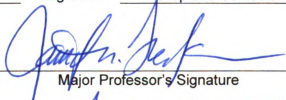This is to certify that the
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AND IMPACTS OF TRANSGENIC PLANTS ON
PHYTOREMEDIATION OF ORGANIC POLLUTANTS

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CLONING AND EXPRESSION OF A BACTERIAL CGTASE
AND IMPACTS OF TRANSGENIC PLANTS
ON PHYTOREMEDIATION OF ORGANIC POLLUTANTS

By

Sarah J Kinder

A DISSERTATION

Submitted to
Michigan State University
In partial fulfillment of the requirements
For the degree of

DOCTOR OF PHILOSOPHY

Department of Crop and Soil Sciences

2007

ABSTRACT

CLONING AND EXPRESSION OF A BACTERIAL CGTASE
AND IMPACTS OF TRANSGENIC PLANTS
ON PHYTOREMEDIATION OF ORGANIC POLLUTANTS

By

SARAH J KINDER

One of the major limitations to biological remediation of persistent organic pollutants is water insolubility and the lack of bioavailability. Surfactants can be used to overcome the limitations on contaminant water solubility for improved biological degradation. Addition of surfactants to soil can be expensive and result in bacterial toxicity. Most surfactants, require a minimum concentration for effectiveness, the critical micelle concentration. Similar in properties to surfactant micelles, cyclodextrins are functional at any concentration due to the toroidal shape of individual molecules, creating a hydrophobic cavity and a hydrophilic exterior. Cyclodextrins can accommodate hydrophobic compounds within the hydrophobic cavity, forming a complex that can improve the water solubility of the "guest" molecule. Cyclodextrins are formed from the degradation of starch by Cyclodextrin Glycosyl Transferase (CGTase, Cgt) secreted into the environment by various *Bacillus* species. We have cloned a novel *cgt* gene from *Paenibacillus sp.* strain C36, *Pl-cgt*. Enzymatic studies showed *Escherichia coli* strains harboring *Pl-cgt* produced quantifiable amounts of βCD in solution. *Pl-cgt* was transformed into the plant species tobacco and Arabidopsis, resulting in transgenic plant lines, which are also capable of degrading starch and producing quantifiable amount of βCD. As a test of transgenically produced CDs, Cgt plants were tested for

phytoremediation of contaminated soils containing polyaromatic hydrocarbons (PAHs) or polychlorinated biphenyls (PCBs). Starch treated transgenic tobacco showed a significant reduction of the highest molecular weight PAH compound, Benzo[ghi]perylene (BGHP) when compared to untreated, unplanted and wild type treatments. Results from PCB studies and total PAHs were inconclusive for Cgt-plants, with multiple treatments, including unplanted, showing significant reductions when compared to untreated soil. Overall, the results showed that Cgt-plants are capable of producing CDs and Cgt-plants can have a positive effect on phytoremediation of some organic pollutants.

laboratory and help me finish my last samples. I would also like to thank my parents and grandparents for their advice, support and visits to the lab for general encouragement and questions. I also thank the Lord for giving me the opportunity to learn so much from those around me and enjoy the company of so many wonderful people during my Ph.D. program, and the providence of my various forms of financial support. I thoroughly enjoyed my studies at MSU and hope that they will enable me to continue learning in new and exciting ways.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PAGE

INTRODUCTION

Environmental contamination by organic pollutants is a pervasive and important threat to the biosphere. Organic pollutants typically originate due to spillage and intentional release of industrial, agricultural or waste products. Once in the environment organic compounds can cause environmental damage, more directly through mutagenicity or indirectly though the disruption of physiological processes.

The problem of organic contaminants in soil has been addressed in the past through a wide variety of methods based around engineering technologies, such as physical soil removal, chemical reactions and stabilization. Physical and chemical technological solutions to polluted land, known as remediation, have been joined by biologically based technologies such as bioremediation, which is using bacteria to decompose or stabilize toxic soil contaminants. Phytoremediation, or the use of plants to degrade or render contaminants harmless, has recently gained favor as a viable technological alternative to engineering and bioremediation installations.

One major property of many organic contaminants that acts as a powerful barrier to remediation technologies that attempt to remove or destroy organic contaminants within soil, is the very low water solubility and bioavailability of many organic contaminants. Organic pollutants are chemically similar to soil organic matter and tend to partition to, essentially dissolving in the organic fraction of soil. Bound organic contaminants can be nearly inert to biological methods of degradation and removal but may still cause environmental harm via slow loss from soil or direct soil consumption by biota. One proposal to help solve the difficulty in removal of organic contaminants from soil, is the use of surfactant or surface active agents on soil to help solubilize organic

1

contaminants. Surfactants are molecules with a hydrophilic "head" and a hydrophobic "tail" portion, enabling the hydrophobic portion of the molecule to associate with contaminant molecules, bringing them into the aqueous phase. However, surfactant molecules are not without problems, they are capable of enhancing biological degradation of organic pollutants. However, surfactants can also cause toxic effects on bacteria capable of biodegradation. Biologically based surfactants or biosurfactants are thought to be a more environmentally friendly, less toxic alternative to synthetic surfactants. Although not a true biosurfactant, one compound stands out as a potential enhancer of biological soil remediation, cyclodextrin. Cyclodextrins (CDs) have similar properties to surfactant micelles and are formed by bacteria. The enzyme CGTase is secreted into the soil by microbes, which acts on available starch molecules forming CDs. CDs are cyclic molecules usually composed of 6-8 glucose units. The hydroxyl groups of cyclodextrins face towards the exterior of the molecule giving cyclodextrins a hydrophilic exterior while the interior remains largely hydrophobic. This property, in common with surfactant micelles, allows the inclusion of hydrophobic compounds inside of the doughnut-shaped molecule, forming a complex. CD complexes can make hydrophobic pollutants more available for both bacterial and plant degradation, speeding up the process of contaminant removal.

The objectives of this project are four-fold:

I. Isolate, clone and characterize a bacterial cyclodextrin glycosyl transferase

II. To create transgenic plants which are capable of secreting CGTase and forming cyclodextrin

III. Testing transgenic plants for improvements in biological degradation of persistent organic pollutants.

IV. Examination of the safety of phytoremediation as a whole, in comparison to standard engineering based remediation practices.

In this project we isolated and cloned a novel CGTase from *Paenibacillus sp.* strain C36. This CGTase was sequenced and compared to known CGTases through different software programs, direct comparison, analysis of signal peptides. The bacterial cgt gene was minimally modified for bacterial and plant expression using PCR based techniques. *Escherichia coli* DH5α was used as the bacterial expression system. Assays were performed using the *E. coli* cgt, and *P. sp.* C36 to determine optimal reaction temperature, quantitative βCD production and qualitative CD production.

PI-cgt was placed into a plant expression cassette, containing a plant functional promoter and terminator sequence. Plant constructs were used to generate transgenic plants, both tobacco and *Arabidopsis*, which were screened for gene integration, *cgt* expression, starch clearing and CD production using similar methods to those used in testing bacterial expression. These plants were then used in experiments with the hydrophobic contaminants, polychlorinated biphenyls (PCBs) and polyaromatic hydrocarbons (PAHs). Chronically contaminated soil as well as spiked soils, were used to test the effectiveness of *cgt*-expressing plants. Some of the soils were starch treated to aid in the *in-situ* production of CDs. Soils were tested for contaminant content after treatment and plants were weight for biomass production. Promising results were seen in the degradation of some contaminants.

As an additional aspect of phytoremediation technology, the safety of phytoremediation technology as a whole was also examined in comparison to standard engineering remediation technologies. The varying and unique risks posed by each were compared and contrasted. Attention was given to transgenic phytoremediation and special risks that are presented by the use of transgenic plants in phytoremediation. A comparison of standard engineering based technologies to their individual phytoremediation counterparts was performed. The ultimate determination of "safety" of phytoremediation technologies was made based on comparison to the existing, accepted engineering technologies.

REVIEW OF LITERATURE

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Environmental Contamination

Anthropogenic environmental pollution is as old as civilization; human and livestock nutrient waste has long polluted rivers and streams, rudimentary metalworking by post-agrarian cultures released toxic metals, and more recently, the industrial revolution rapidly disbursed a vast array of mined and manufactured pollutants. Fossil fuel processing and combustion has caused widespread, persistent impacts to land surface and air quality. Synthetic organic chemistry advances of the 20th century resulted in versatile new chemical products, though also a variety of novel ecotoxicological pollutants. The discovery of unintended effects of manmade organic compounds like polychlorinated biphenyls (PCBs) was largely accidental (Jensen, 1972) and in the United States, led to various regulations such as the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA) (EPA, 2006). These policies are typically called the Superfund and the Clean Water and Air Act and were enacted to reduce ongoing anthropogenic pollution of the environment and force cleanup of existing polluted sites.

Despite laws enacted for the prevention and cleanup, soil and sediment contamination continues to be a common and often persistent problem in many areas of the world, with the United States alone harboring 1,303 contaminated sites on the National Priorities List (NPL) and an estimated 450,000 low level or potentially contaminated sites called brown fields (EPA, 2006). Around the world, contaminated

sites persist and continue to propagate as the rate of industrialization and modernization rapidly accelerates in the world's developing nations.

Due to the human and wildlife health hazards posed by environmental contamination, considerable research has been performed to develop effective methods of cleaning polluted soils, sediments and waterways. This review will discuss the nature and interaction of various pollutants with soils, challenges to their remediation, methods of engineering- and biologically-based remediation, and various approaches to improve biological remediation.

Organic Chemical Pollutants

The ecotoxicity of organic chemicals is dependent on contaminant level, rate of organismal uptake and retention, mechanism of dispersal, and environmental persistence. Some environmental contaminants such as low molecular weight hydrocarbons can be easily degraded or dissipated with little opportunity for biological exposure. Alternatively, chlorinated organic compounds like polychlorinated biphenyls (PCBs) persist in the environment for long periods of time and, even though not acutely toxic, pose widespread biological risk. Due to variation in contaminant biochemical properties, toxicant presence is not synonymous with biological risk: there must be a route of exposure. Contaminants that are tightly bound to the soil matrix may be relatively stable and inaccessible to potential receptor organisms. Hydrophobic organic contaminants and relatively immobile metals such as lead are unlikely to leach to groundwater. More mobile pollutants, such as water-soluble organic compounds and less stably complexed metals, may increase potential risk if conditions favor contaminant leaching. Even if

pollutants are tightly bound to soil, the particles themselves may be moved by wind or water, thus dispersing the pollutants. If humans or animals ingest soil, direct absorption of contaminant molecules from the soil matrix can occur. Other routes of exposure include, direct contact, inhalation, and through contaminated food stocks.

Environmental contaminants are generally grouped into two major classes, organic and inorganic contaminants, with organic compounds being broken into many distinct categories based on their chemical properties. Since the focus of this project is on organic chemicals, these compounds will be the primary focus of this review. Persistent organic pollutants (POPs) include DDT, aldrin, dieldrin, endrin, chlordane, heptachlor, toxaphene, hexachlorobenzene, mirex, PCBs, dioxin, and furans according to the United Nations Environmental Programme (Rodan et al., 1999). POPs can cause considerable environmental problems even at very low concentrations due to a combination of environmental persistence and lipophilicity. POP compounds can be incorporated into the fatty tissues of an organism, where they are typically too hydrophobic to be excreted except at very low levels (Connolly and Glaser, 2002). At only a few parts per million, strongly lipophilic contaminants that incorporate into small organisms will be magnified through each trophic transfer. Eventually, at high trophic levels in apex predators, such as eagles, the toxicological effects become detrimental (Kumar et al., 2002). POPs in oceanic and riverine food chains are thought to be partially responsible for population declines in many marine mammals via immune and reproductive system impairment (Barron et al., 2003; Bitman and Cecil, 1970; Colborn et al., 1993).

Aromatic and aliphatic pollutants include petroleum-based compounds often grouped as total petroleum hydrocarbons (TPHs). TPHs typically possess a range of hydrophobicity, toxicity, and biodegradability characteristics in parallel with increasing molecular weight. TPH compounds are classified by nomenclature and distillation fractions as the lighter weight gasoline-type fractions of benzene, toluene, ethylbenzene, and xylene (BTEX), heavier oils, tar and polyaromatic hydrocarbon compounds (PAH). PAHs are created naturally from incomplete burning of organic materials as well as anthropogenically from industrial processes, such as hydrocarbon burning and coal processing (Wilson and Jones, 1993). High molecular weight PAHs, such as benzo[a]pyrene, are considered carcinogenic and genotoxic (Alexander et al., 2002; Brown et al., 1999). Photomodification may produce oxygenated radicals from PAH molecules, which can react with biological molecules such as DNA (Mallakin et al., 2002).

POPs also include synthetically created pesticides, explosives and assorted compounds used as dielectric fluids, fire retardants, and solvents. One group of POP synthetic compounds is halogenated organic compounds, which contain chlorine, bromine or fluorine bonded to carbon atoms in place of hydrogen. Halogenated aromatic compounds include polychlorinated biphenyls (PCBs), polybrominated biphenyls (PBBs), dichlorodiphenyltrichloroethane (DDT), polychlorinated dibenzofurans (PCDFs) and polychlorinated dibenzodioxins (PCDDs). PCBs were used primarily in electrical transformers and capacitors, carbonless copy paper, paint, plastics and flame retardant materials. PCBs have been shown to possess carcinogenic and estrogenic properties (Safe, 1989). DDT was among the most wide used synthetic pesticides seeing resulting in

unforeseen environmental impacts, such as thinning of bird eggshells (Blus, 1984). POPs, such as PCBs and DDT, persist for many decades in soils and sediments, long after the manufacture and sale of the substances were banned (Erickson, 1993). PCBs and other chlorinated organics are ubiquitous in global distribution, being detected in pristine environments due to volatilization and atmospheric transport (Atlas and Giam, 1981; Risebrough et al., 1968). PCBs, PBBs, PCDDs and PCDFs are mixtures of aromatics compounds with varying chlorination, however only PCBs and PBBs were intentionally synthesized. PCDDs and PCDFs are unintended by products of combustion, pesticide manufacture and are contaminants of concern at 130 sites on the USEPA National Priorities List as of 2006 (EPA, 2006). One PCDD congener, 2,3,7,8-tetrachlorodibenzene-*para*-dioxin, is thought to be one of the most toxic organic compounds known (Steenland et al., 2004).

Chlorinated aliphatic compounds, like trichloroethylene (TCE) and tetrachlorothylene (PCE), are common groundwater contaminants. Chlorinated aliphatics, like most chlorinated organic compounds, are chemically stable and persistent in the environment, more so as the degree of chlorination increases. TCE and PCE are biological harmful compounds, though may be converted to more toxic vinyl chloride by bacterial processes (Nelson, 1988).

Nitroaromatic compounds (NACs), e.g. trinitrotoluene (TNT), cyclotetramethylene-tetranitramine (HMX) and cyclotrimethylenetrinitramine (RDX), are primarily used as explosives, though also as dyes and pesticide intermediates. Environmental contamination from NACs results from manufacture or distribution of

unexploded residuals during detonation. Nitroaromatics are directly toxic, though are also harmful at trace levels via oxidative DNA damage (Homma-Takeda et al., 2002).

Soil and Pollutant Interaction

Soil is a complex physicochemical medium with highly dynamic influences on contaminant fate. Soils are composed of highly varied mineral and organic fractions with large proportions of water and air. Mineral fractions are typically size-classed from largest to smallest as sand, silt and clay, respectively. Chemical composition of the mineral portion of soil includes silica and aluminum oxides arranged in ordered crystalline forms (Dragun, 1998). Soil texture consists of many larger scale structural elements, such as aggregates, cracks and old root tunnels, each of which influences water and air flow. The ratio of soil air space to soil hydration influences mechanical aspects of the soil, such as plasticity. Soil organic matter (SOM) has complex chemical composition and generally serves as the major sorption/partitioning matrix for organic pollutants. Under the International Humic Substances Society Standard, soil organic matter is composed of several fractions, including fulvic acid, humic acid and humin. SOM fractions are largely defined by their extractability. Humin is the acidic, neutral and alkaline insoluble fraction. Fulvic acid is readily soluble in water at neutral pH. Humic acid is only soluble in high pH conditions. Fulvic and humic acid are more labile than humin and turn over more rapidly in the soil (Mobed et al., 1996). Organic compounds, such as PAHs and PCBs, partition to soil organic fractions due to their chemical affinity, becoming largely unavailable for microbial biodegradation. Very hydrophobic organic compounds partition to humin more strongly than the other SOM fractions due to the

similarity in chemical composition (Petruzzelli et al., 2002). Once sorbed to soil organic matter, pollutants may be retained almost indefinitely with extremely slow transfer to the aqueous phase. Permanent binding to soil organic matter, often called irreversible sorption, occurs possibly by covalent linking to SOM molecules. However, irreversibly bound contaminant molecules may be released during decomposition of organic matter by SOM-degrading microbes (Reemtsma et al., 2003).

Water-soluble contaminants may be held within soil pore water or temporarily bound by charged particles such as clays. Some nitroaromatic explosives interact very specifically with certain types of clay (Haderlein et al., 1996). Nearly neutral nitroaromatic compounds (e.g. dinitrotoluene) can be held between smectite clay interlayers in spacing geometries created by the hydration spheres of associated ions such as cesium or potassium. The larger hydration spheres of ions such as calcium and magnesium cause clay layer spacing to increase such that inclusion of nitroaromatics is no longer favorable and the compounds are released into the aqueous phase (Li et al., 2004). Other charged compounds and ions may also be held onto the surface or in-between negatively charged clay layers (Colborn et al., 1993). Nanopores in the soil mineral fraction also sequester hydrophobic pollutants, where they may be inaccessible for microbial bioremediation due to lack of space for microbial entry or growth (Sun et al., 2003).

In addition to the solid soil phase, soil vapor is important for contaminant fate in that it supplies oxygen and other gases for biotic and abiotic chemical reactions. The soil vapor phase is typically in equilibrium with the liquid phase in pore-spaces between solid

soil particles. Volatile contaminants may escape from soil to react with other compounds while in the gaseous state.

Soil Remediation

Conventional Remediation Technologies

In most contaminated sites, engineering based approaches are used to remediate hazardous contaminants. Engineering based remediation largely focuses only on either removing or destroying either the contaminants or the soil that contains them. The most common of these practices is excavation or removal of contaminated soil for off site burial or disposal. Excavation and re-burial of contaminated soil can be extremely expensive and labor intensive with costs usually in the range of $270 to $460 per ton (Deuren et al., 2002; EPA, 2001). Disturbed soils are subject to wind or rain erosion, at least temporarily increasing exposure risk during excavation activities. Excavation may be coupled to a secondary treatment such as soil washing, chemical or physical stabilization, biological treatment or soil incineration followed by offsite disposal or onsite reburial, each step with additional costs.

An alternative to excavation and removal of contaminated soils is *in-situ* soil treatment, which is contaminant stabilization or decomposition on site. There are a wide variety of *in-situ* soil treatment technologies including chemical solidification, vitrification, air sparging, electrokinetic migration, soil flushing, bioremediation as well as other emerging technologies. Chemical solidification immobilizes contaminants by addition of various types of chemicals such as asphalt, concrete and silicate based additives. However, sequestered contaminants may be released as they weather over time

so long-term assessments of stability must be performed (Sellers, 1999). *In-situ* vitrification is similar to chemical stabilization except that electrical energy is applied to melt soil into glass-like blocks, which may retain contaminants for longer periods of time with reduced leaching risk relative to chemical solidification. Air sparging utilizes forced air to remove volatile soil contaminants from the soil for atmospheric dispersal. Many solvents such as TCE and light petroleum compounds may be treated by air sparging, though this practice may be restricted by local or national regulations. Electrokinetics uses electrical energy to mobilize contaminant compounds through the soil matrix for concentration and removal. Electrokinetic effectiveness is influenced by soil moisture and the presence of other conductive soil constituents (Saichek and Reddy, 2005).

Biotic Soil Pollutant Interactions

In addition to abiotic interactions, biological processes are important determinants of environmental persistence of organic contaminants. Bioremediation, the use of living organisms to remove, destroy or detoxify contaminants, is occasionally used alongside standard engineering practices or as a site treatment. The implementation of biological process for pollutant remediation could technically be called bioremediation, but the term is most often used specifically to describe the action of microorganisms. In some cases, bacteria or fungi may utilize organic pollutants as metabolites and completely mineralize these contaminants to CO_2, water, chloride, or nitrate, depending on the compound. Organic molecules may also be transformed to other compounds, some of which may still be environmentally damaging, such as bacterial transformation of trichloroethylene to

13

vinyl chloride by anaerobic dechlorination (Enzien et al., 1994; Freedman and Gossett, 1989).

Organic pollutant biometabolism

Biotic transformation influences incorporation of organic contaminants or metabolic byproducts into humic materials. Bacterial biotransformation of organic compounds occurs either by direct utilization of the pollutant as a growth substrate or via non-specific degradation due to broad specificity of some bacterial enzymes. This latter mechanism is known as co-metabolism, which is the primary process for degradation of many chlorinated compounds, e.g. PCBs. Bacterial cells synthesize dioxygenase and other enzymes directed towards carbon substrates capable of serving as a carbon source. For most PCB degraders PCBs cannot serve as a primary carbon source, and biphenyl or other phenolic compounds fill the role instead. In PCB co-metabolism, the same enzymatic pathway induced by biphenyl also transforms moderately chlorinated PCBs to utilizable products (Brenner et al., 1994).

Polyaromatic hydrocarbons (PAHs) are composed of two or more fused benzene or furan rings. PAHs with less than four rings are termed light PAHs and are potential targets for direct bacterial degradation and assimilation. Light PAHs are typically degraded by dioxygenase enzymes, which catalyze cleavage of the aromatic rings leading to production of ATP and carbon assimilation. Simpler hydrocarbons such as benzene, toluene, ethylbenzene and xylene (BTEX) are common soil and groundwater contaminants and more easily degraded than PAHs. Aerobic or anaerobic BTEX

biodegradation allows rapid contaminant removal under optimal conditions (Tsao et al., 1998).

Highly chlorinated PCBs and tetrachloroethylene (PCE) are not efficiently dehalogenated under aerobic conditions (Enzien et al., 1994). Chlorinated organic compounds may be transformed under anaerobic conditions by bacteria that are capable of reductive dechlorination. In this process, PCBs or trichloroethylene are selectively dechlorinated with microbial use of chlorine as a terminal electron acceptor in place of oxygen, nitrate or other more typical electron acceptors leading to release of the chlorine atom from the molecule (Freedman and Gossett, 1989; Quensen et al., 1988). Reductively dechlorinated organics are more amenable to aerobic degradation due to removal of halogen-caused steric hindrances to dioxygenase enzyme attack.

Nitroaromatic compounds such as 2,4,6-trinitrotoluene and the heterocyclic compounds HMX and RDX can also be biologically degraded, though more typically serve as nitrogen sources rather than as carbon substrates (Esteve-Nunez et al., 2001). Bacteria transform TNT to 2,4- and 2,6-monoamino,dinitrotoluene via nitroreductase activity which converts the nitro groups to amino substituents (French et al., 1998; Labidi et al., 2001).

Fungi that are capable of biodegrading naturally recalcitrant compounds such as lignin have been applied to the degradation of anthropogenic compounds. The lignolytic white rot fungus, *Phanerochaete chrysosporium*, is capable of degrading a wide variety of organic compounds such as PAHs, PCBs and NACs (Sheremata and Hawari, 2000; Yadav and Reddy, 1993; Zheng and Obbard, 2002). Fungal attack, unlike bacterial degradation, is almost always via non-specific enzyme activities, such as by peroxidases

or laccases, which are involved in lignin metabolism degradation and usually released only under lignolytic degrading conditions (Reddy, 1993).

Limitations of Bioremediation

Though it is possible for bacteria and fungi to degrade organic compounds under laboratory conditions, most persistent organic pollutants are very slowly transformed and degraded under field conditions. Slow bacterial degradation rates are frequently linked to low water solubility of the contaminant compounds and slow desorption from soil organic matter. Contaminant desorption from the soil matrix and transfer to the aqueous phase is thought to be the primary limiting factor for biological degradation of hydrophobic contaminants (Bosma et al., 1997). Microbes utilize alternative strategies to overcome mass transfer limitations, including direct colonization on sorbed substrates or production of solubilizing compounds such as biosurfactants (Johnsen and Karlson, 2004). Direct microbial contact with contaminant molecules may be limited due to soil tortuosity, low surface to volume ratios of contaminant globules, and protozoan predation (Bouchez-Naitali et al., 1999). Microbially enhanced solubilization may be limited due to low biosurfactant substrate availability or insufficient bacterial cell density for effective levels of biosurfactant production. Consequently, a large proportion of the bacterial community may access only aqueous phase contaminant prior to degradation. Applied bioremediation is limited by these same factors, as well as poor persistence of introduced microbes, lack of sustained induction of desired biodegradative pathways, or concerns over containment of improved genetically engineered organisms (GEMs) (Giddings, 1998).

<u>Phytoremediation</u>

Phytoremediation is similar to bioremediation, though is focused on the use of plants to remove or detoxify environmental contaminants. Phytoremediation processes include volatilization of contaminants from leaves (phytovolatilization), direct plant decomposition of organic contaminants (phytodegradation) and plant sequestration and concentration of contaminants in the above ground parts (phytoaccumulation). Organic contaminants may be directly metabolized by plant processes, though plant-enhanced biodegradation typically occurs via enhanced bacterial enzymatic activity in the root zone, subsequently termed phytostimulation or rhizodegradation. Plant species that naturally accumulate higher quantities of metals, such as arsenic, nickel, zinc, cadmium and cobalt, in their above ground parts are termed hyperaccumulators (Baker and Brooks, 1989). Plants like the zinc hyperaccumulator *Thlaspi caerulescens* are able to concentrate toxic metals in their tissues above 1% of dry tissue mass, which is many times higher than that found in bulk soil (Brown et al., 1994; Nedelkoska and Doran, 2001; Salido et al., 2003). Hyperaccumulators have the potential to remediate metal contaminated sites by concentrating the disbursed metals in a small quantity of tissue, allowing for easy harvest and removal rather than excavation of the entire volume of contaminated soil (Baker et al., 1994). For economically valuable elements such as nickel, phytoaccumulation also has the potential to provide revenue to defer cleanup costs (Li et al., 2003).

Phytovolatilization is a process in which volatile metals and organic compounds are removed from soil by water uptake followed by evapotranspiration from shoot

tissues. Hybrid poplar tree phytoremediation of hydrocarbon contaminated groundwater is a combination of phytostimulation, phytovolatilization, and phytodegradation processes (Widdowson et al., 2005). Like bioremediation, plant-based cleanup technologies are largely experimental and are limited by environmental and physicochemical factors, though they possess certain ancillary advantages over microbial treatments, including self-sustenance through photosynthesis and containment of contaminated media by stabilization against erosion.

