DECHLORINATION OF THE NATURALLY PRODUCED FUNGAL METABOLITE, 3,5-DICHLORO-*PARA*-ANISYL ALCOHOL BY *BURKHOLDERIA* SP. UW103

BY

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A Thesis Submitted to the Faculty of Graduate Studies in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

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ABSTRACT

Chlorinated anisyl metabolites (CAM), synthesized by lignin-degrading fungi, are found in ecologically significant concentrations in the environment (De Jong et al. 1994; Gribble 1994; Swarts et al. 1994). A bacterium capable of growth on the most common CAM; 3,5-dichloro-*para*-anisyl alcohol (DCA), as sole carbon and energy source was isolated from an enrichment culture derived from commercial compost. When this non-motile, Gram-negative rod, designated UW103, was grown on defined media containing DCA an increase in the chloride ion concentration of the medium was observed equal to 2.1 moles chloride per mole DCA consumed. This ratio suggests that UW103 dechlorinates DCA at both the 3 and 5 positions. Complete mineralization of DCA was observed as measured by 87% CO_2 liberated from the respiration of DCA-utilizing cells.

Cells of strain UW103 are coccobacilli, 1.5x1.2 μm, while growing on DCA and longer, slightly curved bacilli, 4x1.2 μm, when grown on nutrient rich media. Biochemical testing and the results of the Biolog® identification system placed UW103 in the genus *Burkholderia*. The 16S rRNA gene was amplified and the nucleotide sequence of nearly the entire gene was determined. Comparison to the RDB database reveals the greatest sequence homology (97.8%) to the species *B. graminis*. *Burkholderia* sp. UW103 is capable of growth and dechlorination of DCA at concentrations up to 1.5 mM, above which DCA is toxic to the bacterium. The DCA-degrading strain is not capable of utilizing a variety of chlorinated benzoates and phenols, including the

fungicide pentachlorophenol, as sole carbon and energy sources; however, UW103 is capable of growth on the chlorinated herbicide dicamba.

The catabolism of DCA begins with the oxidation of the alcohol component to the aldehyde and acid forms and proceeds through 3-chloro-*para*-anisic acid, detected in the media of DCA-metabolizing cells. Further degradation and mineralization of DCA may occur through protocatechuic acid, a common precursor to aromatic ring cleavage. The presence of DCA and other naturally chlorinated compounds in nature have provided selective pressure on microorganisms to utilize them. The pathways that degrade naturally chlorinated compounds are a likely source of dehalogenases, from which enzymes with xenobiotic-degradative capacity could evolve. The presence of DCA and DCA-degraders in agricultural soil and commercial compost, detected in this study, indicate the ubiquity of DCA-degrading and dechlorinating pathways.

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FOREWARD

This document is submitted to the Faculty of Graduate Studies in partial fulfillment of the requirements for the Degree of Master of Science, in conjunction with an oral defense of the project and this thesis, in the Department of Soil Science at the University of Manitoba in Winnipeg Canada.

The thesis is presented in chapter style, following the format guidelines as outlined in "A Guide to Thesis Preparation for Graduate Students in the Department of Soil Science," (November 1996). The data presented in chapters 3 and 4 has been written in manuscript style and upon further review will be submitted for publication to Applied and Environmental Microbiology, a peer-reviewed journal.

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LIST OF ABBREVIATIONS

2,4-D	2,4-dichlorophenoxyacetic acid
AOX	adsorbable organic halogen(s)
BSA	N, O-bis(trimethylsilyl)acetamide
CAM	chlorinated anisyl metabolite(s)
DCA	3,5-dichloro-*para*-anisyl alcohol; 3,5-dichloro-4-methoxybenzyl alcohol
DCA-YNB	YNB containing 1 mM DCA
DCAcid	3,5-dichloro-*para*-anisic acid; 3,5-dichloro-4-methoxybenzoic acid
DCAld	3,5-dichloro-*para*-anisaldehyde; 3,5-dichloro-4-methoxybenzaldehyde
DHB	3,5-dichloro-4-hydroxybenzyl alcohol
DNA	deoxyribonucleic acid
ECD	electron capture detection
FID	flame ionization detection
ISA	ionic strength adjuster
GC	gas chromatography
Me	methyl substituent
min	minutes
ml	milliters
MS	mass spectrometer
MSD	mass spectrometer detector
PCB	polychlorinated biphenyl(s)
PCDD	polychlorinated dibenzodioxin(s)
PCDF	polychlorinated dibenzofuran(s)
PCP	pentachlorophenol
PCR	polymerase chain reaction
ppb	part per billion
ppt	part per trillion
PTFE	polytetrafluoroethylene
THF	tetrahydrofuran
YNB	DIFCO yeast nitrogen base without amino acids, prepared in 50 mM NaH_2PO_4-Na_2HPO_4 buffer, pH 6.3

CHAPTER 1

INTRODUCTION

Chlorinated aromatic compounds are often used as pesticides, dielectrics, flame retardants, and preservatives due to their chemical and biological inertness. Their biological recalcitrance however often lead to the accumulation and persistence of toxic chemicals in the environment. It may be surprising to note that despite the presence of chlorinated man-made chemicals in the biosphere for less than a century, often less than 50 years, microbes have evolved the ability to utilize them. The presence of naturally produced chlorinated aromatic compounds in the environment suggests that microbes have had the opportunity to develop mechanisms to dechlorinate and biodegrade these compounds for much longer. Catabolic pathways that utilize natural chlorinated aromatic compounds may be the source of genes involved in the biodegradation of recently introduced chlorinated xenobiotics.

The higher fungi, *Basidiomycetes*, have a widespread capacity for organohalogen biosynthesis. At least 68 genera from 20 families of the orders *Agaricales* and *Aphyllophorales* are known to synthesize halogenated organic compounds (De Jong and Field 1997). Chlorinated anisyl metabolites (CAM) are the most common halogenated aromatic compounds produced by at least 18 genera belonging to 4 families of white-rot fungi of the orders *Agaricales* and *Aphyllophorales*. The CAM, 3,5-dichloro-*para*-anisyl

alcohol (DCA), is biosynthesized in the highest concentrations in liquid media and forest litter colonized by *Hypholoma* sp. (De Jong et al. 1994; Swarts et al. 1997).

The physiological function of DCA likely includes a role in lignin degradation. Lignin, second to cellulose, is the most abundant component of plant tissues (Lehninger , Nelson and Cox 1993). Although a large fraction of earth's organic carbon is tied up in lignin, very few organisms are capable of its degradation because it is difficult to hydrolyze and stores little energy. Basidiomycete fungi are the only organisms capable of lignin mineralization in wood and soil (Hatakka 1994; Steffen, Hofrichter, and Hatakka 2000). These fungi secrete lignin-degrading peroxidases, which non-specifically catalyze the oxidation of aromatic components of lignin, utilizing H_2O_2 as electron acceptor (Linko 1992). DCA acts as a recyclable substrate for aryl alcohol oxidase, generating H_2O_2 for lignin-degrading peroxidases (De Jong, Field and De Bont 1994).

The ubiquity of lignin and basidiomycete fungi in the environment suggest that CAM are also widespread in nature. In fact, high concentrations of CAM's can be detected in close vicinity to fruiting bodies of Basidiomycete fungi. However, just outside CAM-producing Basidiomycete colonies, halogenated organic compounds were not detected suggesting biodegradation or biotransformation of CAM (De Jong and Field 1997). Possible environmental fates of DCA include mineralization by soil microbes and biotransformation yielding chlorinated phenols. The resultant chlorinated phenols may undergo biotoxification to dioxins or detoxification by incorporation into chlorohumus (De Jong et al. 1994). Verhagen et al. (1998*c*) demonstrated that under anaerobic conditions, DCA is biotransformed by methanogenic sludge to yield 3,5-dichloro-4-hydroxybenzyl alcohol (DHB). DHB is oxidized then decarboxylated to 2,6-dichlorophenol. Dechlorination was not observed in the study by Verhagen et al. (1998*c*).

DHB is also abiotically dimerized, forming bis(3,5-dichloro-4-hydroxyphenyl)methane. Verhagen et al. (1998*a*) also studied the aerobic degradation of DCA. Under aerobic conditions DCA was rapidly and completely mineralized by incubations with active sludge from a sewage treatment plant, beech forest soil, oak forest soil, and pine forest soil. However, the release of inorganic chloride was not observed, neither in anaerobic incubations with VFA-grown methanogenic sludge, nor in lignin-adapted methanogenic sludge (black liquor derived from wheat straw).

This thesis hypothesizes the existence of aerobic bacteria capable of DCA dechlorination and mineralization. The isolation and characterization of bacteria with the capacity to utilize CAM as sole sources of carbon and energy was attempted. The catabolic pathway of DCA degradation and the mechanism of DCA dechlorination was explored. A DCA-degrader isolated was characterized for its ability to degrade xenobiotic chlorinated aromatic compounds.

CHAPTER 2

LITERATURE REVIEW

2.1 Anthropogenic Chlorinated Aromatic Compounds in the Environment

Chlorinated aromatic compounds constitute one of the most prevalent and recalcitrant classes of xenobiotic chemicals (Häggblom 1992). The chemical and biological inertness of chlorinated aromatic compounds makes them useful as pesticides, dielectrics, flame-retardants, plasticizers, and preservatives. Their toxicity and inertness, however, leads to their accumulation and persistence in the environment. The toxicity, bioconcentration, and ubiquity of chlorinated aromatic compounds in the biosphere have caused considerable public concern over possible health effects.

Polychlorinated biphenyls (PCB) are used as dielectrics in electrical equipment and as plasticizers in paints, plastics, rubber and many other applications [1] (Figure 2-1). Greater than 1 billion kilograms of PCB were produced in North America, until production ceased in 1977 (Minister of Supplies and Services Canada 1984). Although no longer manufactured in Canada or the U. S., many products containing PCB are still in use. As a result of their widespread use and poor biodegradability, PCB are present throughout the environment. Concentration of PCB in contaminated soil can reach one part per thousand (Di Toro, Zanaroli, and Fava 2006; Demnerova et al. 2005). PCB in contaminated waters adsorb to sediments in fresh water (65 ppb; Fish and Principe 1994)

and marine environments (3.4 ppm; Tay et al. 2003). Atmospheric PCB also exist; high mountain lakes, receiving all water from precipitation, contain 20-200 ppt PCB (Vilanova et al. 2001). Although the PCB concentration in sea water can be as low as 7 ppb (Gillan et al. 2005), PCB concentrate in marine animals in a process known as bioconcentration. As a result, PCB are widely found in the aquatic food supply; concentrations in seafood have been measured at 1.6-25 ppb (Johansen et al. 1996; Foran et al. 2005A and 2005B; Sjodin et al. 2000; Fries 1995). PCB have also been found in free-range chicken and eggs (Schoeters and Hoogenboom 2006). Startling findings have shown that human breast milk and human placenta typically contain 1.8 ppb and 5.0 ppb PCB, respectively (DeKoning and Karmoaus 2000).

Pentachlorophenol (PCP) is used widely across North America, mainly as a fungicide on wood to preserve utility poles, fence posts, boats, furniture and log cabins [2] (Figure 2-1). During the peak of PCP production in the mid-1970s, approximately $90x10^6$ kg was produced annually worldwide (Nilsson et al. 1974). In North America, greater than 400,000 metric tonnes of PCP were distributed in the environment between the years 1970 and 1995 (Lorber et al. 2002). As a result, PCP is widespread in the environment and can be detected in freshwater sediments ranging from 1.4 to 34.4 ppb dry weight (Hong et al. 2005), soil (73 to 500 ppm; Hanson et al. 2004; Beaudet et al. 1998) and animals. PCP levels in human urine and seminal fluid range from 20-50 ppb (Dougherty and Piotrowska 1976; Waite et al. 1998) and levels in human cerebrospinal fluid range from 0.24 to 2.0 ppb (Jorens et al. 1991). Even more startling is the detection of PCP in human breast milk at 2.15 ppb (Hong et al. 2005). PCP and PCB levels in human blood plasma correlate strongly with fish consumption, strong evidence for the bioaccumulation of PCP and other toxic organic chemicals (Sjodin et al. 2000).

PCB, PCP and other chlorinated phenols are listed as toxic substances in the Canadian Environmental Protection Act (Department of Justice Canada 1999). Studies on the health effects of PCP show that this chemical is a potent irritant, carcinogen and teratogen (Tisch, Faulde, and Maier 2005; Berkowitz et al. 2004; Dahlgren et al. 2003). PCB have been shown to alter immune, thyroid, and reproductive functions (Kimbrough and Krouskas 2001), increase the risk of cancer (Moysich et al. 2002), and alter neuropsychological function in children (Schantz, Widholm, and Rice 2003).

In addition to PCP and PCB, other chlorinated phenols are used industrially on a large scale as pesticides, antiseptics and intermediates in chemical syntheses (Gribble 1994). They also enter the environment from bleaching fluid effluents (Brownlee, MacInnis, and Noton 1993). Chloroneb [5] (Figure 2-1), trade names Demosan and Tersan SP, is used as a fungicide for treatment of field crop seeds of beans, soybeans and cotton. It is also used on turf grass to control snow mold (*Typhula* spp) or pythium blight. The chlorinated pesticides 2,4-D [3] and dicamba [4] are used extensively in Manitoba as broadleaf herbicides (Figure 2-1).

2,4-D is the most widely used herbicide in world (3rd in Canada and U.S.). Its major uses in agriculture are on wheat and small grains, sorghum, corn, rice, sugar cane, low-till soybeans, rangeland, and pasture. 2,4-D is also used on rights-of-way, roadsides, non-crop areas, forestry, in lawn and turf care, and on aquatic weeds. Dicamba, trade names Banvel®, Banex®, and Trooper®, is used as a selective pre- & post emergence herbicide to control annual & perennial broadleaf weeds in corn, small grains, pastures, and non-cropland areas such as fence rows, roadways & wasteland. Dicamba is often formulated with one or more phenoxyalkanoic acids, including 2-methyl-4-chlorophenoxy acetic acid (DyVel) and 2,4-D (DyVel DS). The dicamba-2,4-D

combination is used to control Canada thistle and other broadleaf weeds in wheat, barley and oats.

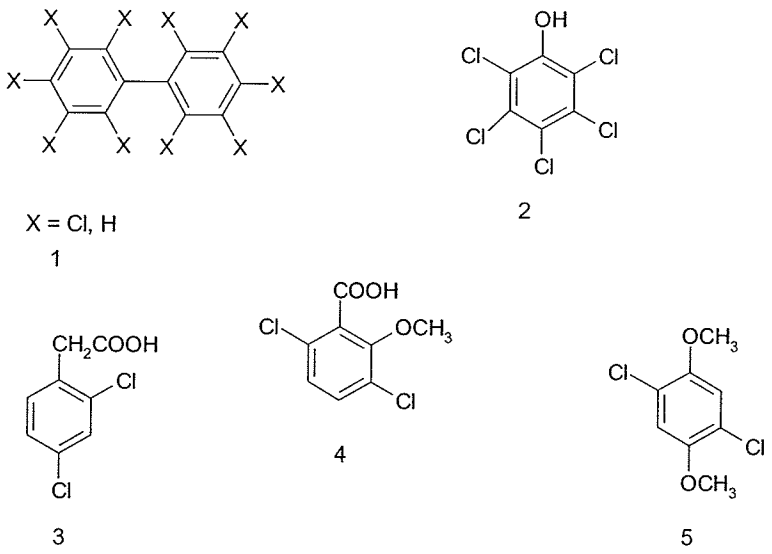


Figure 2-1. Chemical structures of commonly used chlorinated xenobiotics. 1, Polychlorinated biphenyls (PCB); 2, Pentachlorophenol (PCP); 3, 2,4-dichlorophenoxyacetic acid (2,4-D); 4, Dicamba; and 5, Chloroneb®.

It is surprising to note that despite existence in the biosphere for less than a century, microbes have evolved the capacity to degrade chlorinated organic man-made compounds. Although some xenobiotic chlorinated aromatic compounds are resistant to attack or partially broken down to non-degradable, often toxic, products, many others are dehalogenated and completely mineralized by soil microbes. Microorganisms capable of dechlorination or mineralization of chlorinated aromatic compounds are a source of catabolic enzymes to transform these pollutants to harmless end products. Members of the genera *Pseudomonas*, *Burkholderia*, *Arthrobacter*, *Alcaligenes*, *Sphingomonas*, *Hyphomicrobium* and *Rhodococcus* are capable of the dechlorination and mineralization

of a wide variety of chlorinated organic compounds (Fetzner 1998; Copley 1997; Wackett and Hershberger 2000).

2.2 Naturally-Chlorinated Aromatic Compounds

At least 2500 natural halogenated compounds have been isolated from plants, marine organisms, insects, fungi, bacteria, mammals and other natural processes (Gribble 1996). The majority of naturally produced terrestrial halogenated compounds are chlorinated, while some contain bromine, iodine and very rarely fluorine. In contrast, most naturally produced halogenated compounds in the marine environment are brominated. Chlorinated organic compounds are secreted into the environment by organisms for chemical defense as feeding deterrents, irritants, or pesticides.

Even though the existence of natural halogenated compounds has been documented for more than a decade, public knowledge of their existence is still lacking. Halogenated organic chemicals are widely considered to be anthropogenic, and their presence in the environment is assumed to be the result of industrial practices. However, concentrations of natural chlorinated aromatic metabolites often exceed Canadian hazardous-waste norms for analogous chlorophenols in soil, set between 1-10 mg/kg for mandatory remedial action (Field, Verhagen, and De Jong 1995). The natural production of halogenated aromatics has largely been ignored when environmental legislation is formulated. Worldwide, industries are required to comply with stringent environmental standards for chloroaromatic substances even though industry is not the only source of these compounds. Adsorbable organic halogen (AOX) is the bulk parameter used to monitor the extent of man-made pollution in the environment, yet this parameter does not discriminate between natural and xenobiotic organic halogen. Asplund (1989 in Asplund

and Grimvall 1991) demonstrated the level of AOX in an unpolluted bog was at least 300 times greater than that accounted for by anthropogenic sources.

A variety of chlorophenols are synthesized in nature. Drosophilin A [6], isolated from *Drosophila subatrata*, was the first terrestrial natural chlorophenol to be identified and is synthesized by a variety of fungi (Teunissen, Swarts and Field 1997). Drosophilin A and its methylated analog [7] were detected in culture media of 12 species of fungi from 9 genera including *Phellinus*, *Bjerkandera* and *Coprinus*. 3-Chlorogentisyl alcohol [8] is a chlorophenol synthesized by a variety of fungi (Gribble 1996). The soil fungus *Penicillium* sp. produces the growth hormone 2,4-dichlorophenol, a precursor to the commercial herbicide 2,4-D (Ando et al. 1970). Grasshoppers secrete 2,5-dichlorophenol to repel ants (Eisner et al. 1971) and the sex hormone, 2,6-dichlorophenol, is synthesized by female *Amblyomma americanum* and other ticks (Berger 1972 and 1983).

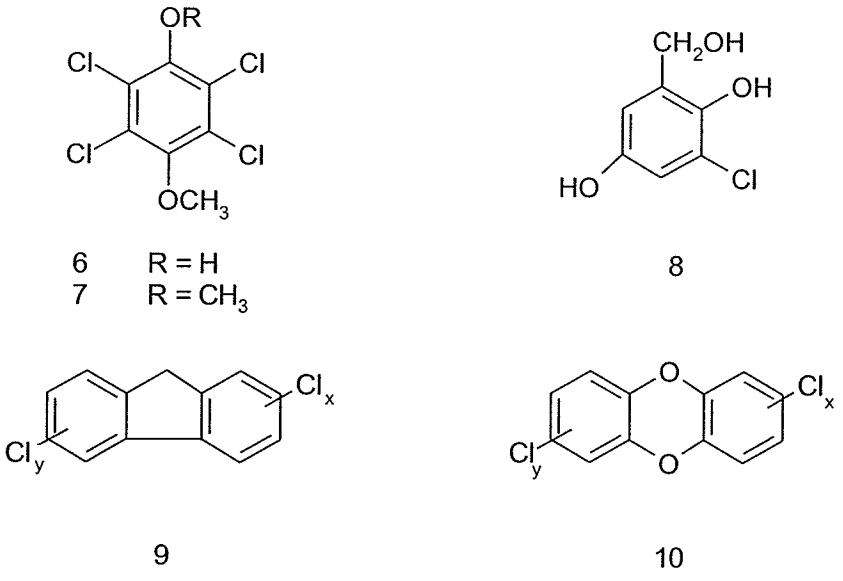


Figure 2-2. Chemical structures of natural chlorinated aromatic compounds. 6, Drosophilin A; 7, Drosophilin A methyl ether; 8, 3-Chloro-gentisyl alcohol; 9, polychlorinated dibenzofurans (PCDF); and 10, polychlorinated dibenzodioxins (PCDD).

Chlorinated phenols, anisoles and fulvic acids have also been detected in soil extracts and ocean waters (Nieden, Pavasars, and Oberg 2000; Walter and Ballschmiter 1991); the source is likely chloroperoxidase enzymes from soil microbes and marine algae, respectively. These microbes produce haloperoxidase enzymes, which catalyze halogenation of organic substrates in the presence of halide and hydrogen peroxide (Hager et al. 1966). Chloroperoxidase activity has been detected in several uncontaminated soils (Asplund, Christiansen and Grimvall 1993; Hoekstra et al. 1999). Furthermore, Niedan et al. (2000) found that chloroperoxidase-exposed fulvic acids contained similar chlorinated aromatic substituents as natural chlorinated fulvic acids found in soil and surface water samples. Their work supports the hypothesis that chlorinated aromatic compounds are formed naturally in soil and water via haloperoxidases. Chlorophenols may also be enzymatically converted to polychlorinated dioxins and dibenzofurans (Nilsson et al. 1974; Svenson et al. 1989; Oberg et al. 1990; Wittsiepe et al. 1999; Hoekstra et al. 1999).

Polychlorinated dibenzofurans (PCDF) [9] and polychlorinated dibenzodioxins (PCDD) [10] are highly toxic to most animal species and products of virtually all combustion processes (Figure 2-2, Gribble 1996). They were first identified as anthropogenic contaminants of chlorinated phenoxy herbicides but have recently been identified in ancient soil samples and lake sediments. Rotard, Christmann, and Knoth (1994) found the level of all chlorinated dioxins and dibenzofurans in forest soils totaled approximately 2.2 μg/kg dry matter, almost 13 times greater than in grasslands. Silk et al. (1997) detected PCDF and PCDD in two peat bogs in New Brunswick; natural synthesis via oxidative coupling of 2,4-dichlorophenol by chloroperoxidases was suggested. Forest

fires and brush fires are the major source of natural PCDF and PCDD production in the environment (Nestrick and Lamparski 1982; Sheffield 1985). It is estimated that 60 kg of dioxins are produced annually by Canadian forest fires (Gribble 1996). Most forest fires are started by lightning, suggesting that PCDD have been present in the biosphere for at least millions of years. PCDD and PCDF are also formed catalytically in sewage sludge and garden compost by peroxidase enzymes acting on chlorinated phenols and hydrogen peroxide.

Svenson, Kjeller and Rappe (1989) demonstrated that 2,4,5-trichlorophenol can be enzymatically converted to PCDD and PCDF by horseradish peroxidase. In vitro formation of PCDD and PCDF, via horseradish and lactoperoxidases in the presence of H_2O_2, can occur from a variety of tri-, tetra- and pentachlorophenols (Oberg et al. 1990; Wittsiepe et al. 1999). The natural formation of chlorinated phenols, PCDF and PCDD was detected in soil of a Douglas fir forest and a peroxidase-mediated mechanism was suggested (Hoekstra et al. 1999).

Chlorinated organic compounds may also undergo mineralization, including dechlorination to chloride ions and degradation to carbon dioxide. The chlorine cycle, until recently, has been overlooked as a significant component of the biogeochemical cycles taking place on earth (Winterton 2000; Oberg 1998; Oberg 2002). Chlorine is one of the most abundant elements in the biosphere and is a major constituent of organic matter. As part of a complex biogeochemical cycle, chlorine atoms are cycled between inorganic chloride and substituents on organic compounds.

Chlorine and chlorinated organic compounds are common in terrestrial ecosystems; however, bromine and brominated compounds are the predominant halogenated compounds in marine ecosystems (Gribble 1999). A variety of marine

organisms, including sponges, polychaetes, and algae are known to produce brominated phenols, including 2-bromophenol, 4-bromophenol, 2,4-dibromophenol, 2,6-dibromophenol, and 2,4,6-tribromophenol (Hassenklover et al. 2006; Gribble 1999). Bromine-containing metabolites can even account for up to 12% of a sponge's dry weight (Turon, Becerro, and Uriz 2000). These compounds are thought to be used as feeding deterrents and are thought to cause the unpleasant sea-like taste and flavour associated with many marine organisms.

