samples using the PowerMaxTM Soil DNA Isolation Kit from MoBio Labs Inc. (Mo Bio, 2011, http://www.mobio.com). This kit is designed to isolate high-quality microbial DNA from large quantities of soil/sediment (up to 10g). It increases the ability to study microbial diversity in even the most difficult sample types – such as those containing high humic levels or low microbial load. The kit provides DNA that is free of PCR inhibitors, including humic substances, and the brown colour often associated with soil DNA.

Six soil/sediment samples were included in this study:

1 High agricultural input soil treated with a variety of chlorinated organic pesticides (Conry and Hogan, 2001) from agricultural plots at Teagasc, Oakpark, Carlow, which had been used to grow either spring barley or winter wheat. Genomic DNA isolated from these high agricultural input samples was compared with genomic DNA isolated from low agricultural input samples from plots used to grow the same crops.

2 PCB-contaminated soil with documented levels of PCBs from Lhenice in the southern Czech Republic that had been used as a model of contaminated soil in a number of other studies (Aguirre de Carcer et al., 2007; Pavlikova et al., 2007; Rein et al., 2007; Liu et al., 2010).

3 Input (sludge) and output (sediment) samples from a constructed wetland associated with an Irish meat-processing plant.

4 Sludge samples from an Irish waste-water treatment plant (WWTP), since a number of bacterial detoxification enzymes had previously been identified and reported from such samples (Gisi et al., 1998; Vuilleumier et al., 2001).

5 PCB-contaminated soil samples from the United States Coast Guard (USCG) Civil Engineering Unit (CEU) based in Honolulu, HI, USA. The USCG CEU in HI is interested in finding ways to detoxify PCB-contaminated soils at the former Long Range Navigation (LORAN) station at Kure Atoll near Midway Island in the Pacific. It is currently developing a gel containing PCB-degrading fungi for use with contaminated soil. Because of the long-term potential of this GST project for bioremediation, PCB-contaminated soil samples at Kure Atoll were collected for analysis of bacterial GSTs that could potentially contribute to degradation and subsequent bioremediation of PCBs in soil. Approximately 50ug DNA was isolated from each 5g soil/sediment sample in line with expected yields and with an OD260/280 ratio of approx. 1.8, indicating minimal contamination with protein.

6 Control garden samples.

An application for access to soil samples (or nucleic acid extracted from these samples) with reported levels of chlorinated organic pesticides (McGrath, 1998; McGrath and McCormack, 1999) from the National Soil Database (NSDB) was submitted but denied in Summer 2009.

2.2 Bioinformatic Analysis of Bacterial Glutathione Transferase

Using bioinformatic techniques, GST sequences were identified in publicly accessible databases (e.g. UniProt), and analysed to identify conserved amino acid residues and regions to be used in the design of (i) site-directed mutagenesis studies (for the development of improved bacterial GST activity for potential use as a biosensor) and (ii) degenerate PCR primers to be used in the identification of bacterial GSTs (known and novel) in environmental soil/sediment samples.

Table 2.1. Glutathione transferase (GST) from bacteria that degrade polychlorinated biphenyl (PCBs) and other organic compounds (with UniProt Accession No.).

Bacterial strain	Degradation properties	UniProt Accession No.	Reference
Burkholderia xenovorans LB400	Polychlorinated biphenyls	Q59721	Hofer et al., 1994
Ralstonia sp. B15	Biphenyl	Q9RAF0	Bartels et al., 1999
Pseudomonas pseudoalocaligenes KF707	Polychlorinated biphenyls	Q52037	Kimura et al., 1997
Pseudomonas sp. B4	Biphenyl	Q9RBS6	Bartels et al., 1999
Cycloclasticus oligotrophus RB1	Biphenyl, phenanthrene, naphthalene	Q46153	Wang et al., 1996
Sphingomonas paucimobilis EPA505	Fluoranthene	O33705	Lloyd-Jones et al., 1997
Sphingomonas sp. KMG425	Biphenyl	Q8RMI1	Shin et al., 2002
Sphingomonas aromaticivorans F119	Toluene, biphenyl, naphthalene	O85984	Romine et al., 1998
Sphingomonas sp. P2	Phenanthrene	Q83VK4	Pinyakong et al., 2002
Pseudomonas sp. B7T	Biphenyl	Q9RBS4	Bartels et al., 1999
Pseudomonas sp. B7T	Biphenyl	Q9RBS4	Bartels et al., 1999
Polaromonas naphthalenivorans CJ2	Naphtalene	A1VUV4	Copeland et al., 2006
Pseudomonas sp. B3B	Biphenyl	Q9RBS8	Bartels et al., 1999
Ralstonia eutropha H850	Biphenyl	Q9RLB9	Bartels et al., 1999

The Uniprot protein databank provided by Expert Protein Analysis System (ExPASY) (Boeckmann et al., 2003) was screened for bacterial GSTs capable of degrading PCBs or other organic compounds using the $BphK^{LB400}$ protein sequence, and a number of bacterial GSTs were identified (Table 2.1). A separate search was also conducted for GSTs showing sequence similarity with $BphK^{LB400}$, with >50% amino acid sequence similarity to $BphK^{LB400}$. Sequence alignments were carried out using the ClustalW2 general purpose multiple sequence alignment program (Larkin et al., 2007) provided by EMBL-EBI. GSTs from 13 bacteria that degrade PCBs or other organic compounds were identified and are listed in Table 2.1.

A ClustalW2 alignment (Fig. 2.1) of nine bacterial GSTs listed in Table 2.1, associated with degradation of PCBs and other organic compounds, showed that 68 amino acids were found to be identical, 40 semi-conserved and 19 partially conserved denoted by *, :, and ., respectively. Sequences Q8RMI1, Q9RBS4, Q9RBS8 and Q9RLB9 were not included in the alignment as there was <76% sequence coverage with $BphK^{LB400}$. However, alignment with these three sequences did not affect conserved amino acids where they aligned, except for an A-T change in the C-terminal region of Q8RMI1, resulting in the conversion of a conserved amino acid to a semi-conserved amino acid.

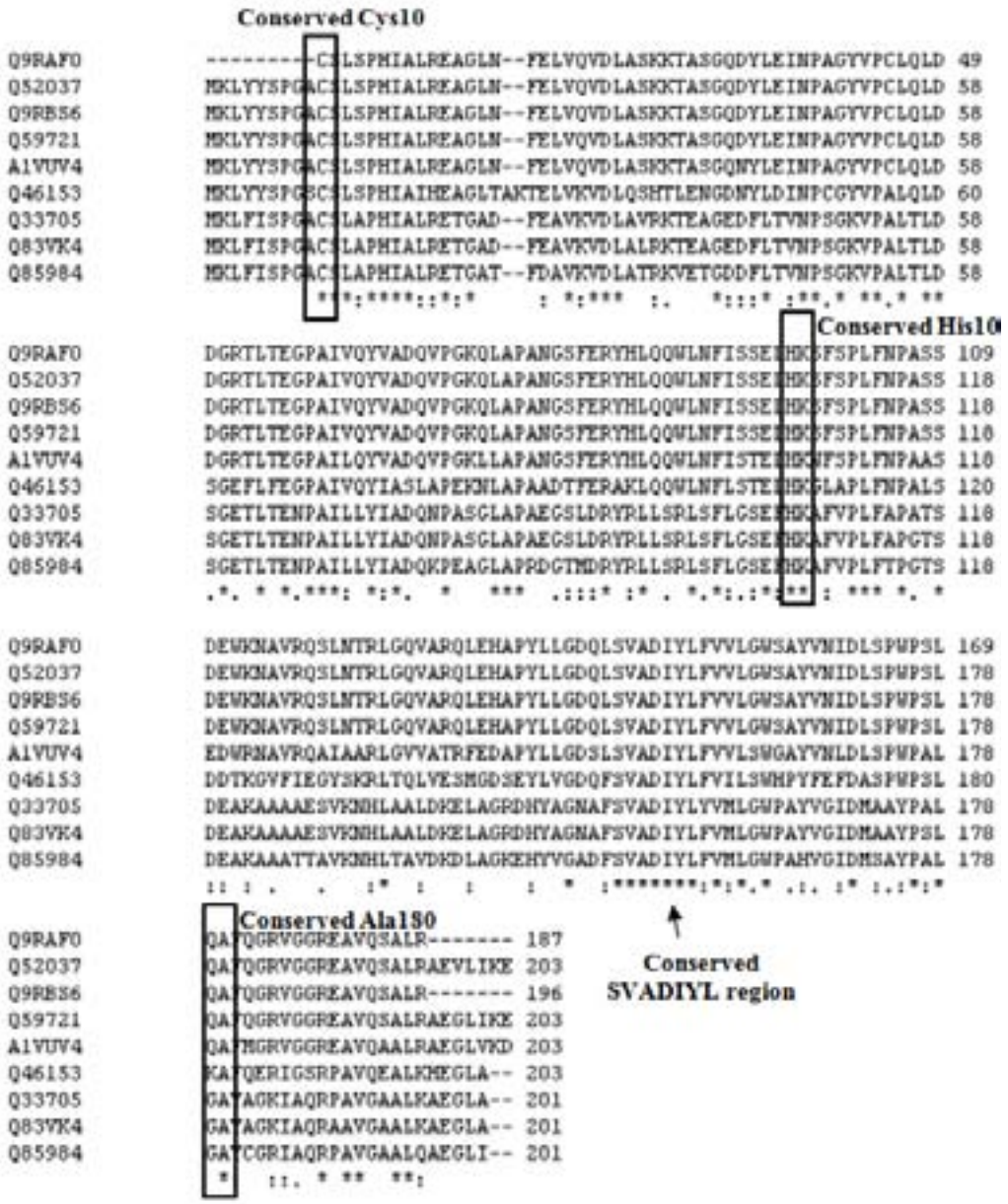


Figure 2.1. A ClustalW2 alignment of bacterial glutathione transferase (GST) amino acid sequences (n = 9) involved in the degradation of organic compounds shows that the Cys10, His106 and Ala180 residues are identical in all sequences.