Bioengineered Rhizosphere Phytoremediation

Crop bioengineering has become a standard technique in crop improvement for agricultural purposes with 56% percent of soybean and 28% of cotton global crop acreage genetically modified (James, 2004). However the application of biotechnological approaches to phytoremediation is still largely in the experimental stages. The most advanced of these approaches is mercury phytovolatilization, which is undergoing field testing in several states (APHIS release #05-045-01). These plants express *mer* bacterial genes for detoxification of bioaccumulative methylmercury and phytovolatilization of less toxic elemental mercury (Rugh et al., 1998; Rugh et al., 1998). Recent advancements in selenium phytoremediation have allowed the genetic enhancement of the innate ability of Indian mustard to accumulate selenium (LeDuc et al., 2004). In another study, plant arsenic accumulation was improved via RNAi silencing of the plant arsenic reductase gene, which normally converts arsenic to an insoluble, immobile form. Without the function of this gene in roots, the plants transported and sequestered soluble arsenic in shoot tissues (Dhankher et al., 2006).

Transgenic phytoremediation of organic pollutants has also been achieved in numerous laboratories. Engineered plants have effective expressed genes for degradation and detoxification of TNT and HMX nitroaromatics (French et al., 1998; Rylott et al., 2006). Mammalian cytochrome P450 monooxygenases has been utilized to enhance transgenic plant degradation of a wide range of organic contaminants (Kawahigashi et al., 2002).

Despite recognized successes in genetically engineered phytoremediation, this approach may present difficulties. Foreign genes may fail to be expressed in transgenic plants due to GC bias or atypical codon usage (Slimko and Lester, 2003), resulting in silenced or low expressing genes (Haseloff et al., 1997; Rugh et al., 1996). *Bacillus* gene sequences are typically A/T rich, which may contain pseudo mRNA splice sites that are recognized by the host transcriptional systems leading to transcript instability and potential silencing. Monocot plants have a considerably higher GC codon bias than dicotyledous species (Kawabe and Miyashita, 2003). In one study, no expression was detected of an unmodified bacterial β-glucanase expressed in barley, but a higher GC, codon-optimized gene was expressed successfully (Jensen et al., 1996). High GC coding regions often contain an abundance of CpG motifs, which are targets for eukaryotic DNA methylases in some hosts (Ingelbrecht et al., 1994; Vanyushin and Kirnos, 1988). Early attempts to express an unmodified bacterial Tn21 transposon *merA* gene in *Arabidopis* were unsuccessful, though gene sequence modification to reduce GC abundance utilizing more common dicot codons allowed transgene expression (Rugh et al., 1996). A highly GC-biased chlorocatechol degradation gene from *Ralstonia eutropha* NH9 was inserted into tobacco BY2 cells without detectable expression, though the GC-rich rice genome

supported expression of the transgene (Shimizu et al., 2002). Chromosomal insertion position may also strongly influence transgene expression, with genes inserted into heterochromatic regions resulting in low expression (Matzke and Matzke, 1998).

Limitations of phytoremediation

Basic limitations on phytoremediation include long treatment time and requirement for agronomically suitable site conditions. Most phytoremediation research on organic compounds has focused on uptake and sequestration or degradation of water-soluble compounds. However, for some strongly non-polar contaminants such as PCBs and PAHs, very small quantities of these compounds will be available to plant tissues for direct degradation (Shrout et al., 2006). One approach to dealing with extremely hydrophobic pollutants using plants is indirect, using root-produced molecules to stimulate available microbes which can more readily access hydrophobic contaminants due to higher surface to volume ratios and potential for direct contact with pollutant globules. For plants to be capable of direct degradation, more creative methods are necessary to increase the bioavailability of hydrophobic compounds. One method of increasing apparent solubility of strongly hydrophobic contaminants is the addition of surfactants.

Surfactants are amphiphilic compounds, which possess both hydrophilic and hydrophobic domains within a single molecule. At low concentrations, surfactants congregate at the aqueous, non-aqueous interfaces, reducing surface tension in immiscible liquid systems. As the concentration of surfactant in an aqueous system is increased, the surface tension will continue to decrease up to a certain point when it will

no longer decrease, which is known as the critical micelle concentration (CMC). Once the CMC is reached, surfactant molecules spontaneously form spherical vesicles called micelles, which are capable of assimilating hydrophobic compounds and increasing their apparent water solubility and bioavailability. Surfactants have been widely used in bioremediation treatment strategies to enhance *in situ* degradation rates of organic compounds or in *ex situ* operations for soil washing procedures. However, due to their solubilizing effect, surfactants have the potential to cause contaminant leaching when used *in situ*. Chemical surfactants may result in bacterial or plant toxicity when used at chemically effective concentrations or become unintended contaminants due to their environmental persistence in soils (Rouse et al., 1995). In some situations, synthetic surfactants may inhibit microbial biodegradation even while enhancing contaminant desorption (Laha and Luthy, 1991) by toxicity to bacterial cells (Volkering et al., 1997) or inaccessibility of entrapped contaminants (Makkar and Rockne, 2003).

Biologically-synthesized surfactants or biosurfactants are considered more environmentally benign than synthetic surfactants due to shorter half-life, lower toxicity, and higher biodegradability. Biosurfactants are grouped in several major classes, glycolipids, lipoproteins, phospholipids, and polymeric biosurfactants. Bacterial biosurfactant production has been proposed as a biological mechanism for transport of less water-soluble compounds, to promote enhanced cell-matrix adhesion, or to function as defense compounds (Maier, 2003; Neu, 1996). Glycolipids are sugar-lipid containing molecules with a well-studied example being rhamnolipids. Rhamnolipids were shown to enhance removal of PAHs in soil washing treatments (Noordman et al., 1998). Rhamnolipids were observed to be similar in desorption effectiveness to the chemical

21

surfactant Triton-X across a wide range of compounds in chronically contaminated soil (Berselli et al., 2004).

Cyclodextrins (CDs) are unique biosurfactant-like molecules unlike most surfactant chemicals. Cyclodextrins are not surface active (do not reduce surface tension) and therefore are not true surfactants. Due to their mono-molecular activity, CDs have no CMC requirement and are capable of contaminant solubilization activity at any concentration. CDs are composed of α-1,4 linked glucose units with the primary hydroxyl groups directed towards the interior of the torus-shaped molecule and secondary hydroxyls directed towards the exterior (Szejtli, 1988). The exterior hydroxyls form the hydrophilic portion of the molecule while the interior portion remains relatively hydrophobic. Hydrophobic compounds can become included into the center cavity of the CD and are called "guest" molecules. CD is typically formed from 6, 7, or 8 glucose units; comprising α, β and γCD respectively. The differing number of glucose units results in a different cavity size, giving each CD a slightly different range of contaminants to solubilize. Cyclodextrin-producing bacteria synthesize mixtures of CDs usually with one or two different cyclodextrin types predominating depending on the specificity of the bacterial cyclodextrin glycosyltransferase (CGTase)(Qi and Zimmermann, 2005). CDs generally cannot solubilize hydrophobic compounds larger than the interior CD cavity but portions of larger molecules may become encapsulated with complex ratios of 1:1, 2:1 or occasionally 3:1 of cyclodextrin to guest molecule. CDs are thought to function in one of two ways, one in which the CD functions as an inert agent for simple solubilization, with another being the specific cellular import and cytoplasmic degradation of intact CD-complexes (Pajatsch et al., 1998). Some microbes

such as *Klebsiella oxytoca* are capable of both producing and utilizing CD as a sole carbon source (Fiedler et al., 1996).

Cyclodextrins have been used for enhancement of environmental remediation, but chemically modified cyclodextrins are frequently utilized, especially modified βCD. This is due to the fact that βCD has relatively low water solubility, which is due to the fact that the hydroxyl groups of βCD tend to hydrogen bond with one another rather than with the surrounding solvent (Szejtli, 1988). To increase the water solubility of βCD, hydrophobic or hydrophilic groups are chemically added to the surface of the molecule to break up the intramolecular hydrogen bonding. Given the nearly unlimited water solubility of most CD derivatives, they are often preferred for exogenous applications. Modified CDs may actually have lower solubilization power than their natural counterparts at lower concentrations, likely due to steric hindrance from functional groups attached to the CD ring (Gao et al., 1998). Since chemical modification of cyclodextrins is unfeasible for *in-vivo* CD production, works concerning modified cyclodextrins must be viewed with recognition that chemically modified CDs could only be added exogenously rather than solely biologically produced. However the solubilization properties of modified CDs may still be indicative of the potential of the parent natural CDs. Modified CDs are considerably more persistent and resistant to degradation than natural CDs; randomly methylated βCD (RAMEB) was found to be fully resistant to biodegradation (Fenyvesi et al., 2005). Low degradation rates may lead to potential problems with persistence of the modified CD compounds similar to those caused by synthetic surfactants, including contaminant leaching or increased toxicity to receptor organisms.

Cyclodextrins are commonly used to increase desorption of various soil contaminants in biodegradation or soil washing treatments. Modified hydroxypropyl βCD (HPCD) was used to enhance the transport of: anthracene, pyrene and a PCB congener (Brusseau et al., 1994). HPCD was found to be very effective in part due to lack of sorption to soil, which has been observed to occur with most true surfactants. HPCD and βCD were both demonstrated to significantly enhance the apparent aqueous solubility of the low molecular weight PAHs naphthalene and phenanthrene (Badr et al., 2004). βCD enhanced biodegradation in liquid cultures spiked with PAHs (naphthalene and anthracene) and linear hydrocarbons (tetracosane and dodecane) (Bardi et al., 2000). CDs were also observed to reduce *Pseudomonas putida* growth inhibition and toxicity by toluene and toluic acid, while increasing biodegradation rates (Schwartz and Bar, 1995). HPCD has been shown to enhance phenanthrene degradation in liquid cultures with 97% of the compound removed in 48 hours in comparison to 54.8% removal in treatments without HPCD (Wang et al., 1998). γCD and HPCD were shown to significantly enhance biodegradation of individual PCB congeners when compared to control treatments lacking cyclodextrin under both slurry and fixed phase column soil bioreactor conditions (Fava et al., 1998). Under greenhouse conditions, several planted systems amended with βCD showed a significant reduction of initial soil PAH levels, ostensibly via enhanced rhizodegradation (Settavongsin, 2005). HPCD enhanced removal of a wide range of compounds, including BTEX and TCE, in field aquifer conditions (McCray and Brusseau, 1998).

Summary & Conclusion

Environmental contamination of soils is a continuing and pervasive problem in many areas of the world. Organic pollutant compounds cause environmental harm even at low levels due to their persistence and bioaccumulation through food webs. These compounds may be mineralized, transformed into their basic constituents of carbon dioxide and water. However transformation of organic compounds is limited by their low solubility in water and resulting unavailability for rapid degradation. Engineering-based approaches to site decontamination, while often quick and effective, may not represent the most cost efficient and environmentally compatible treatment for organic pollutants. Phytoremediation has been proposed as an environmentally friendly alternative to engineering based approaches. Plants are considered to be more environmentally friendly and capable of enhancing the removal of a wide range of compounds. Genetic engineering has also been used to generate plants with improved capabilities for environmental restoration, including genes for biodegradation and contaminant concentration. Despite genetic improvements phytoremediation is still constrained by the physical properties of organic contaminants and soil. Organic compounds tend to partition to the humic materials within soil, protecting them from biological decomposition.

Surfactants are compounds, which are capable of solubilizing organic contaminants, bringing them into the aqueous phase. Surfactants have been used to improve biological degradation of organic compounds, but may be expensive and labor intensive to add to a site. Surfactants may also cause bacterial toxicity and contaminant leaching. Cyclodextrins are biologically synthesized compounds capable of overcoming

the limitations of low water solubility and bioavailability in a similar fashion to surfactants. Instead of relying either on exogenously added cyclodextrins or bacterial synthesis, we propose a more manageable and consistent solution is the plant production of extracellular CGTase coupled with exogenous addition of starch.

In this research project we cloned a novel *cgt* gene was cloned from a soil isolated bacterium. This gene was then expressed in 3 different species, *Esherichia coli*, *Arabidopsis thaliana* and *Nicotiana tabacum*. Functional CGTase was detected in at least one line or clone of all three species. Several plant lines were tested for improvements to degradation of PAHs and PCBs. The transgenic lines examined in this work are a first step towards field implementation of *in-situ* plant-based production of CDs. Contaminated soils containing CDs may exhibit enhanced biological degradation due to both plants and bacteria. Plant produced CD may be more controlled than either direct CD addition or bacterial production due to location within the rhizosphere and direct observation of plant growth. *Cgt*-plants provide potential for acceleration of *in-situ* biological degradation of organic contaminants.

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CHAPTER I

Cloning and Characterization of the Cyclodextrin Glycosyltransferase
from *Paenibacillus sp.* strain C36

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INTRODUCTION

Cyclodextrins

Cyclodextrins (CDs) are versatile surfactant-like molecules used for commercial and analytical purposes including pharmaceutical and pesticide chemical production, plant growth regulator enhancers, chiral chromatographic separation, and many other applications (Apostolo et al., 2001; Gines et al., 1996; Greenberg-Ofrath et al., 1993; Kamiya and Nakamura, 1995; Kilsdonk et al., 1995; Nunez-Delicado et al., 1997; Uekama et al., 1998). CDs are cyclic sugars composed of six, seven, or eight α-1,4 linked glucose units (αCD, βCD and γCD, respectively) produced by bacterial starch degradation. CDs are doughnut-shaped with a hydrophilic exterior and a hydrophobic cavity, which can accommodate appropriately sized hydrophobic "guest" molecules in ratios of 1:1, 2:1, or more rarely, 3:1 CD:guest molecule (Shen et al., 1998). Complexation with CDs has the effect of solubilization, stabilization, or sometimes precipitation of the included compound (Szejtli, 1988). CDs have been found to effectively in enhance dissolution and biodegradation of soil-sorbed organic contaminants (Badr et al., 2004; Bardi et al., 2000; Molnar et al., 2005; Sheremata and Hawari, 2000; Wang et al., 2005).

CGTases

Cyclodextrins are produced from starch by the action of a bacterial enzyme, cyclodextrin glycosyltransferase (CGTase), which is thought to be functionally and phylogenetically linked to α-amylase. Both enzymes act on starch, with CGTase forming cyclic products and α-amylases forming linear products (delRio et al., 1997). The typical reaction of CGTase, cyclization, is the covalent linkage of the non-reducing end of the sugar and another glucose unit of the same oligosaccharide, forming cyclic products (Uitdehaag et al., 1999). CGTases perform other reactions such as disproportionation, coupling, and hydrolysis. The coupling reaction is the reverse of the cyclization reaction. In the hydrolysis reaction a linear oligosaccharide is broken down into smaller linear fragments.

CGTases are used industrially for the production of cyclodextrins and for glycosylation of various sugars and other compounds (Starnes, 1990). CGTases are 60-75 kDa extracellular enzymes secreted into the environment via the action of a diverse array of transit peptides (Schmid, 1989). CGTases have been found in a wide variety of microbes, primarily *Bacillus* and related species but also, *Klebsiella*, *Brevibacillus*, and *Thermoanaerobacter* (Binder et al., 1986; Wind et al., 1995). Full genome sequencing of several microbes has revealed putative *cgt* genes in *Xanthomonas* and *Streptococcus* strains (da Silva et al., 2002; Ferretti et al., 2001).

CGTases contain five recognized domains, most of which are shared with α-amylases. Domain A contains a calcium-binding domain, which comprises the active site of the enzyme. The B and C domains provide stability for the active site during substrate binding, while the function of the D domain remains unknown and is generally limited to

CGTases and not shared with α-amylases (Qi and Zimmermann, 2005). Domain E is found in α-amylases from diverse sources and is thought to function in binding raw starch (Janecek et al., 2003). CGTases are relatively similar across bacterial species with at least 51 completely conserved amino acid residues located primarily within the active site, which contains a $(\beta/\alpha)_8$ barrel tertiary structure (Qi and Zimmermann, 2005). Individual CGTases tend to exhibit differing αCD, βCD or γCD biosynthetic specificities, which may also be influenced by reaction conditions (Szejtli, 1988). Amino acid sequences are generally conserved among βCGTase and αCGTase proteins, both of which are more divergent from the less conserved γCGTases. For example, γCGTases usually contain a deletion of six amino acids at the beginning of the B region, which may serve as a hinge, to open the active site allowing formation of the larger (8 glucose unit) cyclic product (Qi and Zimmermann, 2005).

The objective of this study is to examine the sequence and enzymatic function of a novel CGTase, PI-Cgt, in the original host strain, *Paenibacillus sp.* C36, and as expressed in *Escherichia coli* strain DH5α.

MATERIALS AND METHODS

Gene cloning and modification

Cyclodextrin producing bacteria were isolated from field soil and the *cgt* gene cloned via PCR from genomic DNA isolated from *Paenibacillus sp.* (formerly classified within *Bacillus)* strain C36 (Settavongsin, 2005). To make the *cgt* gene (*PI-cgt*) more amenable to *in vitro* manipulation and allow cloning into the vector pBluescript SK⁻ the

recognition sites for *NotI* and *EcoRI* restriction enzymes (Stratagene, La Jolla, CA) were

introduced into the 5' UTR of the *PI-cgt* using the forward primer:

PIcgt-F1: 5'<u>GAA TTC GGC GGC CCG</u> TTA AAG AGG ATT AAC AAT GTT AAT

GG. The recognition sites of the restriction enzymes *SacI* and *BamHI* were introduced

into the 3' UTR of *PI-cgt* using the reverse primer: PIcgt-R1: 5'CTG TAC <u>GGA TCC</u>

<u>GAG CTC</u> ATT AAG GCT GCC AGT T. These changes allow for the expression of

pBS-*PI-cgt* in *E. coli* DH5α. Primer sections containing engineered restriction sites are

underlined, with remaining DNA sequence of primer PIcgt-R1 complementary to *PI-cgt*.

Primer F1 contains other modifications including an in-frame stop codon in reference to

the pBS LacZ fragment and a new ribosomal binding sequence to replace the original

which was removed by restriction site addition. The in-frame stop codon causes

translation of the LacZ fragment to terminate while the ribosomal binding site attracts the

ribosome to the *cgt* initiation codon, resulting in the translation of PI-cgt.

PCR for cloning and modification were performed in 40μl volume reactions

containing, 0.1μl template, 4μl 10XPCR Buffer, 4μl 25mM MgCl₂, 2μl of 10pm forward

and reverse primers 0.8μl of 100mM dNTPs, 1.6μl BSA (10mg/ml) and 0.4μl of

Amplitaq (Invitrogen, Carlsbad, CA). The PCR conditions were as follows: A primary

denaturation step, 96°C for 3 minutes followed by 40 cycles of 96°C 30seconds, 50°C

annealing temperature for 30 seconds and a 72°C extension temperature for 45 seconds.

The PCR products were first checked for correct size and amplification by agarose gel

electrophoresis. DNA preparations displaying positive PCR reactions were then cloned

directly into pCR2.1 using the TOPO TA cloning kit (Invitrogen, Carlsbad, California)

used according to manufacturer specifications. TA-cloned *cgt* was transformed into *E.*

coli DH5α and were plated on selective LB plates (10g/L NaCl, 5g/L yeast extract, 10g/L

Bacto tryptone, 15g/L Bacto agar; Beckton Dickinson, Sparks, MD) containing 100mg/L

kanamycin. Positive colonies were picked, grown overnight (16hrs) in 5mls LB with

100mg/L kanamycin or 50mg/L ampicillin. DNA was extracted using a truncated

standard alkaline lysis procedure (Maniatis et al., 1982). Cells were spun down at 10,000

X g for 1 minute using 1.5ml micro tubes and 1.5ml of culture. Supernatant was removed

by pouring and the tubes were blotted dry using a paper towel. Cells were then

resuspended by vortexing in 100μl of (50mM glucose, 25mM Tris-HCl, 10mM EDTA)

(pH 8.0). Cells were lysed by 200μl of (0.2% sodium dodecyl sulfate and 0.2M NaOH)

and repeated inversions of the tubes. The resulting mixture was neutralized by 150ml

(60mL 5M potassium acetate, 11.5mL acetic acid, 28.5mL H_2O), mixed well and spun

down for 2 min at 10,000 X g. To precipitate DNA, 200μl of the supernatant was added

to 1ml of 98% ethanol and mixed well. The solution was spun down at 10,000 x g for 5

minutes. Ethanol was carefully decanted, the tubes blotted dry, and tubes were allowed to

stand to air-dry. Once pellets were mostly dry they were resuspended in 27ml of Tris-

EDTA containing approximately 150μg/ml RNase. The resulting plasmid DNA solutions

were screened via restriction enzyme digestion using *EcoRI* (Invitrogen, Carlsbad, CA)

performed according to manufacturer's instructions followed by agarose gel

electrophoresis on a 1% gel in Tris-acetate EDTA at 100 V for 1 hour. Agarose gels were

stained after running with 0.8mM ethidium bromide and visualized on a Bio-Rad

Quantity One Gel Documentation system (Bio-Rad, Hercules, CA).

Positive clones were grown up for 16hrs in LB with selection at 37°C and

extracted using the Wizard DNA minprep kit. DNA solutions were subjected to micro-

dialysis prior to sequence submission, which consisted of placement of the DNA solution

on a13mm, 0.025um pore size nitrocellulose filter (Millipore, Billerica, MA) floating on

sterile water for 5 minutes. Afterwards the DNA solution was placed in a new Eppendorf

tube, and sequenced using Applied Biosystems (Foster City, CA) sequencing technology

in one direction as a primary screen for mutations and was performed by the Michigan

State Research Technology Support Facility (MSU, East Lansing, MI).

A single mutation free, completely sequenced clone was chosen for subsequent

subcloning into pBS using *EcoRI* and *BamHI*. pBS and pCR2.1 containing PI-cgt were

digested using *EcoRI* and *BamHI* enzymes. Both reactions were run on 1% Agarose gels.

The 2100bp PI-cgt gene fragment was excised from the gel using a scalpel and purified

using the QIAquick gel extraction kit according to manufacturer's instructions (Quiagen,

La Jolla, CA). Only a portion of the pBS reaction was run to check for complete

digestion. The remainder of the reaction was heat inactivated at 65°C for 20 minutes. The

purified PI-cgt fragment was combined with the digested pBS in a 3 to 1 molecular ratio

along with DNAligase and fresh ligation reaction buffer as per manufacturers

instructions. Ligations were allowed to proceed overnight and were heat inactivated the

following day at 65°C for 20 minutes. Ligations were digested by an enzyme found

within the polylinker segment of pBS, but not within PI-cgt, to cut self-ligated plasmids.

Linearized plasmids will not be replicated in bacterial cells – biasing the transformation

recovery to cells containing the desired insert. Bacterial transformation was carried out

using *E. coli* DH5α cells prepared to be chemically competent utilizing the following

method. A single colony was used to inoculate an overnight 5mL culture of LB, which

was used to inoculate 1L of LB medium split between 4 1L flasks incubated at 37°C at

200rpm. After the optical density of the cultures reached approximately 0.3 they were spun down for 8 minutes, 10,000 x g at 4°C and resuspended in 0.1M CaCl$_2$ twice. After the second resuspension the cells were held overnight on ice. The next day cells were diluted with 80% glycerol, aliquoted into 1.5ml micro-tubes and flash frozen using liquid nitrogen.

For transformation, DH5α competent cells were thawed and held on ice. The entire ligation was added to 200μl of cells. Cells were heat shocked by placement in a 42°C water bath for 2 minutes followed by 2 minutes on ice. After heat shock 500ml of SOC medium (20g Bacto tryptone, 5g Bacto yeast extract, 2ml of 5M NaCl, 2.5ml of 1M KCl, 10ml of 1M MgCl$_2$, 10ml of 1M MgSO$_4$, 20ml of 1M glucose in 1L) was added and the transformation reaction was incubated at 37°C, 200rpm for 30 minutes.

Transformation reactions, one plate with 10 μl the other with 100μl were plated on solid LB ampicillin plates spread with 50μl X-gal (20mg/ml in dimethyl formamide) to allow for blue-white screening of potential clones. Those clones that were white in color due were more likely to contain an insert due to disruption of the β–galactosidase fragment. Positive clones were screened via restriction analysis using *EcoRI* and *BamHI* as described above. A single positive clone was selected for further experiments.

Sequence Analyses

Alignments, phylograms and bootstrap values were analyzed with Clustal W version 1.83 (Thompson et al., 1994). Trees were drawn by Phylodraw version 0.8 (Graphics Application lab, Pusan National University, South Korea) with the neighbor-

joining method using the Clustal W output. Detection of signal peptide and cleavage site was performed using SignalP analysis software (Bendtsen et al., 2004).

Starch Clearing Analysis

For screening of CGTase producing strains for starch degradation, visual starch clearing was utilized as a diagnostic screen. Bacterial colonies were grown on solid Basic medium containing 1% soluble starch, 0.5%yeast extract, 0.5% tryptone, 0.1%K_2HPO_4, 0.02%$MgSO_4$ * $7H_2O$, 0.02%$CaCl_2$ * $2H_2O$, 1%$(NH_4)_2SO_4$, pH to 7.0 and 15g of Bacto Agar per liter. Media was sterilized prior to use, poured into 100mm X 15mm disposable plates (Fisher Scientific, Hampton, NH) and allowed to solidify overnight. Bacterial colonies were touched to plates and allowed to incubate at their respective temperature optima, 28-30°C for *P. sp.* C36, 37°C for *E. coli* for 16 hr and *P. sp* C36 for 32 hr. After growth was completed, plates were stained with a 1:30 aqueous dilution of an iodine solution consisting of 10% potassium iodide 1% iodine and 50% ethanol with water. Starch forms a deep blue colored complex with iodine. Cyclodextrin fails to form a colored complex with the iodine dye causing areas of starch degradation and CD production appear as colorless, clear zones on agar plates.

Colorimetric Assay of β-CD

A colorimetric dye assay was used to quantify βCD production (Kaneko et al., 1987), modified for use in a microplate. Crude enzyme extract was obtained from overnight cultures of *E. coli* and *P. sp.* C36 respectively, via centrifugation for 1 minute at 10,000xG and filter sterilized to exclude any remaining cells. 50µl of enzyme extract

was added to 200µl of 1.25% starch in 1mM phosphate buffer in 1.5ml microtubes. After incubation of up to 24 hrs for *P. sp* C36 and *E. coli*-PI-cgt, 50µl of the enzymatic reaction was removed and placed in a 96 well plate. 25µl of 0.4mM phenolphthalein and 20µl of 1M $NaCO_4$ were added to the enzyme/starch mixture. CD forms a complex with the colored phenolphthalein dye causing color reduction. The amount of color reduction is proportional to the quantity of CD in solution, with the relationship being a logarithmic reduction in absorbance. Absorbance was measured at 550nm with a Spectra Max 190 (Molecular Devices, Sunnyvale, CA) microplate reader after 2 minutes of incubation accompanied by light shaking. Samples were tested alongside a standard curve done in triplicate, measuring from 15µg/ml to 1000µg/ml.