The abundance of chloride ion in plants, wood, soil and minerals leads to the formation of chlorinated organic compounds during combustion processes. Forest fires and volcanoes produce ecologically significant quantities of chloromethane (Gribble 1994 and 1996). The global emission rate of chloromethane from marine and terrestrial biomasses is approximately 5 millions tons per year (Rasmussen 1980), compared to anthropogenic emissions which are only about 26000 tons per year (Harper 1985). Naturally occurring chloromethane is the most abundant organohalogen responsible for ozone depletion (Neidleman and Geigert 1985; Harper 1994).

Natural halogenation of organic compounds also occurs in the human immune system. Human white blood cells (eosinophils and neutrophils) contain myeloperoxidase, which in the presence of chloride, bromide, or iodide and hydrogen peroxide, rapidly forms halide ions, causing the death of bacteria and fungi by halogenation reactions (Weiss et al. 1986). Many antibiotics used to treat infections are chlorinated aromatic secondary metabolites synthesized by bacteria. The antibiotic chloramphenicol, synthesized by *Streptomyces venezuelae*, was the first halogenated metabolite isolated from bacteria (Ehrlich et al. 1947). Other halogenated antibiotics produced by a number of bacteria include chlorotetracycline and vancomycin. Natural chlorinated chemicals

have widespread antibiotic, antitumor, antifungal, insecticidal, herbicidal, and other potentially valuable biological activities (Gribble 1994).

2.2.1 Chlorinated Anisyl Metabolites

Basidiomycete fungi have a widespread capacity for the synthesis of a variety of halogenated organic compounds at ecologically significant concentrations. Organohalogens, mostly chlorinated, are produced by at least 68 genera from 20 families within the orders *Agaricales* and *Aphyllophorales* (De Jong and Field 1997). Halogenated metabolites play physiological roles as antibiotics and as cometabolites in lignin degradation. Members of the phylum Basidiomycota form close mycorrhizal relationships with plants and many are capable of rotting leaf litter, wood, straw and humic materials. Basidiomycete fungi produce macroscopic fruiting bodies such as mushrooms, toadstools, conks and brackets. Some common examples found worldwide are sulfur tuft (*Hypholoma fasciculare*) and turkey tail (*Trametes versicolor*). *Hypholoma* and *Trametes* species are commonly observed in Manitoba (Bisby 1938). Basidiomycetes are the most ecologically significant group of organisms responsible for lignocellulose decomposition (Verhagen et al. 1996). Basidiomycete fungi produce large amounts of halogenated organic compounds when grown on ligniocelluloic substrates. High producing strains from the genera *Hypholoma*, *Mycena* and *Bjerkandera* produce 1074-30893 mg organohalide per kg dry weight mycelial biomass (Verhagen et al. 1996). Since these fungi play a major role in the conversion of lignocellulose debris and most of the terrestrial biomass is present in the form of lignocellulose, basidiomycete fungi are likely the major source of halogenated organic compounds in terrestrial ecosystems (Verhagen et al. 1996).

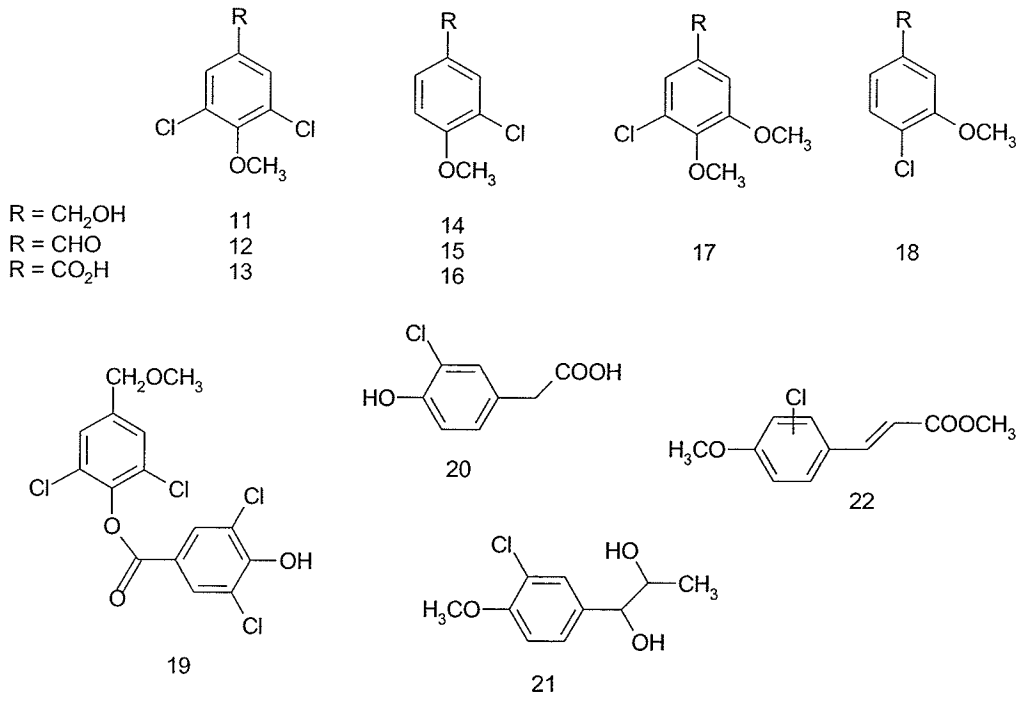


Figure 2-3. Chlorinated anisyl metabolites (CAM) produced by basidiomycete fungi. 11, 3,5-dichloro-*para*-anisyl alcohol; 12, 3,5-dichloro-*para*-anisaldehyde; 13, 3,5-dichloro-*para*-anisic acid; 14, 3-chloro-*para*-anisyl alcohol; 15, 3-chloro-*para*-anisaldehyde; 16, 3-chloro-*para*-anisic acid; 17, 3-methoxy-5-chloro-*para*-anisaldehyde; 18, 4-chloro-*meta*-anisaldehyde; 19, (3-chloro-4-hydroxy phenyl) ethanoic acid; 20, (3-chloro-4-methoxy-phenyl)-1,2-propandiol; 21, (chloro-4-methoxy-phenyl) methyl-propenoic acid.

Most halogenated organic compounds produced by basidiomycete fungi have an aromatic structure; important groups of metabolites include the chlorinated anisyls [11-22] (Figure 2-3), drosophilins [6,7] and other chlorinated hydroquinone methyl ethers, chlorinated orcinol methyl ethers, chlorinated sesquiterpenes, chlorinated anthraquinones (Cohen and Neil Towers 1996), and chlorinated strobulirins and oudemansins (De Jong and Field 1997). The chlorinated anisyl metabolites (CAM) are the most common examples of organohalogens synthesized by basidiomycete fungi because they are produced in high concentrations in nature and are produced by many different basidiomycete species.

CAM produced by basidiomycete fungi include 3-chloro- and 3,5-dichloro-*para*-anisyl alcohols [11,14] (Figure 2-3) and their corresponding aldehydes and carboxylic acids [12,13,15,16]. Other CAM include 3-methoxy-5-chloro-*para*-anisaldehyde [17], 4-chloro-*meta*-anisaldehyde [18], (3-chloro-4-hydroxy phenyl) ethanoic acid [19], (3-chloro-4-methoxy-phenyl)-1,2-propandiol [20], (chloro-4-methoxy-phenyl) methyl-propenoic acid [21], and (methyl-3,5-dichlorobenzyl alcohol)-3,5-dichloro-4-hydroxy phenyl methanoic acid [22].

Chlorinated aromatic metabolite production is a common capacity among basidiomycete fungi. It is estimated that approximately half of all basidiomycete fungi are capable of synthesizing chloroaromatic metabolites. Verhagen et al. (1996) tested 191 basidiomycete strains from 87 genera and observed organohalogen synthesis in 50% of strains and 55% of genera tested. CAM have been detected in a total of 18 genera belonging to 7 families from the orders of the *Agaricales*, *Hymenochaetales*, *Polyporales* and *Russulales* (de Jong and Field 1997). The genera *Hypholoma*, *Bjerkandera*, *Stropharia* and *Oudemansiella* produce the highest concentrations of CAM, ranging from 5 to 108 mg/l liquid culture. Highest producers were especially dominated by species belonging to the genera *Hypholoma*. A culture medium of *H. elongatum* contained 108.4 mg DCA per liter (0.52 mM) and is the highest concentration of CAM produced by basidiomycetes in defined medium so far known (Swarts et al. 1997). Moderate CAM-producing genera include *Ramaria*, *Mycena*, *Peniophora*, *Pholiota, Lepista, Phellinus, Phylloporia, Armillaria*; CAM concentrations range from 2-16 mg CAM per liter liquid culture. Low levels of CAM (< 1mg per liter) have been detected in liquid cultures of the genera *Daedaleopsis*, *Fomes*, *Ischnoderma*, *Marasmius*, *Poria*, and *Trametes*. Many moderate and high producers of CAM are also highly ecologically significant fungal

species. *Hypholoma* sp. fruiting bodies are commonly observed on Manitoba lawns (Bisby 1938). *Bjerkandera*, *Hypholoma*, and *Mycena,* common genera that produce significant quantities of CAM, have a broad geographic distribution and high population densities (Field, Verhagen, and De Jong 1995). These genera have been shown to contain between 1,074-30,893 mg organohalide per kg dry weight mycelial biomass (Verhagen et al. 1996).

Basidiomycetes have been shown to produce CAM in defined liquid media as well as laboratory cultures grown on lignocellulosic substrates. *Hypholoma*, *Mycena* and *Bjerkandera* species produced the highest amount of halogenated organic compounds when grown in liquid media; after 3-4 weeks incubation the cultures were dominated by 3,5-dichloro-*para*-anisyl compounds and after 10 weeks most DCA was oxidized to the corresponding anisic acid (Verhagen et al. 1998*a*,*b*). *Bjerkandera* sp. strain BOS55, grown on autoclaved lignocellulosic substrates at 30 g/l, produced CAM in concentrations ranging from 12 to 61 mg per kg dry weight of wooded material after 30 days (De Jong et al. 1994).

CAM have also been detected in environmental samples colonized with basidiomycete strains that produce CAM in the laboratory. Concentrations of chlorinated metabolites found in nature range from 15-180 mg per kg dry sample weight (De Jong et al. 1994). These values exceed Canadian regulations for analogous chlorophenols in soil, which are set between 1-10 mg/kg for mandatory remediation (Field, Verhagen and De Jong 1995). De Jong et al. (1994) detected CAM in all of several forested sites where random samples of moldy litter and rotten logs were taken. They did not detect CAM in samples of fresh forest litter or intact wood. There are no reports of the presence of CAM in grassland soils nor composts. DCA was the most common CAM detected in

environmental samples from forested sites, in concentrations up to 180 mg per kg dry sample weight. DCA was also the most common CAM synthesized in laboratory cultures, detected in 7 genera of basidiomycete fungi from the orders *Agaricales*, *Polyporales* and *Hymenochaetales*.

CAM play a role in the ligninolytic system of white-rot fungi (De Jong et al. 1994). CAM alcohols are recyclable substrates for extracellular aryl alcohol oxidases, which have a high affinity for chlorinated anisyl alcohols, 3,5-dichloroanisyl alcohol and 3-chloroanisyl alcohol compared to the nonhalogenated secondary metabolites, veratryl alcohol and *para*-anisyl alcohol (de Jong et al. 1994). Chlorination of anisyl metabolites makes them better substrates for the H_2O_2-generating oxidases. CAM alcohols are oxidized to their corresponding aldehydes in order to reduce molecular oxygen to hydrogen peroxide, a necessary co-substrate for the extracellular ligninolytic peroxidases. The aldehydes formed are intracellularly reduced to the corresponding alcohols, generating a physiologically sustainable cycle (De Jong et al. 1994).

Aryl alcohol oxidases also reduce quinones and phenoxy radicals, thereby preventing the repolymerization of the phenolic intermediates of lignin degradation (Marzullo et al. 1995). The chlorinated anisyl alcohols are more resistant to the fungal ligninolytic enzymes than the nonchlorinated analogs because the electron-withdrawing character of the chloro group increases the oxidation potential of the methoxy benzyl ring (Swarts et al. 1996).

2.2.1.1 Environmental Fate of Chlorinated Anisyl Metabolites.

Once biosynthesized by white-rot fungi, we would expect CAM to diffuse away from basidiomycete colonies. CAM can be detected in close vicinity of fruiting bodies of CAM-producing basidiomycetes (De Jong et al. 1994). However just outside the

colonies low-molecular weight organohalogens were no longer detected, suggesting that the compounds are biodegraded or biotransformed (Verhagen et al. 1998*c*). De novo biosynthesized halogenated organic compounds may be fully mineralized or undergo biotransformations to be converted to chlorophenols, which can be incorporated into humus or transformed into PCDD and PCDF (Figure 2-4; De Jong et al. 1994). Fungal metabolites may also enter anaerobic microniches at the boundary of fungal colonies and wetland soils where they undergo anaerobic metabolism (Verhagen et al. 1998*c*).

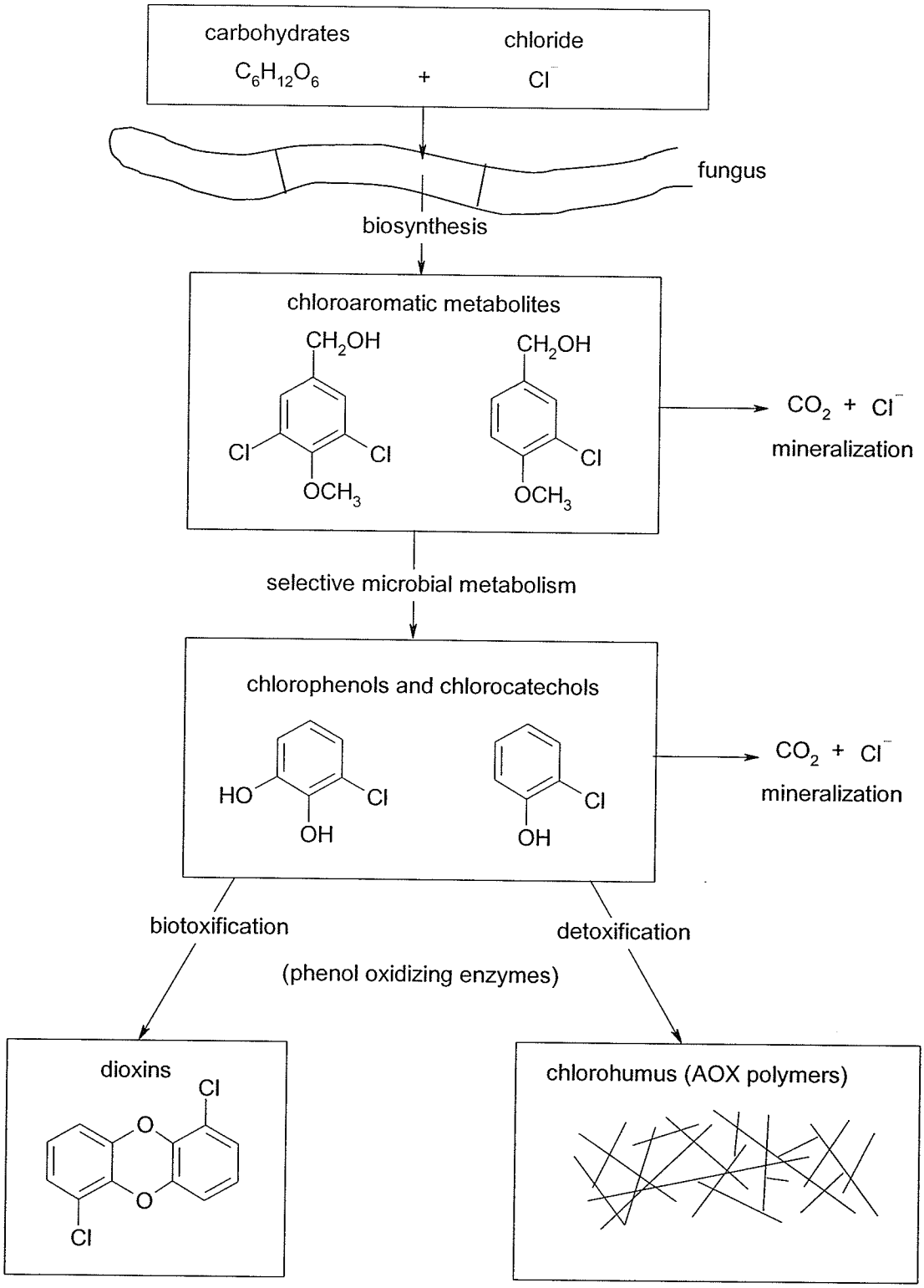


Figure 2-4. Possible environmental fates of chlorinated anisyl metabolites. Modified from De Jong et al. (1994).

Verhagen et al. (1998*c*) studied the anaerobic fate of the CAM, DCA. In methanogenic sludge, DCA was demethylated to 3,5-dichloro-4-hydroxybenzyl alcohol, which was converted via a biotic route leading to the formation of 3,5-dichloro-4-hydroxybenzoate (Figure 2-5). Demethylation was inhibited by oxygen, suggesting the involvement of acetogenic bacteria. Finally, a biotic decarboxylation step yielded 2,6-dichlorophenol. An abiotic route led to the formation of bis(3,5-dichloro-4-hydroxyphenyl)methane, a product resulting from the dimerization of the demethylated fungal metabolite (Figure 2-6). Verhagen et al. (1998*c*) did not observe dechlorination in this study.

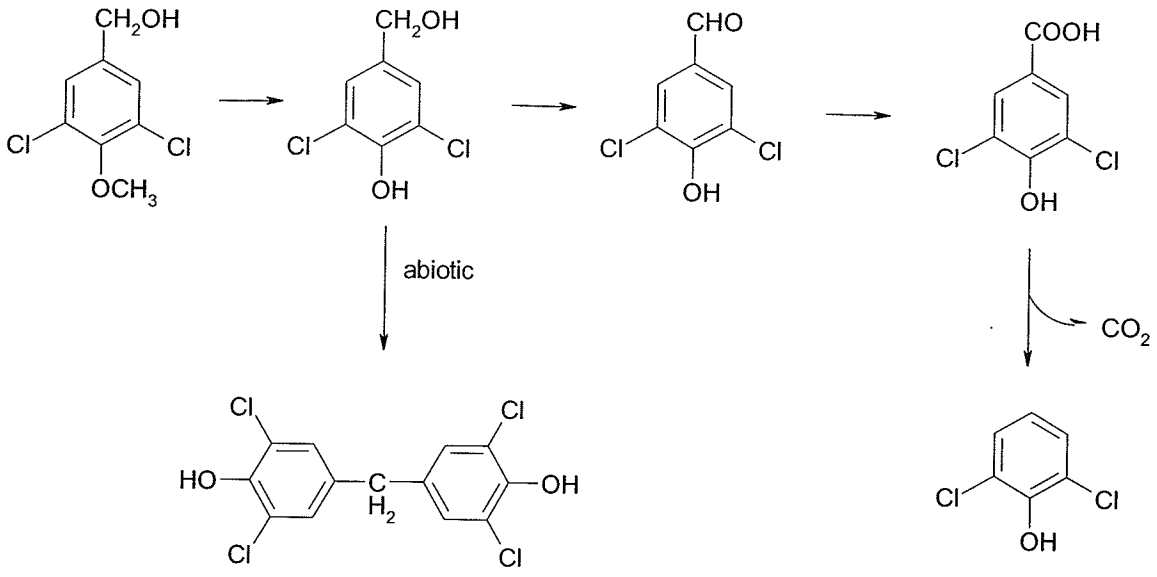


Figure 2-5. Metabolism of 3,5-dichloro-p-anisyl alcohol under anaerobic conditions by methanogenic sludge (Verhagen et al. 1998*c*).

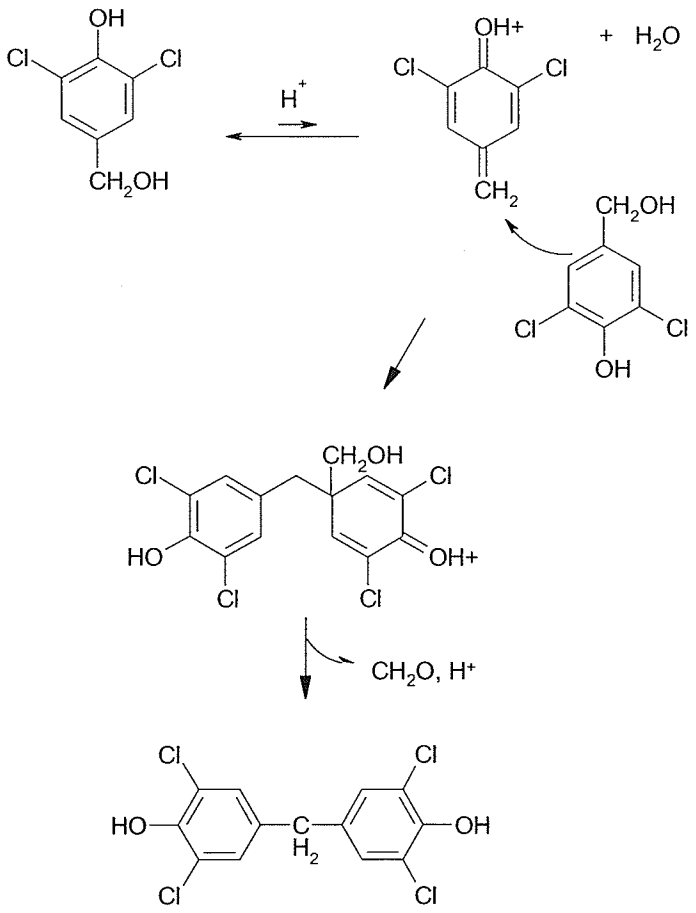


Figure 2-6. Proposed reaction mechanism for the formation of bis(3,5-dichloro-4-hydroxyphenyl)methane by dimerization of 3,5-dichloro-4-hydroxybenzyl alcohol (Verhagen et al. 1998*c*).

Once synthesized, CAM can undergo selective microbial metabolism; demethylation of CAM would yield chlorophenols and chlorocatechols. There are numerous examples of chloroaromatic demethylations as the methoxyl group liberated by demethylation is often used as a carbon and energy source. As mentioned previously, DCA [11] is biologically demethylated by methanogenic sludge (Verhagen et al. 1998*c*). In addition, chlorinated analogs of the phenyl methyl ethers vanillic and syringic acids are examples of low-molecular weight products derived from fungal lignin depolymerization. These compounds are demethylated by acetogenic bacteria belonging

to the genera *Acetobacterium*, *Eubacterium*, *Clostridium* and other anaerobic bacteria (Daniel, Wu and Drake 1988; Cocaign, Wilberg and Lindley 1991; Bache and Pfenning 1981). Products of CAM demethylations, chlorophenols and chlorocatechols, may be mineralized, undergo biotoxification to dioxins or may be detoxified by incorporation into soil humus (Figure 2-4; De Jong et al. 1994). Chlorinated aromatic compounds may also undergo selective microbial metabolism yielding various biotransformation products.

CAM and their demethylation products may also be completely mineralized to carbon dioxide and chloride. A large variety of bacteria, fungi, and microbial consortia are capable of completely mineralizing a wide range of halogenated aromatic compounds under aerobic and anaerobic conditions. Three-chlorobenzoate (Townsend, Ramanand, and Suflita 1997; Becker et al. 2005) and chlorinated hydroquinone metabolites (Milliken et al. 2004B) are a few examples of chlorinated aromatic compounds completely mineralized under anaerobic conditions. Examples of dechlorination and mineralization under aerobic conditions will be discussed in detail in section 2.4.

Ligninolytic Basidiomycetes, synthesizers of CAM, mineralize a wide range of chlorinated aromatic compounds, including chlorophenols, although the chlorinated anisyl alcohols are resistant against oxidative reactions with ligninolytic enzymes (De Jong et al. 1994). There is evidence that a large portion of chlorinated phenols and catechols resulting from CAM catabolism are not mineralized but rather become incorporated into soil humus.