Previously, a number of BphK^{LB400} mutants were generated in the project team's laboratory, including BphK^{LB400} Ala180Pro, which was found to be associated with an increase in GST activity towards a number of chlorinated organic substrates when compared to wildtype BphK^{LB400} (McGuinness et al., 2006). Figure 2.1 shows that amino acid position Ala180 is highly conserved among bacterial GSTs with organic compound degradation capabilities. However, in a ClustalW2 alignment of 61 bacterial GSTs with >50% homology to BphK^{LB400} in UniProt, a number of other amino acid residues in addition to Ala are found at position 180. Interestingly, no change from Ala to Pro was found *in silico* (Fig. 2.2). The naturally occurring amino acid substitutions at Ala180 include Arg, Gly, Asp, Glu, Lys and Gln. Ala is a hydrophobic aliphatic amino acid. The substitutions which have occurred at position 180 range from hydrophobic to hydrophilic, positively to negatively charged, and from basic to acidic to aliphatic side chains.

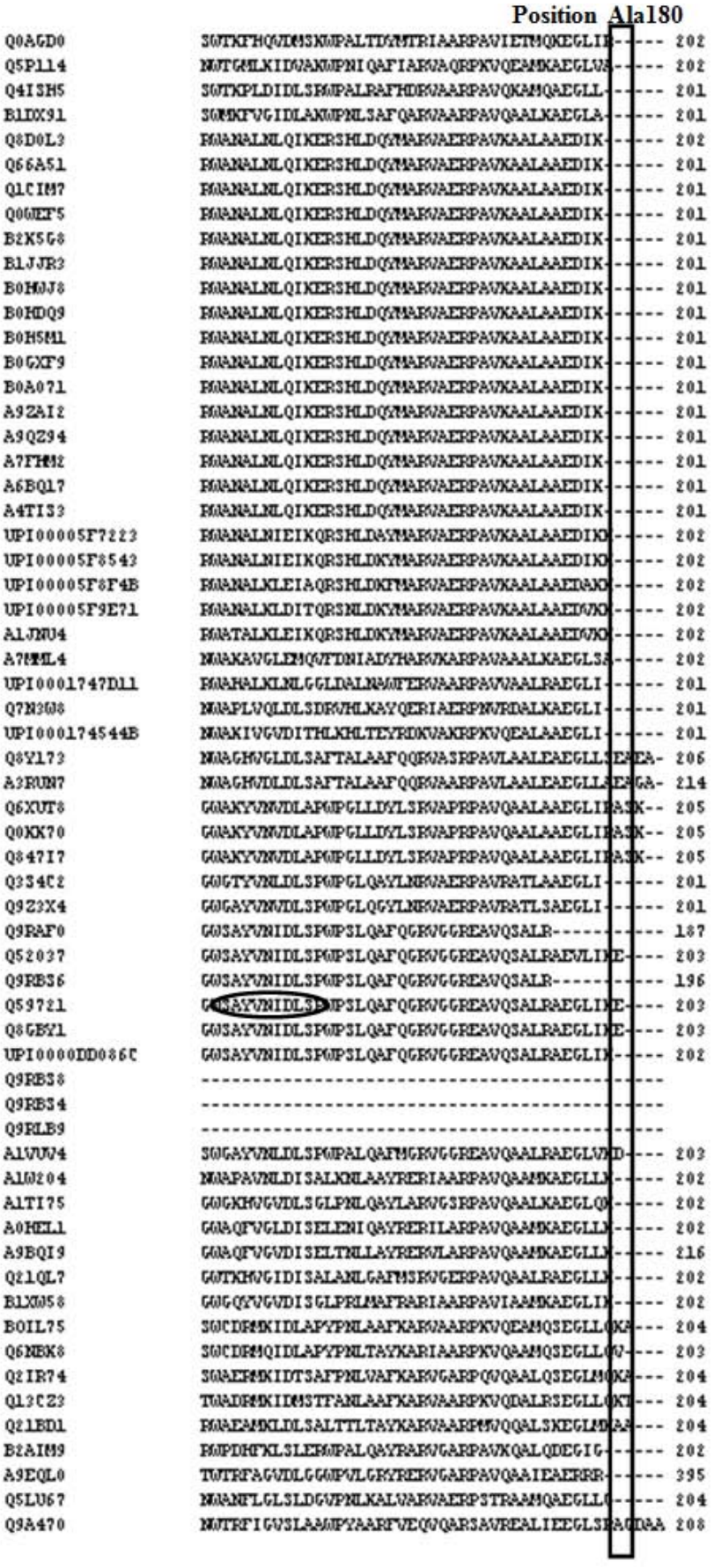


Figure 2.2. A ClustalW2 alignment of bacterial glutathione transferase (GST) amino acid sequences (n = 61) with >50% similarity to BphK^{LB400} (Q59721) in UniProt shows that the Ala residue at position 180 is not conserved.

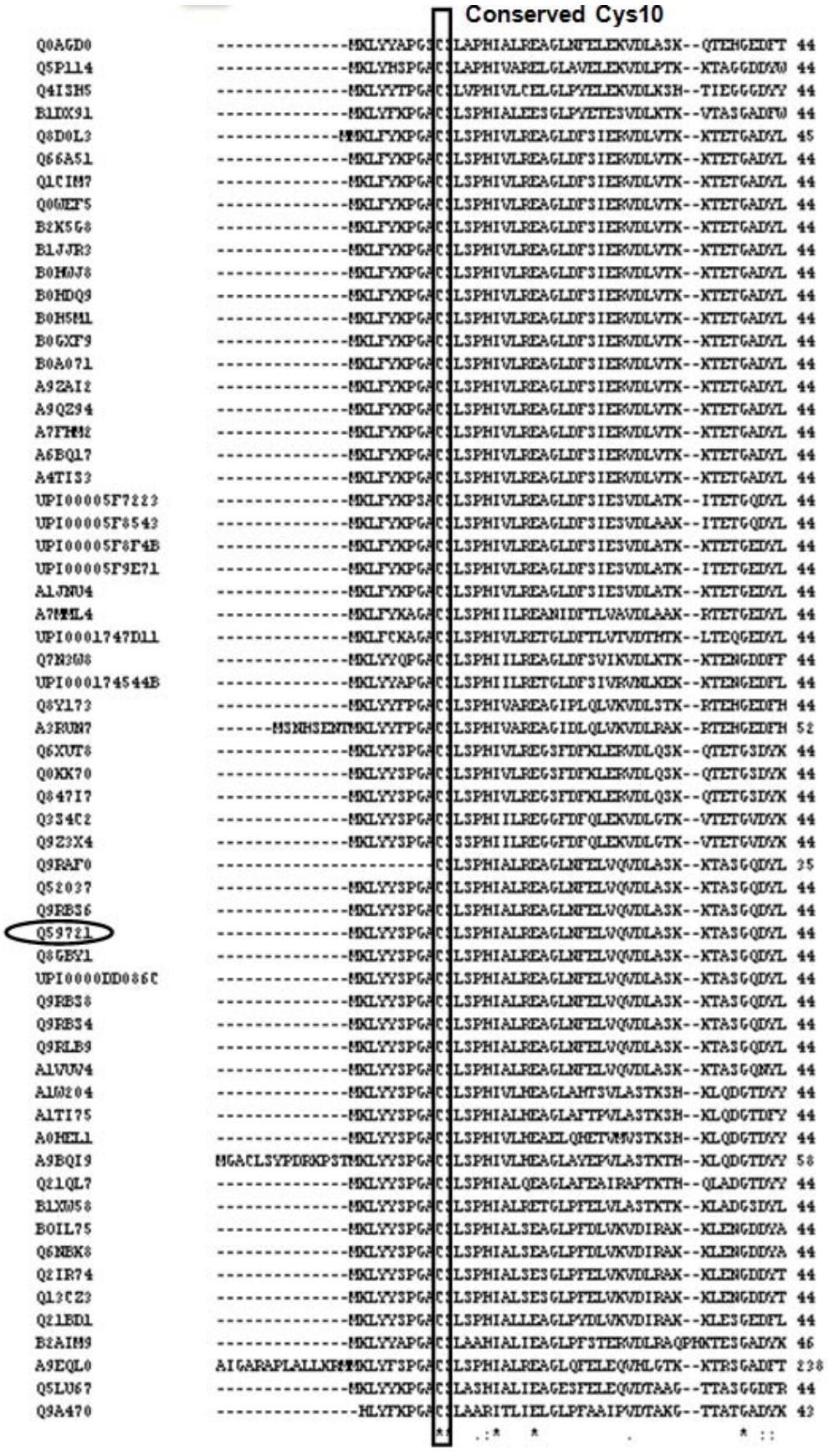


Figure 2.3. A ClustalW2 alignment of bacterial glutathione transferase (GST) amino acid sequences (n = 61) with >50% homology to BphK^{LB400} (Q59721) in UniProt shows that the Cys residue at position 10 is identical in all sequences.