The speed and rate of βCD production by both C36 and pBS PI-cgt were measured. An enzymatic digest was setup as previously described with subsamples being analyzed for βCD content via the colorimetric method. Three replicates of each enzyme source were included, with incubation performed at 50°C. Subsamples were analyzed at zero, one, two, and three hours after incubation start.

Thin Layer Chromatography

Thin Layer Chromatography (TLC) was performed to separate and identify the different CDs. The same *in-vitro* enzymatic reactions as those used for the colorimetric assays, were spotted in 2µl aliquots, 3 times onto the base of a 10cm X 20cm silica gel 60/Kieselguhr $_{F254}$ aluminum TLC sheet (EM Science, Gibbstown, NJ). α, β, and γCDs (Sigma, St. Lois, MO) were used as standards in 1% aqueous solutions and spotted to the same sheet as the enzymatic reactions. The mobile phase was acetonitrile-water-

ammonium hydroxide (6:3:1) with plates being run in a sealed glass TLC tank.

Completed TLC plates were sprayed with Vaugh's solution (1g $Ce(SO_4)_2$, 24g

$(NH_4)_2MoO_4$, 50ml concentrated H_2SO_4 and 450ml H_2O) using a TLC sprayer and

developed by heating on a hot plate until blue spots appeared. Blue spots were compared

to spots generated in lanes containing the standards, if the migration distances were

similar to that of the CD standards, it was deemed a positive result.

RESULTS

Sequence comparisons

Since PI-cgt was not shown to be identical in amino acid sequence to any other

CGTase sequence in the Genbank database, sequence comparisons to known CGTases

were undertaken. A Blast search performed utilizing the entire amino acid sequence of

PI-cgt found the most similar protein to be a CGTase from *Bacillus lichenformis*

(Genbank accession # CAA33763) at 86% identity and 89% similarity using the Blosum

62 amino acid substitution matrix (Henikoff, 1992). Using the same methods, PI-cgt was

73% identical and 78% similar to the well-characterized CGTase of *Paenibacillus*

illinoisensis strain 251. A phylogenetic tree of several bacterial CGTases (archaeal

CGTases were excluded) was created using Clustal W and Phylodraw using the neighbor

joining method (Figure 1.1, Figure 1.2). This method uses the assumption that in any set

of data the quantity of evolution between the various enzymes should be minimized, it is

generally assumed to frequently produce a tree that is very close to the true tree.
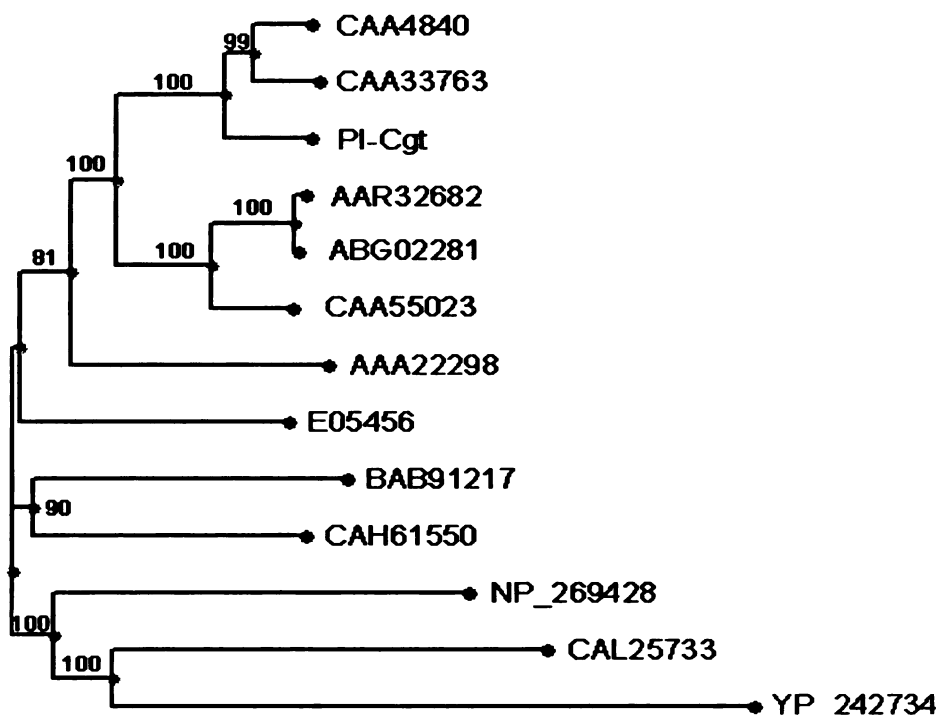
Figure 1. Evolutionary phylogram comparison of Cgts to PI-Cgt. Branch lengths indicate evolutionary distance. Bootstrap values are indicated on the branches.

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CAA48401      --------------------------MFQMAKRAFLSTTLTLGLLAGSALPFLPA
CAA33763      --------------------------MFQMAKRVLLSTTLTFSLLAGSALPFLPA
PI-Cgt        --------------------------MFKWTKRIILSTTLSFSLLAGSALPLFPA
AAR32682      ----------------------------MKRFMKLTAVWTLWLSLTLGLL--
ABG02281      ----------------------------MKRFMKLTAVWTLWLSLTLGLL--
CAA55023      ----------------------------MKKFLKSTAALALGLSLTFGLF--
AAA22298      ----------------------------MKSRYKRLTSLALSLSMALGIS--
E05456        ----------------------------MRRWLSLVLSMSFVFSAIFIVSDT
BAB91217      ------------------------VFRKLLCTLVTIITLSAWIVSHGGE
CAH61550      ------------------MINKKNSIGKAICICLSILLLFGVLSIFQPV
NP_269428     ----------------------------MRELHIKTYKLLTKSAVLLGLISF
CAL25733      ------------------------MTMNRFMKKLFSMFLALALIVGYTAA
YP_242734     MAGRATDLRAGDRRLEPDRGRCVRGAGPKRPGRAMMRSVLMAAMLLYSGA
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CAA48401      SAVYADP------DTAVTNKQSFSTDVIYQVFTDRFLDGNPSNNPTGA--
CAA33763      SAIYADA------DTAVTNKQNFSTDVIYQVFTDRFLDGNPSNNPTGA--
PI-Cgt        ASVFADA------DTAVSNKQNFSTDVIYQVFTDRFLDGNPSNNPTGG--
AAR32682      SPVHAAP------DTSVSNKQNFSTDVIYQIFTDRFSDGNPANNPTGA--
ABG02281      SPVHAAP------DTSVSNKQNFSTDVIYQIFTDRFSDGNPANNPTGA--
CAA55023      SPAQAAP------DTSVSNKQNFSTDVIYQIFTDRFSDGNPANNPTGA--
AAA22298      LPAWASP------DTSVDNKVNFSTDVIYQIVTDRFADGDRTNNPAGD--
E05456        QKVTVEA------AGNLN-KVNFTSDVVYQIVVDRFVDGNTSNNPSGA--
BAB91217      VHASN--------ATNDLSNVNYAEEVIYHIVTDRFKDGDPDNNPQGQ--
CAH61550      TNATQNSLEHIKEHTSVNNQVNYATDVIYQIVTDRFLDGDKYNNPTCEN-
NP_269428     PLTVSAAD-----NASVTNKADFSTDTIYQIVTDRFNDGNTSNNGKTD--
CAL25733      YPLPAVAA-----ASGQSLGPVTSKDVIYQILTDRFYDGDHANNIPPGTP
YP_242734     ACAAPAP------GDYYGTLEPFAADAVYFVVTDRFVNGDTGNDHRDQGG
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CAA48401      ---------AYDATCSNLKLYCGGDWQGLINKINDNYFSDLGVTALWISQ
CAA33763      ---------AFDGTCSNLKLYCGGDWQGLVNKINDNYFSDLGVTALWISQ
PI-cgt        ---------AYDASCSNLKLYCGGDWQGLINKINDNYFSDLGITALWISQ
AAR32682      ---------AFDGTCTNLRLYCGGDWQGIINKINDGYLTGMGVTAIWISQ
ABG02281      ---------AFDGTCTNLRLYCGGDWQGIINKINDGYLTGMGVTAIWISQ
CAA55023      ---------AFDGTCTNLRLYCGGDWQGIINKINDGYLTGMGVTAIWISQ
AAA22298      ---------AFSGDRSNLKLYCGGDWQGIIDKINDGYLTDMGITALWISP
E05456        ---------LFSSGCTNLRKYCGGDWQGLINKINDGYLTDMGVTAIWISQ
BAB91217      ---------LFSNGCSDLTKYCGGDWQGIIDEIESGYLPDMGITALWISP
CAH61550      --------LYSEDGADLRKYLGGDWRGIIQKIEDGYLPDMGISAIWISS
NP_269428     ---------VFDKN--DLKKYHGGDWQGIIAKINDGYLTDMGISAIWISS
CAL25733      PELFNDDNGDGRGDGTDLNKYQGGDWKGIQEKIP--YLKNMGITAVWISA
YP_242734     AHRSFDVPTPCDGGVGDNIGYLGGDFKGIVDHAD--YIRGLGFGAVWITP
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CAA48401      PVEN---------IFATINYSGVTNTAYHGYWARDFKKTNPYFG-TMAD
CAA33763      PVEN---------IFATINYSGVTNTAYHGYWARDFKKTNPYFG-TMTD
PI-cgt        PVEN---------IYSLINYSGVTNTAYHGYWARDFKKTNPAFG-TMTD
AAR32682      PVEN---------IYSVINYSGVHNTAYHGYWARDFKKTNPAYG-TMQD
ABG02281      PVEN---------IYSVINYSGVHNTAYHGYWARDFKKTNPAYG-TMQD
CAA55023      PVEN---------IYSIINYSGVNNTAYHGYWARDFKKTNPAYG-TIAD
AAA22298      PVEN---------IYSVNNTSYHGYWARDFKQTNDAFG-DFAD
E05456        PVEN---------VFSVMN-DASGSASYHGYWARDFKKPNPFFG-TLSD
BAB91217      PVEN---------VFDLHP---EGFSSYHGYWARDFKKTNPFFG-DFDD
CAH61550      PVEN---------IYAVHP---QFGTSYHGYWARDFKRNNPFFG-DLND
NP_269428     PVEN---------IDSIDP--SNGSAAYHGYWARDFKKTNPHFG-TMTD
CAL25733      PYEN---------RENLIAG---MYASYHGYHARNYFATNPHFG-KMQD
YP_242734     IVDNPDEAFTGGKPITCESTLSDHGKTGYHGYWGVNFYRLDEHLPSPGLD
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CAA48401      FQNLITTAHAKGIKIVIDFAPNHTSP------------AMETDTSFAEN
CAA33763      FQNLVTTAHAKGIKIIIDFAPNHTSP------------AMETDTSFAEN
PI-cgt        FQNLINTAHAKGIKVIIDFAPNHTSP------------AMETDTSFAEN
AAR32682      FKNLIDTAHAHNIKVIIDFAPNHTS------------ASSDDPSFAEN
ABG02281      FKNLIDTAHAHNIKVIIDFAPNHTS------------ASSDDPSFAEN
CAA55023      FQNLIAAAHAKNIKVIIDFAPNHTS------------ASSDQPSFAEN
AAA22298      FQNLIDTLTLITSRSDRLRPQPHVSG------------RAGTNPGFAEN
E05456        FQRLVDAAHAKGIKVIIDFAPNHTS------------ASETNPSYMEN
BAB91217      FSRLIETAHDHKIKVIIDFAPNHTS------------VDI-------EN
CAH61550      FRELIAVANEHDIKVIIDFAPNHTSP------------AEVNNPNYAED
NP_269428     FQQLVKVAHQHHIKVVIDFAPNHTST------------AEKEGTTFKED
CAL25733      FTALVDALHDNGIKVVIDFVTNHSGPRPDGDGVRXXPDRDSSGQSVFDPD
YP_242734     FAGFTRSMHANDLKVVLDIVGNHGSP------------AYSMPVAQPGF
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CAA48401      GRLYDNG------TLVGGYTNDTNGYFHHNGGSDFSSLENG--IYKNLYD
CAA33763      GKLYDNG------NLVGGYTNDTNGYFHHNGGSDFSTLENG--IYKNLYD
PI-cgt        GKLYNNG------TLLGGYTNDTNKLFHHNGGSDFSLENG--IYKNLYD
AAR32682      GRLYDNG------NLLGGYTNDTQNLFHHYGGTDFSTIENG--IYKNLYD
ABG02281      GRLYDNG------NLLGGYTNDTQNLFHHYGGTDFSTIENG--IYKNLYD
CAA55023      GRLYDNG------TLLGGYTNDTQNLFHHNGGTDFSTTENG--IYKNLYD
AAA22298      GALYDNG------SLLGAYSNDTAGLFHHNGGTDFSIEDG--IYKNLYD
E05456        GRLYDNG------TLLGGYTNDANMYFHHNGGTTFSSLEDG--IYRNLFD
BAB91217      GKLYDSG------TLLGHYSTDANNYFYHYGGSDFSDYENS--IYRNLFD
CAH61550      GNLYNNG------EFVASYSNDLNEIFYHFGGTDFSTYEDS--IYRNLFD
NP_269428     GALYKNG------KLVGKFSDDKDKIFNHESWTDFSTYENS--IYHSMYG
CAL25733      GNPIDYNGDGKAENRIADILNDTNGFFHHEGNRPDSDTSQFGYRHKELAS
YP_242734     GKLYDAQG-----RLVADHQNLAPAQLDPAHNPLHAFYNTS----GGLAE
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CAA48401     LADFNHNNATIDKYFKDAIKLWLDMGVDGIRVDAVKHMPLGWQKSWMSSI
CAA33763     LADLNHNNSTIDTYFKDAIKLWLDMGVDGIRVDAVKHMPQGWQKNWMSSI
PI-cgt       LADLNHNNSTIDTYFKDAIKLWLDMGIDGIRVDAVKHMPMGWQKNWMSSI
AAR32682     LADLNHNNSSVDVYLKDAIKMWLDLGVDGIRVDAVKHMPFGWQKSFMSTI
ABG02281     LADLNHNNSSVDVYLKDAIKMWLDLGVDGIRVDAVKHMPFGWQKSFMSTI
CAA55023     LADLNHNNSTVDVYLKDAIKMWLDLGIDGIRMDAVKHMPFGWQKSFMAAV
AAA22298     LADINHNNNAMDAYFKSAIDLWLGMGVDGIRFDAVKQYPFGWQKSFVSSI
E05456       LADLNHQNPVIDRYLKDAVKMWIDMGIDGIRMDAVKHMPFGWQKSLMDEI
BAB91217     LASLNQQHSFIDKYLKESIQLWLDTGIDGIRVDAVAHMPLGWQKAFISSV
CAH61550     LAGLNLNNNFVDQYLRDSIKFWLDLGVDGIRVDAVKHMPLGWQKSFVDTI
NP_269428    LADLNNINPKVDQYMKEAIDKWLDLGVDGIRVDAVKHMSQGWQKNWLSHI
CAL25733     LADYSQENGVVIEHLEKAGKFWKAKGIDGFRHDATLHMNPAFVKGFKDAI
YP_242734    LSDLNEDNPAVLDYLAGAYLQWMEQGADAFRIDTIGWMPDRFWHAFVARI
             *:. .  :  :  ::  :    *    * *.:*  *:       : :      :

CAA48401     YA--HKPVFTFGEW■LGS-AASDADNTDFANKSGMSLLD■RFNSAVRNVF
CAA33763     YA--HKPVFTFGEW■LGS-AAPDADNTDFANESGMSLLD■RFNSAVRNVF
PI-cgt       YG--YKPVFTFGEW■LGS-SASDADNTNFANQSGMSLLD■RFNNEVRNVF
AAR32682     NN--YKPVFTFGEW■LGV-NEISPEYHQFANESGMSLLD■RFAQKARQVF
ABG02281     NN--YKPVFTFGEW■LGV-NEISPEYHQFANESGMSLLD■RFAQKARQVF
CAA55023     NN--YKPVFTFGEW■LGV-NEVSPENHKFANESGMSLLD■RFAQKVRQVF
AAA22298     YGG-DHPVFTFGEW■LGA-DQTDGDNIKFANESGMNLLD■EYAQEVREVF
E05456       DN--YRPVFTFGEW■LSE-NEVDANNHYFANESGMSLLD■RFGQKLRQVL
BAB91217     YD--YNPVFTFGEW■TGA-QGSN-HYHHFVNNSGMSALD■RYAQVAQDVL
CAH61550     YN--HKPVFVFGEW■LGK-DEYDPNYYHFANNSGMSLLD■EFAQTTRSVF
NP_269428    YE--KHNVFVFGEW■SGH-TDDDYDMTTFANNSGMGLLD■RFANAIRQLY
CAL25733     DSAPGGPVTHFGEF■IGRPDPKYDEYRTFPDRTGVNNLD■EYYNANRQAF
YP_242734    REK-RPGVFMFGEA■DYD--PAKIAGHTWARNAGVSVLD■PLKQQLSAVF
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CAA48401     R-DNTSNMYALDSMINSTATDYNQVNDQVTFIDNHDMDRFKTSAVNNR-R
CAA33763     R-DNTSNMYALDSMLTATAADYNQVNDQVTFIDNHDMDRFKTSAVNNR-R
PI-cgt       R-DNTSTMVALDSMITSTAADYAQVNDQVTFIDNHDMDRFKTSAVNNR-R
AAR32682     R-DNTDNMYGLKAMLEGSEVDYAQVNDQVTFIDNHDMERFHTSNGDRR-K
ABG02281     R-DNTDNMYGLKAMLEGSEVDYAQVNDQVTFIDNHDMERFHTSNGDRR-K
CAA55023     R-DNTDNMYGLKAMLEGSAADYAQVDDQVTFIDNHDMERFHASNANRR-K
AAA22298     R-DKTETMKDLYEVLASTESQYDYINNMVTFIDNHDMDRFQVAGSGTR-A
E05456       R-NNSDNWYGFNQMIQDTASAYDEVLDQVTFIDNHDMDRFMIDGGDPR-K
BAB91217     R-NQKGTMHDIYDMLASTQLDYERPQDQVTFIDNHDIDRFTVEGRDTR-T
CAH61550     R-NHEKNMFDLYDMLKNTENNYERVVDQVTFIDNHDMDRFHYDGATKR-N
NP_269428    TGFSTFTMRDFYKVLENRDQVTNEVTDQVTFIDNHDMERFATKVANNQTA
CAL25733     G-EFSRSMSDFGQMLVQTSADYMVENQAVTFIDNHDVSRFRYIQPNDK-P
YP_242734    G-HKQAGFEQLATPLYLRKGPYGNPYELMSFYDNHDMARLDAS--DTG--
               :     :          : ::* ****: *:

CAA48401     LEQALAFTLTSRGVPAIYYGTEQYLT-GNGDPDN-----RAKMPSFSKST
CAA33763     LEQALAFTLTSRGVPAIYYGTEQYLT-GNGDPDN-----RGKMPSFSKST
PI-cgt       LEQALAFTLTSRGVPAIYYGTEQYMT-GNGDPDN-----RAKMPSFSKTT
AAR32682     LEQALAFTLTSRGVPAIYYGSEQYMS-GGNDPDN-----RARIPSFSTTT
ABG02281     LEQALAFTLTSRGVPAIYYGSEQYMS-GGNDPDN-----RARIPSFSTTT
CAA55023     LEQALAFTLTSRGVPAIYYGTEQYMS-GGTDPDN-----RARIPSFSTST
AAA22298     TEQALALTLTSRGVPAIYYGTEQYMT-GDGDPNN-----RAMMTSFNTGT
E05456       VDMALAVLLTSRGVPNIYYGTEQYMT-GNGDPNN-----RKMMSSFNKNT
BAB91217     TDIGLAFLLTSRGVPAIYYGTENYMT-GKGDPGN-----RKMMESFDQTT
CAH61550     VEIGLAFLLTSRGVPTIYYGTEQYLT-GNGDPYN-----RKPMSSFDQNT
NP_269428    VNQAYALLLTSRGVPNIYYGTEQYAT-GDKDPNN-----RGDMPSFNKES
CAL25733     YHASLAVLLTSRGIPNLYYGTEQYLNPGHGGSDAGRLFLQAAAPAFSEQT
YP_242734    FIDAHNWLFTARGIPVIYYGSETGFMRGRAEHAGNRNYFGEERVSNAPQS
             .    :*:**:*  :***:*        *          :   :
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50

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CAA48401    TAFNVISKLAPLRKSNPAIAYGSTQQRWINNDVYVYERKFGKS--VAVVA
CAA33763    TAFNVISKLAPLRKSNPAIAYGSTQQRWINNDVYIYERKFGKS--VAVVA
PI-cgt      TAFNVISKLAPLRKTNPAIAYGTTQQRWINNDVYVYERKFGNN--VAVVA
AAR32682    TAYQVIQKLAPLRKSNPAIAYGSTQERWINNDVIIYERKFGNN--VAVVA
ABG02281    TAYQVIQKLAPLRKSNPAIAYGSTQERWINNDVIIYERKFGNN--VAVVA
CAA55023    TAYQVIQKLAPLRKCNPAIAYGSTQERWINNDVLIYERKFGSN--VAVVA
AAA22298    TAYKVIQALAPLRKSNPAIAYGTTTERWVNNDVLIIERKFGSS--AALVA
E05456      RAYQVIQKLSSLRRNNPALAYGDTEQRWINGDVYVYERQFGKD--VVLVA
BAB91217    TAYQVIQKLAPLRQENKAVVYGSTKERWINDDVLIYERSFNGD--YLLVA
CAH61550    KAYKIIQKLAPLRKSNPALAYGTTQERWLNNDVIIYERKFGNN--IVLVA
NP_269428   QAYKVISKLAPLRKQNQALAYGTTEQRWISDHVLVFERKFGNH--VALVA
CAL25733    VAYRLIGKLSALRQSNDALAYGTTDILFSNDDALVYKRQFFDK--QVIVA
YP_242734   PIFGPLQRIATLRRNTPALQRGVQVDLQLRGDQAAFLRVYQHAGMTQTAL
            :   :  ::.**:  .  *:    *       ..     * :       .