Humic acid substances are highly condensed phenolic compounds. Chlorohumus is a high-molecular weight organohalogen present in soil and water; it is non-toxic and poorly biodegraded (Bollag, Shuttleworth and Anderson 1988; Dec and Bollag 1988). It is not known exactly how chlorohumus is formed but the polymerization

of low-molecular weight material biosynthesized by Basidiomycetes and other microorganisms has been suggested (De Jong et al. 1994). Monomeric building blocks in naturally occurring humus include 3-chloro- and 3,5-dichloro-4-hydroxybenzoate and 3-methoxy-5-chloro-4-hydroxybenzoate (Dahlman et al. 1993; Hjelm and Asplund 1995). Partial metabolism of CAM at the methoxy and/or alcohol groups and hydroxylation reactions yields chlorophenols and chlorocatechols which are readily copolymerized into humus with phenol oxidizing enzymes (Bollag, Shuttleworth and Anderson 1988). Consequently, CAM and other halogenated organic metabolites should be regarded as important precursors to the building blocks for the environmentally persistent adsorbable organic halogen polymers present in pristine forest environments (De Jong et al. 1994). Biotransformations of CAM may not only lead to detoxification but biotoxification of CAM demethylation products to dioxins may also occur. Peroxidase-mediated oxidation of chlorophenols, intermediates in CAM degradation, results in the generation of the natural hazardous compounds, PCDD and PCDF (Maloney et al. 1986). Chlorinated dioxins are also formed in sewage sludge and during composting (Rappe 1996).

2.2.1.2 Utility of Chlorinated Anisyl Metabolites. The CAM, including DCA, have antimicrobial activity towards bacteria, yeast and other fungi (Field, Verhagen, and De Jong 1995; Holloway unpublished data). DCA has been shown to inhibit seed germination in plants (Hautzel and Anke 1990; Holloway unpublished data) and inhibit chitin synthase (Pfefferle et al. 1990).

2.3 Aromatic Ring Cleavage

Aerobic degradation of "unactivated" aromatic compounds typically occurs through arene *cis*-diols to common hydroxylated intermediates like catechol or dihydroxybenzoates by dioxygenases (Gibson et al; Fong, Goh, and Tan 1996). One of

the first steps in the degradation of aromatic compounds, therefore, involves the introduction of one or more hydroxyl groups to the aromatic ring. A diverse array of mono- and dioxygenases convert substituted aromatic compounds into prerequisite hydroxylated compounds ("activation") that can be funneled into common aromatic degradative pathways (Wackett and Hershberger 2000). Dioxygenases cleave the aromatic nucleus of catechols and dihydroxybenzoates between two hydroxyl groups (*ortho-* or intradiol cleavage) or at a site adjacent to a hydroxyl group (*meta-* or extradiol cleavage) (Figure 2-7). For gentisate and homogentisate that carry two hydroxyl groups in the *para* position relative to each other, ring fission occurs between the carboxyl or acetyl substituent and the proximal hydroxyl group (Harayama, Kok, and Neidle 1992). *Ortho*-cleavage channels the cleavage product through common β-ketoadipate enol lactone intermediates and is therefore termed the β-ketoadipate pathway. The end products of both the *ortho-* and *meta-* cleavage pathways are then metabolized to intermediates that can enter the tricarboxylic acid (TCA) cycle.

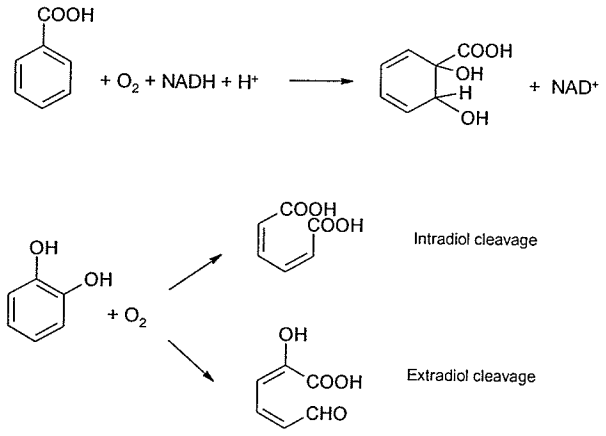


Figure 2-7 Oxygenase-catalyzed reactions of aromatic compounds.

2.3.1 Monooxygenase-Catalyzed Hydroxylation

A variety of monooxygenases catalyze the hydroxylation of substituted aromatic compounds in preparation for ring cleavage. Monooxygenase-catalyzed reactions incorporate one atom of dioxygen into the organic substrate as a hydroxyl group and the other is reduced to water, the reducing power provided by NAD(P)H. Oxidation of NAD(P)H and subsequent hydroxylation of the substrate is most often catalyzed by single-component enzymes but may also be catalyzed by separate polypeptides, linked by an electron transfer chain as in some phenol and toluene monooxygenases (Harayama, Kok, and Neidle 1992). Three classes of monooxygenases that catalyze the hydroxylation of aromatic rings include the flavoprotein, multicomponent binuclear iron, and cytochrome P450 monooxygenases.

2.3.2 Dioxygenase-Catalyzed Aromatic Degradation Reactions

2.3.2.1 Hydroxylation Reactions. Aromatic ring dioxygenases, distinguished from aromatic ring cleavage dioxygenases, catalyze the addition of hydroxyl groups. Multicomponent enzyme systems belonging to a family of enzymes known as Rieske non-heme iron oxygenases catalyze the addition of dioxygen to the aromatic nucleus. NAD(P)H donates electrons connecting dioxygen to the substrate and resulting in the formation of arene *cis*-diols (Whited et al. 1986; Wubbolts and Timmis 1990). Arene *cis*-diols are rearomatized by pyridine nucleotide-dependent dehydrogenases, which replace the NAD(P)H used in the initial dehydroxylation reaction, and form catechols or dihydroxybenzoates which can be funneled into common aromatic degradative pathways.

2.3.2.2 Ring -Cleavage Reactions. The aerobic degradation of aromatic hydrocarbons is facilitated by a second group of dioxygenases which catalyze cleavage of the aromatic ring in a reaction adding both atoms of molecular oxygen to the substrate (Harayama, Kok, and Neidle 1992). Aromatic ring cleavage dioxygenases catalyze cleavage of the aromatic component with incorporation of two hydroxyl groups from both atoms of molecular oxygen and do not require an external reductant. Reactions of dioxygen with transition metals, most commonly iron, facilitate the reaction with organic substrates through formation of metal-oxygen complexes.

2.3.2.2.1 Extradiol (*Meta*) Ring Cleavage. Dioxygenases which facilitate extradiol (*meta*) ring cleavage are typically multicomponent enzymes, containing Fe(II) as cofactor. The substrate range for aromatic ring cleavage dioxygenases is relatively broad; however, some substituted, particularly halogenated, substrates are suicide inhibiters. Suicide inhibition of dioxygenases acting on chlorinated substrates will be discussed in section 2.4. Examples of the various types of extradiol cleavage dioxygenases include catechol 2,3-dioxygenase (Riegert et al. 1998), protocatechuate 4,5-dioxygenase (Kersten, Chapman, and Dagley 1985), dihydroxybiphenyl dioxygenases (Furukawa, Arimura, and Miyazaki 1987), and homoprotocatechuate 2,3-dioxygenase (Jamaluddin 1977).

2.3.2.2.2 Intradiol (*Ortho*) Ring Cleavage. In contrast to extradiol cleavage enzymes which contain Fe(II) as cofactor, intradiol cleavage enzymes contain a nonheme, non-iron-sulfur Fe(III) prosthetic group. Examples of intradiol cleavage enzymes include catechol 1,2-dioxygenase (Dorn and Knackmuss 1978), chloro and dichlorocatechol 1,2-dioxygenases (Van Der Meer et al. 1991), protocatechuate 3,4-dioxygenase (Walsh and Ballou 1983), and gentisate 1,2-dioxygenase (Feng, Khoo, and Poh 1999).

Catechol 1,2-dioxygenases are very poor catalysts in the conversion of halogenated substrates. Chloro and dichlorocatechol 1,2-dioxygenases, however, have broader substrate specificity and are commonly found in degradative pathways for chlorinated aromatic compounds. A detailed discussion of dioxygenases facilitating chlorinated substrates is provided in section 2.4.1.2. Protocatechuate 3,4-dioxygenase catalyzes the intradiol (*ortho*) cleavage of 3,4-dihydroxybenzoate (protocatechuate) and is also capable of cleaving halogenated substrates (Walsh and Ballou 1983). Gentisate 1,2-dioxygenase constitutes a unique group of ring cleavage dioxygenases as its substrate has two hydroxyl positions in the *para* configuration.

Many dioxygenases have evolved broad substrate specificities allowing them to accommodate a wide range of substrates (Wackett and Hershberger 2000). Random genetic mutations result in changes in enzyme folding or catalytic mechanism that, most often, have an adverse affect. Occasionally non-silent mutations result in an advantageous change, broadening substrate range, for example. Soil bacteria will have a selective advantage if they can evolve dioxygenases with broad substrate specificities accommodating a wide range of catabolites (Wackett and Hershberger 2000). A plethora of dioxygenases exist; they may be chromosomally or plasmid-encoded and thus are common in a wide range of aerobic bacteria. The dioxygenases discussed here are found mainly in the genera *Pseudomonas*, *Arthrobacter*, *Comamonas*, *Alcaligenes*, *Brevibacterium*, and *Bacillus* but also *Burkholderia*.

2.4 Microbial Dechlorination

Microbial biodegradation of chlorinated aromatic compounds is complicated by chlorine atoms, which interfere with enzymatic breakdown of the aromatic ring. The biological recalcitrance of chlorinated xenobiotic compounds is thus related to the

number, type and position of the substituent (Fetzner and Lingens 1994). Increased electronegativity and size of the halide substituent (F>Cl>Br>I) relative to a hydrogen atom is correlated with increased recalcitrance of the carbon-halogen bond (Copley 1997). Increased persistence is also observed with an increasing number of halogen substituents. Polychlorinated substances are generally less degradable than mono- or di-chlorinated compounds. In addition, halogenated analogs of intermediary metabolites are often toxic because they inhibit key reactions or form dead-end metabolites. Therefore, early dehalogenation of a halogenated compound is advantageous to the organism because fewer separate enzymes are required, and the downstream metabolic intermediates can be readily degraded by enzymes that function in routine aromatic hydrocarbon catabolism.

Substituted aromatic compounds are often converted to common intermediates like catechol, protocatechuate, and gentisate before proceeding to ring cleavage and common biochemical pathways. Aromatic ring hydroxylation and subsequent ring cleavage reactions of nonhalogenated aromatic compounds are carried out by mono- and di-oxygenases as discussed in the previous section.

Dehalogenation, however, does not always precede ring cleavage. The enzymes and metabolic pathways involved in aromatic catabolism are multifunctional, catalyzing the degradation of a variety of compounds. For example, naphthalene-1,2-dioxygenase from *Pseudomonas* sp. NCIB9816 acts on greater than 70 substrates, primarily catalyzing the dioxygenation of unsubstituted, substituted, and heterocyclic aromatic compounds (Resnick, Lee, and Gibson 1996). It is this relaxed substrate specificity that allows halogenated aromatic substrates to be converted to halogenated non-aromatic substrates

which may undergo spontaneous dehalogenation depending on the stability of the halogenated intermediate, or may be dehalogenated in a further step.

Aerobic dehalogenation of chlorinated aromatic substrates may occur via oxidative, reductive, hydrolytic, or thiolytic mechanisms, in specific dehalogenating reactions or spontaneously after cleavage of the aromatic ring (Fetzner 1998; Copley 1997). In addition, there are enzymes, which because of their relaxed substrate specificity, catalyze the conversion of halogenated analogs of the corresponding unsubstituted substrate, which sometimes leads to "fortuitous" dehalogenation of the substrate analog.

Bacteria metabolize chlorinated hydrocarbons in four different ways. The carbon structure may be assimilated into biomolecules, supporting bacterial growth. Second, after dechlorination the carbon structure may be oxidized to CO_2 generating energy in the form of ATP. Third, chlorinated organic compounds may be used as electron acceptors during metabolic oxidation reactions in the production of ATP. Chloride ion is released but the dechlorinated organic compound may or may not be assimilated. Last, the chlorinated organic compound may be metabolized without energy production, nor assimilation. In the last case the chlorinated hydrocarbon competes with a nonhalogenated growth substrate for the active site. With no apparent benefit to the organism (these reactions may even have a negative effect) this type of metabolism is termed cometabolism.

2.4.1 Oxidative Dechlorination

Oxidative dehalogenation reactions are catalyzed by mono- or dioxygenases, in cometabolic or metabolic reactions (Fetzner 1998; Copley 1997). Oxygenases utilize a cofactor, transition metal, flavin or pteridine, which provide reducing power and interact

with dioxygen producing hydroxylated and often dehalogenated products. Dioxygenases catalyze the addition of both atoms of molecular oxygen to the substrate while monooxygenase-catalyzed reactions incorporate one atom of dioxygen into the organic substrate as a hydroxyl group and the other is reduced to water. Oxidative dechlorination of chlorinated aromatic compounds can occur via specific reactions or fortuitously upon binding the active site of an enzyme designed to catalyze the nonhalogenated analog. Thus dechlorination results indirectly as a result of an oxidative reaction, as the spontaneous chemical dechlorination of an unstable intermediate.

2.4.1.1 Dechlorination Catalyzed By Monooxygenases. Removal of the halide substituent on an aromatic substrate is often accompanied by the addition of a hydroxyl group. Monooxygenases which dechlorinate phenols, including PCP, are well studied.

PCP monooxygenase commonly found in strains of *Sphingomonas chlorophenolica* (previously *Pseudomonas* and *Flavobacterium*) and also Gram positive actinomycetes (often *Mycobacterium* spp.) dechlorinates PCP to tetrachlorohydroquinone which goes on to further dechlorination reactions by another mechanism (Orser et al. 1993; Copley 1997; Fetzner 1998).

Cytochrome P-450 monooxygenases catalyze both oxidative and reductive dehalogenation depending on oxygen availability (Fetzner 1998). A cytochrome P-450 monooxygenase from *Mycobacterium fortuitum* CG-2 catalyzes the *para*-hydroxylation and oxidative dehalogenation of halogenated phenols to their corresponding halogenated quinones while the cytochrome P-450 from *Pseudomonas putida* G786 reductively dechlorinates polychlorinated ethanes (Uotila et al. 1992 and Logan et al. 1993, respectively).

2.4.1.2 Dechlorination Catalyzed By Dioxygenases. Aerobic catabolism of chlorinated aromatic substrates commonly includes the involvement of a dioxygenase (Copley 1997; Fetzner 1998). Dioxygenases act on halogenated aromatic compounds with formation of catechol or hydroxylated benzoates and concomitant loss of the halide ion. Formation of a *cis*-diol product and subsequent spontaneous elimination of HCl or decarboxylation coupled with chloride release, gives a catechol product. Spontaneous re-aromatization of the *cis*-diol intermediate favors chloride elimination, yielding a non-halogenated catechol product which can enter normal aromatic metabolic pathways.

2.4.1.2.1 Dehalogenating Catechol Dioxygenases. The degradative pathways of compounds such as 3-chlorobenzoate, 2,4-D, and chlorinated phenols and benzoates often converge at chlorocatechols as the common intermediate. Subsequently, chlorocatechols may undergo cleavage by catechol dioxygenases. Catechol dioxygenases are comprised of two distinct groups: those that catabolize halogenated substrates (group II) and those whose strict substrate range does not include halogenated compounds (group I). As a result there are separate *ortho*-cleavage pathways for the degradation of catechols and chlorocatechols. Those dioxygenases which accept halogenated catechols, perform oxygenative ring cleavage with the halogen element removed during lactonization and isomerization reactions. Catechol 1,2-dioxygenase activity is monitored by the formation of *cis, cis*-muconates, products of *ortho*-ring cleavage. Catechol 1,2-dioxygenase converts chlorinated catechols to chloro-*cis,cis*-muconates with dechlorination occurring in a subsequent step. *Meta*-cleavage of chlorinated catechols by catechol 2,3-dioxygenases results in the formation of chlorinated hydroxymuconic semialdehydes, which are often dead end or suicide metabolites (Bartels, Knackmuss, and Reineke 1984; Haggblom 1992).

2.4.1.2.1.1 Intradiol (*Ortho*) Cleavage of Chlorocatechols and Subsequent Dechlorination. Arylhalide dehalogenation often involves the removal of aromaticity and labilization (destabilization) of the halogenated molecule by the introduction of oxygen (Slater, Bull, and Hardman 1995; Knackmuss 1981; Harayama, Kok, and Neidle 1992; Pollman et al. 2002). After ring cleavage, the halide ion is removed spontaneously or fortuitously through normal aromatic metabolism. In these cases the chlorinated aromatic compounds are converted to chlorocatechols, which enter the modified *ortho*-cleavage pathway eventually yielding chloromuconic acids, which cycloisomerize releasing chloride spontaneously (Pollman 2002).

In the modified *ortho*-pathway, dioxygenase facilitated aromatic ring cleavage of chlorinated catechols occurs although dechlorination does not take place until after ring cleavage in a subsequent step by chloromuconate cycloisomerases (dehydrodehalogenation). The dechlorinated metabolites subsequently enter nonhalogenated metabolism and the TCA cycle (Fetzner 1998).

Dehalogenation of arylhalides via intradiol cleavage facilitated by dioxygenases is the most common mechanism removing chloride substituents from aromatic substrates (Fetzner 1998). Hence, there are plenty of examples of dioxygenases catalyzing cleavage and associated dechlorination of chlorinated aromatic compounds. Dichlorocatechol 1,2-dioxygenase transforms dichlorocatechol to chloromaleylacetic acid via *ortho*-ring cleavage, in the degradative pathways of 3-chlorobenzoate and 2,4-D (Don et al. 1985). *Pseudomonas* sp. B13 also degrades 3-chlorobenzoate in this manner, releasing chloride upon cycloisomerization of 3-chloro-*cis,cis*-muconate (Dorn et al. 1974; Schmidt and Knackmuss 1980).

2.4.1.2.1.2 Extradiol (*Meta*) Cleavage of Chlorocatechols. The fate of chlorocatechol degradation by extradiol cleavage strongly depends on the position of the chloride substituent. *Meta* cleavage of 3-chlorocatechol, a common catabolite of 2- and 3-chlorobenzoate, results in the suicide inactivation of the *meta* cleavage enzyme, catechol 2,3-dioxygenase. *Meta*-cleavage of 4-chlorocatechol, on the other hand, is accompanied by dechlorination and proceeds to mineralization.

Meta-cleavage of halocatechols is often unproductive because 3-halocatechols inactivate the cleavage enzyme catechol-2,3-dioxygenase (Bartels, Knackmuss, and Reineke 1984). Inactivation of the enzyme is presumed to occur by chelation of the catalytically active ferrous ion or by the production of the toxic, highly reactive acyl halide (5-chloroformyl-2-hydroxypenta-2,4-dienoic acid) which binds macromolecules irreversibly.

It was previously thought that all *meta*-cleavage products of 3-chlorinated substrates were detrimental due to the build up of suicide metabolites. However, a novel type of chlorocatechol 2,3-dioxygenase purified from *Pseudomonas putida* GJ31, upon cleavage of 3-chlorocatechol, hydrolyzes the resulting acylchloride yielding 2-hydroxymuconic acid and is not subject to suicide inactivation (Kaschabek et al. 1998; Mars et al. 1997).

Similarly, proximal *meta*-cleavage of 5-chloroprotocatechuate, a product of 5-chlorovanillate degradation, by a 4,5-dioxygenase from *Pseudomonas testosteroni*, results in spontaneous lactonization and concomitant elimination of chloride yielding 2-pyrone-4,6-dicarboxylate (Kersten, Chapman, and Dagley 1985).

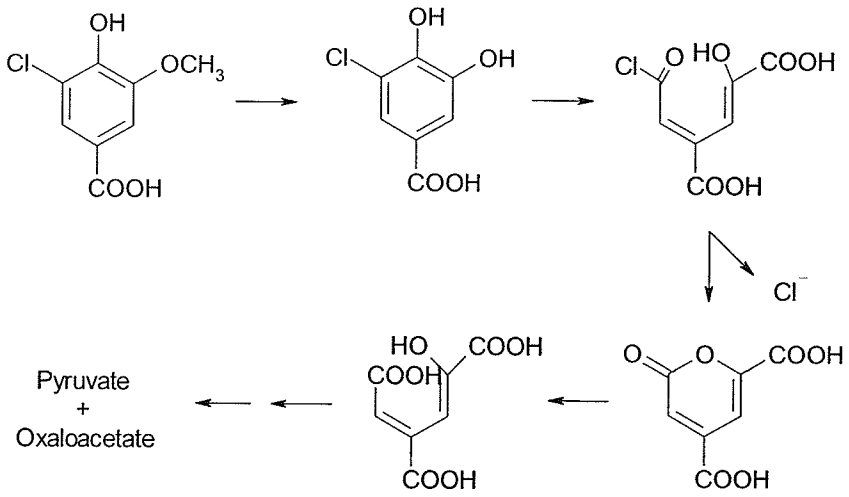


Figure 2-8. Degradation of 5-chlorovanillate with *meta*-cleavage and dechlorination of 5-chloroprotocatechuate. From Haggblom 1992.

Meta-cleavage of 4-chlorocatechols is catalyzed by a separate group of enzymes and does not lead to suicide inactivation as is common with 3-chlorocatechols. Many organisms that degrade 2-chloro and 2,4-dichlorobenzoates and catechols, with *ortho*-elimination of chloride, often do not also degrade 4-chlorocatechols and benzoates. 4-Chlorobenzoates may undergo hydrolytic dechlorination (discussed in section 2.4.5) or alternatively transformation to 4-chlorocatechols and cleavage by 4-chlorocatechol-2,3-dioxygenase yielding 5-chloro-2-hydroxymuconic semialdehyde (Wieser et al. 1994). The chlorinated muconate undergoes dechlorination by 5-chloro-2-hydroxy\muconic semialdehyde dehalogenase whose products can go on to mineralization.

2.4.1.2.2 Dioxygenases Other Than Catechol Dioxygenases That Catalyze Dechlorination Reactions. In addition to the catechol dioxygenases there are a large variety of dioxygenases that catalyze the dehalogenation of other halogenated aromatic substrates. Chlorophenyl dioxygenases, 2(*ortho)*-halobenzoate 1,2-dioxygenases,

3(*meta*)-chlorobenzoate dioxygenases, benzene dioxygenases, and toluene dioxygenases are some examples.

A common example of chlorophenyl dioxygenase is the two-component 4-chlorophenyl acetate 3,4-dioxygenase from *Pseudomonas* sp. strain CBS3 (Schweizer et al. 1987; Haggblom 1992). The reductase component transfers electrons from the cosubstrate NADH to the terminal oxygenase component where mononuclear iron mediates electron transfer to oxygen. $[FeO_2]^+$, the active oxygenating species attacks 4-chlorophenylacetate yielding 3,4-dihydroxyphenylacetate (Figure 2-9).

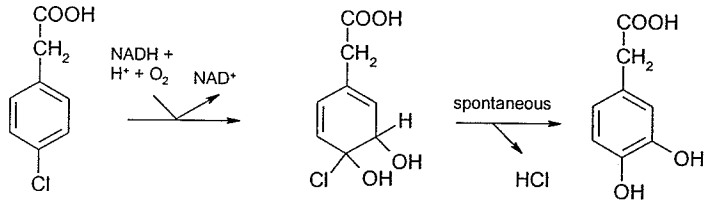


Figure 2-9. Dehalogenation of 4-chlorophenylacetate by a 3,4-dioxygenase system from *Pseudomonas* sp. strain CBS3. From Haggblom 1992.

Three (*meta*)-chlorobenzoate dioxygenase, plasmid encoded, from *Alcaligenes* sp. strain BR60 is involved in chlorobenzoate catabolism yielding protocatechuate in the dioxygenolytic attack of 3- and 4- chlorobenzoate (Nakatsu and Wyndham 1993). Protocatechuate subsequently undergoes normal *meta* aromatic ring cleavage and enters common pathways of non-halogenated compounds. Three (*meta*)-chlorobenzoate dioxygenase is not regiospecific and also catalyzes a 4,5-attack yielding 5-chloroprotocatechuate via an additional dihydrodiol dehydrogenase reaction.

2.4.2 Reductive Dechlorination

Reductive dehalogenation of chlorinated aromatic substrates occurs under both aerobic and anaerobic conditions. Reductive dechlorination can occur during specific reactions involved in carbon metabolism or coupled to energy conservation as some anaerobes use halogenated organic compounds as terminal electron acceptors of a respiratory process (Tiedje and Stevens 1988; Mohn and Tiedje 1992). Alternatively, reductive dehalogenation may also occur as a cometabolic process with no benefit to organism. Reductive dehalogenation involves the release of the halogen substituent as a halide ion and its replacement by hydrogen. A reduced organic substrate or H_2 is the source of reducing power and protons in a one-step transfer of two electrons and one proton. Alternatively, a two-step reduction by a reduced organic substrate as electron donor with proton abstraction from the solvent may occur.