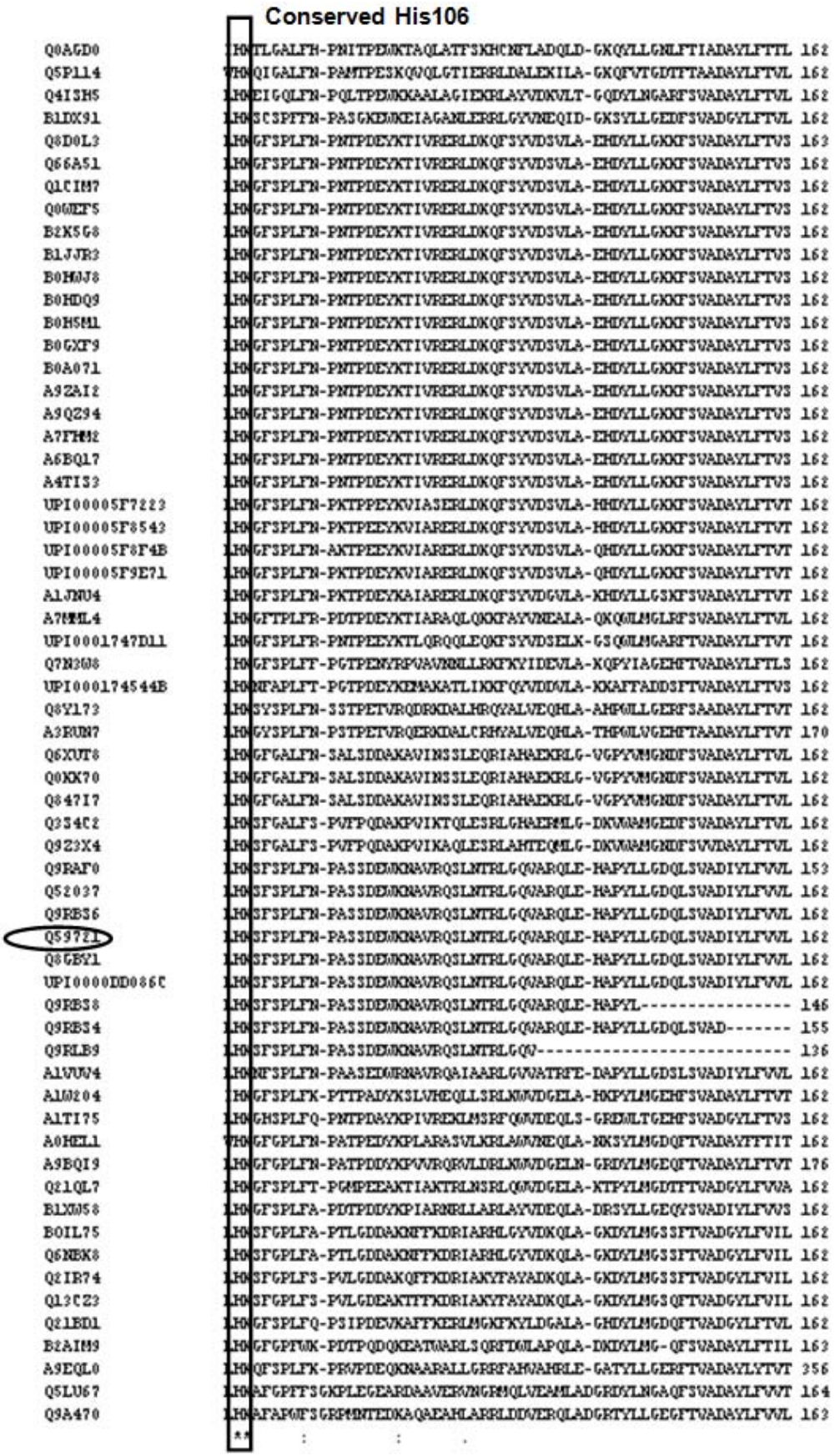


Figure 2.4. A ClustalW2 alignment of bacterial glutathione transferase (GST) amino acid sequences (n = 61) with >50% homology to BphK^{LB400} (Q59721) in UniProt shows that the Histidine (His) residue at position 106 is identical in all sequences.

Two highly conserved amino acids identified in Fig. 2.1 are Cys10 and His106, both of which were identified in the crystalline structures of chlorinated HOPDA-bound BphK^{LB400} (Tocheva et al., 2006). The importance in catalysis of Cys10 and His106 is further suggested by their identity in a ClustalW2 alignment with 61 bacterial GSTs possessing >50% homology to BphK^{LB400} (shown in Figs 2.3 and 2.4, respectively).

Multiple-sequence alignments showed that 68 amino acids were found to be identical, 40 semi-conserved, and 19 partially conserved (data not shown) in this group of 61 GSTs with >50% similarity to BphK^{LB400}. These analyses confirm the importance in catalysis of Cys10 and His106, two highly conserved amino acids, recently identified as playing an important role in catalytic activity of the bacterial GST BphK^{LB400} (Tocheva et al., 2006). *In silico* mutagenesis at Cys10 and His106 identified a number of amino acid substitutions which were predicted to have an effect on the theoretical and crystalline 3D-structure of BphK^{LB400} (Brennan et al., 2009). BphK^{LB400} mutants, Cys10Phe, Cys10Trp, Cys10Tyr, His106Gly, His106Pro and His106Val were generated *in vitro*.

2.3 Dechlorination Activity of a Specific Bacterial Glutathione Transferase, BphK^{LB400}

In vitro site-directed mutagenesis at these two highly conserved residues, Cys10 and His106, of BphK^{LB400} was shown to affect the GST activity towards CDNB, the model substrate for GSTs, when compared to wildtype BphK^{LB400} (Fig. 2.5, and Brennan et al., 2009).

Mutagenesis of His106 to either Gly, Pro or Val, and Cys10 to either Tyr or Trp, resulted in almost a complete loss of dechlorination activity towards CDNB in bacterial cell extracts. In contrast, mutation of Cys10 to Phe resulted in an approximate 4.5-fold increase in activity towards CDNB when compared with wildtype BphK^{LB400}. This increase was found to be statistically significant ($p = 0.003$). The Cys10Trp mutant activity, when compared to wildtype BphK^{LB400}, was found to have a significant decrease in enzyme activity ($p = 0.042$).

Previously, it was reported that BphK^{LB400} has a broad pH range, with the pH optimum between pH 6.0 and 8.0 (Bartels et al., 1999). pH-dependent catalysis studies were carried out to examine whether mutagenesis at the conserved amino acid residues, Cys10 and

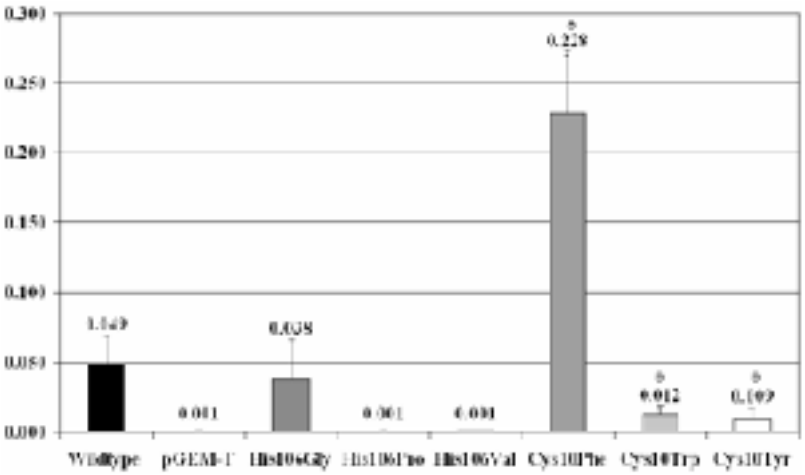


Figure 2.5. Glutathione transferase (GST) activity of bacterial cell extracts containing wildtype and mutant (His106Gly, His106Pro, His106Val, Cys10Phe, Cys10Trp and Cys10Tyr) BphK^{LB400} towards CDNB. Results are from three independent experiments carried out in triplicate, and are expressed as U/mg protein. *E. coli* XLI-Blue containing the pGEM-T plasmid alone was included as a blank in these assays. * denotes $p < 0.05$

His 106, affected the pH optimum of wildtype BphK^{LB400}. BphK^{LB400} mutants generated *in vitro* were purified and examined for their dechlorination activity towards CDNB over a range of pH values and compared to wildtype BphK^{LB400} (Fig. 2.6). Wildtype BphK^{LB400} had no detectable activity at pH 4.0, and at pH 4.5 activity increased to pH 8.5 where maximum activity was achieved. The Ala180Pro mutant was found to have a similar pH stability profile to wildtype BphK^{LB400}. The Cys10Phe and His106Gly mutants had very different pH profiles to wildtype BphK^{LB400}. The His106Gly mutant resulted in low levels of activity at the lower pH range of pH 4.0– 5.5 and increased activity at pH 8.5. The Cys10Phe mutant had maximum activity at pH 8.0, producing activity approximately 7 times the activity found at the standard pH, pH 6.8, used in the *in vitro* assay. Also, maximum activity achieved by Cys10Phe at pH 8.0 was greater than 5 times the activity of wildtype BphK^{L400} at pH 8.5. Purified BphK^{LB400} mutants, His106Pro, His106Val, Cys10Trp, Cys10Tyr, and the double mutant, Cys10Phe/Ala180Pro, had no detectable activity over the pH range 4.0–9.0 studied (data not shown). The increased activity of Cys10Phe at higher pH values compared to wildtype BphK^{L400} suggests that this BphK^{L400} mutant may have the potential for bioremediation in environmental samples with high pH, which might otherwise be unsuitable for bioremediation.

BphK^{LB400} (wildtype and mutant [Ala180Pro, Cys10Phe and Cys10Trp (as a negative control)]) genes were included in studies reported in Section 2.6 on the development of a biosensor for detection, and bioremediation, of chlorinated organic compounds in environmental soil/sediment.

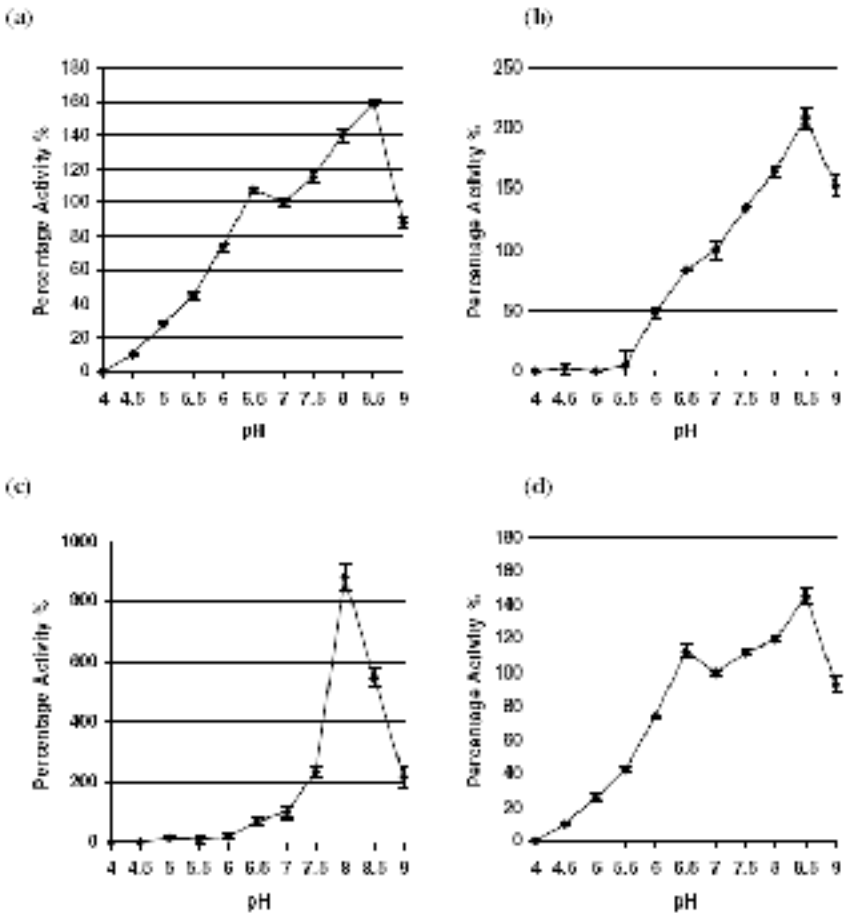


Figure 2.6. pH-dependent catalysis of BphK^{LB400} (wildtype and mutant) towards CDNB using the *in vitro* glutathione transferase (GST) activity assay. Activity at pH 7.0 was taken as 100% activity. (a) BphK^{LB400} wildtype, (b) His106Gly, (c) Cys10Phe, (d) Ala180Pro.