CAA48401    VNRNLSTSASITGLSTSLPTGSYTDVLGGVLNGNNITS----TNGSINNF
CAA33763    VNRNLTTPTSITNLNTSLPSGTYTDVLGGVLNGNNITS----SGGNISSF
PI-cgt      VNRNLSTPTSISGLTTSLPSGTYNDVLAGALSGNNITS----TGGNVANF
AAR32682    INRNMNTPASITGLVTSLPQGSYNDVLGGILNGNTLTVG---AGGAASNF
ABG02281    INRNMNTPASITGLVTSLPQGSYNDVLGGILNGNTLTVG---AGGAASNF
CAA55023    VNRNLNAPASISGLVTSLPQGSYNDVLGGLLNGNTLSVG---SGGAASNF
AAA22298    INRNSSAAYPISGLLSSLPAGTYSDVLNGLLNGNSITVG---SGGAVTNF
E05456      VNRSSSSNYSITGLFTALPAGTYTDQLGGLLDGNTIQVG---SNGSVNAF
BAB91217    INKNVNQAYTISGLLTEMPAQVYHDVLDSLLDGQSLAVK---ENGTVDSF
CAH61550    INRNLSQSYSITGLNTKLPEGYYYDELDGLLSGKSITVN---PDGSVNQF
NP_269428   INRDQTNGYTITNAKTALPQNSYKDKLEGLLGGQELIVG---ADGTISSF
CAL25733    VNRQPDRTVSIPALTTTLPVGTYPDALDGLLYGRTMTVVNQNGALQIPAF
YP_242734   VLLNKGDAAADIAVSRLLQPGSWRDAFS----------------------
            :   .     .      :     : * :

CAA48401    TLAAGATAVWQYTTAE--TTPTIGHVGPVMGKPGNVVTIDGRGFGSTKGT
CAA33763    TLAAGATAVWQYTASE--TTPTIGHVGPVMGKPGNVVTIDGRGFGSAKGT
PI-cgt      TLAAGATAVWQYTANT--TTPTIGHVGPVMGKAGNTVTIDGRGFGTTKGT
AAR32682    TLAPGGTAVWQYTTDA--TAPIIGNVGPMMAKPGVTITIDGR-ASARQGT
ABG02281    TLAPGGTAVWQYTTDA--TAPIIGNVGPMMAKPGVTITIDGRGFGSGKGT
CAA55023    TLAAGGTAVWQYTAAT--ATPTIGHVGPMMAKPGVTITIDGRGFGSSKGT
AAA22298    TLAAGGTAVWQYTAPE--TSPAIGNVGPTMGQPGNIVTIDGRGFGGTAGT
E05456      DLGPGEVGVWAYSATE--STPIIGHVGPMMGQVGHQVTIDGEGFGTNTGT
BAB91217    LLGPGEVSVWQHISESG-SAPVIGQVGPPMGKPGDAVKISGSGFGSEPGT
CAH61550    IINPGEVSIWQFAGET--ITPLIGQVGPIMGQVGNKVTISGVGFGDKKGT
NP_269428   ELGAGQVAVWTYEGED--KTPQLGDVDASVGIAGNKITISGQGFGNSKGQ
CAL25733    TLAGGEVSVWSHNPPADPAEPHIGEVISTMGRPRNTVYIYGTGLGDA-AA
YP_242734   ------------------------------GEQVQVQGR--------
                                          : : *

CAA48401    VYFGTTAVTGAAITSWEDTQIKVTIPSVAAGNYAVKVA-ASGVNSAYNN
CAA33763    VYFGTTAVTGSAITSWEDTQIKVTIPPVAGGDYAVKVA-ANGVNSAYND
PI-cgt      VYFGTTAVTGSAITSWEDTQIKVTIPAVAAGNYAVKVA-ASGVNSNTYNN
AAR32682    VYFGTTAVTGADIVAWEDTQIQVKILRVPGGIYDIRVANAAGAASNIYDN
ABG02281    VYFGTTAVTGADIVAWEDTQIQVKIPAVPGGIYDIRVANAAGAASNIYDN
CAA55023    VYFGTTAVSGADITSWEDTQIKVKIPAVAGGNYNIKVANAAGTASNVYDN
AAA22298    VYFGTTAVTGSGIVSWEDTQIKAVIPKVAAGKTGVSVKTSSGTASNFKS
E05456      VKFGTTAAN---VVSWSNNQIVVAVPNVSPGKYNITVQSSSGQTSAAYDN
BAB91217    VYFRDTKID---VLTWDDETIVITLPETLGGKAQISVTNSDGVTSNGYD-
CAH61550    VNFGEIDAT---IISWTNSVIQIEIPSVPAGNYEITVSSEGGEKSNSYN-
NP_269428   VTFGEISAE---ILSWSDTLITLKVPTVPANYYNISVTTADKQTSNSYQA
CAL25733    VAFGSQQAA---VVSAQDNRIAAVVPNVQAGEYAITVT-KGGKTSNPFR-
YP_242734   --------------------VTLQVPAHG-----VRVLLSDAPVT-----
            :   :          : *           :
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51

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CAA48401   FTILTGDQVTVRFVVNNASTTLGQNLYLTGNVAELGNWSTGSTAIG---P
CAA33763   FTILSGDQVSVRFVINNATTALGENIYLTGNVSELGNWTTGAASIG---P
PI-cgt     FTILSGNQVSVRFVINNASTTLGQNLYLTGNVAELGNWSTGPLAIG---P
AAR32682   FEVLTGDQVTVRFVINNATTALGQNVFLTGNVSELGNWDP-NNAIG---P
ABG02281   FEVLTGDQVTVRFVINNATTALGQNVFLTGNVSELGNWDP-NNAIG---P
CAA55023   FEVLSGDQVSVRFVVNNATTALGQNVYLTGSVSELGNWDP-AKAIG---P
AAA22298   FNVLTGDQVTVRFLVNQANTNYGTNVYLVGNAAELGTWDP-NKAIG---P
E05456     FEVLTNDQVSVRFVVNNATTNLGQNIYIVGNVYELGNWDT-SKAIG---P
BAB91217   FQLLTGKQESVRFVVDNAHTNYGENVYLVGNVPELGNWNP-ADAIG---P
CAH61550   FEVLTNKQIPVRFVVNNAYTSWGQNVYLVGNVHELGNWDP-NRAIG---P
NP_269428  FEVLTDKQIPVRLLINDFKTVPGEQLYLMGDVFEMGANDA-KNAVG---P
CAL25733   YQVLGGDQVQVIFHVNKXRSRDX-----------------CLCRGXX
YP_242734  -DVALRKQLDAQMADQAARDARNK--------------------------
             :  .*  . :  :