Reductive dechlorination is particularly important for the initial catabolic steps of highly chlorinated compounds; reductive dehalogenation is thermodynamically more favourable when there are multiple electron-withdrawing chlorine substituents on the aromatic ring (Copley 1997). Evident in anoxic soils, sediments, and sludges, reductive dechlorination is responsible for the initial transformations of highly chlorinated compounds such as PCB, chlorobenzenes, chloroanilines, chlorophenols, chlorobenzoates, PCDD, PCDF and some chlorinated pesticides (Bunge et al. 2003; Wittich 1998; Fetzner 1998).

2.4.2.1 Reductive Dechlorination Linked To Carbon Metabolism. Reductive dechlorination linked to carbon metabolism is common in aerobic bacteria; strains from the genera *Sphingomonas*, *Pseudomonas*, *Azotobacter*, and *Corynebacterium* have been demonstrated to utilize reductive dechlorination reactions to facilitate carbon metabolism

of chlorinated substrates. For example, *Azotobacter chroococcum* MSB-1 and *Alcaligenes eutrophus* JMP134 utilize the herbicide 2,4-D as a sole carbon and energy source and metabolize it via reductive *ortho*-dechlorination to 4-chlorophenoxyacetic acid (Figure 2-10; Balajee and Mahadevan 1990; Vollmer, Stadler-Fritzsche, and Schlomann 1993).

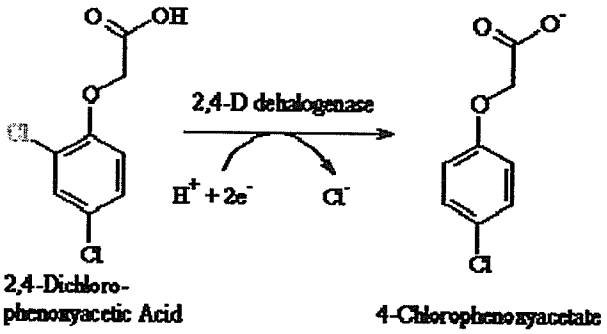


Figure 2-10. Reductive *ortho*-dechlorination of the herbicide 2,4-D. From Ellis, Roe, and Wackett (2006).

In a second example, *Corynebacterium sepedonicum* KZ-4 and coryneform strain NTB-1 aerobically mineralize 2,4-dichlorobenzoate via the NADPH-dependent reductive *ortho*-dechlorination of 2,4-dichlorobenzoyl-CoA to 4-chlorobenzoyl-CoA, which subsequently undergoes hydrolytic dechlorination to 4-hydroxybenzoyl-CoA which is metabolized to 4-hydroxybenzoate and protocatechuate (Van Den Tweel, Kok, and De Bont 1987; Romanov and Hausinger 1996; Fetzner 1998).

Reductive dechlorination may also occur after cleavage of the aromatic ring as part of carbon metabolism. Maleyl reductases play major roles in the dehalogenation and degradation of halogenated aromatic compounds by channeling maleylacetate and some brominated and chlorinated derivatives into the 3-oxoadipate pathway (Figure 2-11).

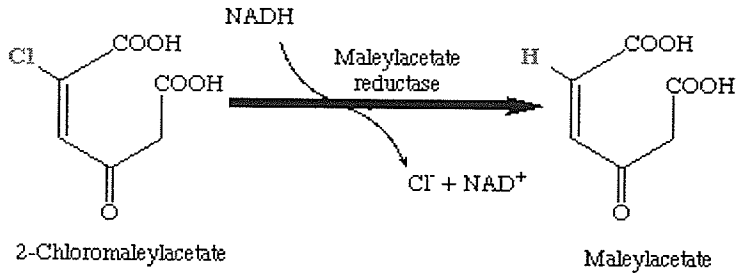


Figure 2-11. Maleylacetate reductase facilitates dehalogenation of substituted maleylacetates for channeling into the 3-oxoadipate pathway. From Ellis, Roe, and Wackett (2006).

Pseudomonas sp. B13, *P. aeruginosa* RHO1 and *Ralstonia eutropha* JMP134 have maleylacetate reductases which catalyze the reaction of 2-chloromaleyl acetate to 5-chloro-3-oxoadipate, using NADH as cosubstrate (Vollmer, Stadler-Fritzsche, and Schlomann 1993; Kaschabek and Reineke 1995; Muller, Schlomann, and Reineke 1996; Padilla et al. 2000; Fetzner 1998). Subsequently 5-chloro-3-oxoadipate undergoes HCl elimination, yielding maleyl acetate.

2.4.2.2 Reductive Dechlorination As A Respiratory Process. Several anaerobic bacteria are capable of coupling reductive dechlorination to energy metabolism by utilizing chlorinated hydrocarbons as final electron acceptors (Fetzner 1998). Dechlorination associated with this process is very specific and occurs at rates that are often several orders of magnitude greater than cometabolic rates (Holliger and Schraa 1994).

A well-studied example of reductive dechlorination as result of a respiratory process is that of the anaerobe *Desulfomonile tiedjei* DCB-1, which uses 3-chlorobenzoate as terminal electron acceptor during respiration (Tiedje and Stevens 1988; Dolfing 1990; Mohn and Tiedje 1992). This process is chemiosmotically coupled to ATP

synthesis. *Meta*-substituted dichlorobenzoates and chlorinated phenols may also act as terminal electron acceptors for this organism and are also dechlorinated in the process.

2.4.3 Thiolytic Dechlorination

Although thiolytic dehalogenation is technically a reductive and hydrolytic process, this unique mechanism warrants discussion in its own section. In an enzyme-catalyzed reaction, glutathione is used as the reduced organic substrate and attacks (nucleophilic) the chlorinated (electrophilic) substrate to form a thioether intermediate. The unstable intermediate decomposes nonenzymatically to regenerate glutathione with release of the chloride ion. Glutathione-*S*-Transferases (GST's) are a large family of enzymes, which catalyze the nucleophilic attack of glutathione on endogenous or xenobiotic electrophilic substrates to form a glutathione conjugate. Cyanobacteria, proteobacteria, phototrophs (Fahey and Sundquist 1991), and some gram-positive bacteria (Newton et al. 1996) are the only bacteria known to contain glutathione (Vuilleumier 1997). During the degradation of PCP by the strict aerobe *Sphingomonas (Flavobacterium) chlorophenolica* ATCC 39723 a glutathione-dependent, cofactor-free reductive dehalogenase converts tetrachlorohydroquinone to 2,3,6-trichlorohydroquinone and 2,6-dichloroquinone (Xun, Topp and Orser 1992). Reducing equivalents are provided by two molecules of glutathione per chlorine removed; nucleophilic attack of glutathione on an electrophilic form of the substrate forms a glutathione conjugate.

Interestingly, this hydroquinone pathway of reductive dehalogenation occurs mainly in organisms that utilize highly chlorinated phenols, such as PCP and PCB. Chlorinated aromatic compounds with fewer chloride substituents are more often degraded through chlorocatechols as discussed in section 2.4.1.

2.4.4 Hydrolytic Dechlorination

Hydrolytic dechlorination is relatively common in the breakdown of *para*-chlorinated benzoic acids by aerobic bacteria, but may occur on aromatic rings bonded to up to three chlorines (Haggblom 1992). Hydrolytic dechlorination is defined by the hydroxylation of the substrate, with the hydroxyl group derived from water as opposed to dioxygen as catalyzed by oxygenases (Muller et al. 1984; Haggblom 1992). Three types of enzymes catalyze hydrolytic dehalogenation: hydrolases, glutathione transferases, and hydratases (Fetzner and Lingens 1994). Hydrolases carry out nucleophilic substitution reactions with water-derived hydroxyl groups as in the degradation of 4-chlorobenzoate, a breakdown product of PCB, whereas hydratases catalyze the addition of water to an unsaturated bond resulting in the dechlorination of vinylic compounds by the chemical decomposition of unstable intermediates.

Several bacterial strains are capable degrading 4-chlorobenzoate under aerobic and anaerobic conditions (Slater, Bull, and Hardman 1995; Copley 1997). The best-studied example is the hydrolytic dechlorination of 4-chlorobenzoate to 4-hydroxybenzoate through a CoA intermediate by *Pseudomonas* sp. strain CBS3 (Scholten et al. 1991; Yang, Liang, and Dunaway-Mariano 1994; van den Tweel, Kok, and de Bont 1987). The process requires three enzymes: 1) 4-chlorobenzoate coenzyme A ligase, 2) 4-chlorobenzoyl-CoA dehalogenase or 4-hydroxybenzoate 3-monooxygenase, and 3) 4-hydroxybenzoyl-CoA thioesterase. This pathway connects to the *ortho*-cleavage pathway and thus to the protocatechuate branch of the *beta*-keto adipate pathway, completing the degradative route of PCB and 4-chlorobenzoate to mineralization.

CHAPTER 3

DCA-DECHLORINATION AND CATABOLISM

3.1 Abstract

The toxicity and widespread use of chlorinated man-made compounds as pesticides, flame retardants, coolants and preservatives has led to research involving the environmental fate of such chemicals. The chlorinated aromatic pesticides dicamba, 2,4-D and chloroneb are structurally similar to the fungal metabolite 3,5-dichloro-*para*-anisyl alcohol (DCA), which is synthesized in nature by basidiomycete fungi. The catabolic pathway of DCA may have been a prelude to the degradation of similarly structured chlorinated pesticides. The study of DCA-degrading microbes and their catabolic pathways may provide insight into the evolution of chlorinated pesticide biodegradation. Strain UW103, isolated from commercial compost and capable of growth on the fungal metabolite as a sole source of carbon and energy, dechlorinated DCA at both the 3 and 5 positions. During one of the first steps in DCA degradation, strain UW103 oxidized DCA to the corresponding anisaldehyde and subsequently to the anisic acid, an isomer of the chlorinated pesticide dicamba. Next, 3,5-dichloro-*para*-anisic acid was dechlorinated yielding monochloro-*para*-anisic acid, identified by GC-MSD analysis of ethyl acetate culture extracts. The remaining steps in DCA-mineralization likely include aromatic ring

cleavage of protocatechuate, a common precursor to ring cleavage in aromatic metabolism. The utilization of a similarly structured chlorinated herbicide by strain UW103 suggests that the catabolic pathway of DCA could be responsible for the degradation of this chemical in agricultural soil.

3.2 Introduction

A wide variety of chlorinated anthropogenic chemicals are used industrially as pesticides, herbicides, fungicides, antiseptics and intermediates in chemical syntheses, and others are released into the environment as industrial waste. Many of these compounds are listed as toxic substances in the Canadian Environmental Protection Act (Department of Justice Canada 1999). PCBs and chlorinated phenols include some of the most toxic examples. In Manitoba, pentachlorophenol, a wood preservative also used widely around the world, leaches into the environment from hydro poles and railway ties. The chlorinated herbicides 2,4-D and dicamba, alone or in combination, are used extensively in Manitoba as broadleaf herbicides.

Microorganisms exposed to man-made chlorinated chemicals will have a selective advantage not only if they can survive the toxicity that these chemicals are known for, but also can utilize the carbon and energy within. One potential source of enzymes necessary for the evolution of bacterial catabolic pathways specific for anthropogenic chlorinated compounds, are pathways involved in the degradation of non-chlorinated analogues. However, the electronegativity and size of a chloride substituent relative to a hydrogen atom is significantly different; as a result chlorinated analogues of intermediary metabolites are often toxic, inhibiting key reactions or forming dead-end metabolites. Therefore dechlorination is often the block or limiting step to detoxification.

Another, more likely, source of catabolic enzymes to dehalogenate and mineralize anthropogenic organic halogenated compounds are those involved in the degradation of naturally-chlorinated compounds. Naturally-produced halogenated organic compounds in terrestrial environments are predominantly chlorinated. One example, the chlorinated anisyl metabolites (CAM), are produced in high concentrations by a large variety of basidiomycete fungi, ubiquitously in terrestrial ecosystems across the planet (De Jong et al. 1994; Verhagen et al. 1996). A variety of CAM are produced by basidiomycete fungi, including the most common examples 3-chloro- and 3,5-dichloro-*para*-anisyl alcohols and their corresponding aldehydes and carboxylic acids.

With the exposure of microorganisms to naturally-produced chlorinated compounds in their environment for millions of years we would expect the presence of catabolic enzymes and pathways to have evolved to utilize the energy and building blocks within. Few studies have focused on the catabolic pathways that dechlorinate and mineralize natural chlorinated compounds. One study by Verhagen et al. (1998*c*) examined the anaerobic fate of the CAM, 3,5-dichloro-*para*-anisyl alcohol (DCA) in methanogenic sludge. DCA was biotically demethylated to 3,5-dichloro-4-hydroxybenzyl alcohol and decarboxylated yielding 2,6-dichlorophenol through the intermediate 3,5-dichloro-4-hydroxybenzoate. Dechlorination of DCA was not observed in this study.

Dechlorination of a wide variety of chlorinated aromatic compounds has been demonstrated by oxidative, reductive, thiolytic, hydrolytic, and spontaneous mechanisms (Fetzner 1998; Copley 1997). Dehalogenation of chlorinated aromatic compounds under aerobic conditions is often catalyzed by mono- or dioxygenases, in cometabolic and metabolic reactions. Oxidative dehalogenation of chlorinated aromatic compounds can

occur via specific reactions or fortuitously upon binding the active site of an enzyme designed to react with the nonhalogenated analog. Thus, dechlorination happens indirectly as a result of an oxidative reaction, as the spontaneous chemical dechlorination of an unstable intermediate.

3.3 Objective of Study

The objective of this study was to isolate a DCA-degrading microorganism and assess its ability to dechlorinate or mineralize DCA while utilizing it as a sole source of carbon and energy under aerobic conditions. The intermediates of DCA catabolism were investigated. Characterization of the ability of strain UW103 to degrade anthropogenic chlorinated compounds was also explored.

3.4 Materials and Methods

3.4.1 DCA Synthesis

DCA was not commercially available and thus synthesized in house beginning with the commercial starting material 3,5-dichloro-*para*-hyroxybenzoic acid, purchased from Sigma-Aldrich (St. Louis MI). Methylation and subsequent reduction using lithium aluminum hydride resulted in the product 3,5-dichloro-*para*-anisyl alcohol (DCA) in a synthesis method modified from De Jong et al. (1994). Eleven ml dimethyl sulfate and 17.4 g anhydrous potassium carbonate were added to a solution of 10.03 g 3,5-dichloro-4-hydroxybenzoic acid in 400 ml acetone, and refluxed under nitrogen for three hours. Once at room temperature the mixture was vacuum-filtered through silica gel and the acetone evaporated from the filtrate under reduced pressure. The remaining white solid was dissolved in 200 ml diethyl ether. This solution was consecutively washed with two

50 ml portions of 5% sodium hydroxide, 50 ml 10% hydrochloric acid, 50 ml saturated sodium bicarbonate, and 50 ml saturated sodium chloride. Anhydrous magnesium sulfate was used to dry the solution and was removed by gravity filtration. Diethyl ether was evaporated under reduced pressure to yield 10.8 g methyl 3,5-dichloro-4-methoxybenzoate. The intermediate was dissolved in hexane and analyzed by gas chromatography with mass spectrometric detection (Figure 3-1).

The intermediate was analyzed with an HP5890II gas chromatograph (Hewlett Packard, Ottawa ON) equipped with a fused-silica capillary column (HP-5MS fused silica gel column; 30 m by 0.25 mm internal diameter; film thickness, 0.25 μm; stationary phase, (5% Phenyl)-methylpolysiloxane) (Sigma-Adrich, Oakville ON). Detection was carried out with an HP 5970 Series Mass Selective Detector (Hewlett Packard, Ottawa ON). The injector temperature was 250°C, the detector temperature was 280°C and the temperature program began at 80°C for 2 min., increased by 10°C per minute to 240°C, and was held for 2 minutes. Sample volumes of 1 μl were injected automatically and helium was used as the carrier gas.

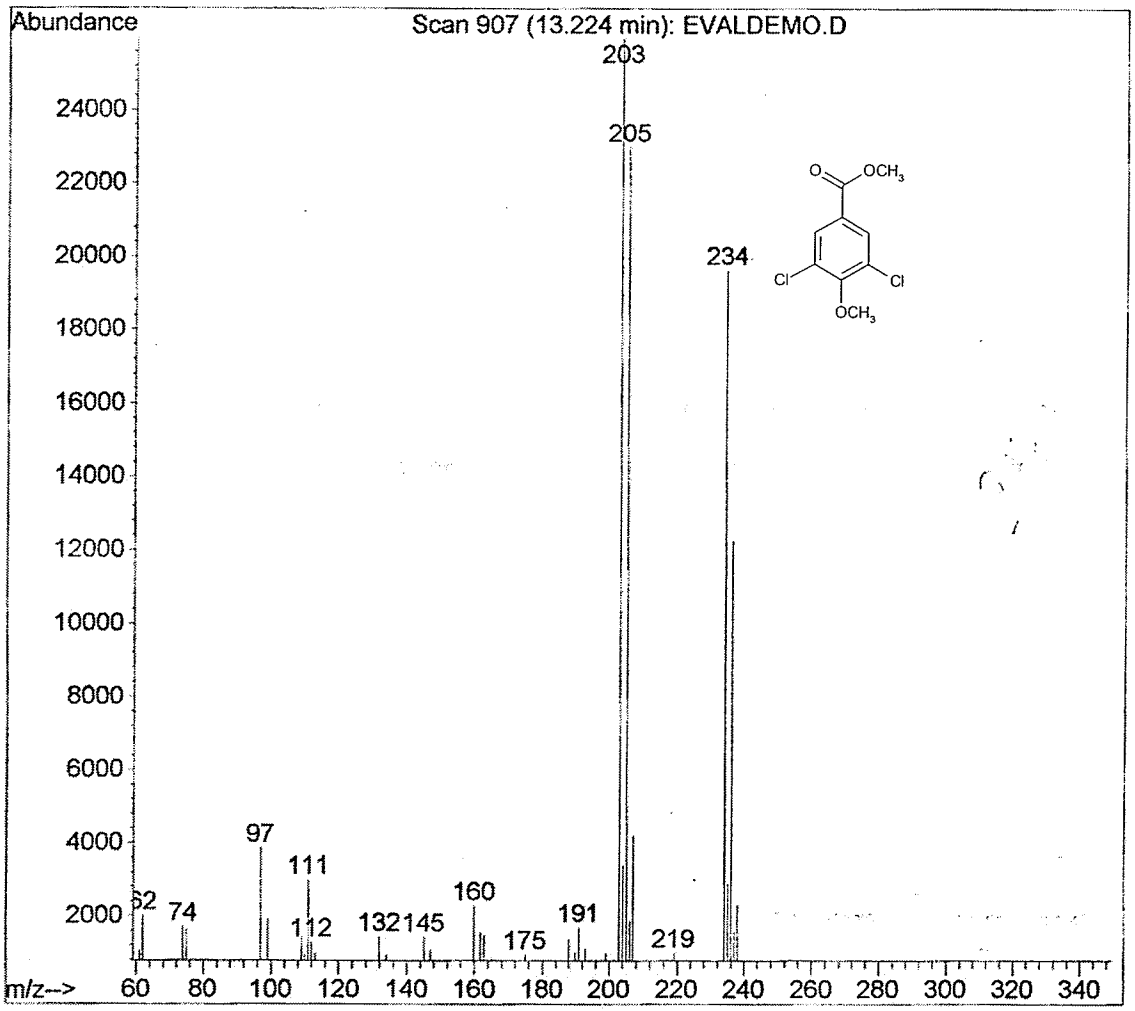


Figure 3-1. Mass Spectrum of methyl-3,5-dichloro-4-methoxybenzoate, an intermediate in the synthesis of DCA.

Lithium aluminum hydride (1.7 g) was added to 360 ml cold anhydrous diethyl ether under a nitrogen atmosphere. Methyl 3,5-dichloro-4-methoxybenzoate dissolved in anhydrous diethyl ether was added drop wise to the cold lithium aluminum hydride solution and the reaction mixture was stirred for 45 minutes. Ice water was added drop wise to the reaction mixture slowly. The remaining $LiAlH_4$ was extracted from the reaction mixture with two 50 ml portions of 5% HCl. The aqueous phase was extracted

with 2-50 ml portions of ether and mixed with organic phase containing product. Organic phase was washed with 50 ml saturated sodium bicarbonate, two 50 ml portions distilled water, and a 50 ml portion of saturated sodium chloride. The solution was dried over anhydrous magnesium sulfate and vacuum filtered through silica gel. Diethyl ether was evaporated and the product, 3,5-dichloro-*para*-anisyl alcohol was recrystallized from a 4:1 solution of ether and hexane. The product was dissolved in hexane and analyzed by gas chromatography with mass selective detection (Figure 3-2; Appendix I).

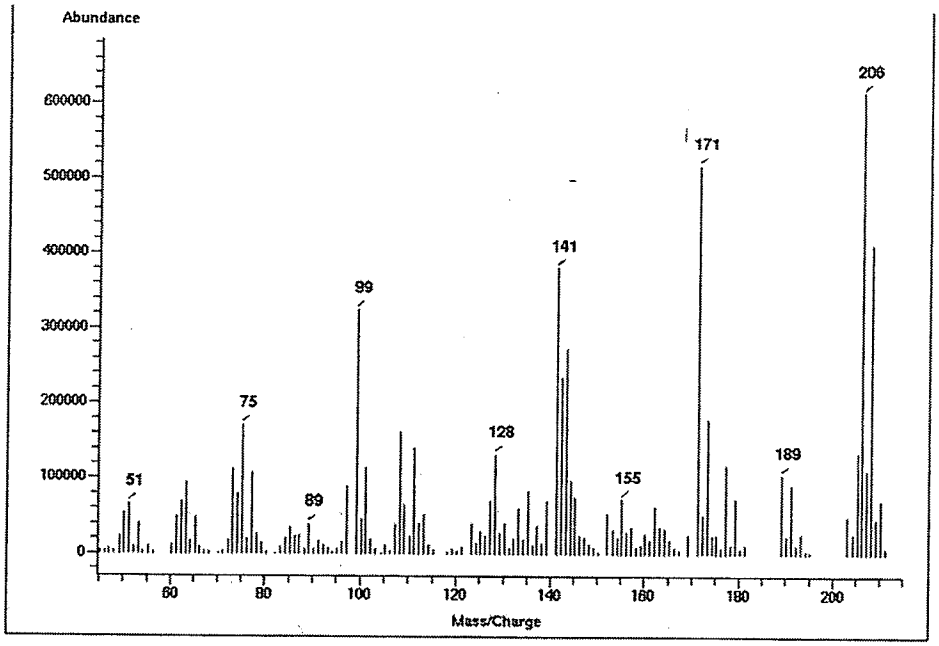


Figure 3-2. Mass spectrum of 3,5-dichloro-*para*-anisyl alcohol (DCA). Refer to appendix I for mass fragment analysis.

3.4.2 Isolation of a Bacterium Capable of Growth on DCA

An enrichment culture capable of growth on DCA as a sole source of carbon and energy was previously isolated from commercial compost (Premier Garden Max compost). The compost contained Canadian Humus, peat moss, and bovine compost. Original enrichment culture was isolated using a sterile medium consisting of 10.5 mg DCA (1.0 mM) in 50 ml Yeast Nitrogen Base without amino acids (YNB), inoculated with 5 grams of compost. The mixture was held at room temperature on a rotary shaker and sub-cultured into the same medium when the absorbance was approximately 0.1 at 600 nm.

After several serial sub cultures the enrichment culture was streaked for isolation on YNB agar containing 1mM DCA. After three sequential single colony isolations, one colony grown on YNB-DCA agar was chosen to prepare set of serial dilutions in physiological saline. Each dilution was plated onto YNB-DCA agar. A single colony growing on the serial dilution plate with the least number of colonies (4) was chosen and cultured on DCA broth at 30°C for use in further experiments. This strain was designated UW103.

Stocks of UW103 were maintained in 50% glycerol at -70°C. Frozen stocks were cultivated on solid YNB media containing 2 mM protocatechuate before being sub-cultured to solid YNB media containing 1 mM DCA.