2.4 Degenerate Polymerase Chain Reaction

2.4.1 Bacterial Glutathione Transferase Sequences used for Degenerate Polymerase Chain Reaction Primer Design

Bacterial GST sequences were analysed bioinformatically as decribed in Section 2.3. Conserved regions were identified and used for the design of degenerate PCR primers with a view to using these PCR primers to identify bacterial GSTs (known and novel) in environmental soil/sediment samples using PCR.

Cytosolic GSTs are subdivided in to seven main classes: alpha, mu, pi, theta, sigma, kappa and zeta on the basis of substrate specificity and primary sequences (Armstrong, 1997). GST enzymes belonging to the same class show 70–80% identity in their primary structure, whereas GST belonging to different classes show less than 30% sequence identity (Mignogna et al., 1993). Bioinformatic analysis of GSTs (with approximately 200 amino acids) shows that there are two conserved regions (Fig. 2.7). The N-terminal region spans approximately amino acids 1–75, is highly conserved, and is associated with glutathione binding. The C-terminal region spans approximately amino acids 90–200, and is much less conserved. Indeed, the sequences of the C-terminal domain are often too different (below 20% identity) to be detected as similar in automated searches of sequence databases. This lends support to the hypothesis that the C-terminal domain of GST enzymes plays a crucial role in determining their functional specificity (Vuilleumier, 1997).

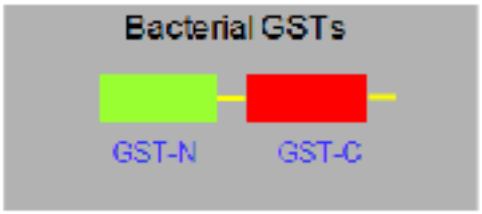


Figure 2.7. Bioinformatic analysis of bacterial glutathione transferase sequences (GSTs) reveals two conserved regions: GST-N: associated with binding to glutathione, GST-C: associated with substrate specificity.

PCR amplification is used in this research project to investigate known and/or novel bacterial GSTs in contaminated environmental soil samples. Degenerate PCR is a powerful tool used to identify novel genes in gene families. By aligning sequences from a number of related proteins, conserved and variable regions are identified, and conserved motifs used for PCR primer design (both manually, and computer aided). Degenerate PCR primers have a set of options ('wobbles') at several positions in the sequence so as to allow the annealing and amplification of a variety of related sequences. Forward PCR primers are chosen from the highly conserved N-terminal region associated with binding to glutathione and reverse PCR primers chosen from the less highly conserved C-terminal region associated with substrate specificity.

2.4.2 Degenerate Polymerase Chain Reaction Primer Design

Primer design is the most important factor to consider when planning to conduct degenerate PCR. When designing degenerate PCR primers, the conserved amino acids, Ser (S), Arg (A), and Leu (L), should be avoided if possible, as these have the most wobbles, that is six codons encode each of these amino acids. The more wobbles introduced into the PCR primer, the more degenerate it becomes. Most successful degenerate PCR experiments have used primers with ≤100-degeneracy. However, primers that were 1000–10000 fold degenerate have also been used successfully (Norwegian University of Science and Technology, 2010, http://boneslab.bio.ntnu.no/degpcrshortguide.htm). Inosine is a purine (which occurs naturally in tRNAs) that can form base pairs with cytidine, thymidine, and adenosine, and is often used in primers where any of the four bases might be required. Therefore, each use of inosine reduces the degeneracy of the primer pool four-fold. However, there is a risk of I:G mismatches, and one must assume that exact base-pairing at other positions in the primer will overcome this problem. Using inosine in the primers requires that the DNA polymerase used in the PCR reaction be capable of synthesizing DNA over an inosine-containing template. *Taq* polymerase is capable of doing this (Koelle, 2010, http://med.yale.edu/mbb/koelle/protocol_degenerate_PCR.html). Degenerate primers with only the first, or first and second, positions of the 3-most codon can be

synthesized giving one or two extra positions of exact base pairs without adding any degeneracy. Degenerate primers should usually be a minimum of 20 bases.

2.4.2.1 Manual degenerate PCR primer design

Both amino acid and nucleotide sequences of a number of multiply-aligned sequences can be studied when designing degenerate PCR primers manually. The sequences are scanned for two regions of similarity separated by an appropriate number of base pairs. Degenerate primers are designed using the IUPAC degenerate code. Primers are then used as input into a primer design programme to check the melting temperatures and presence or absence of predicted secondary structures. The manually designed forward (MF1) and reverse (MR1) PCR primers (Table 2.2) were chosen for commercial synthesis by MWG-Biotech GmbH, Germany.

Table 2.2. M1 polymerase chain reaction (PCR) primers in 5'-3' orientation.

MF1 (20-mer): TGY TCB YTI KCI CCV CAY AT (degeneracy = 256) Tm = 55.9°C
MR1 (18-mer): YAR RTA GRY RTC KGC HAC (degeneracy = 384) Tm = 53.3°C

MF1 = manually designed forward. MR1 = manually designed reverse

2.4.2.2 Computer software-facilitated degenerate PCR primer design

CODEHOP (COnsensus-DEgenerate Hybrid Oligonucleotide Primer)

CODEHOP is a program that designs degenerate PCR primers from conserved blocks of amino acids within multiply-aligned protein sequences (Rose et al., 2003). Each CODEHOP encodes three to four highly conserved amino acids within a 3'-degenerate core. A longer 5' non-degenerate clamp region contains the most probable nucleotide predicted for each flanking codon. The CODEHOP designer (CODEHOP, 2010, http://blocks.fhcrc.org/codehop.html) is linked to BlockMaker and the Multiple Alignment processor within the Blocks database (CODEHOP, 2010, http://blocks.fhcrc.org). The CODEHOP-designed forward (CF1) and reverse (CR1) PCR primers (Table 2.3) were chosen for commercial synthesis by MWG-Biotech GmbH, Germany.

Table 2.3. C1 polymerase chain reaction (PCR) primers in 5'–3' orientation.

C1F (26-mer): TGC TCC CTG TCC CCT cay ath gcn ht (degeneracy = 72) Tm = 61.4
C1R (25-mer): GAG GGG CGA AGG CCt trt gna ryt c (degeneracy = 32) Tm = 61.3

CF1 = CODEHOP-designed forward. CR1 = CODEPHOP-designed reverse

The bacterial GST degenerate PCR forward primers, M1F and C1F, sequences are based on the same amino acids in the more highly conserved N-terminal region of the proteins associated with binding to glutathione. However, the bacterial GST degenerate PCR reverse primers, M1R and C1R, are from different amino acid sequences in the less conserved C-terminal region associated with substrate specificity. M1R, but not C1R, is from the conserved C-terminal SVADIYL region shown in Fig. 2.1.

Primaclade

Primaclade is a web-based application that accepts a multiple-species nucleotide alignment file as input and identifies a set of PCR primers that will bind across the alignment (Gadberry et al., 2005 [program accessible at Primaclade, 2010, http://www.umsl.edu/services/kellogg/primaclade.html]). The Primaclade program iteratively runs the Primer3 application for each alignment sequence and collates the results. Specifically, Primaclade runs Primer3 ten times starting with a primer length of 18 nucleotides and then increasing the length of the primer by one base pair at a time until a maximum length of 27 nucleotides. During each of these runs a maximum of 20 primers are returned, creating a list of 10 X 20 X (number of sequences) primers. This list is then checked to see if the primers meet the user's input criteria, i.e. with respect to degenerate nucleotides and gapped lines, before the primer sequences are written to the output file.

No satisfactory bacterial GST degenerate PCR primers could be designed using Primaclade.

2.4.3 Optimisation of PCR Conditions

Degenerate PCR is a PCR method that uses degenerate primers to amplify unknown DNA sequences that are related to, in this study, known bacterial GST sequences. Degenerate PCR conditions are similar to standard PCR conditions but, as always, for each pair of PCR primers, PCR conditions must be optimized.

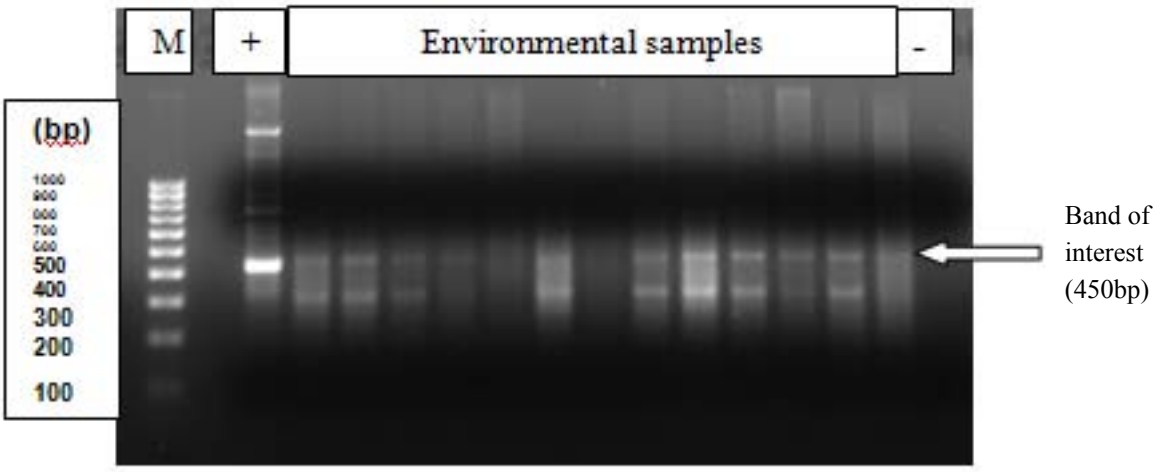


Figure 2.8. Polymerase chain reaction (PCR) product (~450bp) obtained using M1 primers electrophoresed on a 2% agarose gel. M: 100bp ladder; +: positive control; -: negative control.