CAA48401   AFN--QVIHQYPTWYYDVSVPAGKQLEFKFFKKNG-STITWESGSNHTFT
CAA33763   AFN--QVIHAYPTWYYDVSVPAGKQLEFKFFKKNG-ATITWEGGSNHTFT
PI-cgt     AFN--QVIYSYPTWYYDVSVPAGTSLEFKFFKKNG-STITWENGNNHTFT
AAR32682   MYN--QVVYQYPTWYYDVSVPAGQTIEFKFLKKQG-STVTWEGGANRTFT
ABG02281   MYN--QVVYQYPTWYYDVSVPAGQTIEFKFLKKQG-STVTWEGGANRTFT
CAA55023   MYN--QVVYQYPNWYYDVSVPAGKTIEFKFLKKQG-STVTWEGGSNHTFT
AAA22298   MYN--QVIAKYPSWYYDVSVPAGTKLDFKFIKKGG-GTVTWEGGGNHTYT
E05456     MFN--QVVYSYPTWYIDVSVPEGKTIEFKFIKKDSQGNVTWESGSNHVYT
BAB91217   MFN--QVVYSYPTWYYDVSVPADTALEFKFIIVDGNGNVTWESGGNHNYR
CAH61550   FFN--QVVYQYPTWYLDISVPADTTLEFKFIKIDESGNVIWQSGLNRVYT
NP_269428  LFNNTQTIAKYPNWFFDTHLPINKEIAVKLVKKDSIGNVLWTSPETYSIK
CAL25733   -----------PNWGXGIRTX-------RSIRS-----------------
YP_242734  --------------------------------------------------
```

Figure 1.2. Alignment of CGTases. Sources of individual CGTases listed in Table 1 by accession number. PI-cgt is designated by gray box. Stars, * denote identical residues, : denotes conserved strong group residues, . denotes weak group conserved residues. PI-cgt is outlined in gray, the four critical aromatic residues for CGTases are outlined in black.

The host strains for each of the included CGTases are shown (Table 1.1).

Table 1.1. Source organisms and references for CGTases included in Figure 1.1, and 1.2.

Accession Number	Host Organism	Major CD produced	Reference
CAL25733	*Bacillus halodurans*	NI	Unpublished
AAA22298	*Bacillus macerans*	α-CD	(Takano et al., 1986)
CAA48401	*Paenibacillus illinoinensis* strain 8	β-CD	(Nitschke et al., 1990)
CAA33763	*Bacillus lichenformis*	α-CD & β-CD	(Hill et al., 1990)
PI-cgt1	*Paenibacillus sp.* strain C36	NI	This Dissertation
BAB91217	*Bacillus clarkii*	γ-CD	(Takada et al., 2003)

Accession Number	Host Organism	Major CD produced	Reference
E05456 (Translation)	*Geobacillus stearothermophilus*	α-CD	Patent: JP 1993244945-A 1
CAA55023	*Paenibacillus illinoinensis* strain 251	β-CD	(Lawson et al., 1994)
ABG02281	*Bacillus sp.* N-227	β-CD	Unpublished
CAH61550	*Anaerobranca gottschalkii*	NI	(Thiemann et al., 2004)
AAR32682	*Bacillus sp.* I-5	NI	Unpublished
YP_242734	*Xanthomonas campestris pv. Campestris*	NI	(da Silva et al., 2002)
NP_269428	*Streptococcus pyogenes* M1 GAS	NI	(Ferretti et al., 2001)

PI-cgt clusters with most other Bacillus CGTases, and more specifically clusters with the most similar group of CGTases from *Bacillus lichenformis* and *P. illinoisensis* strain 8, although PI-cgt is more different from both of these strains than they are from each other.

Signal Peptide Analysis

CGTase is secreted extracellularly in bacterial strains harboring *cgt*. Since PI-cgt is a novel CGTase and signal peptides are highly variable, SignalP software was used to predict the cleavage location in Gram positive bacteria (the original host strain), Gram negative bacteria and eukaryotic systems. The Gram positive Hidden Markov models (HMM) predicted a cleavage site between position between 34 and 35 with a probability of 0.96.

The Gram positive Neural Networks (NN) gave a slightly different position, between residues 36 and 37 with all scores above the cutoff values, indicating high

probability of an accurate cleavage site prediction. With Gram negative HMM, a position

between 34 and 35 was predicted with a probability of 0.928. The Gram negative NN

gave low scores on the predicted cleavage site (C-score less than cutoff), but high scores

on the presence of a signal peptide (S-scores) with the cleavage site predicted to be

between positions 34 and 35. For eukaryotic HMM, a cleavage site between positions 34

and 35 was again predicted but with a probability of only 0.463. Eukaryotic NN predicted

a cleavage site between positions 23 and 24 with all scores above the cutoff values.

The different positions of the predicted eukaryotic versus bacterial cleavage sites are

shown in Figure 1.3.

M F K W T K R I I L S T T L S F S L L A G S A ^ L P L F P A A S V F A D A * D

Figure 1.3. The predicted PI-cgt signal sequence. Arrow shows predicted eukaryotic

cleavage site of the PI-cgt signal peptide. The asterisk shows the predicted bacterial

cleavage site.

Clear Zone Formation

    P. sp. C36, *E. coli* – PI-cgt and *E. coli* DH5α were all grown on the same plate

containing 1% starch basic medium (Figure 1.4).
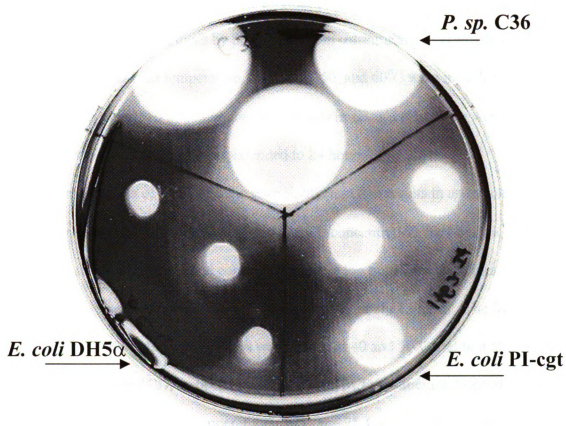
Figure 1.4. Bacterial clear-zone formation. Shown clockwise from top: *E. coli* expressing PI-cgt under control of the lac promoter, *Paenibacillus sp.* C36 parental strain and DH5α negative control.

The plate was incubated first at 30°C for 16hrs then at 37°C for an additional 16 hrs. After iodine staining, C36 showed large clear zones surrounding the colonies, *E. coli* PI-cgt showed smaller clear zones, and the untransformed *E. coli* strain, DH5α, did not show any clear zones.

Enzymatic optimum temperature determination

CGTases are generally considered thermostable enzymes with typical temperature optima in the range of 50-60°C. The temperature optimum for PI-cgt was experimentally

determined for enzyme derived from the original host strain, *P. sp.* C36 via enzymatic reaction with starch and analysis by phenolphthalein colorimetric determination. A set of three replications of each temperature, 25, 30, 40, 50, and 60°C were included. The βCD production was measured at 6 hours but βCD levels in several treatments were still relatively low, so reaction time was extended to 24 hours.

Complexation of βCD with the phenolphthalein dye results in an exponential curve when absorbance at 550nm is related to βCD concentration. To obtain a linear relationship, both absorbance and concentration were log transformed (Figure 1.5). From the graph, the lower detection limit for βCD is approximately 15μg/ml. The βCD production over the tested temperatures was highest at 40 and 50°C yielding an average of 667 and 594μg/ml βCD, respectively (Figure 1.6). βCD production dropped off significantly ($P<0.1$) at lower temperatures, 30°C and 25°C as well as the high temperature, 60°C. There was no significant difference between 40°C and 50°C treatments.

Kinetic Studies of βCD Production

The βCD production increased over time in both *P. sp.* C36 and *E. coli* PI-cgt enzymatic reactions with C36 increasing more rapidly than the *E. coli* strain (Figure 1.7). A spike in βCD concentration was noted at one hour of incubation in C36 but not in PI-cgt *E. coli*. This same spike was observed in repetitions of the kinetic study.
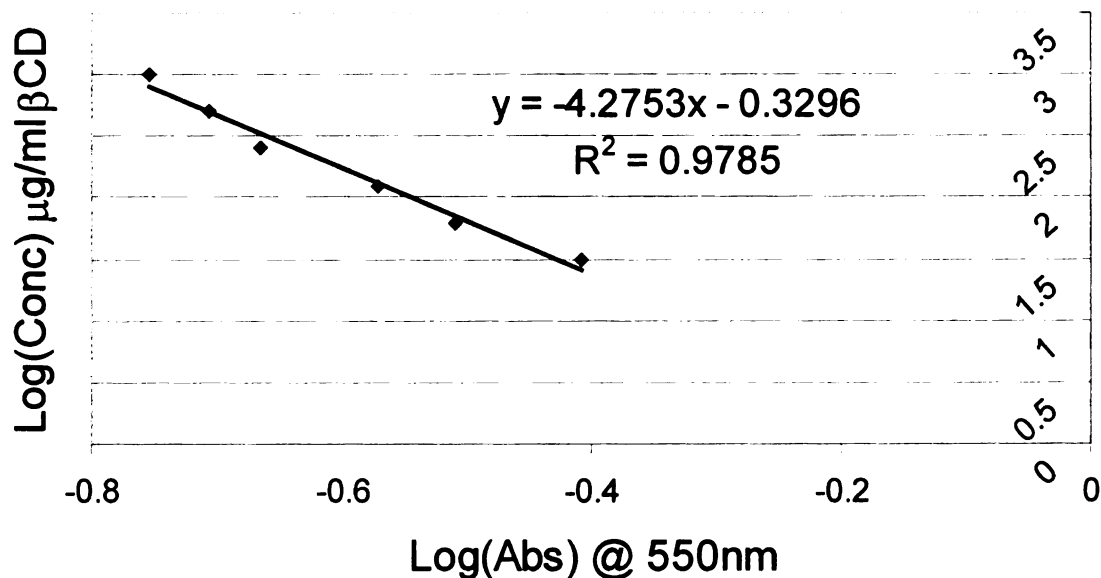
Figure 1.5. Typical standard curve for colorimetric analysis of βCD using

phenolphthalein. Log values were used to generate a linear relationship. The βCD

concentrations used were: 15.6, 31, 62, 125, 250, 500, and 1000 μg/ml.
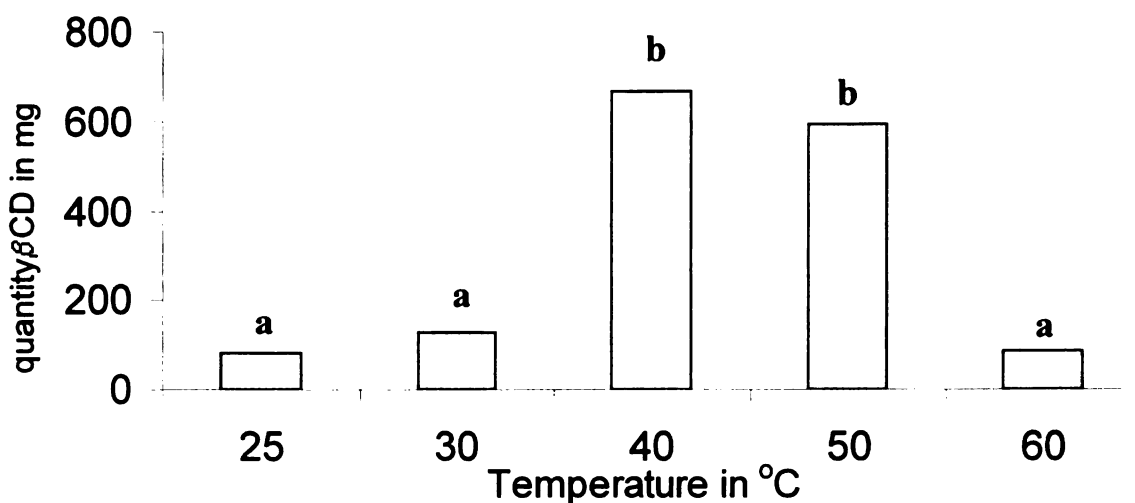
Figure 1.6. Temperature optimum determination for *P. sp.* C36. Samples of 50μl

supernatant were incubated at various temperatures with 200ml starch for 3hours.

Analysed for βCD content via the phenolphthalein method (n=3) Statistically similar

treatments (α < 0.05) are denoted with the same letter.

57

Quantitative Production of βCD by *P. sp.* C36 and *E. coli PI-cgt*

The βCD producing capabilities of both C36 and PI-cgt were measured over a set period of six hours. Reaction conditions were the same as the enzymatic optimum determination, with the temperature being 50°C. *P. sp* C36 and *E. coli* PI-cgt strains produced 7173 ng and 7231 ng βCD per microliter of enzymatic solution respectively. The average βCD production per hour per mg cell dry mass was 897 ng and 1808 ng for C36 and PI-cgt respectively.

TLC of bacterially produced CDs

Thin Layer Chromatography showed that the three cyclodextrins each had slightly different RF values with α, β and γ having 0.46, 0.42 and 0.38 respectively. However, when run together the individual CDs merged into a single spot. Fused spots were found in both bacterial enzymatic reactions at similar locations to the standards, implying that all of the three major CDs were produced, α, β and γ. No spots were produced by the *E. coli* DH5α lacking the *PI-cgt* plasmid, correlating spot production with the presence of *PI-cgt* in the bacterial host. Figure 1.8 shows a representative TLC plate.

Figure 1.7. CGTase Kinetic reaction showing βCD production over time. μg/ml βCD is given per mg of total solution protein, x axis shows minutes of incubation at 55°C. (n = 3)
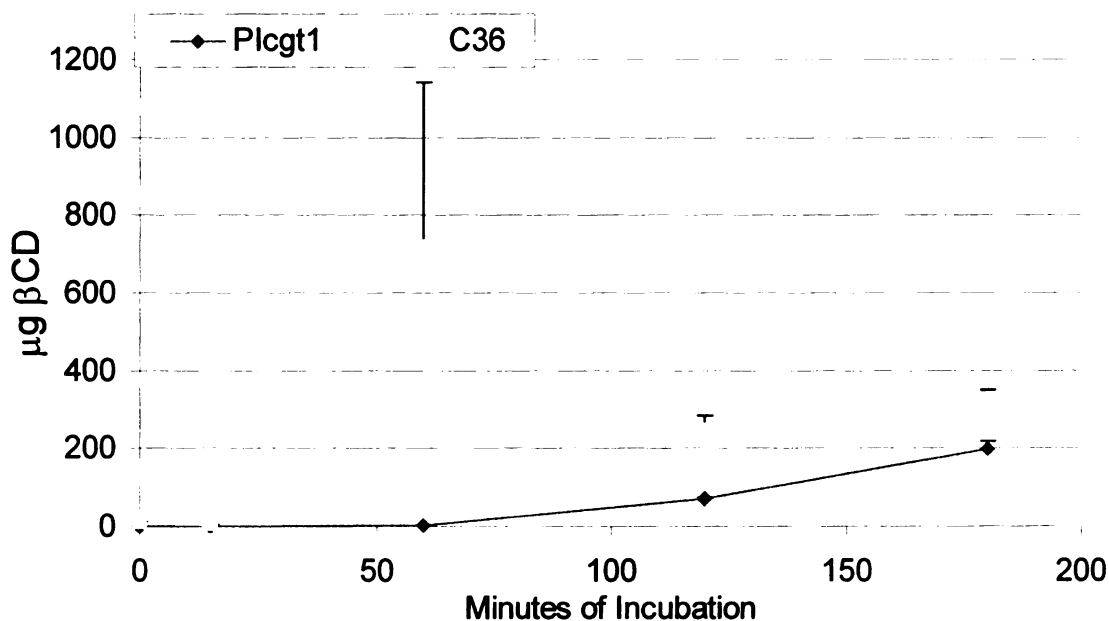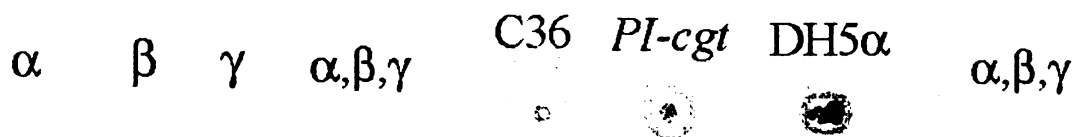

Figure 1.8. Thin layer chromatography analysis of CDs by CGTase producing bacteria. α, β, and γCD are shown as standards. C36, PI-cgt and DH5α were incubated for 5 hours.

DISCUSSION

A novel CGTase has been cloned from *P. sp* C36. This gene clusters with the similar CGTases from *B. lichenformis* and *P. illinoisensis* strain 8, although it is more dissimilar from these two CGTases than they are from one another. The well characterized CGTase from *B. circulans* strain 251 clusters more distantly but most of the CGTases from *Bacillus* species form a single group. Bootstrap values for most branches were high being 99 or 100. The lowest value near the base of the tree was 81. PI-cgt was overall most similar to α and βCGTases and least similar to γCGTases such as that from *Bacillus clarkia* and more distant organisms such as *Xanthomonas* and *Streptococcus*. The *Bacillus halodurans* CGTase also clustered very distantly from the other *Bacillus* CGTases indicating alkaliphiles may impose very different evolutionary constraints on CGTases in mesophilic species. The sequence similarities of PI-cgt to CGTases with known CD products give a strong indication that PI-cgt probably produces primarily βCD and αCD.

A signal peptide sequence was also detected in PI-Cgt that appears to be unique, among CGTases, although signal sequences usually exhibit high variability (Bendtsen et al., 2004). Cleavage site prediction showed the same location and a strong signal for both Gram positive and Gram negative bacteria with much lower scores for eukaryotic prediction. PI-Cgt shares all of the strictly conserved domains found in other CGTases including calcium binding residues present in A Domain, the circular B Domain and the raw starch binding Domain E (Rahman et al., 2006). PI-cgt also contains the four aromatic residues thought to be critical for CGTase function and specificity (Nakamura et al., 1994).

Starch plate clearing showed clear zones produced by both C36 and PI-cgt. However clear zones produced by the original host strain, C36 were much larger than those produced by the *E. coli* strain DH5α containing the cloned CGTase. This may be partially due to the plate having been incubated at a sub-optimal temperature, 30°C for *E. coli*. However, when the experiment was repeated with the bacteria growing separately at their respective optima and preferred medium, the same difference in clear zone size was noted. The smaller clear zone may be indicative of inefficient secretion of PI-cgt from *E. coli* cells. Since PI-cgt originated from *Paenibacillus*, a Gram positive bacterium, secretion through the more complex Gram negative membrane system may be difficult, and smaller clear zones might reflect a difference in secretion efficiency. Effective signal peptide function in a heterologous host is somewhat unusual in CGTases though not unheard of, which commonly show little to no secretion in heterologous expression systems (Lee et al., 2002). The signal sequence from *Brevibacillus brevis* CD162 CGTase could be secreted from *E. coli* cells (Kim et al., 1998). Signal peptide prediction software, using HMM indicated a high probability for the Gram negative cleavage location of PI-cgt to be identical to that of the Gram positive location, a strong indication that the PI-cgt signal peptide would be functional in a Gram negative bacterium such as *E. coli*.

PI-cgt showed a marked preference for higher temperatures in temperature optimum studies, this is typical for CGTases being thermostable enzymes with PI-cgt showing a similar temperature optimum to other CGTases derived from *Bacillus* species (Rahman et al., 2006). However, it is important to note that under field conditions CGTase operates at substantially lower temperatures since many CGTase producing

bacteria are mesophilic soil-dwelling organisms, and strain *P. sp* C36 was isolated from temperate field soil (Qi and Zimmermann, 2005). High temperature stability in CGTases may reflect long-term stability in extracellular environment or perhaps a simple coincidence in enzyme functionality and temperature stability.

The kinetic starch degradation studies showed increased production of βCD over time, with C36 showing the highest rate followed by the recombinant, *E. coli*. It is possible that the lower βCD production by *E. coli* PI-cgt is simply due to lowered secretion of CGTase rather than lower activity of the enzyme itself. The solution protein levels from supernatants both bacterial species used in enzymatic reactions were very similar. Similar total protein concentration may not reflect the relative abundance of PI-Cgt in that solution. Also of note was the very high levels of βCD found in the medium at 1 hour which was immediately followed by lower concentrations measured at 2 hours. This spike is primarily due to a single high concentration sample of the three replications, indicating the degree of increase in concentration is strongly exaggerated by this sample. If this sample were removed, the remaining samples still show an initial spike in concentration somewhat higher than the second time point but not higher than the final time point. Previous experiments also showed a spike in βCD concentration by C36 at approximately the same reaction time. This observation may be because CGTases can degrade CDs as well as synthesize them. CGTases often degrade larger CDs to smaller CDs when incubated over longer periods (Schmid, 1989). The faster reaction seen in the C36 strain could be due to the presence of an additional enzyme such as α-amylase since the enzyme extract was not specifically purified for CGTase. In steady state analysis of the two enzymes over 6 hours, relatively similar βCD production was observed indicating

that while the initial reaction rate of C36 CGTase may be faster, *E. coli* PI-cgt can eventually approach the βCD production of C36.

TLC of bacterial starch reactions showed that both the original host strain and cloned *E. coli* CGTase were capable of producing all of the standard CDs. Lighter spots produced by PI-cgt versus *P. sp.* C36 probably reflect the lower CD production by the *E. coli* expressed CGTase. This again may also simply reflect lower CGTase secretion by *E. coli* cells. Careful examination of the intensity of spots on the plate seem to indicate a in intensity bias towards more quickly migrating CDs, possibly indicating that PI-cgt may produce more α and β CD than γCD.

For improved production of PI-cgt in *E. coli*, modification of the signal sequence may be required. Additionally, the codons of PI-cgt may be sufficiently different from *E. coli* to reduce expression somewhat, since the codon usage of *Bacillus* species and *E. coli* are not identical.

CONCLUSION

In this study, a novel *cgt* gene was cloned from a *Bacillus* relative. Its sequence was examined via several different comparison metrics and unique sequence features were found within PI-cgt. Bacterially produced enzyme from both *Paenibacillus sp.* C36 and *E. coli* transformed with PI-cgt was capable of degrading starch and producing βCD and other CDs. The clear zones produced by *E. coli* PI-cgt give strong evidence that the signal sequence from *P. sp.* C36, a Gram positive bacterium can function in a Gram negative species, confirming signal peptide prediction. However, CD production by *E. coli* was somewhat lower than that produced by strain C36, indicating that either the

signal peptide may not efficiently translocate PI-Cgt through the *E. coli* membrane or the

E. coli produced enzyme may simply be less efficient than the C36.

These bacterial studies are a proof of concept, showing that the cloned *P. sp.* C36

CGTase is functional in a heterologous host. Further characterization and manipulation of

PI-cgt would give a fuller picture of the nature of the enzyme and the precise ratio of α, β

and γCDs. Examination of the CD ratio produced by the *P. sp.* C36 CGTase could give

additional information for understanding how CGTase sequences relate to the production

of specific CDs. Future comparisons of other CGTases may allow for the isolation of

superior enzymes for *in-situ* CD production.

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CHAPTER II

Plant expression of bacterial Cyclodextrin Glycosyltransferase for cyclodextrin production

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INTRODUCTION

Biological production of industrial and pharmacological compounds in plants has become an important emerging technology. While plants have been used primarily for the sources of food and fiber, harnessing them as biological factories is a more recent development. Plants have been used for the production of heterologous proteins with pharmaceutical applications, such as antibodies and enzymes (Berberich et al., 2005; Girard et al., 2006), industrial plastic precursors and other industrial compounds (Conrad, 2005). Production of bioactive proteins, bioplastics and other biologically active materials in plants may be cleaner and more economical than animal cell cultures or synthetic production.

One biological compound with a wide variety of industrial, food and cosmetic uses is cyclodextrin (CD). Cyclodextrins are cyclic sugars synthesized by bacteria from starch and capable of enhancing the solubility of various hydrophobic compounds (Szejtli, 1988). CD molecules orient the primary and secondary sugar hydroxyls along the outer edges of the torus-shaped molecule with the ether linkages positioned in the interior, hydrophobic region. CDs are commonly composed of 6, 7, and 8 glucose units (termed α, β, and γCD, respectively) each capable of associating with different compounds due to the distinct cavity sizes. Host molecules become complexed within the

cyclodextrin, which provides enhanced stability, protection from oxygenating factors and light-induced decomposition as well as solubility and biological absorption (Kamiya and Nakamura, 1995; Loukas et al., 1994; Uekama et al., 1998). CDs can also block bitter tastes in medicines and foul odors in the environment by entrapment of the source compounds and prevention of binding to taste and scent receptors (Szejtli and Szente, 2005). CDs have been used as soil or solution amendments to accelerate environmental remediation treatments, including soil washing and biodegradation applications (Bardi et al., 2000; Molnar et al., 2005; Viglianti et al., 2006; Wang et al., 2005; Wang and Brusseau, 1995).

Cyclodextrin is typically produced on industrial scales using the preparations of the bacterial enzyme cyclodextrin glycosyltransferase (CGTase) in large vat reactors with starch as a feedstock (Starnes, 1990). Starch is first liquefied using either heat or amylase treatment followed by addition of CGTase. CGTases always produce a mixture of α, β, and γ cyclodextrin in ratios dependent on the CGTase source and reaction conditions, so the production of a pure CD form can be difficult. βCD is the easiest to purify since it has low water solubility compared to the other CDs and precipitates at high concentrations. The more water soluble αCD and γCD compounds must be separated by chromatographic techniques to achieve pure reagent forms, though organic solvents may be added as complexing agents to help separate the individual cyclodextrins by enhanced precipitation (Lee and Kim, 1991). Commercial and industrial applications for CDs are increasing with global CD production in excess of 10,000 tons per year and concurrent economy of scale reducing βCD costs to only a few dollars per kg (Szejtli, 2004).

An alternative to bacterial CGTase enzyme production is development and utilization of transgenic plants or plant cell cultures. Plants may be capable of producing higher quantities of CGTase and perhaps more efficiently than bacteria due to the ability of plants to grow in less stringently controlled conditions than bacterial cultures. CD produced by plants would be free of pathogenic contaminants present in animal based culture systems and could require less post-synthesis processing.

For environmental remediation applications, *in situ* CD production would be an effective alternative to labor intensive addition of CD amendments to the soil or maintenance of CD-producing organisms. Since plant starch is typically found only within the sub-cellular plastid compartment, direct plant secretion of CDs from roots would likely involve considerable metabolic engineering. It may be advantageous to engineer plants to secrete CGTase into the rhizosphere essentially in the same manner as CD-producing bacteria. This strategy is in contrast to previous efforts to express CGTase in potato tubers to produce CD within plant amyloplasts as a substitute for conventional industrial CD sources, which resulted in ineffective CD accumulation (Oakes et al., 1991). Rhizosecreted CGTase may offer a more manageable solution to CD production using plants, rather than labor intensive addition to soil or addition of potentially problematic bacterial strains. The similarity of prokaryotic signal peptides to those in eukaryotes should allow plants engineered with the bacterial *cgt* gene to secrete CGTase into the surrounding matrix without further modification (Hall et al., 1990). Using a bioreactor approach, extracellular CGTase would be able to degrade starch in hydroponic system to provide an easily extractable CD.

As a direct application to *in situ* contaminant remediation, CGTase secreting

plants could be planted directly in contaminant soils. Plant produced CGTase could

convert starch present in soil due to exogenous addition or released during root turnover

to cyclodextrin, potentially enhancing the degradation rate of persistent organic

pollutants. The objective of this study is to transfer a bacterial *cgt* gene into laboratory

plants and evaluate *cgt* transgenic expression and product function. If effective, this

approach could provide an efficient alternative source of reagent grade cyclodextrin and

possibly a new tool for environmental bioremediation.

MATERIALS AND METHODS

Cgt Gene Vector Construction

The *PI-cgt* gene was cloned from *Paenibacillus sp.* strain C36 as previously

described (Settavongsin, 2005). The gene was subcloned from the bacterial expression

vector, pBS SK⁻ using *XhoI* and *SacI*, into plant expression vectors, pAPC-9K and

pE1778. The expression cassettes of these vectors each contain different promoters such

as *Arabidopsis* Actin2 and the "super promoter" construct to drive expression of the *cgt*

gene in plants (An et al., 1986; An et al., 1996). The "super promoter" was derived from the

mannopine synthase promoter and multiple octopine enhancer elements originally found

in *Agrobacterium* spp. (Ni et al., 1995). Both promoters are constitutively expressed at

high levels in all tissues, though the "super promoter" is more active in root tissue. The

transcription terminator used in the Actin2 gene expression cassettes is PE21 from *Citrus*

sinensis cv. Valencia (sweet orange) pectinesterase gene (Nairn et al., 1998). The

expression cassette was excised from pAPC-9K using *SpeI*, *XbaI*, and *XmaI* restriction

enzymes cloned into the binary vector pCAMBIA (Gene bank accession #AF234296)

(CAMBIA Institute, Canberra, Australia).
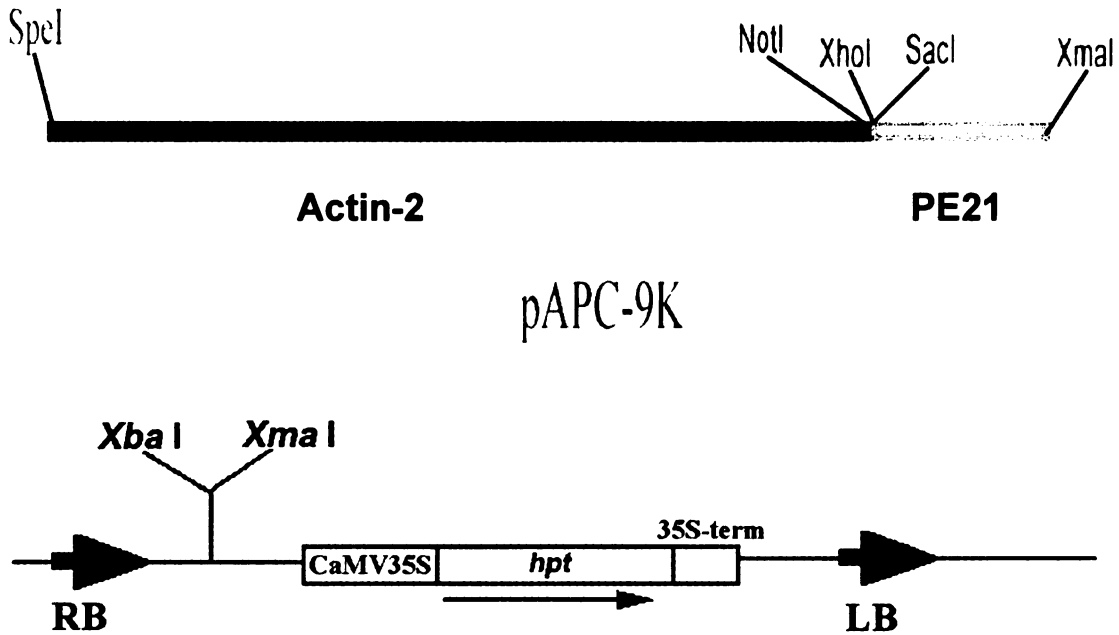
Figure 2.1. Diagram of the pAPC9K and pCAMBIA 1300 cloning vectors.

To create plant expression constructs using the Arabidopsis Actin2 promoter, PI-cgt was excised from pBS-PI-cgt using *NotI* and *SacI* and inserted into pAPK-9K using the same restriction enzymes, resulting in pAPC-9K-PI-cgt. Expression cassettes of pAPC-9K containing *PI-cgt* were excised using *SpeI* and *XmaI* restriction enzymes and inserted into pCAMBIA 1300 cut with *XbaI* and *XmaI*. The resulting construct was named pCAMBIA-Act-PI-cgt. PE1778 constructs were created by excising *PI-cgt* from pBS-PI-cgt using *XhoI* and *SacI* restriction enzymes, pE1778 was cut with these same two enzymes to allow the insertion of *PI-cgt*. Completed pCAMBIA and pE1778 constructs were transformed into electrocompetent *Agrobacterium* LBA4404 (Invitrogen, Carlsbad, CA) via electroporation using a Bio-Rad Micropulser according to the manufacturer's recommendations (Bio-Rad, Hercules, CA). *Agrobacterium*

transformants were screened using semi-solid YM plates containing, 0.4g/L yeast extract, 1% mannitol, 1.7mM NaCl, 0.5mM MgSO$_4$·7H$_2$O, 2.2mM K$_2$HPO$_4$, and 15g/L Bacto agar (Sigma, St. Loius, MO) supplemented with 100mg/L kanamycin for pCAMBIA or pE1778 selection and 100mg/L streptomycin for Ti plasmid selection. Kan^R, Str^R colonies were grown in liquid LB medium (10g/L NaCl, 5g/L yeast extract, 10g/L Bacto tryptone (Beckton Dickinson, Sparks, MD) for plasmid DNA extraction and analysis. *Agrobacterium* colonies displaying appropriate enzyme digested DNA bands fragments via gel electrophoresis analysis were utilized in subsequent plant culture transformation procedures.

Plant Transformation

Tobacco transformation

Tobacco (*Nicotiana tabacum*) cv. Little Havana transformation was carried out using a standard method for co-cultivation of leaf sections with *Agrobacterium* LBA4404 (Invitrogen, Carlsbad, CA) strains harboring the plant expression vector containing the *cgt* gene construct and the selectable marker genes.

Agrobacterium strains were grown in liquid culture for tobacco leaf sections inoculation. Transformation was performed by growing the strain of interest for two days in YM medium. *Agrobacterium* cell preparations were spun down and resuspended in 500µl of fresh YM medium. Semi-solid Murashige and Skoog medium (MSO: 25g sucrose/L, B5 vitamins 100mg/L myoinositol, 10mg/L thiamine-HCl, 1mg/L nicotinic acid, 1mg/L pyroxidine-HCl and 7g/L phytagar) (Invitrogen, Carlsbad, CA) was utilized as a standard basic tissue culture medium (Murashige and Skoog, 1962). Tobacco leaf

sections approximately 1-2cm square were pre-incubated without *Agrobacterium* on MSO medium for 1-2 days then transferred to 1.5mL microtubes containing the resuspended *Agrobacterium* solution, then vortexed for 30 seconds and allowed to stand for approximately 5 minutes. Afterwards the tissue sections were blotted dry using sterile filter paper and placed on non-selective, MSO medium. After a period of two days, the leaf sections were transferred to semi-solid MSO104 which contained, in addition to MSO ingredients, plant growth regulators 1mg/L benzylaminopurine (BA), 0.1mg/L 1-napthaleneacetic acid (NAA) (Invitrogen, Carlsbad, CA), and either 25mg/L hygromycin (for pCAMBIA-*PIcgt*) or 300mg/L kanamycin (for pE1778-*PI-cgt*) as selection agents and 400mg/L timentin (Smithkline-Beecham, Philadelphia, PA) to control the growth of *Agrobacterium*.

After about one month shoots began to form and once true leaves had formed, shoots were excised and placed on rooting media which consisted of MSO medium at 6g/L phytagar, without plant growth regulators, containing antibiotic selection of 25mg/L hygromycin or 100mg/L kanamycin and 400mg/L timentin. Rooted plantlets were placed in 1 gallon round pots containing free draining potting soil (Baccto High Porosity Professional Potting Mix, Michigan Peat, Houston, TX) and grown in a temperature controlled greenhouse (25-30°C) for seed production. Each plant was reproductively isolated from the others via placement of an isolation bag over the flowers before opening. Entire inflorescences were removed from the plant once capsules were filled and beginning to dry. Bags containing inflorescenses were stored under greenhouse conditions until fully dry. One to two dry capsules were harvested from each

inflorescence, seeds placed in 1.5ml microtubes and stored at 4°C until use. These seeds were called the T1 generation.

Arabidopsis Transformation

Arabidopsis thaliana (ecotype RLD, Lehle Seeds, Round Rock, TX) was transformed using the vacuum infiltration method. Vacuum infiltration draws *Agrobacterium* cells into *Arabidopsis* floral tissues, where DNA transfer occurs in developing ovules (Ye et al., 1999). Vacuum infiltration was performed as follows: 4 1/2 inch pots were filled with wet potting soil (Baccto High Porosity Professional Potting Mix, Michigan Peat, Houston, TX). Standard size window screen was cut into squares and placed over the top of mounded soil, held in place by rubber bands. *Arabidopsis* seeds were mixed with sand in an approximate 1:10 ratio, and placed in a salt shaker. The sand and seed mixture was shaken over the pots to evenly distribute seed, with soil being kept moist until seed germination. After approximately 1-2 weeks any excess plants were removed, resulting in approximately 7-10 mature Arabidopsis plants growing in each pot. The plants were allowed to mature until the primary inflorescenses began to emerge and elongated to approximately 3-4 inches. At this stage primary inflorescenses were cut and the plants placed in the plant growth chamber for 1-2 days prior to *Arobacterium* transformation.

Agrobacterium cultured overnight in 5ml YM medium containing 100mg/L streptomycin and 100mg/L kanamycin after which one ml culture was used to inoculate 500ml of the same selective YM medium and grown on orbital shaker 1-2 days at 28°C. After incubation the culture was spun down and resuspended in 1L of infiltration medium

consisting of 1/2X Murashige and Skoog salt mix (Murashige and Skoog, 1962), 1X B5 vitamins (listed in MSO medium above), 50g/L sucrose, 0.5g 2-[N-morpholino]ethanesulfonic acid (MES) and 0.044μM BA. 200μl Silwet L-77 (Lehle Seeds, Round Rock, TX) was added to the *Agrobacterium* suspension and mixed well. The bacterial solution was placed in a 600ml beaker and pots containing *Arabidopsis* to be infiltrated were placed upside down into the filled beakers. The beakers were placed inside of an 18.9L polycarbonate vacuum chamber (Nalgene, Rochester, NY). Vacuum was pulled to approximately 15mm Hg on the system for 5 minutes, then the vacuum source was removed and pressure was held for another 2 minutes. Afterwards pressure was released slowly and pots were removed from the beakers, laid on their sides in a large tub covered with plastic wrap. Pots were left covered for one full day after which they were set upright and watered well. Plants were allowed to develop normally until seed set. Pots containing mature plants were placed on their side and inflorescences were placed in bags until the entire plant was dry. Seeds were collected from infiltrated and non-infiltrated control plants once the siliques appeared fully mature and near dryness. Seeds were then harvested via gentle compression of the bag followed by screening to remove chaff and other dry contaminants. Collected *Arabidopsis* T1 generation seeds were placed in 1.5ml microtubes and stored at 4°C.