3.4.3 Dechlorination

3.4.3.1 Media Sampling. DCA dechlorination was characterized in DIFCO Bacto® yeast nitrogen base medium without amino acids containing 50 mM NaH_2PO_4-Na_2HPO_4 buffer, pH 6.3 and 1 mM DCA (DCA-YNB). Four experimental flasks containing 300 ml DCA-YNB were inoculated with live cells of strain UW103 grown on DCA-YNB and washed

with sterile 50 mM sodium phosphate buffer, pH 6.3. A control flask, containing the same medium, was inoculated with autoclaved cells from the same culture. Experimental and control flasks were shaken on a rotary shaker at 100 RPM for 6 days at 24°C.

Every 8-12 hours, a 5 ml sample was taken from the experimental flasks. Growth was measured by absorbance at 600 nm using a Pharmacia LKB-Ultrospec II spectrophotometer (Pfizer Canada Inc., formerly Pharmacia Canada Inc., Mississauga, ON) and subsequently the sample was filtered through 0.45 μm pore-sized PTFE syringe filter (VWR, Mississauga, ON) to remove cells. Samples were taken less frequently from the control flasks and were not filtered. Samples were stored in polypropylene tubes at –20°C until the end of the experiment. One week after beginning the experiment, samples were allowed to thaw to room temperature. The chloride ion concentrations of each sample were measured and subsequently the same samples were used to measure DCA concentration.

3.4.3.2 DCA Concentration. DCA concentration was estimated in culture media by removing cells by centrifugation followed by filtration through 0.45 μm pore-sized PTFE syringe filter and measuring absorbance at a wavelength of 276 nm using a Pharmacia LKB-Ultrospec II spectrophotometer (Figure 3-3).

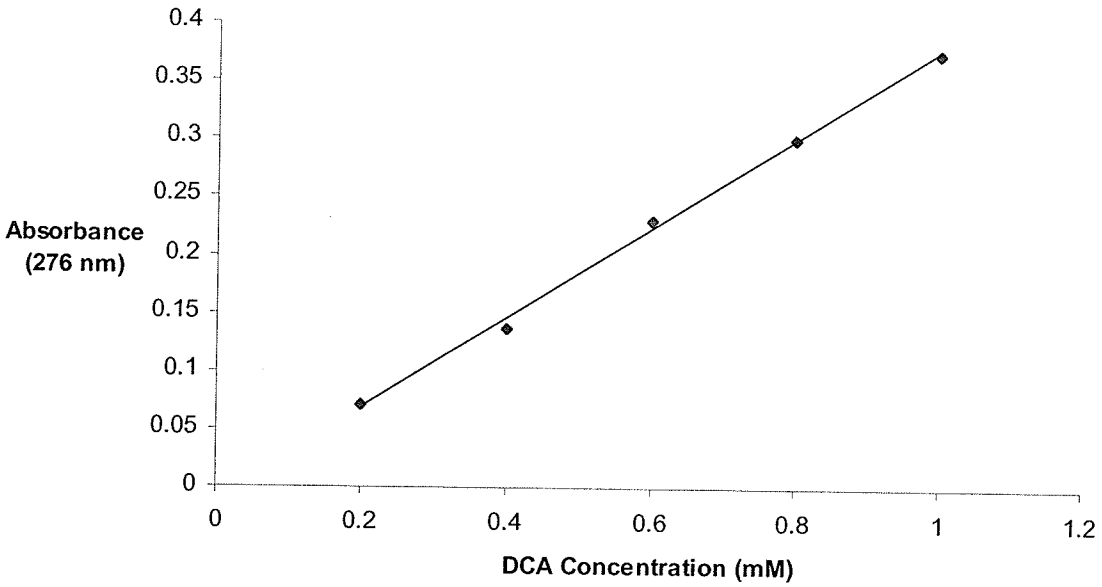


Figure 3-3. Standard curve relating DCA concentration in YNB broth and absorbance at 276 nm. Best fit line equation: $y = 0.3855x - 0.0087$; $R^2 = 0.998$.

Quantitative gas chromatograph analysis provided more accurate and specific measurement of DCA concentration (Figure 3-4). The centrifuged and filtered samples were extracted with two 5 ml portions of ethyl acetate. The ethyl acetate fractions were combined and evaporated under reduced pressure. Extractions were carried out in quadruplicate. The dry sample was dissolved in 2.0 ml of ethyl acetate and analyzed for DCA concentration with a Varian STAR 3400CX gas chromatograph with flame ionization detector (Varian Inc., Mississauga, ON) equipped with a fused-silica capillary column (J&W Scientific Inc., Folsom, CA, DB-5MS; 30m by 0.25 mm internal diameter; film thickness, 0.25 μm; stationary phase, (5% phenyl)-methylpolysiloxane). The injector temperature was 250°C, the detector temperature was 280°C and the temperature program began at 50°C for 2 min. increased by 25°C per min to 250°C and was held for 2 min. Helium was used as the carrier gas. Each sample was automatically injected once

and the concentration of DCA was determined using 4-bromoanisole as an internal standard.

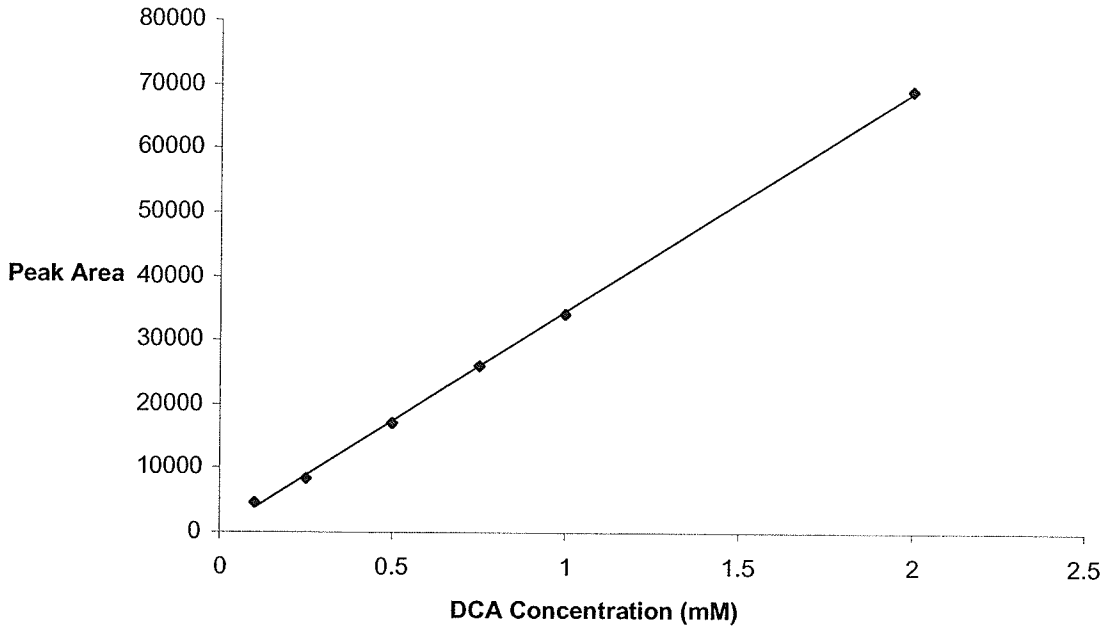


Figure 3-4. Standard curve relating DCA concentration in ethyl acetate to peak area as measured by gas chromatography and flame ionization detection. Best fit line equation: $y = 34053x + 422.36$; $R^2 = 0.9995$.

The extraction efficiency of DCA from YNB-DCA was measured for DCA concentrations of 0.1, 0.5, 1 and 2 mM. Extractions were performed in quadruplicate and each extraction manually injected at least once. Data from a fifth extraction, mechanically injected once, and performed previously, was also included in the extraction efficiency calculations. Extraction efficiency of DCA extracted from YNB broth averaged 77.5% and is presented in Figure 3-5.

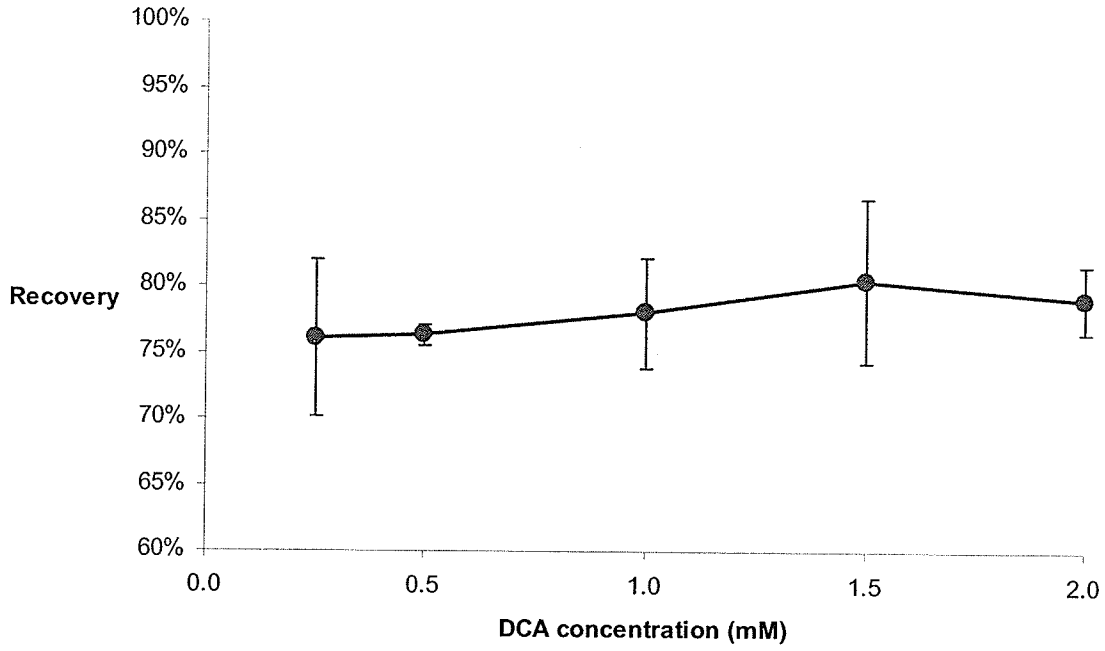


Figure 3-5. Efficiency of DCA extraction from YNB broth. Y Error bars represent +/-1 standard deviation.

3.4.3.3 Chloride Ion Measurement. Chloride ion concentration was measured using an Orion Ionplus model 96-17B combination chloride ion selective electrode (Thermo Electron Corporation, Gormley ON) used in conjunction with an Orion pH/ISE meter model 710A (Thermo Electron Corporation, Gormley ON). Two milliliters of ionic strength adjuster (ISA, 5 M $NaNO_3$) was added to 0.1, 1, 10 and 100 mM NaCl standards. Potential was measured in absolute millivolts and the values plotted against the log of the standard chloride concentrations (Figure 3-6). Experimental and control samples were warmed to room temperature; sample volumes ranged from 2-4 ml. Forty microliters of ionic strength adjuster (ISA) per 2 ml sample was added prior to measurement.

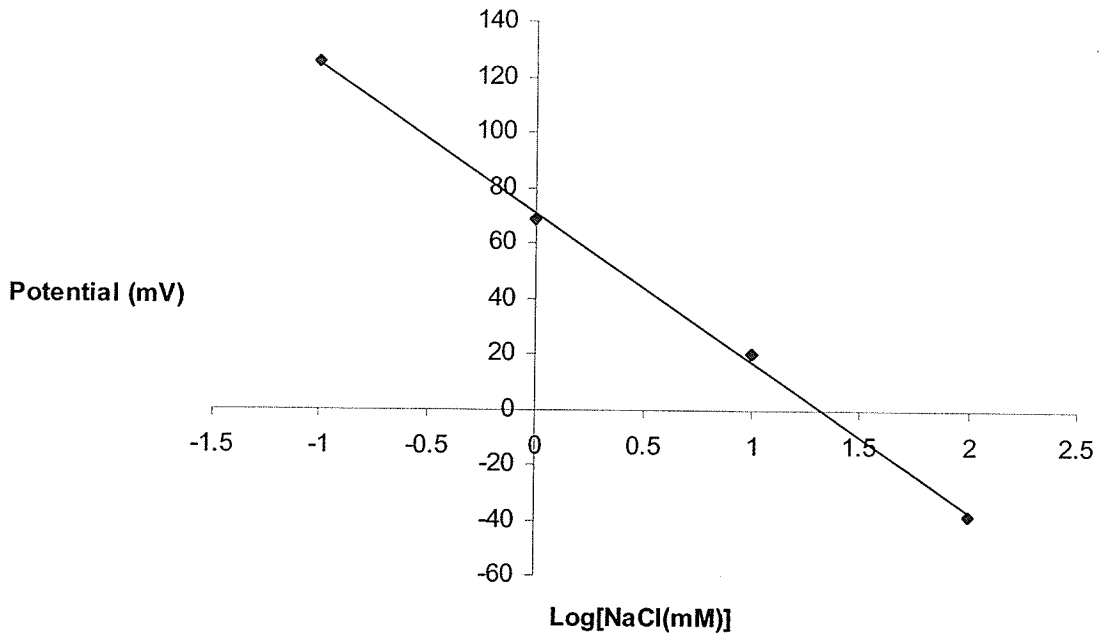


Figure 3-6. Electrical potential (mV), as measured using a chloride ion selective electrode, as a function of chloride concentration. Line equation: $y = -53.76x + 70.63$; $R^2 = 0.9988$.

3.4.4 DCA Catabolism

3.4.4.1 Substrate Utilization. UW103 was cultured in YNB and 1 mM carbon source. Carbon sources were purchased from VWR (Mississauga ON Canada) or Sigma Aldrich (Oakville ON Canada).

3.4.4.2 Culture Extractions for Detection of DCA Metabolites. A 1000ml culture (OD=0.6) was divided into four 250ml sterile centrifuge bottles and centrifuged for 10min at 5000 RPM (Beckman RC-5B centrifuge with GSA rotor, Beckman-Coulter Inc., Mississauga, ON). Supernatant was discarded and each bottle of cells was resuspended in 10ml 50mM sodium phosphate buffer, pH 6.3. Cells were centrifuged and the supernatant discarded. Cells were resuspended in a small amount of buffer and combined into one centrifuge bottle. Cells were washed with buffer once more and added to 100ml

DCA-YNB (Time zero). The optical density of this culture was equivalent to 4 at 600 nm.

At time zero and every 15 minutes, 11 ml of culture was removed into a polypropylene tube and immediately centrifuged at 5000 RPM for 10 min. The supernatant was immediately filtered through a 0.45 μm pore-sized PTFE filter (VWR, Mississauga, ON) into a sterile glass test tube. Samples were extracted with two sequential portions of 5 ml ethyl acetate. Portions were combined and 2 ml was removed for direct analysis by GC-MSD; the remaining extract was evaporated to dryness under ambient conditions, resuspended in 1.5 ml tetrahydrofuran (THF), and derivatized with 5 ul of N,O-bis(trimethylsilyl)acetamide (BSA) and subsequently analyzed by GC-MSD as described previously in section 3.4.1.

3.4.4.3 Mineralization. DCA mineralization was assessed by measuring CO_2 liberation from a large concentration of DCA-fed UW103 cells. Carbon dioxide was captured in standardized NaOH and measured by titration with standardized HCl as described by Zibilske (1994; Figure 3-7). The experiment was performed in triplicate and the weight of CO_2 liberated was averaged. The background respiration was also measured in triplicate by measuring the amount of CO_2 liberated from strain UW103 in YNB without DCA. One apparatus was used for all measurements, which alternated between experimental and control replicates.

Carbon dioxide weight liberated from DCA was calculated as described by Zibilske (1994), expressing equivalent weight in terms of CO_2 (E=22) in the following equation:

$$C = (B - V) N E$$

where, C = weight of CO_2 (mg)

V = volume acid to titrate base in experimental CO_2 traps

B = volume acid to titrate base in control CO_2 traps

N = normality of acid

E = equivalent weight

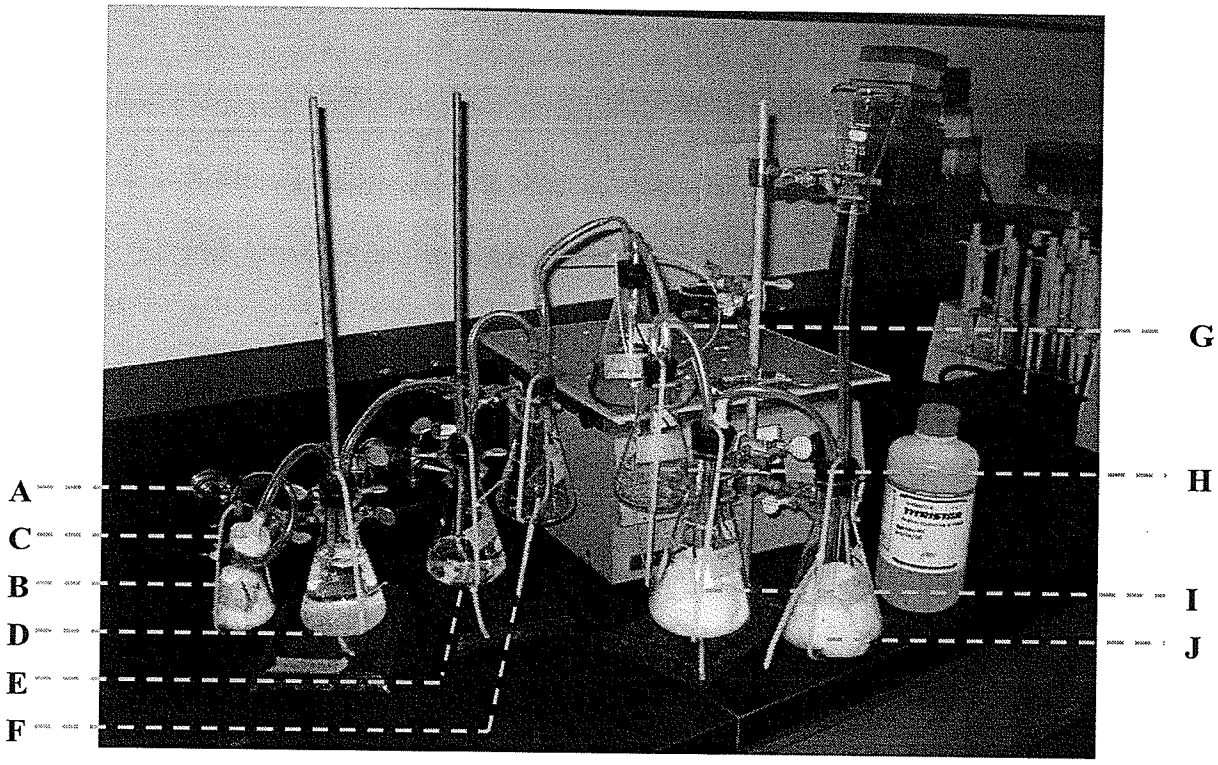


Figure 3-7. CO_2 capture apparatus. Modified from Zibilske (1994). A, air source; B, sterile cotton filter; C, 0.45 µm PTFE filter; D, 4+ M NaOH; E, CO_2-free water; F, flow trap; G, culture flask; H, flow trap; I, 1.0 M NaOH quantitative trap; J, 1.0 M NaOH secondary quantitative trap.

3.5 Results

3.5.1 DCA Utilization

A Gram negative bacillus, designated strain UW103 and isolated from a DCA enrichment culture from commercial garden compost, was capable of growth in DCA-YNB broth, where DCA was the sole carbon and energy source, but did not grow in YNB medium without DCA. The DCA concentration declined to less than 50 μM from an initial concentration of 1.0 mM in cultures of strain UW103 cultures containing DCA as the sole carbon and energy source. DCA-grown cells were inoculated into 1 mM DCA-YNB and over the following six days three additions of 1 mM DCA were made. DCA utilization and degradation was detected by UV analysis and gas chromatography (Figures 3-3 and 3-4, respectively).

DCA has an absorption peak at 276 nm, while the YNB medium absorbs at this wavelength only slightly. DCA-YNB media inoculated with UW103 had a UV absorbance of 0.41, and after 72 hours incubation the absorbance decreased to less than 0.1 and remained below 0.3 for the duration of the experiment despite three additions of 1 mM DCA (Figure 3-8).

A control flask, inoculated with non-viable cells, showed an increase in absorbance at 276 nm from 0.28 to greater than 1.6 reflecting a UV absorption of greater than 1 mM DCA, while the flasks inoculated with viable UW103 cells remained low, equivalent to less than 1 mM DCA. Absorbance of the control culture increased to greater than 1.6 reflecting UV absorption of greater than 1 mM DCA. Similarly, disappearance of DCA in inoculated flasks was confirmed by GC-FID analysis (Figure 3-8). Ethyl acetate extracts of the medium inoculated with viable UW103 cells demonstrate

that the level of DCA dropped to less than 50 μM after 60 hours and remained below this level for the duration of the experiment despite three additions of 60-70 mg DCA, equivalent to 1-1.5 mM DCA. GC-FID analysis of controls demonstrates the presence of DCA at concentrations consistent with the amount of substrate added. Superimposing the UV absorption and DCA concentration analysis by gas chromatography, we see that UV absorption provides a good estimate of DCA concentration for DCA concentrations less than 1 mM (Figure 3-8).

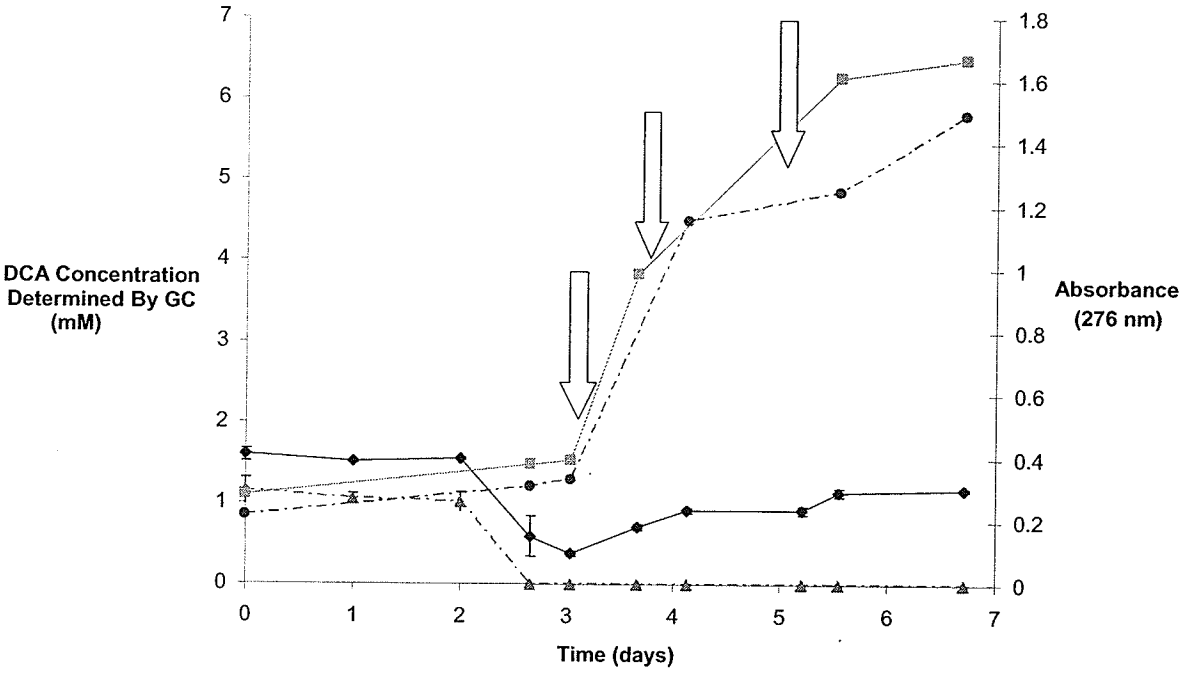


Figure 3-8. DCA concentration as estimated by UV spectrophotometry and gas chromatography. Arrows (⇨) represent the addition of 60-70 mg DCA (equivalent to 1 - 1.5 mM). Y Error bars represent +/-1 standard deviation for 4 replicate flasks. —▲—, DCA concentration determined by GC (average of 4 inoculated flasks); —◆—, absorbance at 276 nm (average of 4 inoculated flasks); —●-, DCA concentration by GC (non-viable control flask); —■—, absorbance at 276 nm (non-viable cell control flask).

Growth of the DCA-utilizing strain was measured as optical density with the assumption that absorbance at 600 nm is directly proportional to growth (Figure 3-9). An increase in absorbance at 600 nm was only observed in flasks inoculated with viable cells. Colonial morphology and Gram stains of samples taken near the beginning, midpoint, and end of experiment and inoculated onto DCA-YNB agar confirmed the purity of experimental broth cultures. An increase in CFU/ml was also observed, confirming growth as measured by absorbance at 600 nm.