PCR conditions were optimized using the manually designed degenerate bacterial GST primers, M1F and M1R (sequences shown in Table 2.2) to amplify a PCR product of the expected size (i.e. ~450bp) in environmental genomic DNA samples (PCR products shown in Fig. 2.8 above).

Optimal PCR conditions for M1 primers involved denaturation at 95°C for 5 mins, 50 cycles of denaturation, annealing and extension at 95°C for 1 min, 43°C for 1 min, and 72°C for 1 min, respectively, followed by a final extension at 72°C for 7 mins. The PCR reaction mix contained 0.5µM of each primer, 2.5mM $MgCl_2$, and 200µM dNTP. PCR products can be cloned and sequenced so as to determine whether or not they are known or novel GSTs.

2.5 Analysis of Environmental Samples using Gas Chromatography-Mass Spectrometry

In the initial research proposal, chlorinated organic compounds in environmental soil samples were to be analysed commercially. However, with the arrival of an Enterprise Ireland-funded GC-MS at ITCarlow in 2009, it was decided to carry out GC-MS analysis in the project team's own laboratory. To date, toxic chlorinated organic pesticides of concern as environmental pollutants (see Section 1.1) (2,4-dichlorophenoxyacetate [2,4-D] and atrazine [as standards made up in ethanol]), and polychlorinated aromatic compounds in soil/sediment samples (extracted into acetone, hexane and heptane) have been identified using GC-MS.

The GC column used was a JVA Factor Four™ DB-5 capillary column (30m with 0.2mm internal diameter, coated with 0.25um stationary phase).

A number of extraction methods for the toxic chlorinated organic pesticides (2,4-dichlorophenoxyacetate [2,4-D] and atrazine) in soil/sediment samples were tested. Optimal conditions were as follows: (i) 10µg/ml pesticide was made up in 100% ethanol, (ii) 1ul of sample was injected onto the column with oven temperatures as follows:

- 250°C for 10 min;
- 270°C for 20 min;

and (iii) elution was with hexane. Carrier gas was helium and mass spectrometry was using electron impact. Analysis of atrazine, using the conditions described above, is shown below in Fig. 2.9.

A number of extraction methods for the polychlorinated aromatic compounds in soil/sediment samples were tested. Optimal conditions were as follows: (i) 1g soil/ sediment samples was extracted into 5ml each of ethanol, hexane and heptane, (ii) samples were filtered (0.2µ) before being injected onto the column, (iii) 1ul of sample was injected onto the column with oven temperatures as follows:

- 50°C; held for 2 mins;
- Increase to 250°C @ 10°C/min; held for 1 min;
- Increase to 280°C @ 10°C/min; held for 1 min;
- Increase to 300°C @ 10°C/min; held for 2 mins;

and (iv) elution was with hexane. Carrier gas was helium and mass spectrometry was using electron impact detection.

Analysis of a WWTP-pressed sludge sample, using the conditions described above, showing the presence of tri-chlorinated aromatics is shown in Fig. 2.10.

Atrazine identified using the National Institute of Standards and Technology (NIST) database

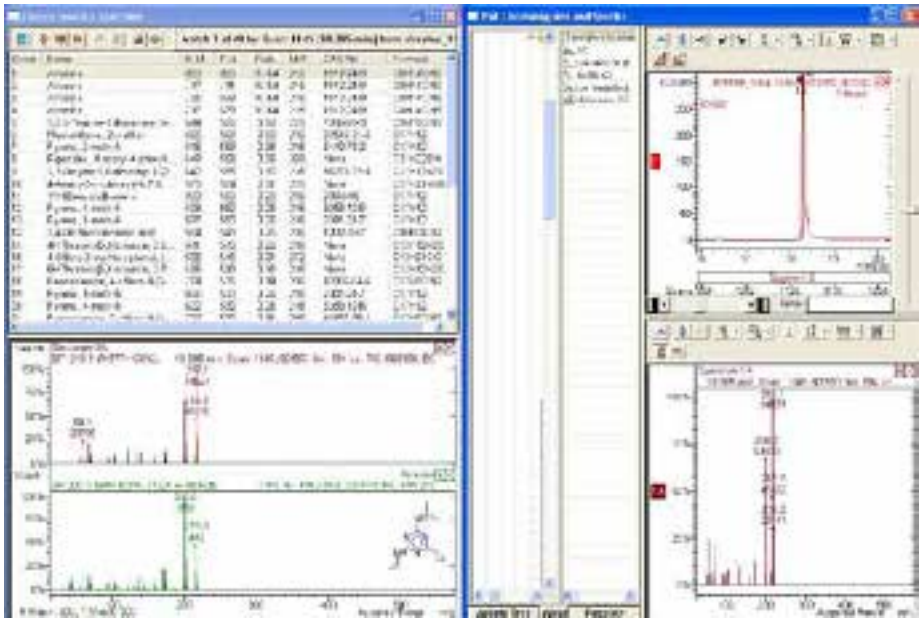


Figure 2.9. Gas chromotography-mass spectrometry (GC-MS) profile of atrazine.

2,4,5-Trichlorobiphenyl identified using the National Institute of Standards and Technology (NIST) database

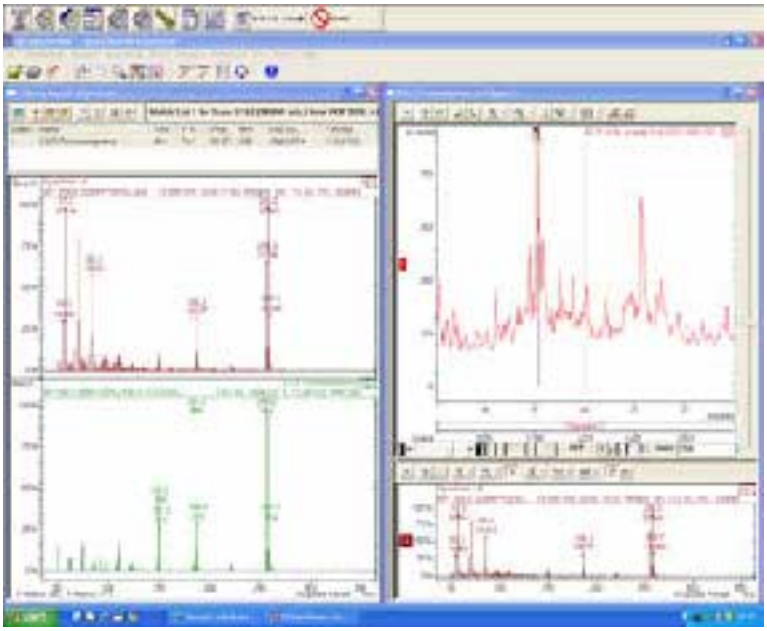
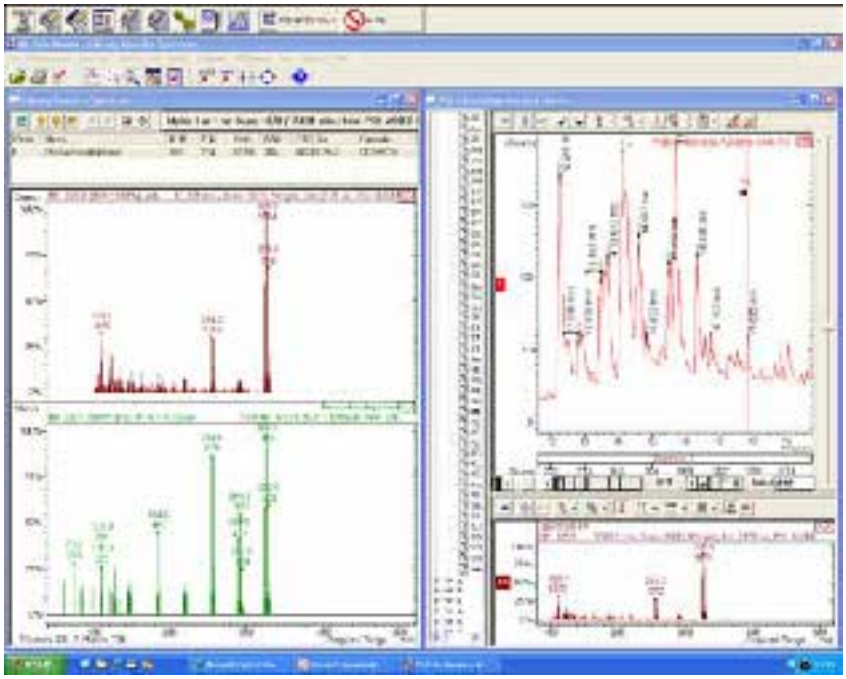


Figure 2.10. Gas chromotography-mass spectrometry (GC-MS) profile of waste-water treatment plant (WWTP) pressed sludge showing the presence of tri-chlorinated aromatics.



Pentachlorobiphenyl identified using the National Institute of Standards and Technology (NIST) database

Figure 2.11. Gas chromotography-mass spectrometry (GC-MS) profile of pentachlorophenal (PCB)-contaminated soil showing the presence of penta-chlorinated aromatics.

Analysis of PCB-contaminated soil from Lhhenice in the southern Czech Republic showing the presence of penta-chlorinated aromatics is shown in Fig. 2.11. Up to seven hepta-chlorinated aromatic compounds were detected using this methodology in this PCB-contaminated soil sample.

When compared to commonly used methods for detection of these compounds, the GC-MS method for detecting chlorinated organic compounds in soil/sediment described in this report has a number of advantages. It is (i) relatively inexpensive (compared with the commonly used method for detection of these compounds, i.e. LC-MS), (ii) less complicated than methods requiring derivatization of compounds of interest in samples, and (iii) uses a common GC column found in most laboratories carrying out GC-MS analysis, i.e. a JVA Factor Four™ DB-5 capillary column (30m with 0.2mm internal diameter, coated with 0.25um stationary phase). This methodology has the potential for the rapid monitoring of environmental samples for chlorinated aromatics of concern as environmental pollutants, e.g. toxic pesticides and PCBs. Small samples (1g) of environmental soil could be collected in the field, added to a vial containing 5ml each of ethanol, hexane and heptane, and brought back to the laboratory for analysis, which takes less than an hour using the conditions described above.

2.6 Microbial Community Profiling

Since only approximately 1% of microorganisms in environmental samples are culturable, polymerase chain reaction–denaturing gradient gel electrophoresis (PCR-DGGE) can be used to characterize microorganisms since it can detect and quantify both culturable and non-culturable microorganisms.