Transgenic Seed Screening and Selection

Seeds of tobacco and *Arabidopsis* were sterilized with 15% household bleach (6.15% sodium hypochlorite, Clorox Company, Oakland, CA) for 15 minutes followed by two rinses with sterile water.

For *Arabidopsis*, plating of seed represented the initial screen for transformed lines. Seedlings showing resistance to hygromycin had incorporated the marker *hpt* gene and likely the *cgt* gene as well. Seeds were germinated on selective medium – MS salts only with 7g/L phytagar plus 25mg/L hygromycin. Resistant seedlings were scored as putative transformants and removed from selective medium and planted in 2 1/2 inch pots as soon as they were recognized. Resistant seedlings were allowed to grow and set seed, which was named the *Arabidopsis* T2 generation.

Ratios of resistant to susceptible seedlings in T1 tobacco seed lots were used to estimate the copy number of the *PI-cgt* gene. A three to one ratio of resistant to susceptible seedlings is consistent with a single site of genome integration. Resistant seedlings were removed from selection promptly and greenhouse grown as previously described, with the resulting seeds being labeled the tobacco T2 generation. Seeds from subsequent generations were also screened on antibiotic containing media. The results of seedling screening on *Arabidopsis* and tobacco are shown in Tables 1a and 1b.

Transgenic Plant Genomic PCR Screening

Genomic DNA extracts from putative transgenic plantlets of both species were screened for integration of the gene of interest using *PIcgt* specific primers in PCR. Genomic DNA extracts were generated using the DNeasy plant kit (Qiagen, La Jolla, CA) according to manufacturer instructions. PCR reactions were carried out using 0.5 – 1.0µl genomic DNA template, 2µl 10X PCR Buffer (500mM KCl, 100mM Tris*Cl (pH 8.8) 1% TritonX), 0.6µl 50mM magnesium chloride, 0.5µl of 10pm forward and reverse

primers 0.25μl of 100mM dNTPs, and 1.0μl of crude Taq polymerase extract with the total reaction volume being 20μl.

Crude Taq polymerase was generated by growing *Esherichia coli* expressing Taq polymerase in Super Broth, which contains (16g Tryptone, 10g Yeast Extract, 2.5g NaCl 2.5ml of NaOH with the final volume being 500ml. Overnight cell cultures were spun down and resuspended in Buffer A consisting of, 50mM Tris (pH 8.0), 50mM dextrose, 1mM Ethylenediaminetetraacetic acid (EDTA) pH8.0. Buffer B consisting of, 10mM Tris pH 8.0, 50mM KCl, 1mM EDTA pH 8.0, 0.5% Tween 20, 0.5% NP-40, was added to lyse the cells, which were then incubated at 75°C for one hour. Cells were again spun down with the supernatant being mixed 1:1 with storage buffer (50mM Tris pH 8.0, 100mM NaCl, 0,1mM EDTA pH 8.0, 0.5mM DTT, and 50% glycerol). This solution was diluted again 1:1 with 80% glycerol, resulting in the working solution of crude Taq polymerase which was stored at –20°C until use.

RT-PCR-expression analysis

RNA was extracted from leaves using the RNeasy kit (Qiagen, La Jolla, CA) along with on-column DNase digestion using the RNase-free DNAse set. The cDNA synthesis was performed using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. PCR reactions for cloning were performed in reactions containing, 0.5 – 1.0μl template, 2μl PCR buffer (500mM KCl, 100mM Tris-HCl (pH 8.8) 1% TritonX), 0.6μl 50mM MgCl₂, 0.5μl of 10pm forward and reverse primers 0.25μl of 100mM dNTPs, and 1.0μl of crude Taq polymerase extract with the total reaction volume being 20μl. The thermocycler conditions were as follows:

A primary denaturation step, 94°C for 3 minutes followed by 40 cycles of 94°C 30 seconds, 55°C annealing temperature for 30 seconds and a 68°C extension temperature for 45 seconds. RT-PCR products were run on agarose gels of concentration from 0.8 – 1% in Tris-acetate EDTA at 100 V for 1 hour. Reactions using primers designed for the selectable marker gene were run in parallel as an expression and cDNA preparation control. Gels were stained with 0.8mM ethidium bromide in water for 15 minutes and visualized under UV light using a Bio-Rad Quantity One Gel Documentation system (Bio-Rad, Hercules, CA).

Starch Agar Clearing

The simplest CGTase expression assay is the clearing of starch. Putative CGTase expressing plants were placed in 1X Murashige and Skoog media containing 1.0-0.1% starch and 7g/L Phytagar (Murashige and Skoog, 1962). After growing on the medium for 2-3 weeks, starch agar is stained with a 1:30 aqueous dilution of an iodine solution consisting of 10% potassium iodide 1% iodine and 50% ethanol by mass with water. Starch forms a deep blue colored complex with iodine. Iodine dye does not form a colored complex with cyclodextrin, causing areas of starch degradation and CD production appear as clear regions on a blue-black background of agar.

Arabidopsis and Tobacco Plant Tissue Preparation

Tobacco plants were grown individually in 300ml Erlenmeyer flasks filled with 1/5X MS salt medium. Plants were watered once weekly and allowed to grow to near maturity. Samples of approximately 15ml of hydroponic medium were removed and

concentrated using a filtration/concentration apparatus (Millipore, Billerica, MA) using a centrifuge at 2000Xg for 20 min, resulting in approximately 500µl crude preparation which was used directly in enzymatic reactions with starch.

Sterilized tobacco and *Arabidopsis* seeds in aqueous solution were added to 30mls of sterile MS salts medium in sterile 150ml Erlenmeyer flasks capped with aluminum foil. These flasks were placed on a rotary shaker inside of a growth chamber under 16h day and 86 µmol/s*m^2 and approximately 25rpm. After 15 - 30 days of growth 15ml of hydroponics medium were concentrated using the same method as for adult plants to between 250µl and 500µl. Crude preparations were removed from the apparatus and used immediately or stored at 4°C for future use. Plant tissue was removed from the flask and weighed to determine the biomass present in the system, plant tissue was dried in an oven at 60°C and weighed again after drying to determine dry biomass.

Colorimetric βCD Assay

Enzymatic reactions were carried out by placing 50µl of the concentrated plant enzymatic solution in 200µl of 1.25% starch in pH 6.0 1mM phosphate buffer. The enzymatic reactions were placed in a 50°C incubator shaking at 200 rpm for a minimum of 2 hours up to several days. After the incubation time had elapsed a 50µl aliquot of the enzymatic reaction was analyzed for phenolphthalein color reduction by addition of 25µl of 0.4mM phenolphthalein and 20µl of 1M NaCO$_4$ mixed in a 96 well microplate. After light shaking and standing for a few moments, the plates were read at 550nm with a Spectra Max 190 (Molecular Devices, Sunnyvale, CA) spectrophotometer equipped with a microplate reader. The βCD produced was quantified via comparison of absorbance to a

standard curve of known quantities of βCD. The curve produced was an exponential

reduction in color in response to increasing βCD concentration. Both concentration and

absorbance were log transformed to result in a linear relationship.

Thin Layer Chromatography

Thin layer chromatography (TLC) was performed to separate and identify the

different CDs. 2μl of starch reaction was spotted 4 times to a 10cm X 20cm silica gel

60/Kieselguhr $_{F254}$ aluminum TLC sheet (EM Science, Gibbstown, NJ). α, β, and γCD

(Sigma, St. Lois, MO) were used as standards and spotted from 1% solutions to run

adjacent to starch reactions, these standards were spotted only once. The mobile phase

was acetonitrile-water-ammonium hydroxide (6:3:1) with plates being run in a sealed

glass TLC tank. Completed TLC plates were sprayed with Vaugh's solution (1g

$Ce(SO_4)_2$, 24g $(NH_4)_2MoO_4$, 50ml concentrated H_2SO_4 and 450ml H_2O) using a TLC

sprayer and developed by heating on a hot plate until blue spots appeared.

RESULTS

Selection of cgt Plant lines

Both the Super Promoter and Actin2 Promoter constructs were transformed into

tobacco (*Nicotiana tabacum*) cv. Little Havana. The transformation resulted in the

production of 12 independent transgenic lines with seven of these being selected for

further screening. These lines were allowed to grow and set seed. Using the appropriate

antibiotic marker, seed lots were screened by sterile plating. Most transgenic lines

displayed segregation ratios consistent with a single–copy transgene integration (Table

2.1a).

Table 2.1a. Selective marker segregation analysis for T2 generation tobacco seeds. PC

lines use hygromycin selection, PE lines use kanamycin selection.

Line	Hyg/Kan^R	Hyg/Kan^S	Segregation ratio
PC8A7	55	12	~3:1
PC1B2	56	0	All positive
PE3A1	39	14	~3:1
PE11A1	49	17	~3:1
PE11A4	50	14	~3:1
PE13A2	53	15	~3:1
PE2B4	54	15	~3:1

Kan^R= Transformed lines resistant to kanamycin

Kan^S= Transformed lines susceptible to kanamycin

Only the Actin2 promoter construct was utilized in *Arabidopsis* transformation.

Six lines of *cgt*-Arabidopsis were generated. Of these lines, only one was found to be

homozygous after the T2 generation, cgt1-5 (Table 1b).

Table 2.1b. Selective marker segregation analysis for T2 generation *Arabidopsis* seeds

Line	Hyg^R	Hyg^S	Segregation ratio
PIcgt1-3	27	15	~3:1
PIcgt1-5	31	0	All positive
PIcgt13	18	10	~3:1
PIcgt14	19	10	~3:1
PIcgt1-6	15	10	3:2
PIcgt1-1	15	26	1:1

Hyg^R= Transformed lines resistant to hygromycin

Hyg^S= Transformed lines susceptible to hygromycin

This result may indicate multiple insertion events in line cgt1-5 but confirms

single integration events for the cgt1-1, cgt14, and cgt13 with ratios of resistant to

susceptible seedlings near 3:1. Several lines exhibited low ratios, with some lines such as cgt1-1 showing a 1:1 ratio.

Starch Clearing

Several transgenic lines of both tobacco and *Arabidopsis* have exhibited enhanced ability to degrade starch by the formation of a zone of clearing in the blue iodine-starch region of the medium. Wild type strains of either species either produced only faint clear zones or none at all. *Arabidopsis* transformants appeared to be capable of forming larger clear zones than tobacco plants of similar size. Clear zone formation by *Arabidopsis* seedlings also appeared to be both more rapid and extensive than with tobacco seedlings. Interestingly, smaller *Arabidopsis* seedlings also seemed capable of forming larger clear zones than their more developed counterparts (Figure 2.2).

PCR and RT-PCR

All seven lines chosen for further testing have been shown to be positive for hygromycin or kanamycin resistance. Six of these tobacco lines were show to be positive for genomic PCR. The seven tobacco lines were tested in RT-PCR, with the 5 tested showing a positive reaction. Out of 3 *Arabidopsis* lines tested for RT-PCR, 2 showed a positive result for *Plcgt* expression and *hptII* expression. RT-PCR results from Arabidopsis correlated well with the observed quantity of cyclodextrin produced with those exhibiting a positive result in RT-PCR also producing detectable quantities of βCD. Example gels of tobacco and *Arabidopsis* RT-PCR reactions are shown in Figures 2.3a and 2.3b.
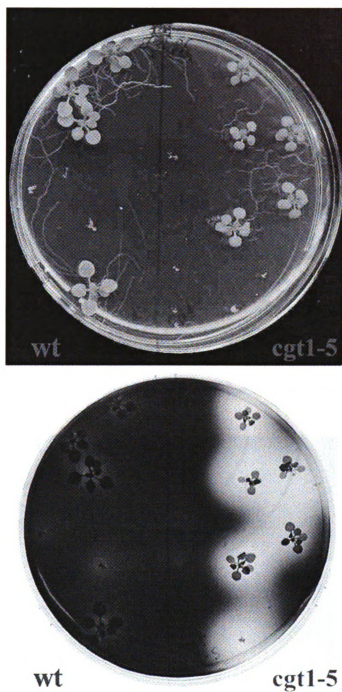
Figure 2.2. Clear zone formation by cgt-arabidopsis. Upper plate shows before iodine staining. Lower plate shows clear zones in 0.1% starch agar formed by Cgt-expressing *Arabidopsis* line, cgt1-5

Figure 2.3a. RT-PCR of *Arabidopsis* lines. Left gel is with cgt primers Right gel is with hygromycin control primers. Arrows denote expected fragment size

Figure 2.3b. RT-PCR of tobacco lines. Left gel contains hygromycin primers, right gel is *cgt* primers. Arrows denote expected fragment sizes.

βCD production

Adult tobacco hydroponics failed to produce detectable quantities of βCD. Concentrated Arabidopsis seedling hydroponic solution did produce detectable quantities of βCD after 48 hours of incubation. Quantities of βCD continued to increase over extended incubation time in *Arabidopsis* seedling exudates. Of 6 tested tobacco lines, seedling hydroponics also failed to produce detectable quantities of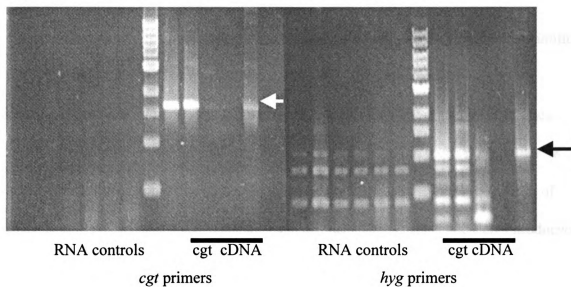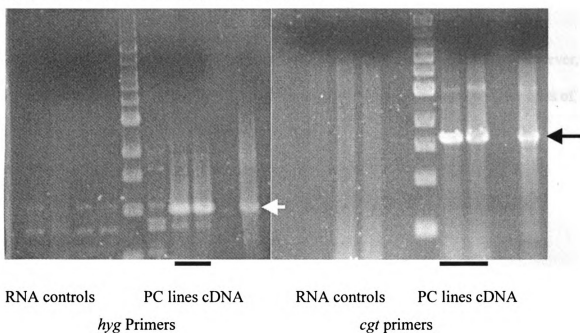 βCD even after 48 hours of incubation. Only one tested tobacco line did produce detectable quantities of βCD despite positive RT-PCR reactions from all lines. Lines cgt1-5 and cgt13 produced the highest quantities of βCD with cgt1-3 producing little to no βCD confirming the negative RT-PCR result in this line (Figure 2.4). The quantity of βCD produced using the enzymatic assay was 5300ng/mg dry tissue averaged across functional arabidopsis lines at 120 hrs of incubation and 1120ng/mg tissue in the single functional tobacco line at 72 hrs. Both values are much higher than the reported in-tuber production of transgenic potatoes, which was approximately 5 - 25ng/mg dry tissue (Oakes et al., 1991). However, comparisons between the two types of transgenic plants may be unfair due to the facts of in-tuber production versus an in-vitro incubation at the optimal temperature for the enzyme.

The TLC assay for the various CDs showed that plant produced CGTase is capable of synthesizing all of the usual CDs as evidenced by the multiple fused spots found in plant enzymatic reactions. The R_f values were determined for each of the CDs when run separately, 0.49, 0.42 and 0.40 for α, β and γCD. However, when run together the CDs fused into a single long spot. There did appear to be less quantity of αCD compared to the other CDs as shown by the reduced spot intensity at the highest RF.
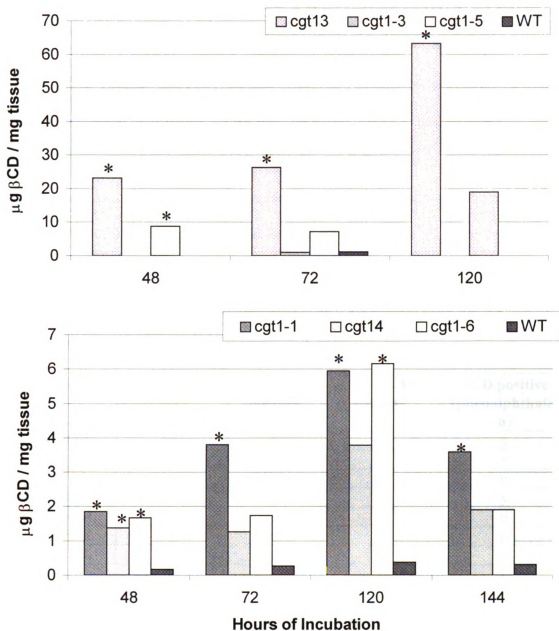
Figure 2.4. Production of βCD by *Arabidopsis* seedlings as shown by colorimetric assay. WT is the parental RLD strain. Cgt lines are independently transformed lines all under control of the Actin promoter. Values are shown as micrograms of βCD per mg of plant tissue. Samples marked with a star are higher than wt control for that time point, $\alpha \leq 0.1$.

However the presence of spots in the transgenic *Arabidopsis* lines, cgt1-1 and cgt14 and

lack of spots in RLD provide additional evidence that *cgt Arabidopsis* are capable of

producing CDs. Tobacco line PE11A-1 also displayed a fused spot on TLC plates with

spots being absent in lines which did not produce detectable βCD as well as the

hygromycin only transformed control line P1-1 (Figure 2.5).

Table 2.2a and 2.2b show the compiled results of antibiotic resistance testing,

genomic PCR, RT-PCR and phenolphthalein analysis methods for all *Arabidopsis* and

tobacco lines.

Table 2.2a. Gene integration, expression, function summary of Tobacco lines, y =

positive result, n = negative result, nt = not tested

Line	Hyg/Kan^R	gPCR positive	RT-PCR Positive	βCD positive (phenolphthalein)
PC8A7	y	y	y	n
PC1B2	y	y	y	n
PE3A1	y	y	y	n
PE11A1	y	y	y	y
PE11A4	y	y	nt	n
PE13A2	y	nt	y	n
PE2B4	y	y	nt	n

Table 2.2b. Gene integration, expression, function summary of *Arabidopsis* lines, y =

positive result, n = negative result, nt = not tested

Line	Hyg/Kan^R	gPCR positive	RT-PCR Positive	βCD positive (phenolphthalein)
PIcgt1-3	y	y	n	n
PIcgt1-5	y	y	y	y
PIcgt13	y	y	y	y
PIcgt14	y	nt	nt	y
PIcgt1-6	y	nt	nt	y
PIcgt1-1	y	nt	nt	y

Figure 2.5. TLC analysis of plant-produced cyclodextrins. Standards are shown in the first 4 lanes, a,b,g CDs with the 4th lane being an equal mixture of all 3 CDs. *Arabidopsis* lines, cgt1-6,cgt14 and the wildtype RLD. Lanes 8-10 show the tobacco lines PE11A1, PC1B2 and the transformed control P1-1.

DISCUSSION

The production of a biologically based surfactant via extracellular secretion of a bacterial enzyme has potential utility for a wide variety of applications. From a contaminated soil remediation standpoint, in-situ production of a relatively non-toxic compound with surfactant–like properties will, along with the expression of novel genes for contaminant degradation, enhance the efficacy of plant-based remediation. *Arabidopsis* production of CGTase is a step towards field implementation of plant-produced solubilizing compounds.

Several challenges are still in place for *cgt*-expressing plants. *In vitro* production of βCD from plant-generated enzyme was generally low, but appeared to increase slowly over incubation time. Despite the presence of positive bands in RT-PCR reactions of almost all tobacco lines, βCD could be detected via the phenolphthalein method in only one line, PE11A1. This line was able to produce detectable quantities of βCD from seedling exudates. The failure of so many tobacco lines to produce CGTase that was active under standard enzymatic conditions, despite production of cgt-mRNA may indicate a basic problem with CGTase expression in tobacco specifically. The fact that two different promoters gave similar results in tobacco makes it unlikely that the promoter itself is the major limitation. It could be that tobacco plants may produce functional transcript in many cases but nonfunctional CGTase protein. Modification of foreign proteins in plant systems through the attachment of various sugars, glycosylation has been documented and might account for the difference in the two species (Samyn-Petit et al., 2003). It is also possible that in tobacco CGTase is poorly expressed in or transported through root epidermal tissues. *Arabidopsis* has much finer roots than tobacco

and the increased surface to volume ratio may allow for enhanced protein diffusion to culture media. Different temporal or spatial expression between *Arabidopsis* and tobacco may cause differences in detectable CGTase activity. Or tobacco may contain a nonspecific protease, released in hydroponic solution that degrades CGTase, which is lacking in Arabidopsis. Degradation of foreign proteins expressed in plant systems, especially when secreted into growth media, has also been a frequent occurrence (Doran, 2006).

The CGTase signal peptide sequence also seems to indicate the potential for low efficiency secretion of CGTase due to a sub-optimal signal peptide for eukaryotic systems, due lack of clarity in splice location. Replacement or modification of the signal peptide to a more plant-like version might assist in further increasing the quantity of secreted CGTase. The *cgt* gene itself, being originally from a bacterial host, may need to be optimized for better expression. While genes from *Bacillus sp.* are generally of relatively high AT content, and usually less of a problem than those from high GC bacteria, they can contain regions that are detrimental to expression such as sequences that appear to be intron splice sites, repeated ATTTA sequences, or a very extreme AT skew (Perlak et al., 1991). PI-cgt is 51% AT and may be relatively suitable as is. But optimization of the main *cgt* sequence may still enhance *cgt* expression to levels capable of providing more CD production.

The low ratios of viable seeds in seedling antibiotic resistance screening, observed in some *Arabidopsis* lines may be due to an overall weakness in the seed lots tested, since hygromycin is a very strong selection agent and genetically resistant but physiologically weak seedlings still might be overcome by the added stress of the selective agent.

Once optimized for efficient production, hydroponically produced CGTase could also be a cheaper, alternative source of industrial enzyme for the commercial production of cyclodextrins. The current bacterial enzymatic systems may continue to out-compete plant produced enzyme until higher expression and/or activity levels can be achieved.

Modification of PIcgt or use of other CGTases with altered CD production profiles, biased towards γCD or αCD may be useful for increasing the range of contaminants that may solubilized by *cgt*-expressing plants. With greater manipulation of the plant biochemical machinery and utilization of more bacterial genes, direct secretion of cyclodextrin into soil may be possible – thus eliminating bacterial competition with freely available exogenous starch substrate. CD uptake mechanisms could also be co-opted from bacteria and inserted into plants to allow for plant uptake of the intact CD-contaminant complex. Used in concert with intracellular CD and contaminant degradation genes, plants could become an improved system for organic contaminant degradation or metal sequestration. Uptake of intact CDs also might allow for very specific nutrient, pesticide or growth regulator delivery to plants via plant absorption of complexed agents added to the soil. Cyclodextrin technologies will continue to grow and evolve, and plants capable of producing cyclodextrins may have applications to very diverse fields.

CONCLUSION

Cgt-Arabidopsis is capable of expression and secretion of bacterial CGTase. Some tobacco transformants are capable of secreting CGTase, but the βCD production is lacking or low in most lines. Plant produced CGTase is capable of producing βCD from

starch. Plants expressing CGTase may be useful in environmental restoration and production of industrial or food grade CGTase enzyme. Future work can enhance the production of CDs from plant sources and potentially combine the benefits of CGTase production with the expression of genes for contaminant degradation, creating a self contained system for biologically based remediation. Further genetic manipulation should yield improved expression and function of the *Pl-cgt* gene in plants.

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CHAPTER III

Effect of Cyclodextrin Glycosyl Transferase expressing plants on the remediation of PAHs and PCBs

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INTRODUCTION

Persistent organic pollutants (POPs) are widespread environmental toxicants and important threats to the global ecosystem. POPs are capable of global atmospheric transport as well as long-term persistence in the environment (Rodan et al., 1999). POPs are strongly hydrophobic and resistant to most forms of degradation, remaining in the environment for decades after deposition partly due to a strong tendency to sorb or dissolve into the organic fraction of the soil matrix. This similarity of chemical properties to large portions of the soil itself contributes to the pollutants' resistance to solar radiation and chemical reactions.

Among the most more notoriously toxic and damaging POPs are the polyaromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs). PAHs occur if organic material is burned with insufficient oxygen and PCBs are synthetically created via chlorination of biphenyl molecules. High molecular weight PAHs are considered both carcinogenic and mutagenic, particularly after some photoconversion processes (Alexander et al., 2002; Brown et al., 1999; Yan et al., 2004). PCBs are thought to be toxic to the endocrine systems of many creatures due to similarity to hormones, causing abnormal developmental effects (Colborn et al., 1993). Both PCBs and high molecular weight PAHs are capable of accumulating in food chains through the process of

biomagnification in which each trophic level concentrates a contaminant at a higher level than the one before (Gobas et al., 1999).

Persistent organic contaminants are most commonly remediated by standard engineering-based techniques such as excavation and off-site disposal, which is labor intensive, expensive, and ecologically disruptive to the treated site (Sellers, 1999). One alternative to standard remediation practices is biologically-based remediation. Unlike engineering-based techniques, biological degradation can transform organic contaminants to less harmful compounds or even mineralize organic pollutants to non-toxic components such as CO_2, chloride and water. A wide variety of microbes have been isolated that are capable of degrading many POPs, including both PCBs and PAHs. Low molecular weight PAHs, such as those with three benzene rings or less, may serve as a carbon source for microbes (Ahn et al., 1999; Daane et al., 2001). PAH degradation becomes more difficult with increasing molecular weight and typically proceeds co-metabolically, requiring the presence of additional substrates for microbial growth (Ho et al., 2000). Highly chlorinated PCBs cannot generally be used as a carbon substrate for microbial metabolism (Boyle et al., 1992; Quensen and Tiedje, 1997).

Plants have been proposed as a potential enhancer of biologically based environmental restoration. Plants have been used to treat a wide variety of pollutants including metals and organic compounds of various types, including PCBs and PAHs (Arthur et al., 2005; Cunningham and Ow, 1996). Phytoextraction is a process in which plants remove and concentrate soluble metals within their tissues. Alternatively, plants may be used to stabilize inorganic contaminants in the soil matrix through the process of

phytostabilization (Berti and Cunningham, 2000). In phytodegradation applications, organic pollutants are transformed to less toxic products after uptake into plant tissues.

Low contaminant bioavailability can severely reduce contaminant biodegradation by plants and microbes (Feng et al., 2000). Bioavailability is a measure of the accessibility of a contaminant to biological systems and is typically related to its water solubility. Microbes, with their high surface area to volume ratios, can more easily utilize compounds with low bioavailability by adsorption to solid phase pollutants and rapid uptake of solubilized molecules (Johnsen and Karlson, 2004).

Many strategies have been proposed to help overcome bioavailability limitations of organic contaminants including various soil amendments intended to liberate strongly sorbed organic molecules. Surfactants, or surface-active agents, are compounds used for the enhancement of hydrophobic compound bioavailability. Surfactants contain a charged hydrophilic portion, which is soluble in water, and a hydrophobic "tail", which is chemically similar to nonpolar organic contaminants. The head to tail organization allows the organic contaminant to associate with the hydrophobic portion of the surfactant, while the hydrophilic portion of the molecule pulls the complex into the aqueous phase, making the compound soluble and therefore bioavailable. Most surfactant molecules must be present in a solution at or above a certain concentration called the critical micelle concentration (CMC) for maximum effectiveness in solubilization (Kile and Chiou, 1989). Micelles are spherical arrangements of surfactant molecules with the charged "head" groups facing outwards towards the aqueous solution and the non-polar "tail" groups facing inwards, creating a hydrophobic cavity to contain hydrophobic pollutant compounds.

Several studies have shown that surfactants can enhance biological degradation of organic pollutants (Bury and Miller, 1993; Fava and Di Gioia, 1998). However, the effects of surfactants on biological degradation can be variable. While micelles can raise the apparent concentration of organic contaminants in the aqueous phase, surfactant-solubilized compounds may still be inaccessible to bacteria and bacterial enzymes (Makkar and Rockne, 2003). Surfactants themselves may cause bacterial toxicity and enhanced movement of toxic compounds through soil, potentially limiting the effectiveness of surfactants (Berselli et al., 2004).

Biologically synthesized surfactants, called biosurfactants, may be a more environmentally friendly alternative to synthetic surfactants (Cameotra and Makkar, 2004). Biosurfactants are generally classed into groups such as glycolipids, phospholipids, fatty acids, surface active antibiotics and polymeric microbial surfactants (Maier, 2003). Biosurfactants, such as rhamnolipids, have been shown to enhance the biological degradation of organic contaminants (Herman et al., 1997; Zhang et al., 1997).

Cyclodextrins (CDs) are another group of biologically synthesized compounds that have been examined for solubilizing effects on contaminants in soil. Cyclodextrins are not true surfactants but are unique cyclic oligosaccharide compounds synthesized by bacteria from starch, capable of enhancing the solubility of hydrophobic compounds in a similar fashion to surfactant micelles (Szejtli, 1988). Unlike surfactants, CDs do not exist as monomers and have no critical micelle concentration, making them largely non-toxic to both plants and bacteria (Apostolo et al., 2001; Bar and Ulitzur, 1994). CDs are enzymatically formed from starch in a mixture of products primarily made up of three CD forms composed of 6, 7, and 8 glucose units, classified as α-, β-, and γCD,

respectively. CDs are structured in such a way that the hydroxyl groups of the glucose subunits face outward leaving the interior of the molecule relatively hydrophobic. While surfactant micelles can grow to almost any size, cyclodextrins are limited by the numbers of glucose units forming the hydrophobic cavity consequently limiting the size of inclusion molecule. Cyclodextrins have been used to enhance the dissolution and biological degradation of various contaminant compounds, including PCBs and PAHs (Fava et al., 1998; McCray and Brusseau, 1998; Wang et al., 1998). Previous work has shown βCD can reduce soil sorption of the PAH phenanthrene (Settavongsin, 2005). CDs have been tested for their ability to enhance desorption and biological degradation of various organic contaminants. Chemically modified CDs are commonly used for applied soil treatments due to the relatively low water solubility of naturally occurring CDs. However, at biologically relevant concentrations, natural CDs have sufficient water solubility to perform as complexing agents (Gao et al., 1998).

Many bacteria are capable of producing cyclodextrins via extracellular secretion of the CD biosynthetic enzyme cyclodextrin glycosyltransferase (CGTase)(Binder et al., 1986; Larsen et al., 1998; Takano et al., 1986). Bacterially produced CGTase creates cyclodextrins by degradation and circularization of starch molecules. For cyclodextrins to be a useful *in-situ* treatment for contaminated soil, starch and CD-producing organisms need to be present in sufficient quantities or CD must be added exogenously. Direct addition of CD can be expensive and labor intensive. βCD has become relatively inexpensive due to easier chemical production, though the price of α and γCD is still relatively high. βCD may have a more limited range of compound solubilization than a mixture of all three CDs. *In-situ* production of cyclodextrins at a contaminated site could

be an effective method of enhancing biologically-based site cleanup if the process of βCD production could be controlled and enhanced. CD producing bacteria may be present, but may be insufficient to produce the needed quantities of cyclodextrin. Starch addition may not promote bacterial CGTase production since *cgt*, like the expression of many secondary bacterial metabolic genes, is tightly regulated (Nishida et al., 1997). The presence of alternative substrates in soil, possibly due to starch degradation by other microbes, may inhibit *cgt* expression, CGTase production, and subsequent CD production. Addition of CGTase producing microbes to soil is subject to the same difficulties of other types of bioaugmentation, including strain persistence and preferred metabolic activity (vanVeen et al., 1997).