3.5.2 Dechlorination

Complete dechlorination of DCA was observed in replicated cultures of strain UW103 (Figure 3-9). DCA-grown cells were inoculated into 1.2 mM DCA-YNB medium and after 2 days an increase in the chloride ion concentration of 2.1 mM was observed, a ratio of 1.9:1. After a total of 7 days, and the equivalent of 5.7 mM of DCA fed to the broth culture of *Burkholderia* sp. UW103, the chloride ion concentration increased by 12.0 mM, a 2.1:1 ratio of chloride elimination to DCA consumed. In the non-viable cell control flask chloride concentration remained at initial levels despite the same additions of DCA as in the inoculated flasks.

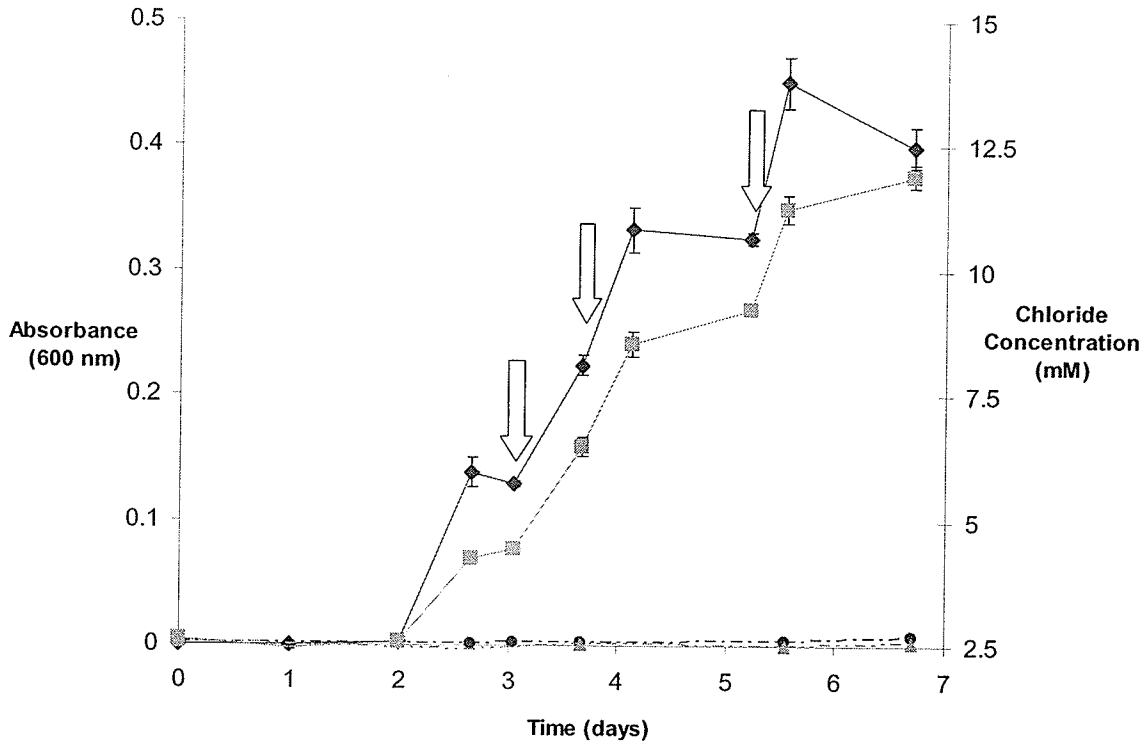


Figure 3-9. Growth of strain UW103 in DCA-YNB and chloride concentration as measured using a chloride ion selective electrode. Arrows (⇨) represent the addition of 60-70 mg DCA (equivalent to 1 - 1.5 mM). Y Error bars represent +/-1 standard deviation for 4 replicate flasks. —◆—, absorbance at 600 nm (average of 4 inoculated flasks); —■—, chloride concentration (average of 4 inoculated flasks); —▲—, absorbance at 600 nm (non-viable control flask); —●-, chloride concentration (non-viable control flask).

3.5.3 Mineralization

Evidence of DCA mineralization was detected using a modified apparatus and system, originally designed for the measurement of CO_2-liberating respiration from soil (Figure 3-7). With this system we measured $48.8^{+}/_{-}14.0$ mg CO_2 ($^{+}/_{-}$ 1 standard deviation) liberated from a culture incubated in 1 mM DCA YNB broth (Figure 3-10). Respiration under the same conditions without the presence of a carbon source yielded $18.3^{+}/_{-}6.7$ mg

CO_2 . The difference, 30.5 mg and assumed to be sourced from DCA, is 86.6% of the theoretical value (35.2 mg). Theoretically 1.0 mM DCA will yield the equivalent of 8.0 mM CO_2 (35.2 mg in 100 ml broth).

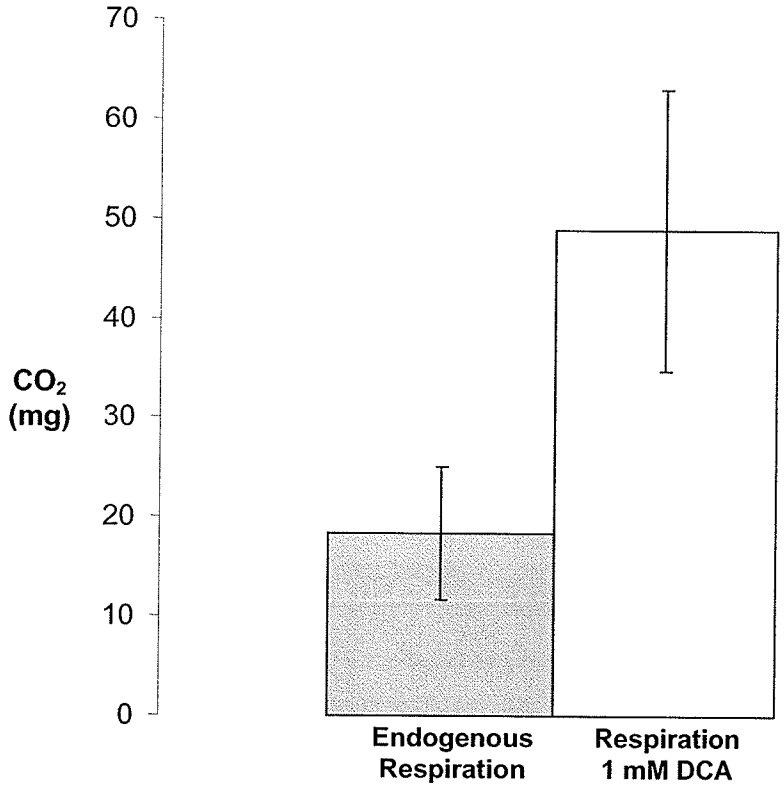


Figure 3-10. Respiration from *Burkholderia* sp. UW103, measured as weight of CO_2, in YNB with and without 1 mM DCA. Y error bars represent +/-1 standard deviation for 3 replicate flasks. Grey, endogenous respiration; white, respiration from cells utilizing 1 mM DCA.

3.5.4 Catabolites

Ethyl acetate extracts from DCA-grown UW103 cultures revealed the presence of at least four catabolites. The first catabolite, identified in both directly analyzed extracts and BSA-derivitized extracts, shares a nearly identical retention time (Table 3-1) and highly similar mass spectrum to that of 3,5-dichloro-*para*-anisaldehyde (DCAld) (Figure 3-11). The second metabolite, also identified in both directly analyzed extracts and BSA-

derivitized extracts, shares nearly identical retention times and highly similar mass spectra to 3,5-dichloro-*para*-anisic acid (DCAcid) (Table 3-1; Figure 3-12). A third catabolite, detected in ten-fold lower quantity as the second catabolite, was only detected in derivitized extracts. The mass spectrum of this catabolite has a characteristic isotope mass-ratio pattern of a monochlorinated compound and resembles the spectrum of BSA-derivitized 3-chloro-*para*-anisic acid (Figure 3-13). Figure 3-14 provides a time course demonstration of DCA and catabolites detected in sequence.

Table 3-1. Gas chromatography retention times (RT) for DCA, DCA catabolites, and reference compounds. Values are the average of 2-3 replicates run on different days.

	Direct		BSA-derivitized	
	RT (minutes)	Standard Deviation	RT (minutes)	Standard Deviation
DCA standard	9.404	0.047	9.702	0.057
Catabolite 0	9.456	0.062	9.675	0.038
DCAld standard	8.298	0.021	8.280	0.102
Catabolite 1	8.283	0.104	8.296	0.088
DCAcid standard	10.064	0.075	9.957	0.129
Catobolite 2	10.112	0.023	9.895	0.140
MCA standard	-	-	9.775	0.124
Catabolite 3	-	-	9.767	0.151

Various mono-, di- and non-chlorinated aromatic compounds, possible DCA catabolites, were assessed for strain UW103's ability to utilize them as sole sources of carbon and energy (Table 3-2). All compounds were initially tested at 1 mM concentration. Strain UW103 was capable of growth on all of the catabolites detected by GC-MS, a variety of hydroxylated benzoic acids, and catechol. Compounds that were negative for growth at 1 mM were also tested at a concentration of 0.1 mM. Strain UW103 was not capable of growth on a variety of chlorinated benzoic acids and non-chlorinated anisyls at neither concentration.

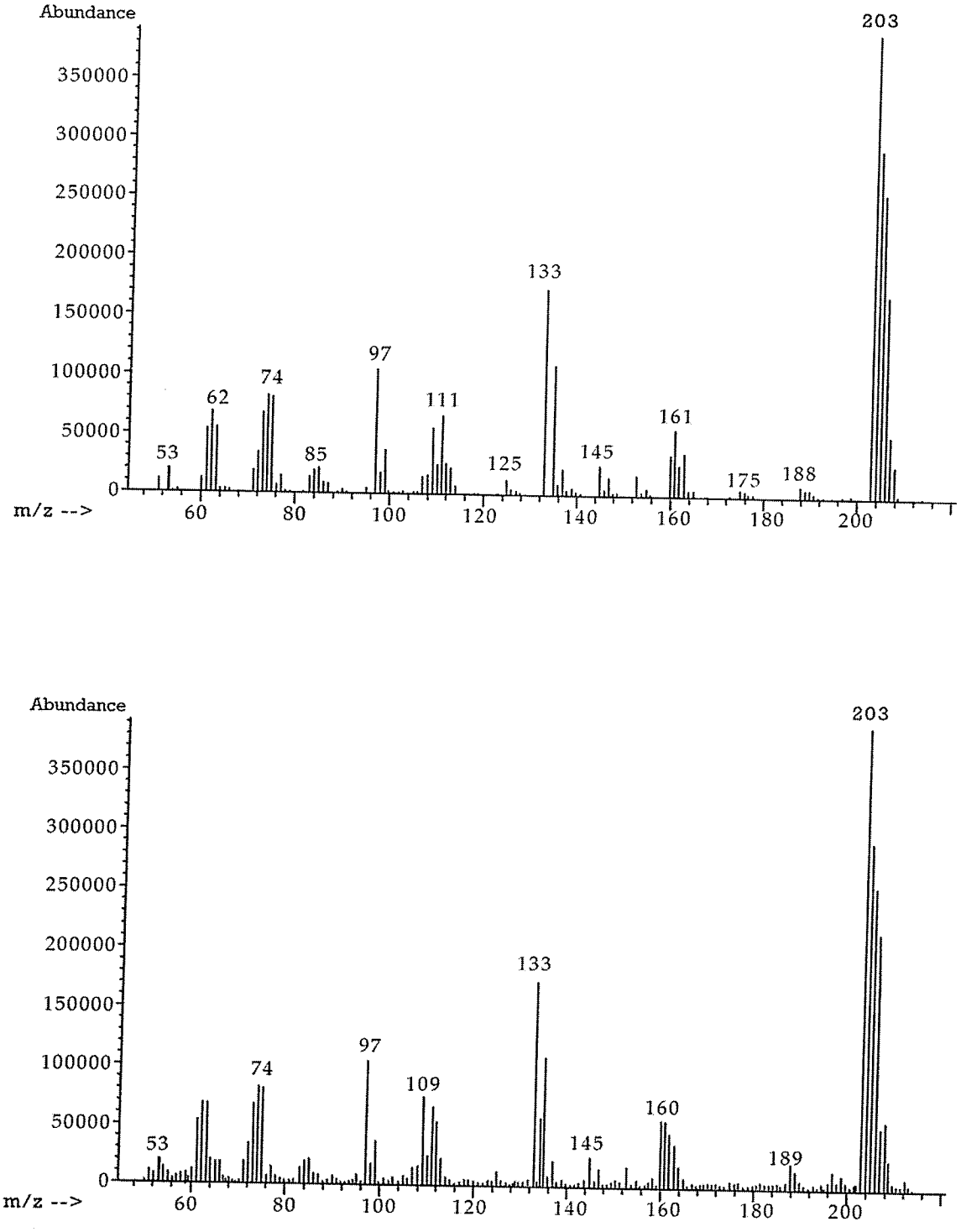


Figure 3-11. First DCA catabolite detected in a DCA grown culture of strain UW103 and reference spectrum. Top, mass spectrum of first DCA catabolite detected; bottom, mass spectrum of 3,5-dichloro-*para*-anisaldehyde (DCAld). Refer to Appendix III for mass fragment analysis.

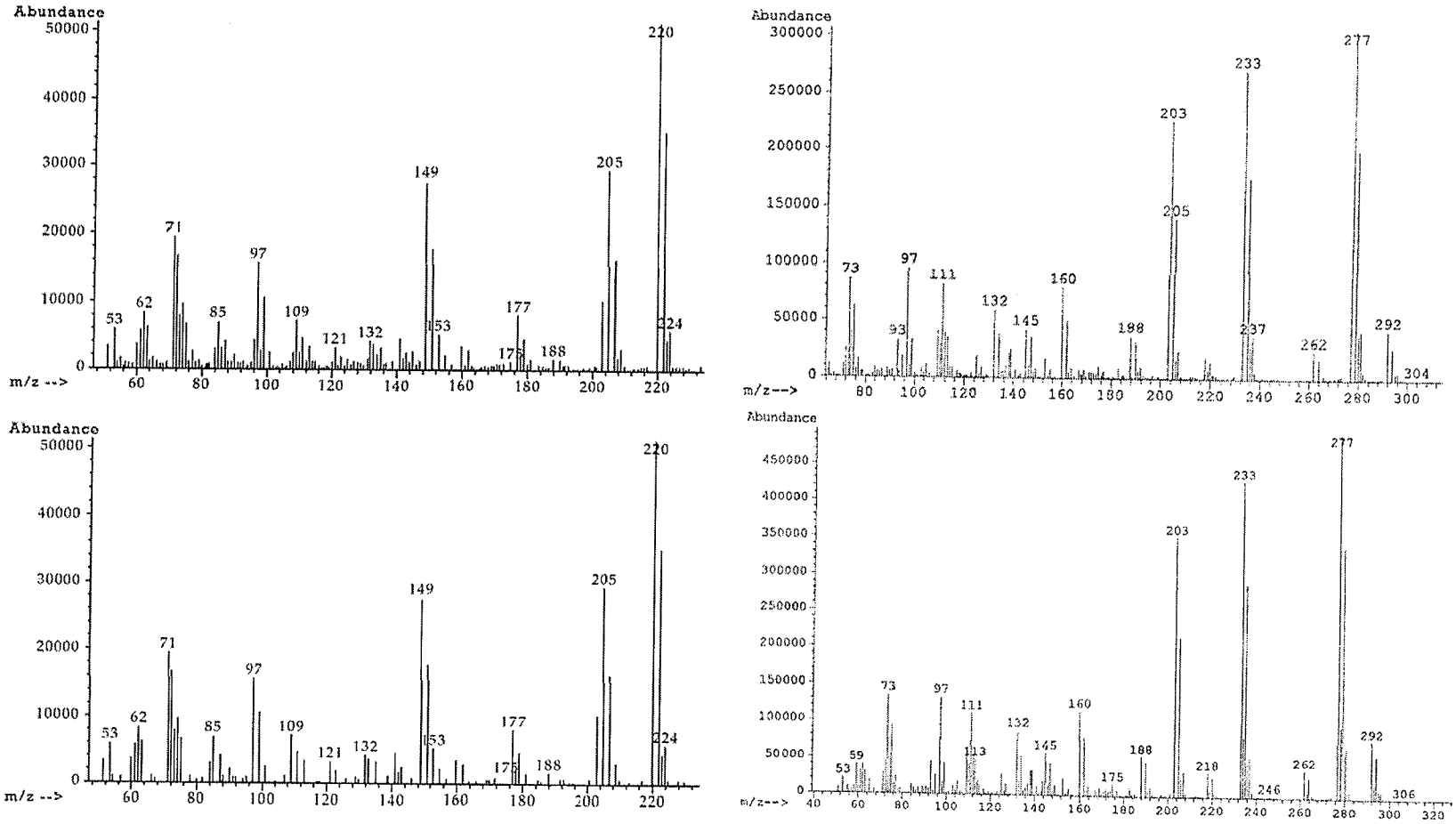


Figure 3-12. Mass spectra of second DCA catabolite detected in a DCA grown culture of strain UW103 and reference spectrum. Top left, mass spectrum of second DCA catabolite detected in directly analyzed extracts; bottom left, mass spectrum of 3,5-dichloro-*para*-anisic acid (DCAcid); top right, mass spectrum of second DCA catabolite detected in BSA-derivitized extracts; bottom right, mass spectrum of BSA-derivitized 3,5-dichloro-*para*-anisic acid (DCAcid). Refer to Appendices IV and V for mass fragment analyses.

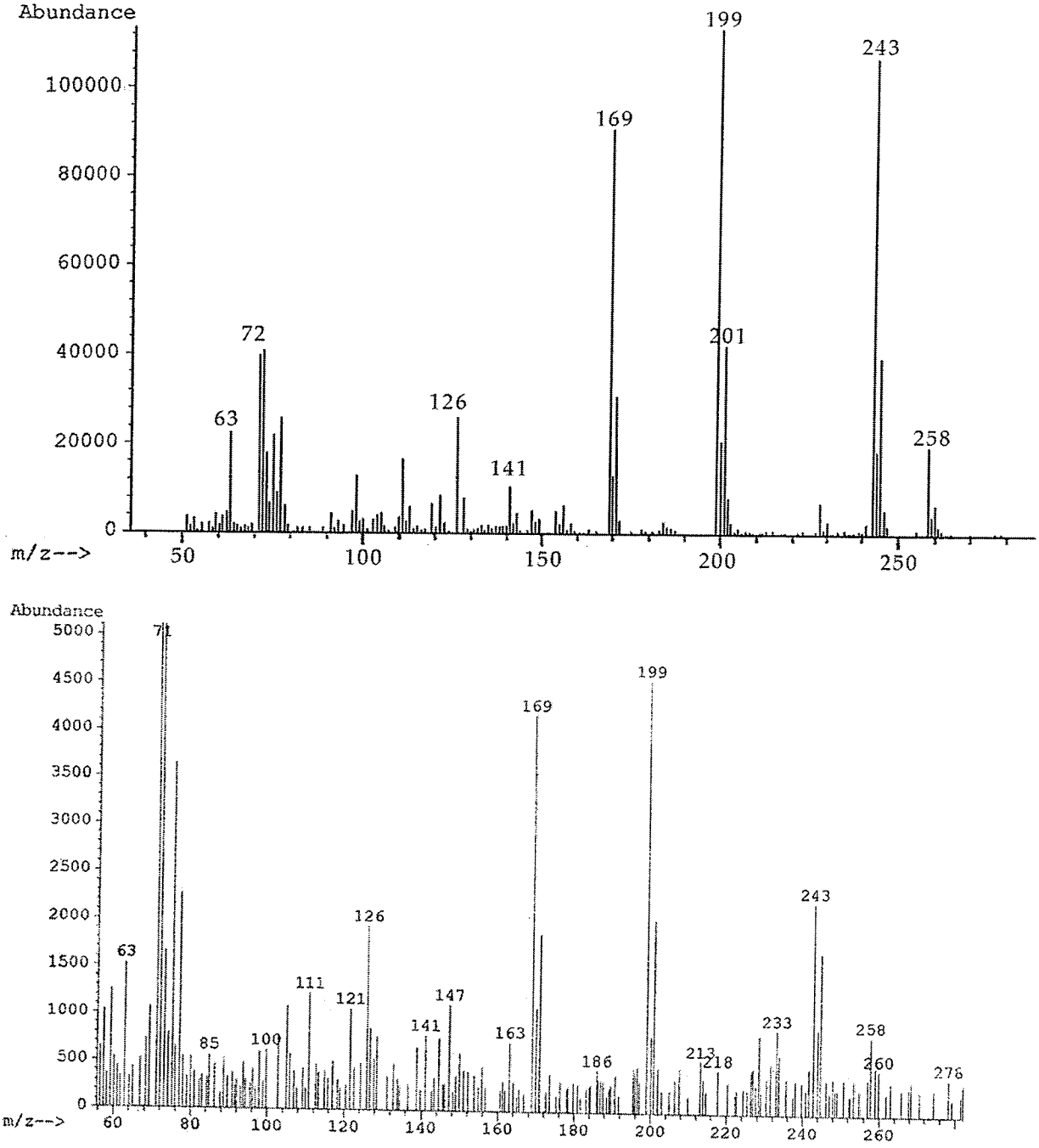


Figure 3-13. Mass spectrum of third DCA catabolite detected (bottom; BSA-derivatized) and reference spectrum of BSA-derivatized 3-chloro-*para*-anisic acid (top). Refer to Appendix VI for mass fragment analysis.

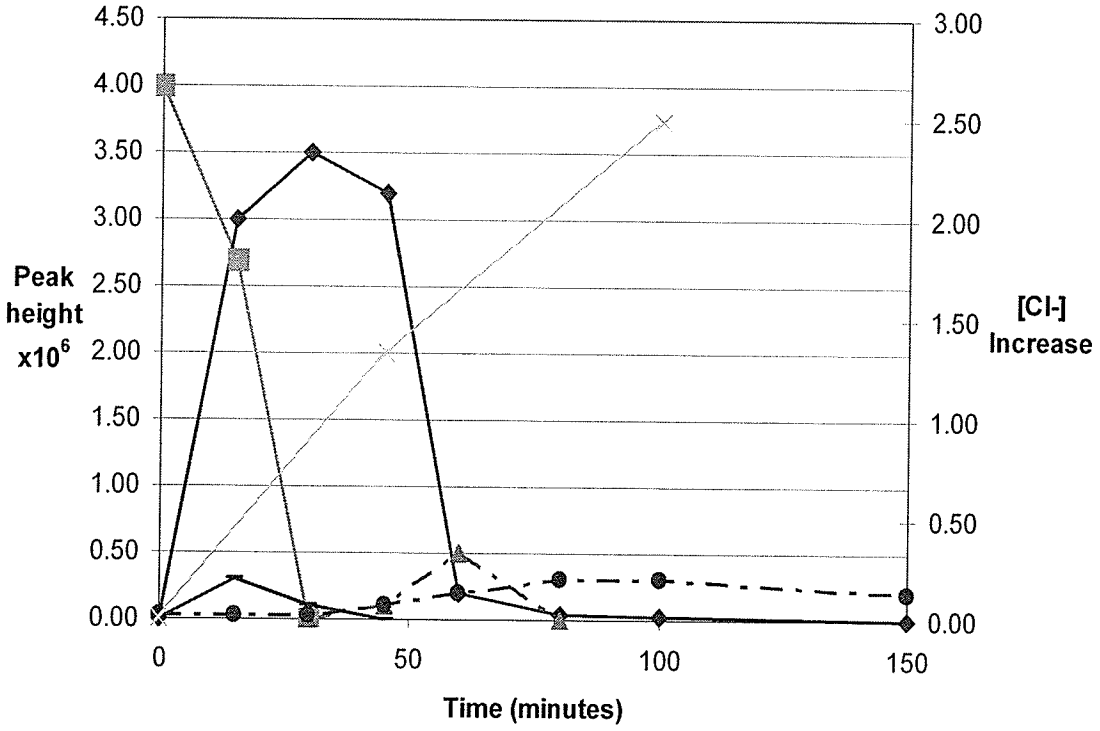


Figure 3-14. Relative quantity of DCA-catabolites after inoculation of DCA-YNB medium with large quantities of strain UW103, time zero. Separated by gas chromatography and identified by mass spectrometry. —■—, DCA; —◆—, DCAld; ———, DCAcid; —▲—, monochlorinated catabolite; —● -, unidentified catabolite; ·····, chloride ion concentration.