This profiling technique is based on the melting behaviour of the 16S rRNA gene on a polyacrylamide gel containing a urea gradient, which is dependent on the G+C content and the nucleotide sequence. The primers used to amplify the 16S rRNA genes in the community contain a GC clamp; as the DNA fragment moves down the gradient it will denature except for the terminal GC clamp. The denaturation or melting reduces the mobility of the DNA fragment in the gradient gel dramatically. Amplification of the 16S rRNA genes within a community and subsequent analysis by DGGE gives rise to a banding pattern in which each band corresponds to a single species. The advantage of this technique is that individual bands may be recovered from the gel for sequencing, facilitating the potential

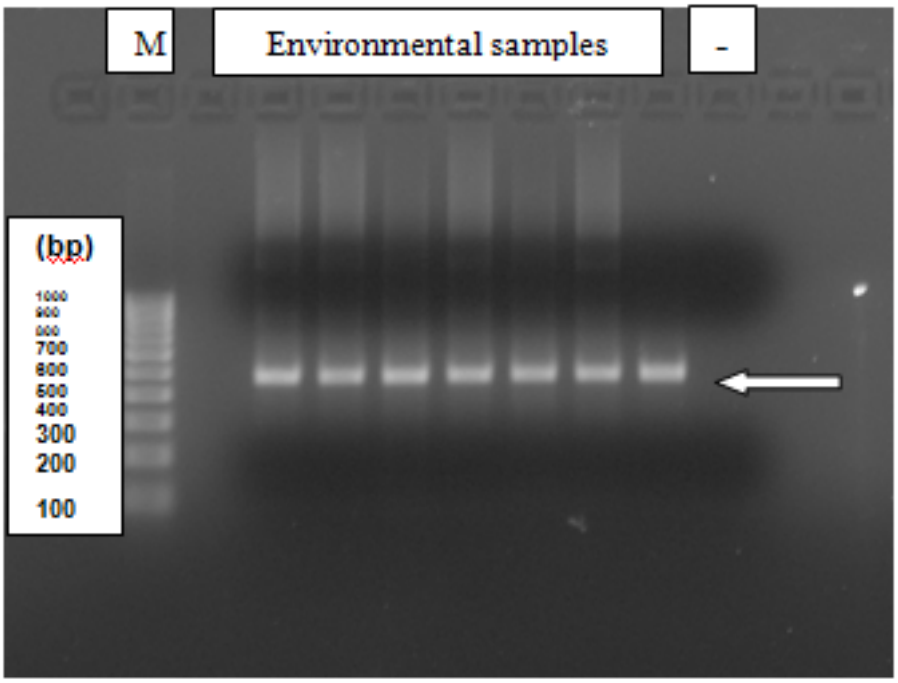


Figure 2.12. Polymerase chain reaction (PCR) product (433bp) obtained using F984GC and R1378 primers electrophoresed on a 2% agarose gel. M: 100bp ladder; -: negative control.

identification of microorganisms by comparing the sequence(s) obtained with those already deposited in a ribosomal database.

16S rRNA DNA PCR amplification was carried out using the fD1 and rD1 primers described by Weisburg et al. (1991), followed by the nested F984GC (with a GC-clamp) and R1378 primers described by Heuer et al. (1997). The resulting 16S rRNA PCR products (433bp) (Fig. 2.12) are currently being used for analysis of microbial diversity in each environmental soil sample using DGGE.

2.7 Developing a Specific Bacterial Glutathione Transferase, BphK^{LB400}, as a Biosensor for Detecting Chlorinated Organic Compounds in the Environment and for Bioremediation

In the initial research proposal, the development of BphK^{LB400} as a biosensor for detecting chlorinated organic compounds in the environment was to have been carried out in collaboration with Prof. Anthony Campbell from the University of Cardiff in Wales. Prof. Campbell has developed a range of (i) whole-cell biosensors, and (ii) colourful proteins incorporating a sensing element (e.g. an enzyme capable of detecting various substrates) and a reporting element (bioluminescent reporter protein, e.g. luciferin). The project team's aim was to construct a biosensor using BphK^{LB400} (wildtype or mutant), and perhaps subsequently other bacterial GSTs, as the sensing element with a view to using the recombinant protein in an *in vitro* assay optimized to detect levels of chlorinated organic compounds in environmental samples. However, although initial experiments in collaboration with Prof. Campbell got under way in his laboratory in Wales in 2008, due to unforeseen circumstances they did not advance beyond the early stages. As this approach would have been difficult to undertake independently in the project team's own laboratory at ITCarlow, an alternative approach to the development of a specific bacterial GST, BphK^{LB400}, as a biosensor for detection of chlorinated organic compounds in the environment and for bioremediation was taken. This is described below.

Previously, in the laboratory, a specific bacterial GST, BphK^{LB400}, was over-expressed in *E. coli* and the dehalogenating activity of BphK^{LB400} towards a number of synthetic chlorinated organic compounds associated with varying levels of ecotoxicity was measured biochemically (McGuinness et al., 2006, 2007). These compounds included PCB metabolites (3-CBA and 4-CBA) and commonly used pesticides (2,4-D, atrazine,

pentanochlor, Clean-up®, CMC and triphenyltin chloride).

Site-directed mutagenesis at a number of highly conserved amino acid positions in BphK^{LB400} was carried out to study the effect of mutation on dechlorination acitivity with a view to developing improved activity for potential use as a biosensor (see Section 2.3).

Two BphK^{LB400} mutants, Ala180Pro and Cys10Phe, demonstrated a 2.5- and 4.5-fold increase in GST activity in bacterial cell extracts when compared with wildtype BphK^{LB400} (McGuinness et al., 2006; Brennan et al., 2009) and could have potential for use as biosensors and for bioremediation. The *bphK^{LB400}* gene (wildtype and mutant [Ala180Pro, Cys10Phe, Cys10Trp]) was inserted into a plasmid vector containing an antibiotic (chloroamphenicol [Cm]) resistance gene to facilitate selection (see Fig. 2.10). The Cys10Trp mutant was included as a negative control.

This construct was inserted into a mini Tn5 transposon containing the gene encoding green fluorescent protein (*gfp*) (see Fig. 2.13) followed by conjugation of the transposon, via horizontal gene transfer, with two strains of endophytic bacteria, *Pseudomonas sp*. L23 and *Pseudomonas sp*. L228 isolated from the internal plant tissue of the energy crop *Miscanthus* (Keogh, 2009), and a single strain of rhizospheric bacteria, *Pseudomonas fluorescens* F113RIF, a spontaneous Rif resistant mutant of the well-characterized rhizospheric strain isolated from the rhizosphere of sugar beet (Brazil et al., 1995). The use of a transposon facilitated the generation of more stable transformants than would have been obtained using plasmids for rhizospheric and endophytic bacterial transformation.

Transconjugants had *gfp* expression (see Fig. 2.14), resistance to *Kan* and *Cm*, and dechlorination actiivity toward CDNB (data not shown). BphK^{LB400} expression was regulated by the constitutive *Cm* promoter. Plant seeds (rapeseed [Brassica napus]) were inoculated with endophytic and rhizospheric transconjugants. Plants were grown in a plant-growth chamber for approximately four weeks. L23 and L228, and F113 control and transconjugants, were found to colonize rapeseed plants (McGuinness et al., 2010; Brennan, 2010), suggesting that these genetically enhanced rhizospheric and endophytic bacteria could have the potential for the sustainable bioremediation of toxic chlorinated organic compounds in environmental soil.

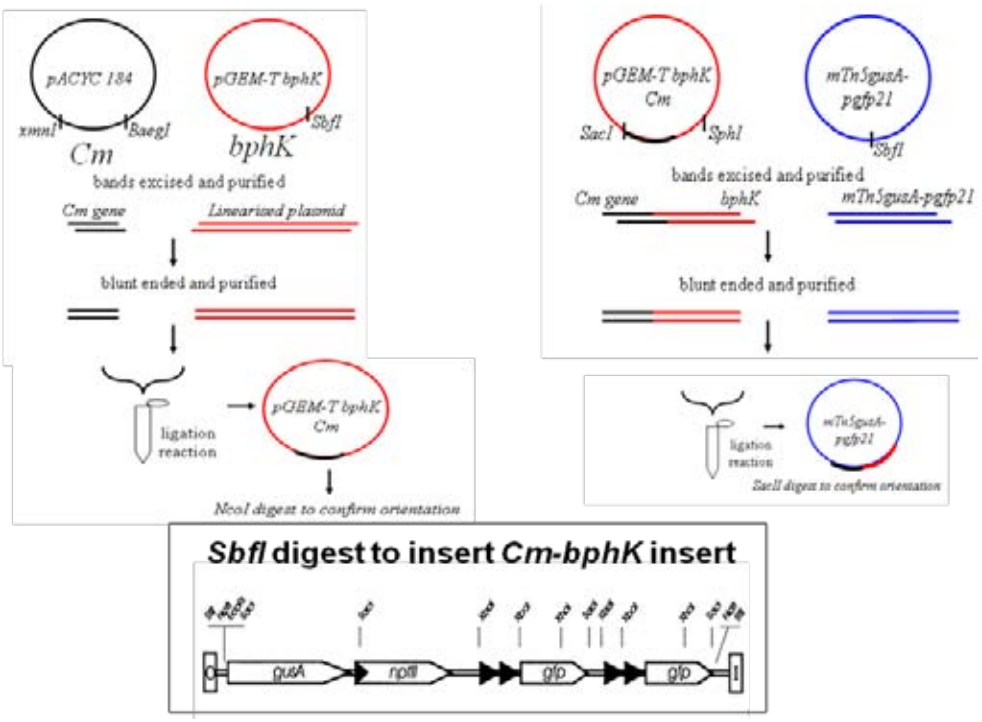


Figure 2.13. Construction of mini-transposon.