We propose that plant secretion of CGTase into the rhizosphere for *in-situ* production of CD would be a cost effective alternative to microbial bioaugmentation or direct addition of CD. Bacterial CGTase is secreted into the soil environment and plants engineered to express this gene may also secrete CGTase. Plant roots are a source of starch in the rhizosphere and could be easily available on a contaminated site, remaining in place for the duration of treatment. If root-produced starch were found to be insufficient, exogenous starch could be added to the soil at considerably lower cost relative to addition of CD. In an effort to generate an alternative *in-situ* source of CGTase, transgenic tobacco and Arabidopsis plants capable of secreting CGTase from their roots were generated through biotechnological procedures. Genetically engineered *cgt*-plants were tested for their effect on PCB and PAH biodegradation rates in contaminated soil.

MATERIALS AND METHODS

PAH Soil Phytoremediation

<u>PAH soil X Tobacco</u>

PAH-contaminated soil was obtained from the Rouge Manufacturing Complex (Dearborn, MI) coke oven facility. The "PAH soil" contained, 13% organic matter, 81% sand, 11% silt and 8% clay as analyzed by A&L Great Lakes Analytical Labs (Fort Wayne, IN). PAH soil was sieved through a stainless steel mesh (2.36 mm) to remove large rocks and debris and thoroughly homogenized. The resulting PAH Soil contained approximately 2000ppm total PAHs (tPAH) – the sum of concentrations of 15 of the EPA priority PAHs found in detectable concentrations. The PAH Soil was placed in 25 X 150 mm glass test tubes, bottom wrapped with aluminum foil to exclude light from the roots. A 10" Pasteur pipet was placed in the test tube before soil addition to facilitate bottom watering. Tubes were well watered and planted with a single 2-week-old tobacco seedling of either wild type or one of two *cgt*-tobacco lines PE13A1 (Line A) or PC1B2 (Line B). Unplanted controls were also included. Plastic wrap was loosely wrapped around loaded test tube racks placed in a plant growth chamber maintained at 25^o +/-3^oC with 16-hour day length (150-230$\mu E*s^{-1}*m^{-2}$). Treatments were split in half with one half being watered weekly with 2ml of autoclaved 1% starch and the other half being watered with distilled, deionized water. Test tube treatments were harvested approximately 50 days after planting with total plant shoot tissue and approximately 40cc of soil from each tube collected.

<u>Soil PAH analysis</u>

For soil PAH determination, 6g of soil were measured into a 40ml vial to which

6ml of aqueous saturated KCl were added, followed by 20ml dichloromethane. Vials

were then capped, using Teflon liners, vortexed for 20 seconds, sonicated in an ultrasonic

water bath for 10 minutes and then shaken on a rotary shaker at 150 RPM for 16 hours.

After overnight shaking the vials were removed and set upright for 10- 20 minutes. A

portion of the lower layer of solvent but above the settled soil was removed with a

Pasteur pipet and filtered through a glass wool stuffed Pasteur pipet. Filtrate was placed

in GC vials and analyzed via an Agilent GC 6890 equipped with an Agilent 3396 B/C

integrator and Agilent 7683SLS auto sampler and injector, ICB PAH column and flame

ionization detector. The GC conditions were: ICB-PAH capillary column 15m in length

250µm i.d. 0.15µm film thickness (J&K scientific, Milton, Ontario), with helium carrier

gas at 41Kpa constant pressure, 270°C inlet temperature, flame ionization detector

maintained at 330°C. Column initial temperature was 80°C, followed by elevation to

220°C at 40°C per minute, and a second ramp including elevation to 285°C at 8°C per

minute. The injected volume was 4µl with a split ratio of 7:1. The PAHs measured and

summed to determine the total soil PAH concentration (tPAH), listed in order of

increasing molecular weight and retention time were, napthalene, acenapthylene,

fluoranthene, phenanthrene, anthracene, fluorene, pyrene, benzo[a]anthracene, chrysene,

benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, indeno [1,2,3-CD]pyrene,

and benzo[ghi]perylene. tPAH is the sum of the 14 compounds typically found in the

Rouge soil in highly unequal proportions (Table 3.1).

Table 3.1. Percentage of relative concentration of each of the individual PAH compounds in reference to the total PAH content of the soil.

Compound	Percentage of tPAH
napthalene	1.8
acenapthylene	3.6
fluoranthene	0.9
phenanthrene	5.7
anthracene	2.8
fluorene	15.3
pyrene	15.1
benzo[a]anthracene	9.7
chrysene	6.6
benzo[b]fluoranthene	11.5
benzo[k]fluoranthene	4.8
benzo[a]pyrene	11.9
indeno[1,2,3-CD]pyrene	2.5
benzo[ghi]perylene	7.7

Plant Biomass and Soil Moisture determination

Fresh weight of plant aboveground tissues was determined immediately after sample harvest and dry biomass was determined after drying to static weight in a 60°C oven. Soil moisture determination was accomplished by weighing 5g treatment soil subsamples into aluminum weigh pans, oven drying at 105°C for 24 hrs, and reweighed for dry weight.

PCB Soil Phytoremediation

PCB soil X Arabidopsis

Industrially contaminated PCB soil was obtained from the Kalamazoo River Basin Superfund site (EPA ID# MID006007306). The collected soil was stored in steel cans at room temperature for approximately 6 months prior to use. In preparation for the experiment, soil was sieved first through 5mm steel mesh and then through 2.36 mm stainless steel mesh. Soil was thoroughly homogenized and poured into aluminum foil wrapped 25 X 150 mm glass test tubes. Soil was watered and tubes planted with wildtype or *cgt*-Arabidopsis seedlings or left as unplanted controls. Plastic wrap was loosely wrapped around loaded test tube racks placed in a plant growth chamber maintained at 25° +/-3°C with 16-hour day length (150-230μE*s^{-1} *m^{-2}). Test tubes were watered with 2ml autoclaved 1% starch in water 10 days after planting (DAP), 1/4 X MS salts at 19 DAP, 3ml 1% starch at 33 DAP. Two treatment times were used prior to sampling; 72 DAP (N = 4 tubes each treatment) and 92 DAP (N = 7 tubes).

Spiked PCB Soil X Tobacco

Soil was obtained from Wayne County, Michigan and classified as a Hoytville series, a silty clay loam. This soil was spiked with PCB by addition of Aroclor 1248 diluted in acetone resulting in a calculated final concentration of 100ppm. The spiked PCB soil was stored dry at room temperature for a period of 2 months and then added to 25 X 150 mm glass test tubes. Transgenic *cgt-* or wild type tobacco seedlings were planted in each tube with left unplanted. Plastic wrap was loosely wrapped around loaded test tube racks placed in a plant growth chamber maintained at 25° +/-3°C with 16-hour day length (150-230μE*s^{-1} *m^{-2}). At 4 days after planting (DAP) all tubes were watered with 2ml 1% starch. All treatments were destructively sampled after 74 DAP.

In order to examine the effect of bioaugmentation with PCB soil extract inoculum on the spiked PCB treatments, a soil extract was made by washing an aliquot of Kalamazoo River Superfund site soil in 1% tetrasodium phosphate buffer (1:1000 :: soil:buffer). To determine total colony forming units (CFUs), soil extract samples were plated on YEPG agar (Per liter: 1g glucose, 2g polypeptone, 0.2g yeast extract, 0.2g NH$_4$NO$_3$, and 15g Bactoagar) and visible colonies enumerated after 12d incubation at 25°C.

PCB analysis

PCB extraction was conducted using an accelerated solvent extractor, ASE 200 (Dionex, Sunnyvale, CA). Ten grams of dry soil were mixed with an equal amount of sodium sulfate (Sigma, St. Louis, MO) and placed into a 33ml extraction cell with a glass fiber filter (P/N 047017, Dionex, Sunnyvale, CA) on the bottom side of the cell. Void

space in the cell was filled with Ottawa sand (S23-3, Fisher Scientific, Pittsburg, PA). Cells were capped and placed on the machine with pre-weighed 60ml glass collection vials for retention of solvent extract. The ASE program was: 1 minute preheat, 5 min heat, 5 min static time, 60% flush and a 60 second purge. The extraction solvent was 50% acetone and 50% hexane. After extraction, the collection vials were weighed to determine extract mass and recapped with undamaged septa. ASE samples were transferred to GC vials and analyzed on an Agilent GC 6890 (Agilent, Santa Clara, CA) equipped with a Supelco Equity 5 column (Supelco, Bellefonte, PA), 30m in length 320μm i.d. and 0.25μm film thickness. The GC program consisted of a 4μl splitless injection with the inlet temperature at 250°C, helium carrier gas with pressure set at 59KPa. The initial run temperature was 120°C increased by 9°C per minute to 300°C. The detector was an Agilent Micro ECD set at 310°C with 30ml/min nitrogen makeup. Ten representative peaks were chosen based on fraction of total area and uniqueness in the Aroclor 1248 standard as compared to the Aroclor 1254 standard (Supelco, Bellefonte, PA). The sum of these peaks was taken to represent the total PCB content of the soil.

RESULTS and DISCUSSION

PAH Soil Phytoremediation

The effects of *cgt*-tobacco plants with and without supplemental starch addition on soil PAH degradation were examined. Starch appeared to enhance tPAH reduction in two of the nine treatments: unplanted (26.3% soil [tPAH] reduction) and *cgt*-tobacco line B (24.9% soil [tPAH] reduction) compared with the untreated control soil (Figure 3.1).
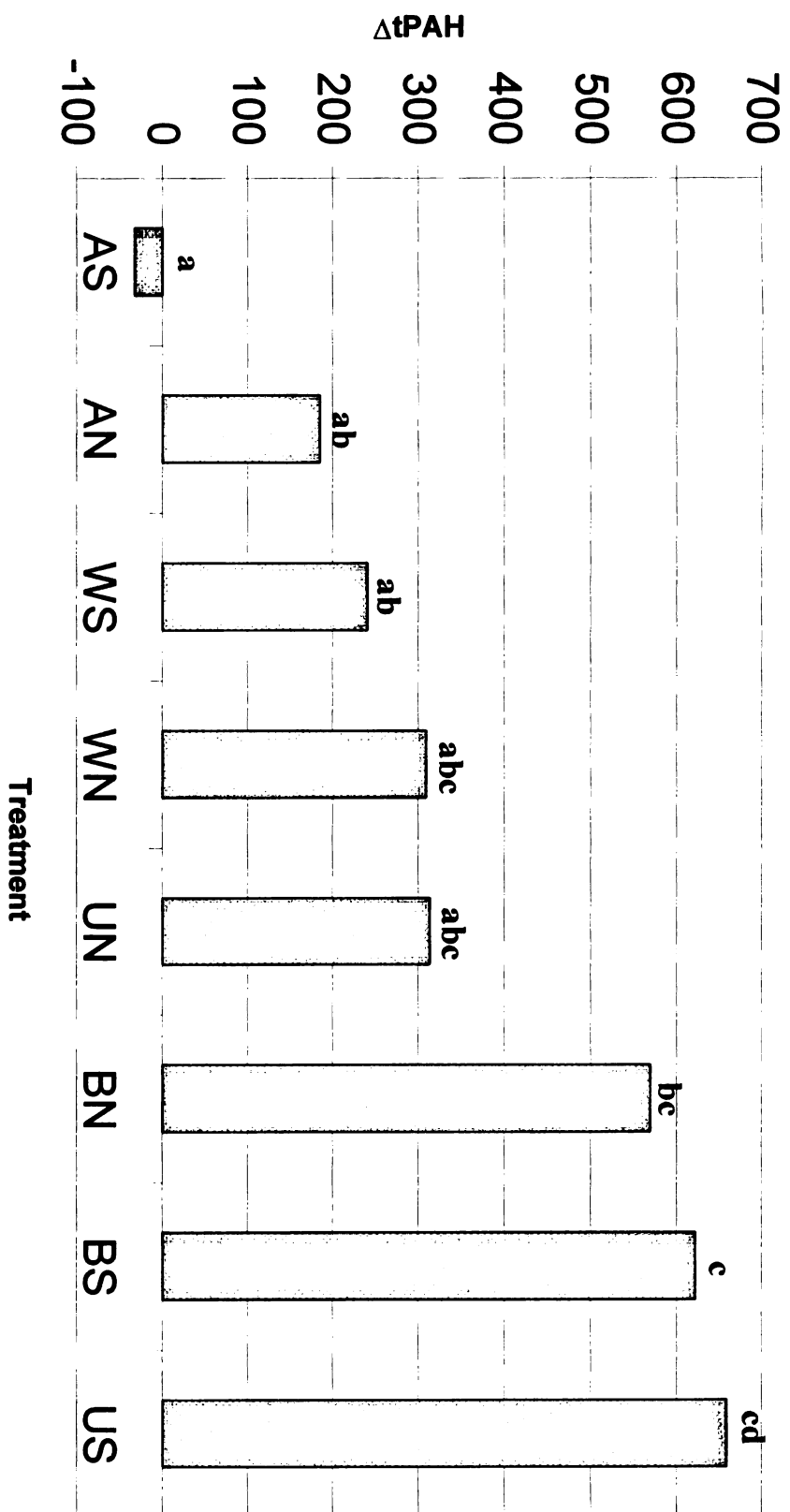
Figure 3.1. tPAH content of soil from PAH Soil Cgt-Phytoremediation treatments. Treatment codes: A = Cgt-Tobacco-PE13A2, B = Cgt-Tobacco-PC1B2, W = Wildtype, U = Unplanted, N = No starch addition, S = Starch added, TZ=Untreated control. ΔtPAH shows percentage change in soil [tPAH] relative to untreated control. Statistically similar treatments (α < 0.05) are denoted with the same letter.

However, these two treatments were also statistically similar to the unamended and unplanted treatments. Unamended *cgt*-tobacco line B also showed a significant reduction in tPAH content (28.1%). Other than these three treatments, no significant reduction in tPAH content was observed in any other plant genotype-amendment treatment combinations compared to untreated control.

While most individual compounds exhibited similar patterns of reduction to that shown by tPAH, a few compounds displayed interesting differences. Benzo[ghi]perylene (BGHP), the highest molecular weight PAH measured by our methods, also showed a similar pattern with all treatments being significantly lower than the untreated (Figure 3.2).
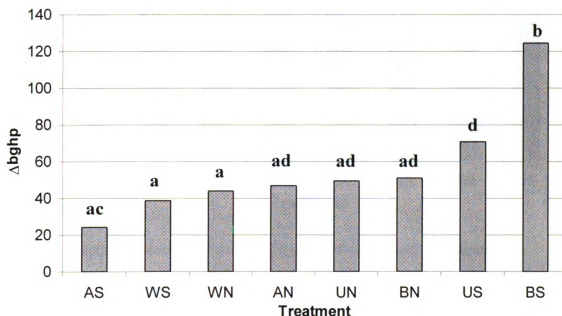

Figure 3.2. Benzo[ghi]perylene (BGHP) content of soil PAH Soil Cgt-Phytoremediation treatments. Treatment codes: A = Cgt-Tobacco-PE13A2 B = Cgt-Tobacco-PC1B2 W = Wildtype U = Unplanted N = No starch addition S = Starch added. Δbghp shows

percentage change in soil [BGHP] relative to untreated control. Statistically similar treatments ($\alpha \leq 0.05$) are denoted with the same letter.

Starch-watered *cgt*-tobacco line B showed a significantly lower content of BGHP than unplanted and wildtype treatments both starch treated and water only, with a 55, 53, 43 and 51 percent reduction, respectively. Napthalene, the lowest molecular weight PAH, had significantly reduced levels in all treatments when compared to the untreated control (Figure 3.3).
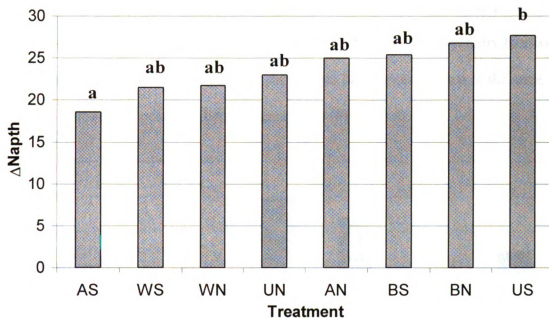

Figure 3.3. Napthalene content of soil PAH Soil Cgt-Phytoremediation treatments. Treatment codes: A = Cgt-Tobacco-PE13A2 B = Cgt-Tobacco-PC1B2 W = Wildtype U = Unplanted N = No starch addition S = Starch added. ΔNapth shows percentage change in soil [naphthalene] relative to untreated control. Statistically similar treatments ($\alpha \leq 0.05$) are denoted with the same letter.

Napthelene was most likely lost by a combination of volatilization and biodegradation, since its low molecular weight makes it more susceptible to biological degradation and loss by volatilization.

The high levels of PAHs and poor agronomic qualities of the Rouge soil resulted in highly stressed plants in all treatments, with yellow leaves and accelerated leaf senescence, although this appeared to be less of a problem in treatments with starch addition, potentially indicating some protective effects of starch on plant health. Interestingly the dry biomass of line B *cgt* and wildtype treatments seemed to positively correlate, although not significantly, with the addition of starch. However, dry biomass between treatments showed no significant differences between treatments of the same genotype, or the same treatment between genotypes (Figure 3.4).
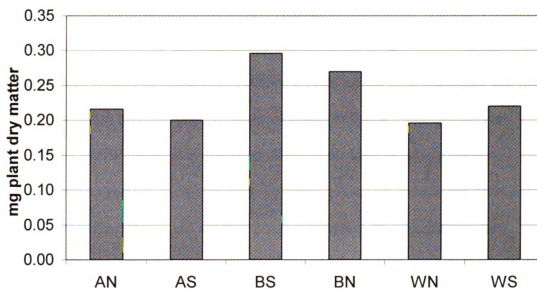

Figure 3.4. Plant dry biomass from soil PAH Soil Phytoremediation treatments. Treatment codes: A = Cgt-Tobacco-PE13A2, B = Cgt-Tobacco-PC1B2, W = Wildtype, U = Unplanted, N = No starch addition, S = Starch added. No significant differences were observed ($\alpha \leq 0.05$).

Plant survival rate appeared to be enhanced in the starch amended wild type treatment. Starch amended wild type had 17 surviving plants versus only 9 in the unamended treatment. Both transgenic tobacco lines showed similar survival rates in starch treated and unamended treatments.

One problem associated with this study was the choice of tobacco lines. Neither PC1B2 (Line B) nor PE13A1 (Line A) have been shown to produce detectable quantities of CD under enzymatic digest conditions. It is possible that both lines were producing small amounts of CGTase at levels, which are undetectable by direct enzymatic analysis. Or *in-vitro* enzymatic analysis may also not reflect the ability of plant produced CGTase to function in soil, or the potential for plant production of CGTase when grown in soil. *cgt* line B was shown to produce light clear zones on starch containing media. However, it is likely that the quantity of CDs produced by these tobacco lines in soil is quite low. A significant decrease in contaminant levels by a starch watered cgt line B was observed for at least one high molecular weight PAH (BGHP), compared to both unplanted and wild type treatments. It is possible that low levels of plant-produced CD promoted the degradation of BGHP, perhaps by partly solubilizing the high molecular weight compound. The extremely low water solubility of BGHP might be such that even a slight increase in bioavailability would result in a large increase in biodegradation and a subsequently noticeable reduction in concentration. The reductions seen in BGHP concentration by starch treated *cgt*-tobacco line B may hint that positive effects on other PAHs may be possible with increased CD concentration or incubation time. The presence of cyclodextrin alongside the effects of starch may still provide benefits for degradation even if not quantitatively observable for most of the PAH compounds.

Starch addition seemed to be a contributing factor in promoting tPAH degradation in Rouge soil, especially notable in the unplanted treatment. The highest reduction in tPAH and BGHP specifically were seen in starch-amended treatments. Enhancement of biodegradation promoted by the addition of a substrate such as starch has been seen in many other treatment studies when carbon sources are added to impoverished soils (Haby and Crowley, 1996; Leigh et al., 2002; Rao et al., 1995).

The effect from starch alone on tPAH reduction was similar to any effects of cyclodextrin produced from starch, at least by *cgt*-tobacco line B, in total PAH degradation. Even so, the presence of cyclodextrin alongside the effects of starch may still provide benefits even if not quantitatively observable for the majority of PAH compounds. Starch combined with plants was not always effective as both *cgt*-tobacco line A and wild type plants treated with starch showed no significant reduction of tPAH when compared with untreated soils. It is possible that these plant lines were in some way presenting a hindrance to PAH degrading microbes, perhaps by maintaining unfavorable soil conditions, when compared to *cgt*-tobacco line B. These lines may have been producing exudates that were unfavorable to microbes while *cgt*-tobacco line B was not, and the CD production of this line was able to mitigate the apparent negative effects of tobacco on PAH degradation.

Future analysis of the effects of starch and CD producing plants on the microbial community, specifically starch and PAH degrading microbes might provide additional information on the effects of CGTase expressing plants on PAH impacted soil and clarification of the results of the experiments. The use of single spiked compounds might

be another way of teasing apart the interactions of the plants, cyclodextrin, and PAH molecules.

PCB Soil Phytoremediation

     Cgt-Arabidopsis was tested with supplemental starch addition for enhancement of PCB biodegradation. The *cgt*-Arabidopsis line chosen for the PCB phytoremediation experiment was demonstrated to produce CGTase with subsequent conversion of starch to CD in bioindicator assays (as described in Chapter 2). These observations make it likely *cgt*-Arabidopsis line 14 was producing βCD *in-situ* for some if not all of the treatment period.

     After 72 days of growth, all of the treatments had significantly lower PCB content than the untreated control. However, no individual treatment was significantly different from any other treatment, with soil [PCB] reductions of 34%, 32%, and 32% for *cgt*-Arabidopsis, wild type Arabidopsis, and unplanted treatments, respectively (Figure 3.5).
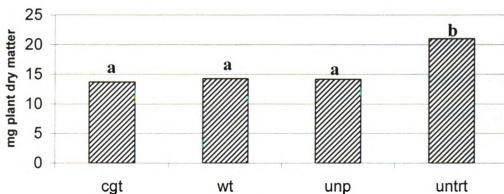

Figure 3.5. PCB content of soil from PCB Soil Cgt-Phytoremediation treatments. *Arabidopsis* cgt14, wildtype, unplanted and untreated are listed from left to right. Values are after 72 days of plant growth. Statistically similar treatments ($\alpha < 0.05$) are denoted with the same letter.

Plants would have to make a very large difference over unplanted treatments in what is already a miniscule amount of contaminant with many individual PCB peaks being less than one ppm in soil concentration. Additionally, the chronic contamination of the soil may be very resistant to large changes in contaminant content, due to long-term sorption of some proportion of the PCBs. Low levels of CDs produced by cgt-Arabidopsis may have partly counteracted the plant induced effect resulting in the slightly lower PCB content in cgt-Arabidopsis treatments. The effects of cgt-plants on PCB degradation appears to be minimal in this experiment, in either time point as unplanted samples were lower and statistically indistinguishable from planted treatments. The simple mixing of soil and the addition of water may explain the overall loss of PCBs. These actions may have stimulated preexisting microbes, by increased exposure to oxygen due to breakup of large aggregates through sieving. The low levels of PCBs in the Kalamazoo soil may have contributed to the inability to detect significant differences between the treatments.

Overall, resulting plant biomass of the cgt-Arabidopsis line was significantly lower (25% less dry mass) than that of the wild type control plants (Figure 3.6). This may be explained by the presence of the transgene causing pleiotropic effects or possibly lower seed vigor from the seed stocks used due to previous antibiotic selection of the parental plants.

Spiked PCB Soil Phytoremediation

The spiked PCB experiment was distinct from the other trials in that it used freshly spiked soil rather than aged, industrially contaminated soil. The relatively low

117

levels of PCBs observed in the untreated sample (25 ppm) compared to the calculated concentration of PCBs (100 ppm) was likely due to volatilization during the aging period.

The spiked PCB soil treatments were highly variable in contaminant removal, though bioaugmentation with microbial extract from industrially contaminated soil appeared to improve effectiveness (Figure 3.7).

Extract-inoculated soils of the unplanted and *cgt*-tobacco line B planted treatments displayed greater [PCB] reduction that uninoculated soils. These results suggest that the soil extract supplied PCB biodegrading microbes to the inoculated soils, which may have been lacking in the "clean" field soil initially spiked in this study. Soil PCB reduction was significant in one treatment with a 45% reduction by inoculated *cgt*-tobacco. However, the inoculated unplanted treatment was statistically similar to inoculated *cgt* treatments suggesting that soil inoculation may have a larger impact on PCB reduction than the presence of plants or the production of CGTase. Given the probability of low levels of CGTase present in the soil and the potential for a nearly total lack of PCB degrading organisms in spiked soil, this result may be somewhat expected. Soil spiking very often results in a significant mortality in soil microbes such that few native microbes may have remained to carry out degradation (Brinch et al., 2002). The total number of culturable microbes added from the soil inoculum was 7746 per tube, 193 per gram based on an average of 40 grams (DW) soil per tube. Since the spiked soil was maintained in a dry state for several years and was never contaminated, it is also likely the overall microbial population as well as potential PCB degraders was very low. A sudden appearance of PCB degrading microbes in contact with relatively labile contaminants, due to "fresh" spiking, might have resulted in relatively rapid degradation.
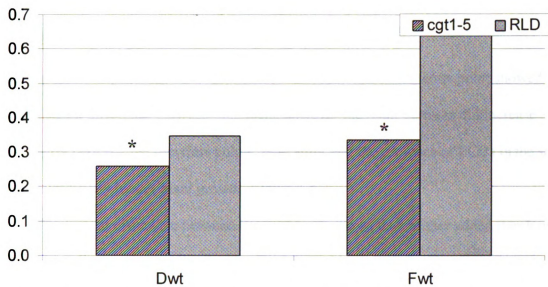
Figure 3.6. Biomass of Arabidopsis shoot tissues in PCB Soil Cgt-Phytoremediation treatments, 92 days after planting. Dry (Dwt) and fresh (Fwt) biomass are shown. Stars denote treatments significantly lower biomass ($\alpha < 0.05$).
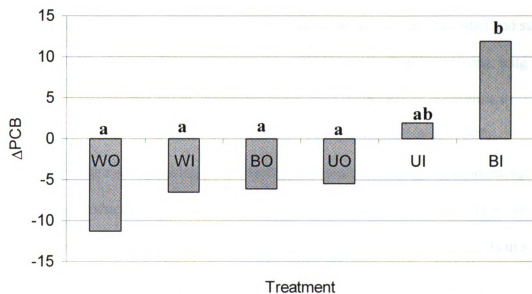
Figure 3.7. Percentage change in soil PCB content from spiked PCB Soil Cgt-Phytoremediation treatments as compared to untreated control. U = unplanted, W = wildtype, B = PC1B2 cgt-tobacco, I = inoculated, O = not inoculated. Statistically similar treatments ($\alpha < 0.05$) are denoted with the same letter.

Unequal dispersal of these microbes in soil inoculum might have resulted in the observed scattered, but high degradation rates in some samples.

In terms of plant dry biomass, none of the treatments of any time point showed any significant difference between the others, however the plant biomass did increase significantly between the two time points, indicating that the presence of PCBs in the soil did not excessively hinder plant growth (Figure 3.8).

The lack of difference between the treatments shows that neither added starch nor the presence of the transgene in tobacco affected plant growth.

CONCLUSION

Several studies on two different contaminant classes, PCBs and PAHs, were done to examine the effects of *cgt*-expressing plants, starch addition, and bioaugmentation for soil remediation. All but one of the tested soils was chronically contaminated and such soils are less likely to see large reductions in contaminant levels due to strong, long term sorption of contaminants to soil components and high variability of contaminant concentration within those soils (Burgos et al., 1996; Carmichael et al., 1997). Additionally, the *cgt*-expressing plants used in this study are first generation transgenics and may not produce sufficient CGTase to make substantial quantities of CD *in-situ*. Despite these limitations, several of the studies yielded some interesting results in soil contaminant loss and plant biomass production.

In this study *cgt*-Arabidopsis and *cgt*-tobacco were tested for their effects on PAH and PCB remediation. The results of the biodegradation experiments showed positive effects by one tobacco line on at least one PAH compound and under specific treatment
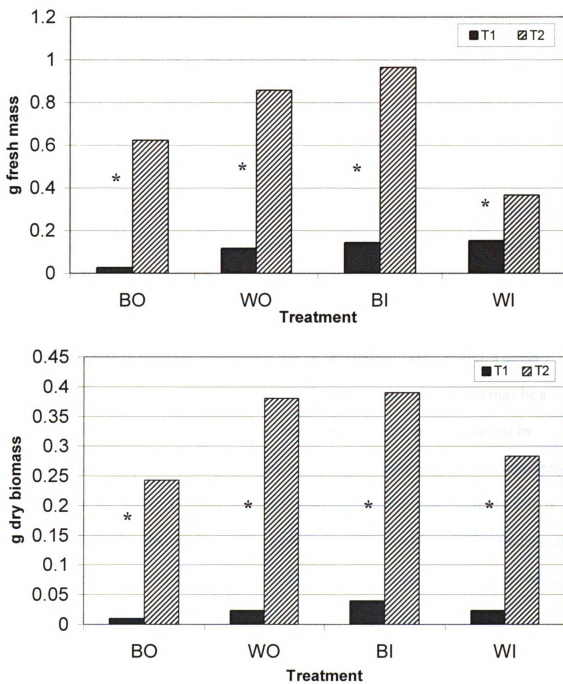
Figure 3.8. Tobacco fresh and dry biomass in spiked PCB Soil Cgt-Phytoremediation treatments. W = Wildtype, B = PC1B2, I = Inoculated, O = Not inoculated. Stars denote treatments significantly lower at $\alpha < 0.05$

conditions for PCBs. Starch addition was beneficial for soil PAH reduction and contaminated soil extract inoculation appeared to enhance PCB biodegradation. *Cgt*-tobacco enhanced dissipation of the highest molecular weight PAH compound, benzo[g,h,i]perylene, in the phytoremediation study, though most of the other PAH compounds did not show clear reduction. The lack of strength in the results may be due to a large number of factors including low expression/production of CD in soil. The high variability of contaminants in many of the soils also may have hampered efforts to show significant reductions on contaminant concentration that might be a direct result of transgenic plants. Future experiments may be able to show the positive effects of *cgt*-plants on a wider range of compounds and confirm the effects of *cgt*-plants on BGHP. Given the difficulty in showing quantitative loss of organic pollutants in soil, a direct assessment of mutagenicity or toxicity reduction in treated contaminated soil may be a better measure of phytoremediation success. In addition to being less constrained by contaminant variability, direct toxicity/mutagenicity experiments would more completely show whether remediation, in terms of true reduction of the toxic qualities of the soil, is occurring. Information about the microbial community might also be useful in finding out what sort of effect CDs and plant produced CGTase might have on numbers of microbial degraders. Hopefully, with better lines and expression, coupled with additional experiments the effects of *cgt*-plants on the degradation of contaminants in soil will become clearer.

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CHAPTER IV

Is Phytoremediation Safe? A Comparison of Risks and Management Strategies of Plant-Based Environmental Remediation Technologies and Their Engineering-Based Counterparts

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INTRODUCTION

Phytoremediation is the use of plants to remove, stabilize and/or detoxify soil or aqueous contaminants. Phytoremediation is a relatively young technology initially focused on the removal of toxic metals from soil but broadened to include organic contaminants (Baker et al., 1994; Bell, 1992). Public perception of phytoremediation as an ecologically compatible, cost effective and aesthetically pleasing alternative to more disruptive standard remediation approaches has helped fuel and even driven the growth of the technology.

Engineering-based remediation which, are technologies that use purely physical and abiotic chemical means to stabilize, destroy, remove or contain pollutants, is the most commonly implemented treatment for contaminated sites. While the potential risks of most of these traditional treatments have been thoroughly evaluated (Wickramanayake et al., 2000), risks associated with biologically based technologies, especially phytoremediation, have received relatively limited consideration (Angle and Linacre, 2005; Linacre et al., 2003). Because public perception of phytoremediation is almost invariably positive, this paper will focus on actual risks posed by the technology rather than perceived risks. This paper will address the risks and benefits associated with the various phytoremediation technologies by elaborating on an approach to risk-benefit

considerations that should be taken into account when deciding on a technology. Exploration of potential hazards inherent in applied phytoremediation will allow preemptive management and containment of those risks, while maximizing its effectiveness as an environmental rehabilitation tool. In thus study we will define phytoremediation as "safe" if it can be determined to be at least as safe as widely accepted and utilized engineering based approaches.

RISK ASSESSMENT AND RISK MANAGEMENT

Risk has been defined in various ways, and has generally been thought of as both a chance for a bad outcome and the bad outcome itself. Multiplying a numerical probability of a particular risk by the potential severity of the risk mathematically derives a standard measure of risk. Risk analysis is a tool that looks for the approach that presents the lowest overall risk. However, a more obviously logical approach can be the risk benefit analysis, which combines the assessment of risks alongside of the benefits and the probabilities of those benefits. A risk benefit approach will be used to compare phytoremediation and engineering based technologies. Exposure is the key component of risk on a contaminated site. If there is no route of exposure, risk from contaminants is minimized. A technology's effects on routes, frequency, duration and degree of exposure of the pollutants to receptors are all important factors in determining the nature of a risk from a particular contaminated site. Considerable research has been done on methods of environmental risk assessment for quantitative risk analysis, which utilize numerical measurements of probability usually in very specific situations (Alexander, 2000; Al-Yousfi et al., 2000; Oberg and Bergback, 2005).

Despite the obvious ease in decision making when utilizing numeric comparisons of risk, there are instances where quantifiable probabilities of bad outcomes are difficult to obtain. Locations of high levels of pollutants are frequently completely unpredictable, especially when they are the result of individual spillage events. Even after thorough mixing individual soil particles may hold chunks of highly concentrated contaminant. Complicating remediation, the histories of contaminated sites may be unknown and new impacted areas, higher levels of contamination or new pollutant types may be uncovered during remediation. These factors make it difficult to obtain a precise value for risk probability from phytoremediation installations.

RISKS AND BENEFITS FROM STANDARD REMEDIATION TECHNOLOGIES

Contaminated sites are currently most frequently remediated via standard engineering based techniques. Engineering based remediation techniques can be broken into two groups, treatments that remove or destroy pollutants in soil, sediment or water and those that stabilize contaminants within the matrix. Technologies that stabilize contaminants in soil are, excavation and off site disposal, stabilization and solidification. Excavation and off site disposal is the most commonly used treatment for contaminated soils, which involves removal of the soil and disposal at an approved landfill. But this type of treatment generates some new risks; disturbance of contaminated soil and sediment during excavation operations may, at least in the short term, increase wind and rain erosion of hazardous particulates or bulk soil material. Excavation activities can also pose a significant risk to workers dealing with the contaminated soil (Cohen et al., 1997; Proctor et al., 1997). Landfilling of excavated wastes is not a permanent solution for