Table 3-2. Growth of the DCA-degrader, strain UW103, on various chlorinated and/or aromatic compounds, as sole carbon and energy sources. Tested at 1 mM concentration in YNB. Compounds that did not support growth at 1 mM were additionally tested at 0.1 mM.

Compound	**Growth**
2-chlorobenzoic acid	-
3,4-dichlorobenzoic acid	-
3,5-dichlorobenzoic acid	-
3,5-dichlorobenzyaldehyde	-
3,5-dichloro-*para*-anisaldehyde (DCAld)	+
3,5-dichloro-*para*-anisic acid (DCAcid)	+
3,5-dichloro-*para*-anisyl alcohol (DCA)	+
3,5-dihydroxy benzoic acid	-
3-chlorobenzoic acid	-
3-chloro-*para*-anisic acid (MCA)	+
3-chloro-4-hydroxybenzoic acid	+
3-hydroxy benzoic acid	+
3-hydroxy-*para*-anisic acid (isovanillic acid)	-
4-chlorobenzoic acid	-
4-hydroxy-benzyl alcohol	-
4-hydroxy-benzoic acid	+
catechol (3,4-dihydroxybenzene)	+
gallic acid (3,4,5-trihydroxy benzoic acid)	+
ortho-anisic acid	-
para-anisaldehyde	-
para-anisic acid	-
para-anisyl alcohol	-
phloroglucinol (1,3,5-trihydroxy benzene)	-
protocatechuate (3,4-dihydroxybenzoic acid)	+
pyrogallol (1,2,3-trihydroxybenzene)	-
salicylic acid (2-hydroxybenzoic acid)	+
vanillic acid (4-hydroxy-3-methoxybenzoic acid)	-
vanillin (4-hydroxy-3-methoxybenzaldehyde)	-

3.6 Discussion and Conclusions

Chlorinated compounds of anthropogenic origin have been present in the environment for only a few decades and, remarkably, some microorganisms have evolved the ability to dechlorinate and degrade them. Dechlorination of such xenobiotics as PCBs, PCP, and other chlorinated phenols have been well-studied. This study examined the dechlorination and mineralization of a naturally-produced chlorinated compound, the chlorinated anisyl metabolite (CAM), dichloro-*para*-anisyl alcohol (DCA).

Strain UW103 was isolated from an enrichment culture of commercial compost and is capable of growth on DCA as sole carbon and energy source. DCA utilization and degradation was detected by UV spectrophotometry and gas chromatography (Figures 3-10 and 3-18). The disappearance of DCA from UW103 cultures was confirmed by GC-FID analysis and decreased absorbance in the UV range suggests cleavage of the aromatic ring. DCA degradation was also accompanied by dechlorination. This was exciting because we are interested in bacterial dechlorination mechanisms. UW103 cultures grown on DCA showed an increase in moles of chloride equivalent to two times the moles of DCA added (Figures 3-12 and 3-18). This ratio suggests that UW103 has the ability to dechlorinate DCA at both the 3 and 5 positions. Furthermore, growth of UW103 paralleled DCA dechlorination (Figure 3-15), suggesting that energy could be gained from DCA catabolism.

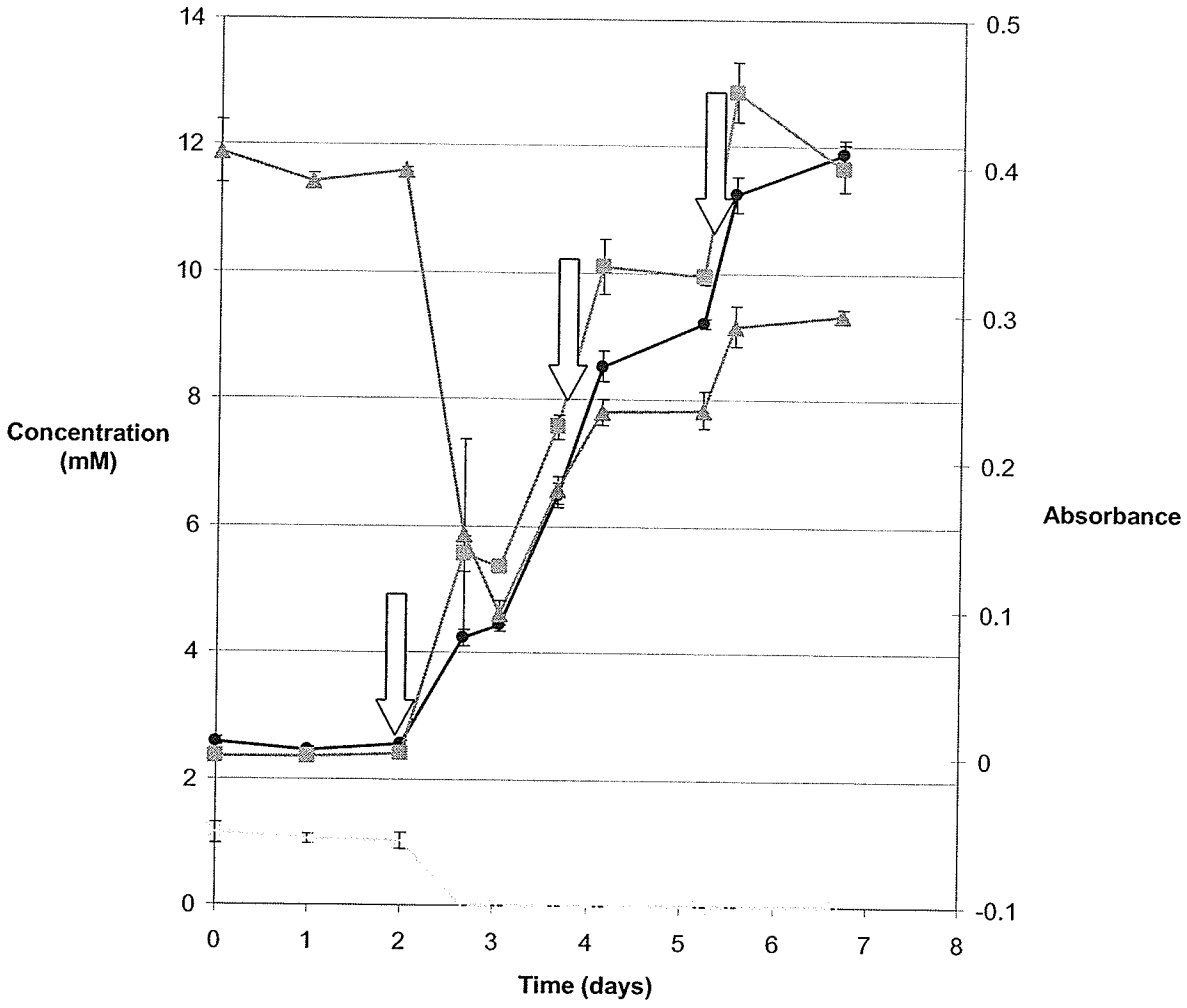


Figure 3-15. DCA-grown strain UW103. Arrows represent the addition of 1mM DCA. Y Error bars represent +/-1 standard deviation for 4 replicates. —●-, Chloride concentration; ⋯⋯, DCA concentration; —■—, absorbance at 600 nm; —▲—, absorbance at 276 nm.

Absorbance at 276 nm provided a good estimate of DCA concentration; however, measurement of DCA concentration by gas chromatography provided a more accurate measurement. The increase in UV absorbance did not appear to be quantitative with the

increase in DCA concentration. There are two explanations for this observation. Although we know that the relationship between DCA concentration and UV absorbance is linear up to at least 1 mM (Figure 3-3), we do not know that this relationship remains linear above 1 mM DCA. The standard curve was measured only up to 1 mM due to the limited solubility of DCA in YNB. Second, the sample taken from the control culture to measure UV absorbance, may not reflect the true amount of DCA in the media as the solubility of DCA in an aqueous medium is limited. Therefore we cannot quantitatively assess the concentration of DCA in the control culture. However, we can say that the concentration of DCA in the control culture is well above 1 mM, while the concentration of DCA in the inoculated flasks remain below 1 mM for the duration of the experiment despite three additions of 1 mM DCA.

The experimental flasks, containing DCA-grown UW103, maintained an absorbance at 276 nm below 0.3, equivalent to less than 0.8 mM DCA despite three additions of at least 1 mM DCA. Since the aromatic component of DCA is largely responsible for the its absorption in the UV range (Friedel and Orchin 1951), the low UV absorbance maintained in the experimental flasks suggests that the aromatic component of DCA is no longer present.

Dehalogenation and aromatic ring cleavage, the major stumbling blocks to mineralization, appear to be accomplished in DCA-grown cultures of strain UW103. Throughout this project extracts from DCA-grown *Burkholderia* sp. UW103 cultures, using a variety of extraction solvents and methods, never revealed the build-up of a DCA-metabolite. Our extraction methods, designed for aromatic compounds of varying polarities, would at least have detected the build up of an aromatic dead-end metabolite.

The two greatest hurdles for bacteria to mineralize halogenated aromatic compounds are dehalogenation and aromatic ring fission. Since we confirmed complete dechlorination of DCA we would expect the only remaining hurdle to be the breakdown of the aromatic ring. Since no aromatic compounds appeared to be accumulating in DCA-grown cultures mineralization was probably taking place.

To support our argument for DCA mineralization we measured CO_2-liberation (Figure 3-7). Our CO_2 capture experiment recovered 86.6% of the theoretical value of CO_2 for complete mineralization. This is evidence that the aromatic ring is cleaved, and in conjunction with dechlorination, is strong evidence that mineralization of DCA is taking place in cultures of *Burkholderia* sp. UW103.

Without demonstrating the release of C^{14}-radiolabelled CO_2 from ring-radiolabelled DCA we cannot conclusively say that the aromatic ring was cleaved. The difference in structure of DCA from common synthetic precursors amplified by the high cost of ring-radiolabelled aromatic compounds unfortunately precluded the project from including ring-radiolabelled DCA in its budget. Analysis using radio-labelled carbon within the aromatic ring of DCA would provide concrete evidence of complete mineralization.

A proposed pathway for DCA catabolism leading to dechlorination and ring cleavage via protocatechuate is presented in Figure 3-16. The first three catabolites presented, DCAld, DCAcid, and 3-chloro-*para*-anisic acid were identified in culture extracts analyzed by GC and mass spectrometry.

Characteristic chlorine isotope patterns were observed in mass spectra for all of these compounds (Lee 1998; Safe and Hutzinger 1973). The relative abundance of chlorine isotopes in nature is 3:1 Cl^{35}: Cl^{37}. Compounds with one chlorine atom in their

structure exhibit "double" peaks in their mass spectra, each peak in the pair corresponding to the ion containing each isotope. The two peaks are spaced 2 mass/charge units apart and in a relative abundance of 3:1. The mass spectrum of the proposed DCA catabolite, 3-chloro-*para*-anisic acid (MCA; Figure 3-13) contains a series of peak pairs corresponding to ion fragments containing one atom of chlorine. Molecules containing two chlorine atoms demonstrate "triple" peaks spaces 2 mass/charge units apart in a relative abundance of 9:6:1.

Growth of strain UW103 on possible DCA catabolites would support their role in the pathway to DCA mineralization. Strain UW103 is capable of growth on all compounds in Figure 3-16 as sole carbon and energy sources, except isovanillic acid (nor its isomer, vanillic acid). Therefore, the pathway likely proceeds through 3-chloro-4-hydroxy-benzoic acid (MCA).

The aerobic pathway of DCA differs from the degradation of DCA in anaerobic methanogenic sludge by Verhagen et al. (1998*c*). Under anaerobic conditions DCA was initially demethylated to 3,5-dichloro-4-hydroxybenzyl alcohol, which was converted via a biotic route leading to the formation of 3,5-dichloro-4-hydroxybenzoate (Figure 2-5). Verhagen et al. Demethylation (1998*c*) determined that this reaction was inhibited by oxygen and suggested the involvement of acetogenic bacteria. Finally, a biotic decarboxylation step yielded 2,6-dichlorophenol. Neither decarboxylation nor demethylation were observed under aerobic conditions with strain UW103; however we proposed that the DCA-degradation intermediate, MCA, is demethylated and dechlorinated to protocatechuate prior to aromatic ring cleavage (Figure 3-16). Verhagen et al. (1998*c*) did not observe dechlorination in their study of DCA degradation in methanogenic sludge.

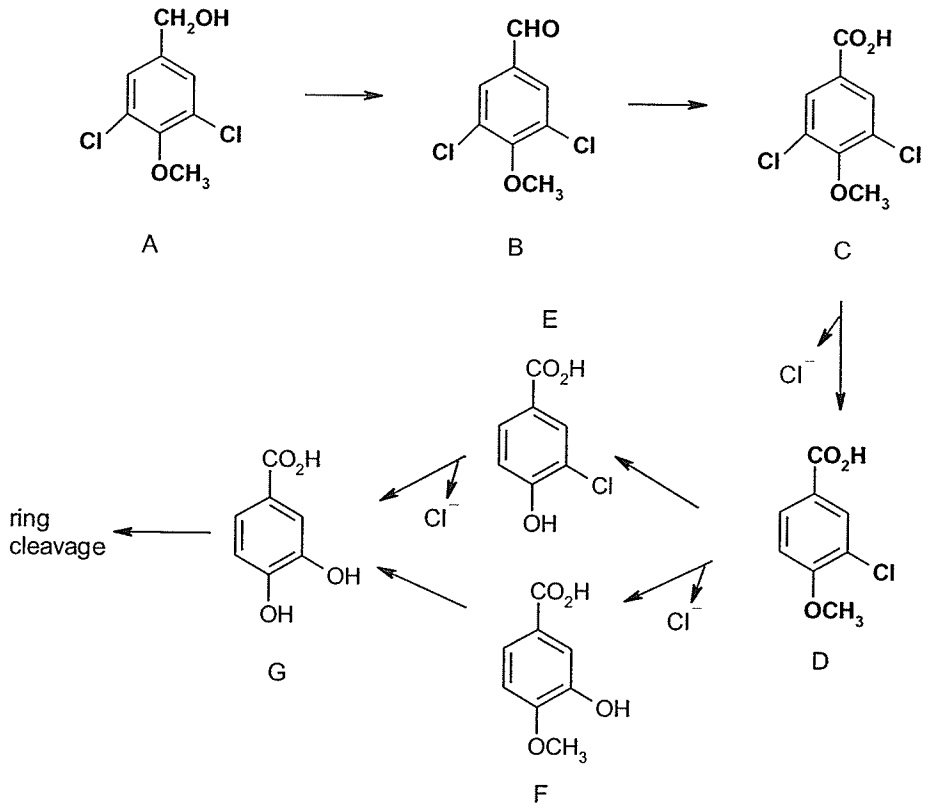


Figure 3-16. Proposed pathway for DCA catabolism to the ring cleavage precursor, protocatechuate. A, DCA; B, DCAld; C, DCAcid; D, 3-chloro-*para*-anisic acid; E, 3-chloro-4-hydroxybenzoic acid; F, isovanillic acid (3-hydroxy-*para*-anisic acid); G, protocatechuate (3,4-hydroxy benzoic acid).

After the initial decarboxylation of DCA, Verhagen et al. (1998*c*) observed an abiotic dimerization reaction in methanogenic sludge. In our study, we did not observe evidence of dimerization. Since we could account for all chloride molecules liberated from DCA in UW103 cultures, it is unlikely that dimerization occurred as was observed by Verhagen et al. (1998*c*).

The structure of MCA predicts that the initial dechlorination step proceeds by a reductive mechanism and is either followed or preceeded by demethylation (Figure 3-16). Reductive dechlorination linked to carbon metabolism is common in aerobic bacteria

(Fetzner 1998). Strains from the genera *Sphingomonas*, *Pseudomonas*, *Alcalingenes*, *Azotobacter*, and *Corynebacterium* have been demonstrated to utilize reductive dechlorination reactions to facilitate carbon metabolism of chlorinated substrates. *Azotobacter chroococcum* MSB-1 and *Alcaligenes eutrophus* JMP134 utilize the herbicide 2,4-D as a sole carbon and energy source and metabolize it via reductive *ortho*-dechlorination to 4-chlorophenoxyacetic acid (Balajee and Mahadevan 1990; Vollmer, Stadler-Fritzsche, and Schlomann 1993). *Corynebacterium sepedonicum* KZ-4 and coryneform strain NTB-1 aerobically mineralize 2,4-dichlorobenzoate via the NADPH-dependent reductive *ortho*-dechlorination of 2,4-dichlorobenzoyl-CoA to 4-chlorobenzoyl-CoA, and after a second dechlorination step by another mechanism proceed to ring cleavage through protocatechuate (Van Den Tweel, Kok, and De Bont 1987; Romanov and Hausinger 1996; Fetzner 1998).

The probable fate of MCA, the product of the oxidation and dechlorination of DCA, is mineralization; however, the chemistry after the initial oxidation and dechlorination of DCA to MCA is not clear. The 2:1 molar ratio of chloride ions to DCA is strong evidence that MCA is dechlorinated. In addition, the measurement of 86.7% CO_2 from DCA-fed UW103 cultures is suggestive of mineralization to CO_2 and chloride.

MCA may undergo dioxygenolytic attack yielding protocatechuate, a common precurser to aromatic metabolism. Dechlorination catalyzed by dioxygenases is common in the degradation of chlorinated aromatic substrates by aerobic bacteria and dioxygnease-catalyzed intradiol cleavage of the aromatic ring is the most common mechanism removing chloride substituents from aromatic substrates (Fetzner 1998; Copley 1997). For example *Pseudomonas* sp. B13 degrades 3-chlorobenzoate via *ortho*-

ring cleavage, releasing chloride upon cycloisomerization of 3-chloro-*cis*,*cis*-muconate (Dorn et al. 1974; Schmidt and Knackmuss 1980).

Genetic investigation of DCA dechlorination by UW103 would provide further evidence for the catabolic pathway of DCA proposed in this study and may provide insight into dechlorination mechanisms. PCR-amplification and sequencing of dehalogenase genes, using primers designed from publicly-available sequences of *Burkholderia* spp., may yield new information about the dechlorination mechanisms involved in DCA degradation. Mutational analysis of the potential genes involved in DCA-degradation could provide strong support for the DCA catabolites suggested in this study.

CHAPTER 4

IDENTIFICATION AND CHARACTERIZATION OF *Burkholderia* sp. UW103, A DCA-DEGRADER FROM COMPOST

4.1 Abstract

A non-motile, gram-negative, oxidase and catalase positive bacterium was isolated from an enrichment culture of commercial compost and was capable of growth on 3,5-dichloro-*para*-anisyl alcohol (DCA) as a sole source of carbon and energy. Strain UW103 is a coccobacillus, 1.5x1.2 μm, while growing on DCA and longer, slightly curved bacillus, 4x1.2 μm, when grown on nutrient rich media. Colonial morphology on trypticase soy agar was circular, flat, entire and smooth with a buttery consistency. Traditional biochemical tests, API-20NE, and Biolog® reveal that the bacterium belongs to the genus *Burkholderia*. The 16S rRNA gene was PCR-amplified and a 1454 bp portion was sequenced. Comparison to the NCBI database revealed the greatest sequence homology (98.5%) to *B. caledonica*. *Burkholderia* sp. UW103 was capable of growth on DCA at concentrations up to 1.5 mM, above which DCA was toxic to the bacterium. The DCA-degrading strain was capable of utilizing a limited range of chlorinated phenols and benzoates.

4.2 Introduction

It is estimated that the white rot (basidiomycete) fungi evolved approximately 300 million years ago with the remarkable capability to utilize lignin as a source of carbon and energy (Berbee and Taylor 1993). Chlorinated anisyl metabolites (CAM) function as a source of electrons for extracellular aryl alcohol oxidases in the reduction of molecular oxygen to produce hydrogen peroxide, a necessary cosubstrate in the degradation of lignin by extracellular peroxidases. The role of CAM in lignin degradation suggests that they have existed for as long as the white rot fungi.

The presence of CAM in the environment for millions of years allows ample time for microorganisms to evolve catabolic pathways specific to the degradation of CAM. Similarly structured xenobiotic compounds, like the pesticides chloroneb®, dicamba and 2,4-D, have been present in the biosphere for less than a century, a very short time on the evolutionary scale. Therefore biodegradation of these chlorinated pesticides may occur via catabolic pathways evolved for the utilization of CAM.

CAM are synthesized in significant concentrations by basidiomycete fungi in the natural environment (De Jong et al. 1994). The most common CAM, 3,5-dichloro-*para*-anisyl alcohol (DCA), was detected in wood and forest litter colonized by *Hypholoma* sp. in concentrations up to 180 mg per kg dry sample weight. Although DCA is detected in close vicinity of fruiting bodies of CAM-producing basidiomycetes, low molecular weight organohalogens are not detected just outside CAM-producing basidiomycete colonies (Verhagen et al. 1998*c*). The lack of DCA detection outside basidiomycete colonization suggests that DCA is rapidly biodegraded or biotransformed.

Aerobic degradation studies of DCA demonstrated a ubiquitous mineralization capacity in forest soils (Verhagen et al. 1998*a*). Verhagen et al. (1998*a*) isolated

Burkholderia cepacia from enrichment cultures and suggested that this organism may be responsible for the fast degradation of DCA in oak forest soils.

Verhagen et al. (1998*c*) also studied the anaerobic fate of DCA. In methanogenic sludge, DCA was demethylated to 3,5-dichloro-4-hydroxybenzyl alcohol, which was converted via a biotic route leading to the formation of 3,5-dichloro-4-hydroxybenzoate (Figure 2-8). Demethylation was inhibited by oxygen, suggesting the involvement of acetogenic bacteria. Finally, a biotic decarboxylation step yielded 2,6-dichlorophenol. An abiotic route led to the formation of bis(3,5-dichloro-4-hydroxyphenyl)methane, a product resulting from the dimerization of the demethylated fungal metabolite (Figure 2-9). Verhagen et al. did not observe dechlorination in this study.

4.3 Objective of Study

The main objective of this study was to isolate a bacterium capable of the degradation of DCA by enrichment culture, containing DCA as a sole source of carbon and energy under aerobic conditions. The second objective was to characterize the bacterium's ability to utilize man-made chlorinated organic compounds as sole sources of carbon and energy.

4.4 Materials and Methods

4.4.1 DCA Synthesis

DCA was synthesized beginning with the commercial starting material 3,5-dichloro-*para*-hyroxybenzoic acid, purchased from Sigma-Aldrich (St. Louis MI). Methylation and subsequent reduction using lithium aluminum hydride resulted in the pure product 3,5-dichloro-*para*-anisyl alcohol (DCA), as previously described.

4.4.2 Isolation of a Bacterium Capable of Growth on DCA

Strain UW103 was isolated from commercial compost as previously described in section 3.4.2. The culture was routinely cultivated on 1 mM DCA in YNB media at 30°C and stocks were maintained as previously described.

4.4.3 Biolog® and Substrate Utilization

GN2 Biolog® plates and API20NE strips were purchased from Biolog Inc. (Hayward CA). GN2 Biolog® plates and API20NE were inoculated according to manufacturer's instructions with axenic UW103 cells grown on trypticase soy agar (TSA) for at least 2 subcultures. Biolog ® plates were incubated at 30°C for 18 hours and measured using a Biolog® plate reader and confirmed visually. API20NE strips were incubated for a total of 1 week and read at 24 and 48 hours and 7 days.

Growth of strain UW103 on selected chlorinated and/or aromatic substrates, as sole sources of carbon and energy, was attempted in YNB broth without amino acids. The medium contained 50 mM phosphate buffer pH 6.3 and 0.1 to 1 mM of the substrates listed in Table 4-5. An absorbance at 600 nm greater than 0.1 after three 1% serial subcultures was used as an indication of substrate utilization as sole carbon and energy source. A control flask containing no carbon source was also inoculated and the

absorbance at 600 nm was zero for the duration of the experiment. All substrates were purchased from Sigma-Aldrich (St. Louis MI).

4.4.4 16S rRNA Gene Analysis

4.4.4.1 DNA Extraction. Crude DNA was obtained by suspending UW103 in an aqueous solution of 0.5% N-lauroyl sarcosine, purchased from Sigma-Aldrich (St. Louis MI). After 1 minute incubation at room temperature cellular debris was removed by centrifugation at 5000xg for 10 minutes. Cells for DNA extraction were grown in DCA-YNB for 72 hours before harvested.