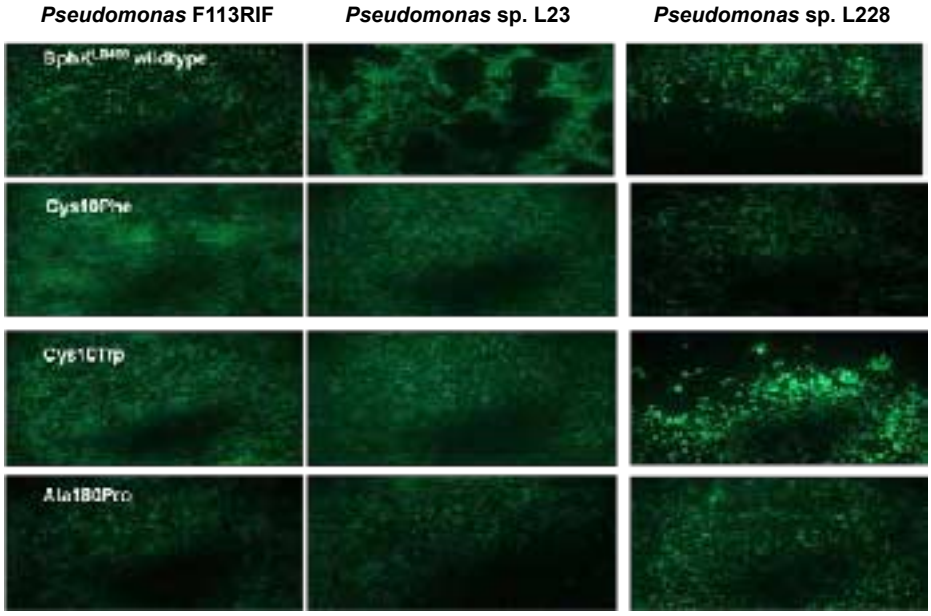


Figure 2.14. Green fluorescent protein (GFP) fluorescence of transconjugants.

Bacteria expressing inducible *bphK*^{LB400}, and *gfp*, could have potential as biosensors for environmental soil contaminated with chlorinated organic compounds.

The use of molecular biological techniques to identify additional GSTs capable of dehalogenating toxic organic pollutants could contribute to the construction of novel transposons for conjugation, via horizontal gene transfer, with bacteria.

3 Discussion

Because of the ambitious nature of a number of these initial goals, not all the specific goals of this project were reached. However, data obtained from this project has the potential for the development of biosensors and for the bioremediation of chlorinated organic compounds in the environment, and for the development of a relatively simple and inexpensive method of detecting chlorinated organic compounds in environmental samples.

Molecular biological techniques were used to study microbial diversity in environmental soil/sediment samples from a number of polluted sites in Ireland as well as from sites in the USA and the Czech Republic. Standard techniques were used to PCR amplify 16S ribosomal RNA sequences for analysis using DGG electrophoresis. In a parallel study in the laboratory, which also used 16S rRNA analysis from high- and low-input agricultural plots at Teagasc Oakpark, Carlow (Chhabra et al., 2010, submitted for publication), investigating microbial diversity of wheat and barley monocultures under different pesticide input regimens, analysis of bacterial diversity suggested only a small difference ($p>0.05$) between pesticide input regiments. In this study, a total of 270 sequences were compared with sequences in the Ribosomal Database Project and the Genbank database, and the major bacterial groups identified were *Acidobacteria*, *Proteobacteria*, *Verrucomicrobiae*, *Gemmatimonadetes* and *Planctomycetes* (Chhabra et al., 2010, submitted for publication). Chhabra et al. (2010, submitted for publication) suggest that the finding of only a small difference between the high- and low-input samples may be caused by the limited number of 16S rRNA sequences analysed within data sets. However, it should be noted that a study by Hartmann and Widmer (2006) on three differently managed agricultural soils, involving the analysis of approximately 1900 16S rRNA sequences, found a level of diversity similar to Chhabra et al. (2010, submitted for publication) within their soils. These studies suggest that a large number of bacterial 16SrRNA sequences might be required for analysis before a difference between environmental soils/ sediments is detected. In the current study, preliminary comparisons were made only between microbial populations in environmental samples.

Molecular biological techniques were also used in the current study in an attempt to characterize bacterial GSTs in environmental samples. Degenerate PCR was used to amplify bacterial GSTs for subsequently cloning, sequencing and identifying GSTs (known and novel) in environmental samples. This approach has been used successfully to detect biphenyl dioxygenases in the PCB contaminated soil from the Czech Republic also used in the current study (Aguirre de Carcer et al., 2007). However, there are no reports to date of studies involving the detection of bacterial GST sequences using degenerate PCR in genomic DNA isolated from environmental samples. This could reflect the fact that these bacterial GST sequences are difficult to PCR amplify using degenerate primers as there are many difficulties inherent in developing a PCR-based detection system using novel degenerate PCR primers. More work needs to be done to identify specific bacterial GSTs (known and/or novel) in the environmental samples collected in this study.

The problems associated with degenerate PCR, in addition to the specific sequences of bacterial GSTs, may mean that this approach to characterizing bacterial GSTs in environmental samples might not be the best approach. Other approaches may prove more successful. A metagenomics library constructed using the high- and low-input samples from Teagasc Oakpark Carlow, Carlow, which was constructed in a collaborative effort between ITCarlow and University College Cork (UCC) could be used to screen for GST activity and the isolation of clones of interest for analysis of microbial GST gene sequences.

Future evaluation of microbial profiles and/or bacterial GSTs associated with the environmental samples collected in this study could have the potential for the development of bioindicators of environmental toxins.

A specific bacterial GST, $BphK^{LB400}$, was developed as a biosensor for the detection of chlorinated organic compounds and for the bioremediation of these pollutants in the environment. Specific $BphK^{LB400}$ mutants were generated by site-directed mutagenesis and assayed for GST activity. Data showed that two $BphK^{LB400}$ mutants, Ala180Pro and Cys10Phe,

demonstrated increased GST activity in bacterial cell extracts when compared with wildtype BphK^{LB400}. The Cys10Phe mutant BphK^{LB400} protein had increased GST activity at higher pH, compared with wildtype BphK^{LB400} and the Ala180Pro mutant, suggesting that the Cys10Phe BphK^{LB400} may have the potential for the bioremediation of alkaline soil/sediment in the environment.

Wildtype and mutant (Ala180Pro and Cys10Phe) BphK^{LB400} genes were inserted into endophytic and rhizospheric bacteria. The transconjugants generated in this study have the potential for use as whole-cell microbial biosensors and in the bioremediation of chlorinated organic compounds in environmental soil/sediment. Currently, the BphK^{LB400} gene in the transconjugants generated in this study is under the expression of the constitutive *Cm* promoter. However, if BphK^{LB400} was under the expression of an inducible promoter, these rhizospheric and endophytic transconjugants could be used as whole-cell biosensors of chlorinated organic compounds in environmental soil/sediment. While some work has been carried out previously at ITCarlow in developing genetically modified rhizospheric *Pseudomonas* biosensing biodegraders to detect PCB and CBA bioavailability and biodegradation in contaminated soils (Liu et al., 2010), the work carried out in this project describes the development of endophytic biosensing degraders, as well as more sensitive rhizospheric biosensing degraders, to detect chlorinated organic compounds of concern as environmental pollutants.

4 Recommendations

Below are a number of recommendations relating to the work carried out in this research project.

4.1 Development of an Assay for Gas Chromatography-Mass Spectrometry Analysis of Chlorinated Organic Compounds in Environmental Soil/ Sediment

Using the GC-MS conditions described in Section 3.4, small samples (1g) of environmental soil could be collected in the Irish environment, added to a vial containing 5ml each of ethanol, hexane and heptane, and brought back to the laboratory for investigation (in less than an hour) and detection of chlorinated organic compounds, PCBs and commonly used pesticides, which are of concern as environmental pollutants. This methodology provides a relatively simple and inexpensive assay for samples collected in the field in laboratories with GC-MS facilities. In 2006, the European Commission published the final *Thematic Strategy for Soil Protection* and a proposal for a directive which would establish a framework for the protection of soil involving the identification of the location and extent of soil contamination and the requirements for remediation. As an output of this project, it is recommended that this assay be developed further so that when the Soil Framework Directive is introduced, and legally implemented in all EU member states, a large number of Irish environmental soil/sediment samples can be analysed relatively quickly and inexpensively in Irish laboratories with standard GC-MS facilities.

4.2 Development of a Specific Bacterial Glutathione Transferase, BphK^{LB400} (Wildtype and Mutant) for Bioremediation

In this project a specific bacterial GST, BphK^{LB400} (wildtype and mutant), was introduced into two strains of endophytic bacteria, *Pseudomonas sp*. L23 and *Pseudomonas sp*. L228 isolated from the internal plant tissue of the energy crop *Miscanthus* (Keogh, 2009), and a single strain of rhizospheric bacteria, *Pseudomonas fluorescens* F113Rif, a spontaneous Rif resistant mutant of the well-characterized rhizospheric strain isolated from the rhizosphere of sugar beet (Brazil et al., 1995). Further study of the dechlorination activity BphK^{LB400} mutant Cys10Phe in a high pH environment towards toxic pollutants could provide information on the potential of this BphK^{LB400} mutant in bioremediation in alkaline soil/sediment. Much work remains to be done in carrying out field studies based on laboratory-scale experiments before commercially viable systems are available using plant-associated endophytic and rhizospheric bacteria to degrade a wide range of toxic organic compounds of concern in environmental soil. Plant-associated endophytes may offer more potential for bioremediation than plant-associated rhizospheric bacteria since (i) the use of endophytes that are native to the host plant reduces competition between bacterial strains and may eliminate the need for reinoculation, (ii) toxic organic contaminants can remain in the plant xylem for up to two days facilitating their degradation by endophytes, and (iii) endophytes can be isolated from host plants of interest and genetically enhanced with genes encoding degradation enzymes of interest before reinoculation for bioremediation. Further work could involve the use of endophytes specific to crops of relevance in Ireland, for example, crops grown as a source of bioenergy. The main energy crops that can be grown in Ireland include oilseed rape, cereals, hemp (annual crops) and willow, *Miscanthus* and reed canary grass (perennial crops) (EPA, 2008b). Emphasis should be placed, when developing bioremediation systems using plant-associated bacteria, on choosing wildtype bacteria, or bacteria enhanced using natural gene transfer, to avoid the complications of national and international legislation restricting and monitoring the use of GMMs.

4.3 Improved Access to National Soil Database Samples

The National Soil Database (NSDB) was set up in Ireland as a result of a collaborative project between the EPA, Institute of Technology (IT) Sligo and the National University of Ireland (NUI) Galway. The National Soil Archive contains approximately 1300 bulk soil samples taken on a 10 x 10 km grid. An application to the NSDB

for access to soil samples (or nucleic acid extracted from these samples) with reported levels of chlorinated organic pesticides (McGrath, 1998; McGrath and McCormack, 1999) from the NSDB was made in Summer 2009. However, this application was denied. Because of the difficulty in the Republic of Ireland in accessing soil contaminated with documented levels of chlorinated organic compounds, an output from this project is the recommendation that improved access to NSDB samples should be available to Irish researchers where possible, with an explicit opportunity for applicants to provide additional information, if necessary, as part of the application-evaluation process.