treatment of soil contamination. At some point hazardous waste landfills will leak and pose recurring and complex hazards to adjacent communities and natural resources. Many sites awaiting remediation are former landfills, with over 200 of the approximately 2000 sites listed on the EPA national priorities list, being former landfills (EPA, 2006).

Methods for removing or destroying contaminants in soil sediment and water include, chemical extraction, soil washing, soil incineration, thermal desorption, soil vapor extraction, air sparging, pump and treat, reactive barriers and bioremediation. Table 4.1 contains a list of accepted technologies, along with potential risks, costs and target contaminants.

Benefits of Standard Remediation Technologies

Engineering technologies have their own set of benefits and advantages, these tend to vary within individual technologies. The main benefit of most engineering based operations and especially that of excavation based techniques is the nearly complete and immediate removal of risk from a site, this particular quality is the primary reason for the popularity of excavation as a treatment solution. Some engineering based technologies are capable of completely removing a contaminant from the soil, usually resulting in destruction of or damage to the soil as a result of treatment.

RISKS AND BENEFITS FROM PHYTOREMEDIATION

Phytoremediation technologies are divided into two basic approaches based on target pollutant chemistry. An organic contaminant can be broken down into its constituent elements. PCBs, for example, can be degraded to carbon dioxide and chloride

131

Table 4.1. Summary of Accepted Remediation Technologies and Associated Potential Risks

Technology	Description	Target Contaminants*	Estimated Costs (US $ per cyd or per 1000 L)	Potential Risks	References
Air-sparging	Forcing air into the vadose zone to reduce subsurface contaminant concentration	NAPL, TPH	$10.36 (assumes no offgas treatment)	contamination spread into groundwater vapor migration required aquifer clogging	(Miller, 1996)
Soil Vapor Extraction (SVE)	Removal of volatile contaminants via removal of saturated air.	NAPL, TPH	$134 (average)	loss of contaminants to air	(EPA, 2001)
Bioremediation	Degradation of contaminants by bacteria	PCB, PAH, TPH, SVOC, POP	$270 (average)	Bacterial escape Failure of treatment	(EPA, 2001)
Chemical washing	Removal of contaminants by solvent extraction	PCB, PAH, SVOC	$110-$540	Remaining chemical solvent, contaminant/solvent evaporation to atmosphere	(EPA, 1997)
Incineration	Treatment of excavated soil, sediment, sludge by high temperature burning	PCB, PAH, TPH, SVOC, POP	$546 (average)	Noxious fumes/loss of contaminants to air	(EPA, 2001)
Landfilling	Excavation and transport to approved area for piling or burial	All soil and sediment contaminants	$100-$600	Wind, rain, or mechanical erosion during excavation, Leaching runoff from landfill site.	(EPA, 1997)
Pump & treat	Groundwater pumping followed by physical or chemical treatment	NAPL	$32 average annual cost per 1000 gal groundwater treated	Release of fumes Containment failure	(EPA, 2001)
Reactive barrier	Underground barrier against leaching contaminants – degrades or immobilizes	HM, NAPL	$5.30 per 1000 gal groundwater treated	Clogging or failure of barrier	(Schnoor, 2002)
Solidification	Immobilizes contaminants by chemical solidification	All soil and sediment contaminants	$240-$340	incomplete immobilization/later breakdown of solidified material	(Schnoor, 2002)
Surfactant washing	Removal of contaminants by surfactant treatment	HM, PCB, PAH, SVOC	$80-$200	Leaching of contaminants Remaining surfactant	(Schnoor, 2002)
Vitrification	High temperature thermal treatment causing solidification of soil structure	All contaminants in dewatered soil	$100-$1000	Noxious fumes/loss of contaminants to air incomplete immobilization	(EPA, 1997)
Thermal Desorption	Thermal treatment to cause volatilization of contaminants from soil	PCB, PAH, POP	$199/ton (average)	Noxious fumes/loss of contaminants to air	(EPA, 2001)

* DNAPL = dense non-aqueous phase liquid, HM = heavy metal, NAC = nitroaromatic compounds, NAPL = non-aqueous phase liquid, NP = nutrient pollutant (or nitrogen and phosphorous), PAH = polyaromatic hydrocarbon, PCB = polychlorinated biphenyl, TPH = total petroleum hydrocarbon, SVOC = semi-volatile organic compounds

ions, but inorganic contaminants such as lead and mercury cannot be changed and can only be converted to alternate valence states or integrated into different compounds.

Inorganic Phytoremediation

Phytoextraction is one of the favored approaches in phytoremediation of metals, due to the unique ability of some plants, which are capable of extracting and concentrating metals within their tissues. Plants that naturally accumulate up to 2% of a metal in their leaf tissues are called hyperaccumulators (Baker et al., 1994). In phytoextraction, plants capable of concentrating metals to some degree, preferably hyperaccumulators, are planted on a contaminated site. After a period of growth and biomass accumulation, the plant material can be subsequently harvested and either landfilled or the metals reclaimed in cases where it is economically viable (Li et al., 2003). Despite the very high tissue concentrations some hyperaccumulators can achieve, the rate of remediation can still be quite slow. An early study using *Thlaspi caerulescens* showed it would take a minimum of 13 years to remediate the soil under treatment (Baker et al., 1994).

Consumption of contaminated plant material by wildlife is another potential risk of phytoextraction using hyperaccumulators. Herbivory from large animals can be controlled by means of a fence, however more stringent means might be necessary to control insect feeding and prevent dissemination of toxins through the food chain. In most studies, given a choice, herbivores tend to reject contaminated plant material if uncontaminated plants are available. This pattern has been observed plant material containing Zinc and Selenium (Hanson et al., 2004; Pollard and Baker, 1997). But the

degree of feeding choice within an industrial setting, where many contaminated sites are found, might be very low, with very little to no other surrounding vegetation. One potential mitigating factor in the potential for hyperaccumulator escape is that many hyperaccumulating species are specifically adapted to grow on metal enriched soils and do not thrive elsewhere (Baker and Brooks, 1989). This makes the likelihood of a plant species being capable of both high metal content and vigorous growth on uncontaminated soils relatively low.

Increased bioavailability is another potential risk from phytoremediation, but exposure via direct contact might actually be reduced, since metals in soil may be present in the aqueous soil solution whereas plant tissues usually sequester metals within the vacuole of the cell using various transport proteins (Elbaz et al., 2006; Kupper et al., 1999). Although the likelihood of exposure from direct contact and inhalation may be reduced due to plant coverage of the soil and moisture retention, remaining leaf litter would provide a more concentrated source of the contaminant and a new risk when compared to the unaltered soil.

After hyperaccumulation has been utilized in the field and the plants harvested, there still exists the problem of dealing with the plant tissue. In situations where the removed metals are economically valuable, incineration or pyrolysis may be used to release the stored metals from dry plant tissue so that they may be recycled and utilized for manufacture (Li et al., 2003). In other situations where metals are not intended for reclamation, the dry tissue may be stored or landfilled.

Some metals are difficult for plants to take up naturally into the above ground tissues. Chelators such as ethylenediaminetetraactetic acid or EDTA can enhance plant

accumulation of insoluble metals. In lead phytoremediation, chelators are typically utilized to help solubilize the lead and allow plants to take up the metal (Cooper et al., 1999). When chelators enhance the water solubility of metals they also increase mobility in soil and bioavailability. The enhanced bioavailability of metals will also cause any fauna that ingests the metal contaminated soil to be at higher risk due to the EDTA facilitating absorption. Chelators may also cause the movement of metals lower into the soil profile and even into groundwater. Movement of lead into groundwater has been a documented occurrence on at least one chelator assisted phytoremediation installation, however the problem of leaching on this particular site was probably exacerbated by the short growing season and poor plant growth (ESTCP, 2001). This study shows chelator enhanced phytoremediation should be carefully managed and planned, taking into account rainfall events, sorption of contaminants to soil particles, preferential flow of water through the soil and distance to groundwater. The application of a chelator may not only enhance the mobility of the target metal it may also enhance the movement of other metals in the soil. This necessitates that analyses be performed on other metals so that their movement into groundwater would not go unobserved. Technological combinations such as phytoextraction with permeable reactive barriers could reduce the risk of chelate-induced leaching considerably, by over 60 times in one lead phytoremediation study (Kos and Lestan, 2003).

An alternative treatment strategy for inorganic contaminants is phytostabilization, in which plants stabilize the elements within the soil and prevent their dissemination by wind and rain. Plants can also alter the pH of soil by secreting organic acids, potentially affecting the sorption of contaminants (Neumann and Romheld, 1999; White et al., 2003;

Zhou et al., 2003). These alterations may in turn provide metal resistance or aid in metal uptake by plants (Ma et al., 2000; Tolra et al., 1996). Phytostabilization may be at highest risk from attractive nuisance since phytostabilization is typically permanent and potentially subject to less management and monitoring.

Phytovolatilization, or the delivery of soil-based pollutants to the atmosphere via plant transpiration, is a component of some phytoremediation installations by design, however it may potentially occur in any situation where volatile contaminants are present. Phytovolatilization can pose risks similar to those found in hyperaccumulation and phytoextraction since the contaminant is moved from the soil to another medium. Rather than being contained within the plant tissue, phytovolatilization moves the contaminants into the air. For phytovolatilization to considered an appropriate solution, a significant reduction in toxicity must be expected or a very high dilution effect. Conversion of methyl mercury in the soil to elemental mercury in transgenic plant tissue and subsequent release the atmosphere may be an acceptable reduction in toxicity, due to the extreme toxicity of methylmercury and the much lower toxicity of elemental mercury (Bizily et al., 2000; Rugh et al., 1996). Volatilization and atmospheric transport of selenium will simply cause dispersal of an overly concentrated nutrient to areas where selenium may be deficient (De Souza et al., 2000). In cases of uncertainty actual contaminant levels in the atmosphere, leaves and stems could be analyzed to determine if phytovolatilization has the potential to produce a high concentration in the atmosphere surrounding the site such that it would be overly risky to be within or near the site. However atmospheric dilution would probably be able to compensate for relatively low levels of contaminants released from plant leaves.

Organic Phytoremediation

Phytoremediation of organic contaminants can be accomplished by the plant alone
(phytodegradation) or in concert with soil bacteria (phytostimulation) and in some cases
phytovolatilization. Water-soluble compounds may be moved from the soil into plant
roots or even aboveground tissues and degraded by plant-produced enzymes or
volatilized. Alternatively, extracellularly secreted enzymes may degrade less water-
soluble compounds. This has been especially well documented in TCE phytoremediation
in which TCE can be converted to the less toxic dichloroethylene or trichloroethanol
within plant cells (Shang et al., 2001). Quantitative studies of the amount of TCE
transpired by poplar trees growing on TCE contaminated aquifers have been conducted
which showed that the release of TCE from leaf tissue was less than 9% of the total
quantity of uptaken TCE (Newman et al., 1999).

Phytodegradation is the transformation or degradation of organic compounds by
plants. Hydroxylation of xenobiotic compounds or conjugation with varying sugars is a
frequent result of exposure studies using plant cell cultures (Huckelhoven et al., 1997;
Wilken et al., 1995). However, uptake through the transpiration stream might result in a
somewhat different set of metabolites than direct cell culture exposure. Some studies
have already begun to characterize these metabolites and future studies should help
clarify xenobiotic metabolic pathways for a wider variety of compounds (Subramanian et
al., 2006). Chemical modifications can transform organic compounds to be more water-
soluble and therefore more mobile or possibly more toxic. Transformation to more toxic
compounds is known to occur in microbes that degrade trichloroethylene (TCE). When

degraded anaerobically, TCE can be converted to the more toxic and carcinogenic vinyl chloride (McCarty, 1997). It is not impossible that similar increases in toxicity could occur in plants. The optimal outcome for the degradation of organics is mineralization or conversion back to basic elements such as CO_2, chlorine and water. Even if contaminants are uptaken and modified, their loss from intact plant tissue via volatilization, or mineralization may be variable and likely to not be 100%, with the maximum observed for RDX being 25% in one study (Yoon et al., 2006).

Ideally the major metabolites produced by phytoremediating plants be identified and assessed for their individual impact. Barring actual data, potential metabolites could be imagined based on typical plant metabolic patterns and estimated for toxicity. While the potential for plant-induced changes to organic chemicals to be harmful, the variety of plant produced metabolites is likely to be high meaning that the concentration of any one metabolite is likely to be below thresholds for toxicity. Although the combination of many metabolites could be more toxic than any individual compound or an individual compound could be at extremely increased toxicity, this seems to be relatively low likelihood. Direct toxicity tests of exposed plant tissue and soils post-treatment on indicator species would likely be considerably easier and more appropriate than chemical analysis of metabolites, and should provide sufficient proof of lowered toxicity. If it is found that plants are increasing toxicity through their metabolic activities they may need to be removed and alternative remediation methods found, or methods of reducing bioavailability through chemical addition could be implemented (Chen et al., 2000). Comparisons to contact toxicity from the soil or medium itself may be useful in determining the potential costs and benefits of the phytoremediation technology.

Transgenic Phytoremediation

Transgenic plants are those that are transformed with bacterial genes for enhanced contaminant degradation, solubilization or stabilization. All of the risks from non-transgenic phytoremediation are also a factor in remediation installations using transgenic plants as the processes are typically the same as with non-transgenic plants, including the potential for genetic escape. Any introduction of a non-native organism poses a potential risk to the local region and the environment as a whole. Instances of damaging invasive species resulting from well-intentioned introductions are not unusual and invasive species are now considered a major threat to the global ecosystem (Vitousek et al., 1997). However, the simple introduction of a non-native species or transgenic plants does not necessarily spell disaster, since many thousands of plant species have been imported with little effect, but those that do cause problems may do so on a massive scale. The severity of this risk is largely dependent on the gene and plant species involved. The degree of risk posed by any particular transgene is highly variable and depends on the nature of the transgene. The escape of a gene for PCB degradation might have a very different ecological impact than a gene to enable arsenic hyperaccumulation.

The presence of compatible relatives for the introduced plants to potentially cross with or permissible conditions for pollen or seed dissemination can enhance the risk. Lack of compatible relatives nearby to a site of implementation greatly reduces potential risk of gene escape but does not eliminate it, if the transgenic plant is able to propagate through viable seeds or vegetatively such as suckers, rhizomes or runners. Transgenic plants may also have new properties not present in unmodified plant species, posing some

139

risk to wildlife simply by being placed on a site. Plants containing and secreting new compounds for uptake of novel contaminants represent unique risks over non-transgenic phytoremediation. Engineered mercury volatilization, overexpression of phytochelatins has shown that the creation of novel hyperaccumulators is possible (Pilon-Smits and Pilon, 2002). The chemistries in these plants could bypass normal avoidance mechanisms by herbivores, leading to increased risk over natural hyperaccumulator species.

An already proposed method of assessing the risks presented by transgenic crops could be applied to the assessment of plants genetically modified for enhanced phytoremediation and even the introduction of new species (Hancock, 2003). Variables such as dispersal distance, potential for crossing to native relatives and weedy/invasiveness of the transgenic or novel species can be weighed as to importance in a particular instance of implementation. The chance that a known plant species that is non-invasive will be made invasive via transgenic manipulation for phytoremediation is probably quite low, since only 1-2 genes are typically added to the transgenic plant.

But the risk of escape cannot be discounted entirely and the consequences for a single escape could be very damaging to public perception of phytoremediation even if a documented escape does not actually pose a high risk to the community or environment. There are quite a number of methods to reduce the risk of plant escape, primarily the use of sterile cultivars so that dissemination via seed or pollen is prevented or cultural practices such as harvesting plants before flowering can occur (Singh et al., 2006). There may still be situations in which the potential risk from the introduction of a new species or a genetically modified plant would still be overly risky from the standpoint of escape. In these cases phytoremediation, using species that need to be avoided, can be conducted

ex-situ in greenhouses, or avoided altogether. Any new introductions to an outdoor area, transgenic or no, should be carefully examined for ecological impact prior to large scale planting. Table 4.2 lists the costs and associated risks of most phytoremediation approaches.

Benefits of Phytoremediation

Phytoremediation has a number of advantages and benefits over engineering based remediation approaches. Phytoremediation is typically lower cost than engineering operations as the planting of plants on soil is usually considerably cheaper than excavation or intensive soil treatment. Phytoremediation is generally considered environmentally friendly and aesthetically pleasing, potentially capable of restoring habitat when implemented in degraded locations. Phytoremediation may also be the only technology, which is suitable for certain types of contaminated sites, large regions such mine tailings or locations downwind from smelter operations would benefit from phytostabilization installations, since excavation and other forms of treatment are frequently economically unviable in very large areas of contamination. Phytoremediation also has unique capabilities for the specific removal and containment of certain metals, including the concentration of metals into a much smaller volume and the potential for metal reclamation from plant tissue. The combination of lowered cost and greater public acceptance of phytoremediation technologies can also lead to a more pro-active cleanup effort by responsible parties.

Table 4.2. Summary of Phytoremediation Technologies and Associated Potential Risks

Technology	Description	Target Contaminants*	Estimated Costs (US $ per cyd or per 1000 L)	Potential Risks	References
Constructed wetlands	Filtration of runoff from non-point sources	NP, low levels of other contaminants	$1.36/Kgal over 10 yr period	Contaminant accumulation, Contaminant escape into other watersources	(Berghage et al., 1999; ESTCP, 1999)
Hydraulic control	Plants used as a barrier against contaminated groundwater movement	NAPL, HM	1 acre site ($250,000)	Contaminant accumulation Contaminant escape	(EPA, 2000)
Phytodegradation	Plant degradation of organic contaminants to non-toxic forms.	NAPL, SVOC, NAC	$174 - $2,322 depending on site size and difficulty	Potential formation of toxic intermediates or accumulation of undegraded material	(Cunningham and Berti, 1993; Deuren et al., 2002)
Phytoextraction	Plant uptake and foliar accumulation for harvest and disposal or recycling	HM, NP, PCB, POP	$40-$50 per cubic yard	Contaminant mobilization by deciduous litter or during chelated-assisted methods. Herbivory.	(Eidenspace, 1998)
Phytostabilization	Plant stabilization of soils by chemical and/or physical means	HM	$0.02 - $1.00 per cubic meter	Contaminant accumulation attractive nuisance	(Cunningham et al., 1995)
Phytovolatilization	Plant root uptake and aerial volatilization of volatile pollutants	NAPL, Se, Hg	$174 - $2,322 depending on site size and difficulty	Conversion of water/soilborne contaminants to airborne contaminants	(Chaney et al., 1997; Deuren et al., 2002)
Rhizofiltration	Plant root uptake/sorption of contaminants from waste water streams	HM, NAC	$2-$6 per thousand gallons of water	Plants become contaminated and must be disposed of But, this technology is usually not field implemented	(Dushenkov et al., 1997)
Rhizostimulation	Plant roots stimulate bacterial community to degrade contaminants	NAC, PAH, PCB, POP, TPH, SVOC	$174 - $2,322 depending on site size and difficulty	Incomplete degradation, contaminant or contaminant intermediate accumulation	(Deuren et al., 2002; Kuiper et al., 2001)
Riparian barrier	Barrier to runoff from non-point sources	NP, low levels of other contaminants	$13,000 - $30,000 per acre	Contaminant accumulation, Contaminant escape into other watersources	(Blanco-Canqui et al., 2004)

* DNAPL = dense non-aqueous phase liquid, HM = heavy metal, NAC = nitroaromatic compounds, NAPL = non-aqueous phase liquid, NP = nutrient pollutant (or nitrogen and phosphorous), PAH = polyaromatic hydrocarbon, PCB = polychlorinated biphenyl, POP = persistent organic pollutant, TPH = total petroleum hydrocarbon, SVOC = semi-volatile organic compounds

IS PHYTOREMEDIATION SAFE?

Is Phytoremediation Suitable?

The first step in an assessment of any technological application is to determine if it is suitable for a particular impacted site. Suitability assessment includes an implicit risk reduction strategy, since technological failure due to placement in an improper location, would almost certainly cause increased risk, even over no action at all. A decision tree to determine phytoremediation suitability has already been constructed by the Interstate Technology and Regulatory Cooperation (ITRC) (ITRC, 2000). The general limitations of phytoremediation are; long treatment times, shallow treatment depth (13 feet or less usually) poor performance in short growing seasons or arid conditions, and high contaminant levels (potential plant toxicity). Once it has been determined that phytoremediation is an otherwise viable option, the safety implications of its implementation on a particular site can be considered.

Is Phytoremediation Safe?

To define an approach or a technology as "safe", probabilities of different outcomes can be used as a basis, but they do not work in every circumstance nor can they be applied to every situation, especially in cases where uncertainty is high as is often the case in phytoremediation installations. While some determination of exposure can be made, the nature of a phytoremediation installation makes these factors hard to quantify. The degree of contaminant uptake by plants may be difficult to predict in some instances and the degree to which plants reduce or accelerate contaminant dispersal may also be highly variable. Because of the difficulty in assigning numerical values to risks from

phytoremediation we will compare standard engineering practices to phytoremediation practices. Since these technologies are currently in use, these technologies are generally considered by regulatory bodies as well as the public at large as "safe". If phytoremediation is "safe" it should have similar or fewer risks and potentials for risk to standard engineering technologies. A simplified tabular comparison of basic risks of phytoremediation and engineering based approaches is shown in (Table 4.3).

One basic difference between the most common form of engineering based remediation, excavation, and off site disposal is the nature of contaminant treatment. While immediate risk to an area or group of people is removed via excavation, ultimately the risk has been transferred spatially and temporally to a controlled location. Phytoremediation, especially of organic contaminants and phytoextraction of metals

Table 4.3. Direct comparison of exposure based risks from remediation technologies.

Risk	Phytoremediation	Engineering based technologies
Leaching	Reduced Risk in most cases due to plant root containment, except in chelate or surfactant assisted phytoremediation	Enhanced by excavation based treatments, reduced by stabilization
Soil Particle Dissemination	Reduced risk, some contribution by planting operations	Risk increased by excavation/soil disruption
Volatilization	May decrease except in phytovolatilization	May increase risk due to disturbance or volatilization based technology
Altered Contaminant Toxicity	Potential risk of almost any phytoremediation installation, lower for contaminants with low water solubility.	Generally unlikely except in cases of chemical treatment
Failure of Technology	Phytoremediation often unpredictable. Failure or longer than anticipated treatment times common	Technologies typically well known, risk of failure generally low

Risk	Phytoremediation	Engineering based technologies
Concentration of Contaminants	By design in phytoaccumulation, potential for increased risk	Only in technologies designed for concentration such as soil washing
Dissemination of assimilated compounds	Risk specific to phytoremediation	Only occurs in the case of pre-existing biota
Plant Escape	Risk specific to phytoremediation	Not Applicable

actually cleanses the soil by removing or destroying the contaminants in the soil while not damaging the soil and even potentially improving the soil's agronomic qualities. Despite this seemingly overwhelming advantage in terms of risk removal rather than risk transfer, phytoremediation cannot always perform complete remediation. Sometimes plants may not be able to remove or detoxify contaminants up to a regulatory standpoint that is sufficient to reduce risk to an acceptable level.

The decision between phytoremediation and engineering technologies comes down to a choice between removing all or most of the risk from a particular area immediately with the understanding that the risk may reoccur at some other place and time, or accepting some level of ongoing risk in the hopes that over a long period of time the risk will be substantially reduced or removed completely. In most cases regulatory agencies find the idea of immediate risk removal to be superior, especially in cases where contamination levels are high. However, if a site is not an immediate threat to a surrounding area, phytoremediation as a potential permanent solution should gain favor. In terms of overall risk reduction risk removal is preferable to temporal or spatial risk transfer. Although the risk from gene and plant escape stands out as a novel and

somewhat unknown risk, it is potentially highly manageable with sterile clones and cultural practices. The sum total of risks and benefits from phytoremediation make it no less safe than engineering practices, when implemented under appropriate circumstances. Phytoremediation should by no means replace engineering approaches, as there are situations where phytoremediation is of limited utility. However especially in situations of low level contaminants, phytoremediation can be particularly safe and effective.

CONCLUSION

Phytoremediation is still an emerging treatment technology, slowly gaining market share and becoming more broadly implemented. Understanding the risks posed by a phytoremediation installation is an important step forward for responsible choices concerning phytoremediation and increased regulatory acceptance of the technology. Phytoremediation is not devoid of risk and bears some different risks than standard engineering-based remediation technologies. Careful analysis and comparisons of risks from both technological types shows that, when properly implemented, phytoremediation does not appear to pose any more risk than currently accepted technologies. However, like any remediation technology careful management and monitoring is necessary to avoid unforeseen complications and unnecessary risks.

Several of the special risks posed by phytoremediation are worthy of consideration for further study to clarify and obtain quantifiable probabilities where possible. These areas include the risks posed by phytoremediative gene escape and the potential for phytoremediation species to become invasive. More methods for preventing gene escape and the production of sterile clones will allow for the implementation of

genes or plant species that might pose an overly high risk without stringent controls. Information concerning uptake and transformation of various environmental toxicants would be especially valuable in assisting in assessment of risks of contaminant conversion for particular pollutant and plant species combinations.

Assuming careful management and appropriate implementation of phytoremediation, even given some gaps in knowledge is no less safe than currently accepted remediation technologies and may offer the public a superior level of protection in some situations. Continued research and creation of improved plant varieties with reduced potential for escape will ensure the future of phytoremediation as a safe and effective technology.

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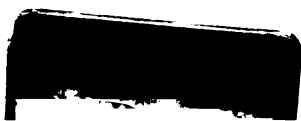
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