4.4 Relevance to Policy

In 2006, the European Commission published the final Thematic Strategy for Soil Protection and a proposal for a directive which would establish a framework for the protection of soil involving the identification of the location and extent of soil contamination and the requirements for remediation. As noted above, as an output of this project, it is recommended that the GC-MS assay be developed further so that when the Soil Framework Directive is implemented legally in all EU member states, it will be possible to analyse a large number of Irish environmental soil/sediment samples relatively quickly and inexpensively in Irish laboratories with standard GC-MS facilities.

Although many environmental regulatory measures have been adopted in Ireland in recent decades, there has been little regulation regarding the protection of soil, and the remediation of contaminated soil. With environmental technologies playing a key role in the Irish government's *Building Ireland's Smart Economy* (2008), and a global political shift towards sustainable and green bioremediation technologies, the development of the modified plant-associated bacteria (generated as an output of this project) to degrade toxic synthetic organic compounds in environmental soil is in line with current policy to provide an efficient, economic, and sustainable green remediation technology for the twenty-first century environment.

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Acronyms and Annotations

2,4-D	2,4-dichlorophenoxyacetic acid
Ala	Alanine
bp	Base pairs
CBA	Chlorobenzoate
CDNB	1-chloro 2,4-dinitrobenzene
Cm	Chloroamphenicol
CMC	Chloromequat chloride
Cys	Cysteine
DDT	Dichlorodiphenyltrichloroethane
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxy-nucleotide triphosphate
GC-MS	Gas chromatography–mass spectrometry
GFP	Green fluorescent protein
GMM	Genetically modified microorganism
GSH	Glutathione
GST	Glutathione transferase
His	Histidine
HOPDA	2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate
HPLC	High pressure liquid chromatography
kan	Kanamycin
LC-MS	Liquid chromatography–mass spectrometry
NSDB	National Soil Database
PCB	Polychlorinated biphenyl
PCP	Pentachlorophenol
PCR	Polymerase chain reaction
PCR -DGGE	Polymerase chain reaction –denaturing gradient gel electrophoresis
Phe	Phenylalanine
POP	Persistent organic pollutant
Pro	Proline
Rif	Rifampicin
rRNA	Ribosomal ribonucleic acid
Sm	Streptomycin
sp.	Species
TBT	Tributylin
TCP	Tetrachlorophenol
Trp	Tryptophan
WWTP	Waste-water treatment plant

An Ghníomhaireacht um Chaomhnú Comhshaoil

Is í an Gníomhaireacht um Chaomhnú Comhshaoil (EPA) comhlachta reachtúil a chosnaíonn an comhshaol do mhuintir na tíre go léir. Rialaímid agus déanaimid maoirsiú ar ghníomhaíochtaí a d'fhéadfadh truailliú a chruthú murach sin. Cinntímid go bhfuil eolas cruinn ann ar threochtaí comhshaoil ionas go nglactar aon chéim is gá. Is iad na príomh-nithe a bhfuilimid gníomhach leo ná comhshaol na hÉireann a chosaint agus cinntiú go bhfuil forbairt inbhuanaithe.

Is comhlacht poiblí neamhspleách í an Ghníomhaireacht um Chaomhnú Comhshaoil (EPA) a bunaíodh i mí Iúil 1993 faoin Acht fán nGníomhaireacht um Chaomhnú Comhshaoil 1992. Ó thaobh an Rialtais, is í an Roinn Comhshaoil agus Rialtais Áitiúil a dhéanann urraíocht uirthi.

ÁR bhFREAGRACHTAÍ

CEADÚNÚ

Bíonn ceadúnais á n-eisiúint againn i gcomhair na nithe seo a leanas chun a chinntiú nach mbíonn astuithe uathu ag cur sláinte an phobail ná an comhshaol i mbaol:

- áiseanna dramhaíola (m.sh., líonadh talún, loisceoirí, stáisiúin aistrithe dramhaíola);
- gníomhaíochtaí tionsclaíocha ar scála mór (m.sh., déantúsaíocht cógaisíochta, déantúsaíocht stroighne, stáisiúin chumhachta);
- diantalmhaíocht;
- úsáid faoi shrian agus scaoileadh smachtaithe Orgánach Géinathraithe (GMO);
- mór-áiseanna stórais peitreail.
- Scardadh dramhuisce

FEIDHMIÚ COMHSHAOIL NÁISIÚNTA

- Stiúradh os cionn 2,000 iniúchadh agus cigireacht de áiseanna a fuair ceadúnas ón nGníomhaireacht gach bliain.
- Maoirsiú freagrachtaí cosanta comhshaoil údarás áitiúla thar sé earnáil - aer, fuaim, dramhaíl, dramhuisce agus caighdeán uisce.
- Obair le húdaráis áitiúla agus leis na Gardaí chun stop a chur le gníomhaíocht mhídhleathach dramhaíola trí comhordú a dhéanamh ar líonra forfheidhmithe náisiúnta, díriú isteach ar chiontóirí, stiúradh fiosrúcháin agus maoirsiú leigheas na bhfadhbanna.
- An dlí a chur orthu siúd a bhriseann dlí comhshaoil agus a dhéanann dochar don chomhshaol mar thoradh ar a ngníomhaíochtaí.

MONATÓIREACHT, ANAILÍS AGUS TUAIRISCIÚ AR AN GCOMHSHAOL

- Monatóireacht ar chaighdeán aeir agus caighdeáin aibhneacha, locha, uiscí taoide agus uiscí talaimh; leibhéil agus sruth aibhneacha a thomhas.
- Tuairisciú neamhspleách chun cabhrú le rialtais náisiúnta agus áitiúla cinntí a dhéanamh.

RIALÚ ASTUITHE GÁIS CEAPTHA TEASA NA HÉIREANN

- Cainníochtú astuithe gáis ceaptha teasa na hÉireann i gcomhthéacs ár dtiomantas Kyoto.
- Cur i bhfeidhm na Treorach um Thrádáil Astuithe, a bhfuil baint aige le hos cionn 100 cuideachta atá ina mór-ghineadóirí dé-ocsaíd charbóin in Éirinn.

TAIGHDE AGUS FORBAIRT COMHSHAOIL

- Taighde ar shaincheisteanna comhshaoil a chomhordú (cosúil le caighdéan aeir agus uisce, athrú aeráide, bithéagsúlacht, teicneolaíochtaí comhshaoil).

MEASÚNÚ STRAITÉISEACH COMHSHAOIL

- Ag déanamh measúnú ar thionchar phleananna agus chláracha ar chomhshaol na hÉireann (cosúil le pleananna bainistíochta dramhaíola agus forbartha).

PLEANÁIL, OIDEACHAS AGUS TREOIR CHOMHSHAOIL

- Treoir a thabhairt don phobal agus do thionscal ar cheisteanna comhshaoil éagsúla (m.sh., iarratais ar cheadúnais, seachaint dramhaíola agus rialacháin chomhshaoil).
- Eolas níos fearr ar an gcomhshaol a scaipeadh (trí cláracha teilifíse comhshaoil agus pacáistí acmhainne do bhunscoileanna agus do mheánscoileanna).

BAINISTÍOCHT DRAMHAÍOLA FHORGHNÍOMHACH

- Cur chun cinn seachaint agus laghdú dramhaíola trí chomhordú An Chláir Náisiúnta um Chosc Dramhaíola, lena n-áirítear cur i bhfeidhm na dTionscnamh Freagrachta Táirgeoirí.
- Cur i bhfeidhm Rialachán ar nós na treoracha maidir le Trealamh Leictreach agus Leictreonach Caite agus le Srianadh Substaintí Guaiseacha agus substaintí a dhéanann ídiú ar an gcrios ózóin.
- Plean Náisiúnta Bainistíochta um Dramhaíl Ghuaiseach a fhorbairt chun dramhaíl ghuaiseach a sheachaint agus a bhainistiú.

STRUCHTÚR NA GNÍOMHAIREACHTA

Bunaíodh an Ghníomhaireacht i 1993 chun comhshaol na hÉireann a chosaint. Tá an eagraíocht á bhainistiú ag Bord lánaimseartha, ar a bhfuil Príomhstiúrthóir agus ceithre Stiúrthóir.

Tá obair na Gníomhaireachta ar siúl trí ceithre Oifig:

- An Oifig Aeráide, Ceadúnaithe agus Úsáide Acmhainní
- An Oifig um Fhorfheidhmiúchán Comhshaoil
- An Oifig um Measúnacht Comhshaoil
- An Oifig Cumarsáide agus Seirbhísí Corparáide

Tá Coiste Comhairleach ag an nGníomhaireacht le cabhrú léi. Tá dáréag ball air agus tagann siad le chéile cúpla uair in aghaidh na bliana le plé a dhéanamh ar cheisteanna ar ábhar imní iad agus le comhairle a thabhairt don Bhord.

NDP
National Development Plan 2007 - 2013

Science, Technology, Research and Innovation for the Environment (STRIVE) 2007-2013

The Science, Technology, Research and Innovation for the Environment (STRIVE) programme covers the period 2007 to 2013.

The programme comprises three key measures: Sustainable Development, Cleaner Production and Environmental Technologies, and A Healthy Environment; together with two supporting measures: EPA Environmental Research Centre (ERC) and Capacity & Capability Building. The seven principal thematic areas for the programme are Climate Change; Waste, Resource Management and Chemicals; Water Quality and the Aquatic Environment; Air Quality, Atmospheric Deposition and Noise; Impacts on Biodiversity; Soils and Land-use; and Socio-economic Considerations. In addition, other emerging issues will be addressed as the need arises.

The funding for the programme (approximately €100 million) comes from the Environmental Research Sub-Programme of the National Development Plan (NDP), the Inter-Departmental Committee for the Strategy for Science, Technology and Innovation (IDC-SSTI); and EPA core funding and co-funding by economic sectors.

The EPA has a statutory role to co-ordinate environmental research in Ireland and is organising and administering the STRIVE programme on behalf of the Department of the Environment, Heritage and Local Government.

epa
Environmental Protection Agency
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