4.4.4.2 Polymerase Chain Reaction (PCR). 1µl DCA-degrader DNA, 43 µl PCR supermix (Invitrogen), 1 µl 25mM magnesium chloride, 1 unit Taq polymerase and 2 µl each of 50 µM forward (27F) and reverse (1492R) primers were combined in 15 replicate 500 µl PCR tubes (Lane 1991). Three controls were run simultaneously: one control using 100ng *E. coli* strain 2206 DNA, one control without DNA and one control with 100ng *E. coli* strain 2206 DNA without primers. Two drops of sterile mineral oil was added to each tube. All PCR reactions were prepared in a laminar air flow hood. Thirty amplification cycles were performed in a Robocycler 96 (Stratagene; La Jolla CA), using a denaturing temperature of 96°C (1.5 minutes), annealing temperature of 55°C (1 minute) and polymerization temperature of 72°C (2 minutes). Gel electrophoresis was performed, using 1% agarose, for 35 minutes at 150V (Figure 4-3). PCR product was purified using BioRad PCR Product Purification Kit (Biorad; Mississauga ON) and electrophoresed through a 1% agarose gel for 35 minutes at 150V. Band intensity after gel electrophoresis of 2, 5 and 15 µl of PCR product were compared to band intensities of known concentrations of lambda DNA (Boehringer Ingelheim Canada Ltd.; Burlington

ON) (Table 4-1; Figure 4-4). Total weight of PCR-product was obtained by multiplying the estimated DNA concentration of the purified PCR amplification product by the total volume. Purified PCR product was lyophilized to dryness using a Savant Speed Vac Model SC 210A and brought to a concentration of 50 ng DNA/μl molecular grade H_2O.

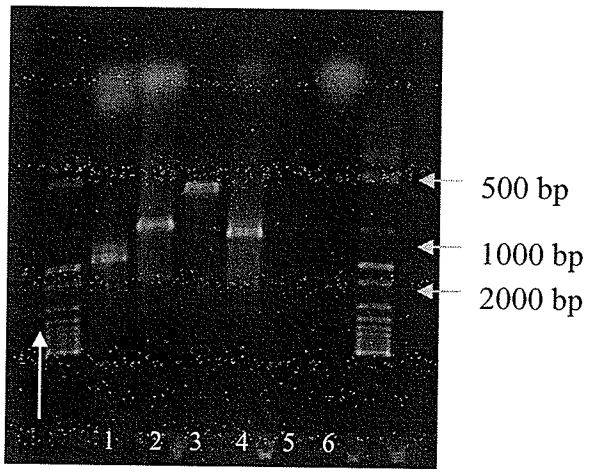


Figure 4-1. Gel image of 16S PCR product of DCA-degrader UW103 and controls. Lane 1, primer set 27F and 1492R; lane 2, primer set 530F and 1492R; lane 3, primer set 530F and 1100R; lane 4, primer set 27F and 1100 R; lane 5, control containing template without primers; lane 6, contamination control containing primers 530F and 1100R and no template.

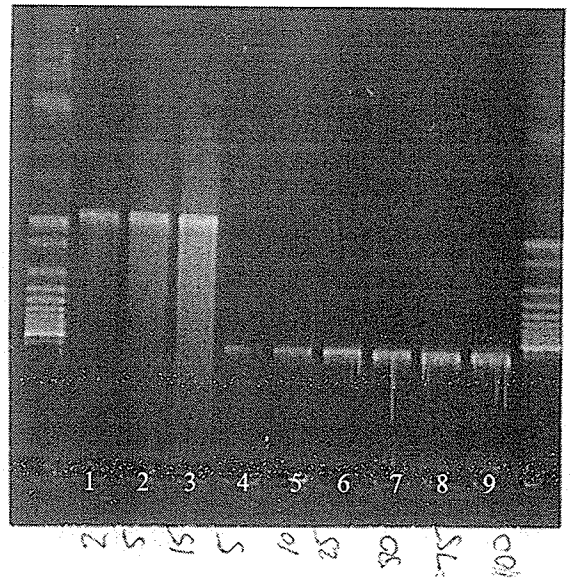


Figure 4-2. DNA quantification. Lanes 1-3, PCR-product; Lanes 4-9, lambda DNA standards.

Table 4-1. Estimation of PCR product quantity by comparison to lambda DNA standards.

Volume run (µl)	Estimated DNA weight (ng)	Calculated DNA concentration (ng/µl)	Average
2	10	5	
5	25	5	
15	80	5.3	5.1

4.4.4.3 DNA Sequencing. Sequencing primers, suggested by Lane (1991), (27f, 75f, 519r, 530f, 619r, 787r, 805f, 926f, 1100r, and 1492r) were purchased from the Molecular Supercentre at the University of Guelph (Guelph ON). Primers and purified PCR products were shipped on dry ice to Guelph ON and sequenced by Laboratory Services at the University of Guelph.

4.4.5 DNA Sequence Alignment and Database Search

The sequences of the PCR product generated from the different sequencing primers were aligned using the computer program Seqman (Burland 2000; Figure 4-4). The resulting 1550 bp sequence was shortened by eye to remove poor sequencing data at each end to give the sequence of a 1454 bp portion of the 16S rRNA gene, which was used for phylogenetic analysis.

4.4.6 Phylogenetic Analysis

The 1454 bp rDNA sequence was compared against sequences in the NCBI database. Eight of the most homologous sequences from the database, and sequences from all 42 *Burkholderia* species, were chosen and aligned along with the rDNA sequence of strain UW103 using Clustal X (Jeanmougin et al. 1998). Clustal X sequence alignments were performed using parameters recommended by Hall (2004). Default pairwise alignment parameters were used except gap opening and gap extension, modified to 10.0 and 0.1, respectively. Multiple alignment parameters were defaults except modifications to gap opening and gap extension to 10.0 and 0.2, respectively.

The aligned sequences were used to create a phylogenetic tree using MEGA (Kumar, Tamura, and Nei 2004). Figure 4-5 illustrates the phylogenetic tree of the near complete 16S rRNA gene from 40 of 42 valid *Burkholderia* species and strain UW103. B. vietnamThe phylogenetic tree was created using the neighbor-joining method with bootstrap values of 1000 replicates provided at nodes. The tree is rooted to the type strain of the type species of the closely related genus, *Pseudomonas*.

4.4.7 DCA Utilization Profiles and Toxicity

To assess strain UW103's ability to utilize DCA at various pHs, growth in DCA-YNB was measured by the absorbance at 600 nm. DCA-grown cells were inoculated into a series of DCA-YNB media with pH varying from 4.0 to 9.2. After 96 hours, the absorbance at 600 nm was measured and taken to indicate growth. Control flasks, containing no carbon source, were also inoculated for each pH tested.

To assess strain UW103's preference for temperature, DCA-grown cells were inoculated into a series of DCA- YNB, pH 6.3, incubated at temperatures ranging from 4 to 45°C. After 96 hours, growth was measured as absorbance at 600 nm.

The toxicity of DCA towards strain UW103 was estimated by inoculating a series of DCA-YNB media containing a range of DCA concentrations from 0.5 to 5 mM. After 96 hours, the absorbance at 600 nm was measured and taken to indicate growth.

4.5 Results

4.5.1 Cellular and Colonial Morphologies

A Gram negative bacterium capable of growth on DCA as sole carbon and energy source was isolated from a DCA enrichment culture derived from commercial compost. The DCA-degrader formed circular, entire, convex colonies on trypticase soy and nutrient agar (Table 4-2). Growth on R2A was exhibited as colonies having a mucoid, opaque, smooth and entire morphology. Upon Gram staining it appeared to have an invagination in the middle that did not stain and was barely visible under phase contrast microscopy of unstained cells. This was in contrast to the dark ends which stained well and were clearly visible under phase contrast microscopy. A transmission electron micrograph (TEM) and a scanning electron micrograph (SEM) were obtained to closer observe the cellular

morphology (Figure 4-3). In the TEM, UW103 cells appeared as bacilli 1.2 μm by 3.5-4.5 μm in length, slightly curved, with an invagination at center. The ends of the cell were darkly stained and lighter in the center. The plasma membrane or cell wall were not visible; the margin of the bacteria were not well defined. In a SEM UW103 DCA-grown cells were coccobacilli, 1.2 μm x 1.5-1.8 μm, in length.

Table 4-2. Morphology of strain UW103 grown on various media for 72 hours at 30°C.

Medium	Cellular morphology and dimensions	Colonial morphology
DCA-YNB agar	Coccobacilli, 1.2 μm x 1.5-1.8 μm	<0.5 mm diameter, white, circular, flat, entire, smooth
R2A agar	Bacilli, 1.2 μm x 3.5-4.5 μm, slightly curved with invagination at center	3.0 mm diameter, white, circular, raised, undulate, concentric
Trypticase soy agar	Bacilli, 1.2 μm x 3.5-4.5 μm, slightly curved with invagination at center	1.0 mm diameter, pale yellow, circular, convex, entire, smooth
Nutrient agar	Bacilli, 1.2 μm x 3.5-4.5 μm, slightly curved with invagination at center	3.0 mm diameter, pale yellow, circular-irregular, raised, undulate, smooth

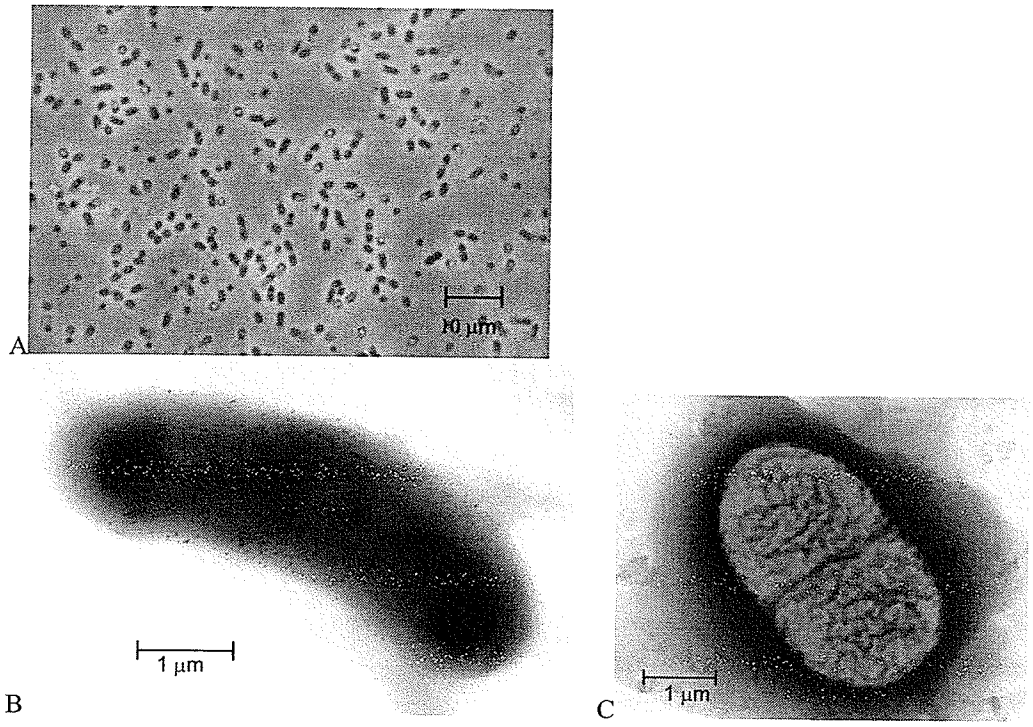


Figure 4-3. DCA-degrading strain UW103. A, wet mount viewed under phase microscopy (1000X magnification); B, TEM of section (21000X magnification); C, negative stain TEM of whole cell (15000X magnification).

4.5.2 Biochemical tests including Biolog®

The bacterium was oxidase and catalase positive by conventional biochemical tests and was identified as a *Burkholderia*-like species by Biolog® GN2 analysis. A total of 146 biochemical tests (including the 95 from Biolog®) were performed to identify the bacterium (Table 4-3). All tests were negative for motility including the sulfide-indole-motility (SIM) medium test and wet mounts of DCA and nutrient rich medium grown cells.

Table 4-3. Biochemical test results for strain UW103 and comparison to *Burkholderia* species (Achouak et al. 1999; Coeyne et al. 1999, 2001*a* and 2001*b*; Zhao et al. 1995; Segonds et al. 1999; Yabuuchi et al. 1992; Viallard et al. 1998 and Holt 1984). +, 75-100% strains positive; v, 25-74% strains positive; -, 0-24% strains negative; w, weakly positive.

		DCA-degrader UW103	B. fungorum	B. caribiensis	B. caledonica	B. graminis	B. phytofirmans	B. phenazinium	B. caryophylli	B. cepacia	B. gladioli	B. pseudomallei	B. mallei
16S rDNA sequence homology		100	98.0	96.6	98.5	97.5	98.5	96.1	96.6	96.1	96.2	96.7	96.7
Test results in common with DCA-degrader		100%	92%	90%	89%	88%	88%	83%	74%	70%	70%	64%	58%
Physiological and biochemical characteristics:													
arginine, Moeller	API20 E and NE	-							-	-	-	+	+
B-hemolysis	blood agar	-	-	-	-	-	-	-	-	v	-	+	-
casein hydrolysis	casein agar	-									+		
catalase	TSA	+	+	+	v	+	v	-	+	+	+	+	+
esculin hydrolysis	API20NE	-							-	v	v	-	-
flagella	negative stain on SEM	0					1		>1	>1	>1	>1	0
gelatin liquification	API20 E and NE	-	-	-	-	-	-	-	-	v	+	v	v
growth at 37oC	DCA-YNB	+	+	+	v	+	-	v					
growth at 42oC	TSA, R2A, DCA, NA	-	-	-	-	-		-	-	v	-	+	-
H2S	API20E and SIM tube and TSI slant	-	-	-	-	-		-	-	-	-	-	+
indole	API20NE	-	-	-	-	-	-	-					
oxidase	TSA	+	+	-	-	v	+	v	-	v	v	+	+
MacConkey	MacConkey agar	+							-	-	+	-	+
MR	broth	-											
motility	SIM and wet mount	-	+	+	+	+	+	+	+	+	+	+	-
nitrate reduction	API20 E and NE	-	+	-	-	+	-	-	+	-	v	+	+
N2	API20 E and NE	-	-	-	-	-	-	-	-	-	-	-	-
pigment blue		-							-	-	-	-	-
pigment yellow		-							-	+	-	-	-
p-nitrophenyl-B-D-galactopyranoside	API20NE	+	-	v	-	-	-	-					
starch hydrolysis	starch agar	-							-	-	-	+	v
Tween 40	BIOLOG	+											
Tween 80	BIOLOG	+	+	+	+	+	+	+	-	+	-	-	-
VP	broth	-								-		-	-
urease	broth, API20 E and NE	-	-	-	-	+	-	-	-	-	+	-	-
Acid from:													
Amygdalin	API20E	-											
L-Arabinose	API20E	-							+	+	+	+	+
Glucose	API20E & NE & OF & TSI	-	+	-	+	-	+	-	+	+	+	+	+
Inositol	API20E and BIOLOG	-							-	+	+	+	+
Lactose	TSI slant	-	-	-	-	-	-	-	-	+	-	+	+
Mannitol	API20E	-							-	+	+	+	+
Melibiose	API20E	-							-	-	+	-	-
Rhamnose	API20E	-							+	-	-	-	-
Sorbitol	API20E	-							-	+	+	+	+
Sucrose	API20E and TSI slant	-	-	-	-	-	-	-	+	+	-	-	-
Growth in OF medium w/													
D-glucose	OF media	+	+	+	+	v	+	v					

Table 4-3 Continued..

		DCA-degrader UW103	B. fungorum	B. caribiensis	B. caledonica	B. graminis	B. phytofirmans	B. phenazinium	B. caryophylli	B. cepacia	B. gladioli	B. pseudomallei	B. mallei
Carbohydrate assimilation/respiration:													
N-Acetyl-D-galactosamine	BIOLOG	-											
N-Acetylglucosamine	API20NE & BIOLOG	+	+	+	v	+	+	+	+	+	+	+	+
Adonitol	BIOLOG	+	v		v	+	v		+	v	+	-	-
L-Alaninamide	BIOLOG	+											
2-Aminoethanol	BIOLOG	+											
D-Arabinose	API20NE	+				+			+	+	-	v	v
L-Arabinose	BIOLOG	+	v	+	+	+	+	v	+	+	+	-	-
D-Arabitol	BIOLOG	+				+			+	+	-	+	-
2,3-Butanediol	BIOLOG	-											
Caprate	API20NE	-	v		v		+						
Cellobiose	BIOLOG	-				v			-	+	-	v	v
alpha-Cyclodextrin	BIOLOG	-											
dextrin	BIOLOG	+											
Erythritol	BIOLOG	-				-			-	-	-	+	-
Fructose	BIOLOG	-	+		+		+		v	+	+	+	v
L-Fucose	BIOLOG	-				+			-	+	-	+	-
Galactose	BIOLOG	-							+	+	+	+	+
Gentibiose	BIOLOG	+				v			-	+	-	-	-
Gluconate	API20NE	+	+	+	+	+	+	+	+	+	+	+	v
Glucose	API20NE & BIOLOG	+	+	+	+	+	+	+	+	+	+	+	+
Glucose-1-Phosphate	BIOLOG	-											
Glucose-6-phosphate	BIOLOG	+											
glucuronamide	BIOLOG	+		+		+		-	-				
Gylcerol	BIOLOG	+							+	+	+	+	+
D,L-alpha-Glycerol Phosphate	BIOLOG	+											
Glycogen	BIOLOG	+							-	-	-	+	-
Inositol	BIOLOG	-							+	+	+	+	+
Lactose	BIOLOG	+		-		+		-	-	v	-	-	-
Lactulose	BIOLOG	+											
Maltose	API20NE/ BIOLOG	-/+	-	-	-	-	v	-	-	+	-	-	-
Mannitol	API20NE & BIOLOG	+	+	+	+	+	+	+	+	+	+	+	+
Mannose	API20NE & BIOLOG	+	+	+	+	+	+	+	+	+	+	+	+
Melibiose	BIOLOG	-							-	+	-	-	-
Beta-Methyl-D-Glucoside	BIOLOG	-							-	-	-	-	-
D-Psicose	BIOLOG	+											
Putrescine	BIOLOG	-											
Raffinose	BIOLOG	+		-		+		-	-	+	-	-	-
Rhamnose	BIOLOG	+							+	-	-	-	-
Sorbitol	BIOLOG	+							+	+	+	+	-
L-Sorbose	YNB broth	-				- all *Burkholderia* negative -							
Starch	starch agar	+							-	-	-	+	v
Sucrose	BIOLOG	+	-	v	v	+	-	+	+	v	-	+	+
Trehalose	BIOLOG	-	-	+	+	+	+	-	+	+	-	-	+
Xylitol	BIOLOG	-				+			+	+	-	-	-
D-Xylose	YNB broth	+	v		+	+	+		+	+	+	v	v

Table 4-3 Continued..

		DCA-degrader UW103	B. fungorum	B. caribiensis	B. caledonica	B. graminis	B. phytofirmans	B. phenazinium	B. caryophylli	B. cepacia	B. gladioli	B. pseudomallei	B. mallei
Amino acids assimilation/respiration:													
D-Alanine		+							+	+	+	-	+
L-Alanine		+							+	+	+	+	+
DL-4-Amino-butyrate	BIOLOG	+							+	+	+	+	+
L-Alanylglycine	BIOLOG	+											
L-Arginine	YNB culture	+	v	+	+	+	+	+	+	+	+	+	-
L-Asparagine	BIOLOG	+											
L-Aspartate	BIOLOG	+							+	+	+	+	+
DL-Carnitine	BIOLOG	+		+		+		-	-				
L-Cysteine	YNB culture	-							+	+	+	+	-
L-Glutamate	BIOLOG and culture	+							+	+	+	+	+
L-Glutamine	YNB culture	+											
Glycyl-L-Glutamic Acid	BIOLOG	+											
L-Histidine	BIOLOG	+							+	+	-	+	+
Hydroxy-L-Proline	BIOLOG	+											
Inosine	BIOLOG	+											
L-Isoleucine	YNB culture	-							-	+	+	+	-
L-Leucine	BIOLOG	+							-	+	+	+	-
lysine	API20E	-	-	-	-	-	-	-	-	+	-	-	-
L-Lysine	YNB culture	-							-	+	+	-	-
L-Ornithine	BIOLOG	-		-		+		+	+				
L-Ornithine	API20E	-	-	-	-	-	-	-	-	+	+	-	-
L-Phenylalanine	BIOLOG	+							+	+	+	+	-
Phenylethylamine	BIOLOG	-											
L-Proline	BIOLOG	+							+	+	+	+	+
L-Pyroglutamic acid	BIOLOG	+											
L-Serine	BIOLOG	+							+	+	+	+	-
L-Threonine	BIOLOG	+							+	+	+	+	-
L-Tryptophan	API20E	-							-	+	+	+	-
L-Tyrosine	YNB culture	+							+	+	+	+	-
D-Serine	BIOLOG	+											
Thymidine	BIOLOG	+											
Uridine	BIOLOG	+											
Urocanic Acid	BIOLOG	+											

Table 4-3 Continued..

		DCA-degrader UW103	B. fungorum	B. caribiensis	B. caledonica	B. graminis	B. phytofirmans	B. phenazinium	B. caraphylli	B. cepacia	B. gladioli	B. pseudomallei	B. mallei
Organic acids assimilation/respiration:													
acetate	BIOLOG	+							-	+	+	+	-
Cis-Aconitic Acid	BIOLOG	+											
adipate	API20NE	-	v	-	v	+	v	-	-	+	+	+	+
benzoate	YNB broth	+							+	+	+	+	-
bromo-succinic acid	BIOLOG	+											
citrate	API20 E and NE/BIOLOG	-/+	+	+	-	+	+	-	+	+	+	+	-
Formic Acid	BIOLOG	+											
D-Galactonic Acid Lactone	BIOLOG	+											
D-Galacturonic Acid	BIOLOG	+											
D-Gluconic Acid	BIOLOG	+											
D-Glucosaminic Acid	BIOLOG	+											
D-Glucuronic Acid	BIOLOG	+											
alpha-Hydroxy Butyric Acid	BIOLOG	+											
DL-3-Hydroxy-Butyrate	BIOLOG	+							+	+	-	+	+
g-hydroxy Butyric acid	BIOLOG	-											
p-Hydroxy-phenylacetic acid	BIOLOG	+											
Itaconate	BIOLOG	-							-	-	-	-	-
alpha-keto butyric acid	BIOLOG	+											
2-Ketoglutarate	BIOLOG	-							+	+	+	-	-
alpha-Keto valeric acid	BIOLOG	+				-							
DL-Lactate	BIOLOG	+	+	+	+	+	+	+	+	+	+	+	+
DL-Malate	YNB broth	+											
L-Malate	API20NE / YNB broth	-/+	+	+	+	+	v	+	+	+	-	+	-
Malonate	BIOLOG	+		-		+		+	+	+	-	-	v
Phenylacetate	API20NE	+	+	+	+	+	+	+	+	+	-	-	-
Propionate	BIOLOG	+							+	+	+	+	-
Pyruvate	YNB broth and BIOLOG	+							+	+	+	+	-
Quinic acid	BIOLOG	+											
D-Saccharic Acid	BIOLOG	+							+	+	+	-	-
Sebacate	BIOLOG	+							-	-	+	-	-
Succinamic acid	BIOLOG	+											
Succinate	BIOLOG	+							+	+	+	+	-

4.5.3 PCR Sequences and Alignment

The 16S rRNA gene of strain UW103 was sequenced utilizing 11 primers and the individual sequences used to create a consensus sequence, aligned with Seqman (Burland 2000; Figure 4-4). The resultant sequence, a 1454 base pair portion of16S rDNA, was compared to sequences in the NCBI Blast database (Table 4-4). The sequence shares high homology with numerous unspeciated *Burkholderia* species and uncultured eubacteria (1-30 mismatches per 1454 bp, 100 – 98.7% homology). The sequence is

98.5% homologous to the type strains of *Burkholderia caledonica* and *B. phytofirmans* (Table 4-4; Figure 4-5).

The DCA-degrader has the most biochemical test results in common with *Burkholderia fungorum, B. caribiensis*, and *B. caledonica*. (92, 90, and 89%, respectively). All of these species are motile with at least one polar flagellum while strain UW103 is non-motile.

Flagella were not observed in a negative stain viewed under electron microscopy at 40,000X magnification. All biochemical tests were negative for motility. According to the description of the *Burkholderia* genus, *B. mallei* is the only *Burkholderia* species that is non-motile without a flagellum (Yabuuchi et al. 1992; Palleroni 2001). *B. mallei* has only 58% biochemical test results in common with strain UW103 and the 16S rDNA sequence homology is 96.7%. Coeyne et al. (2001), in their validation of *B. caledonica* and *B. fungorum*, among the species sharing the highest homology with UW103, did not provide motility data.

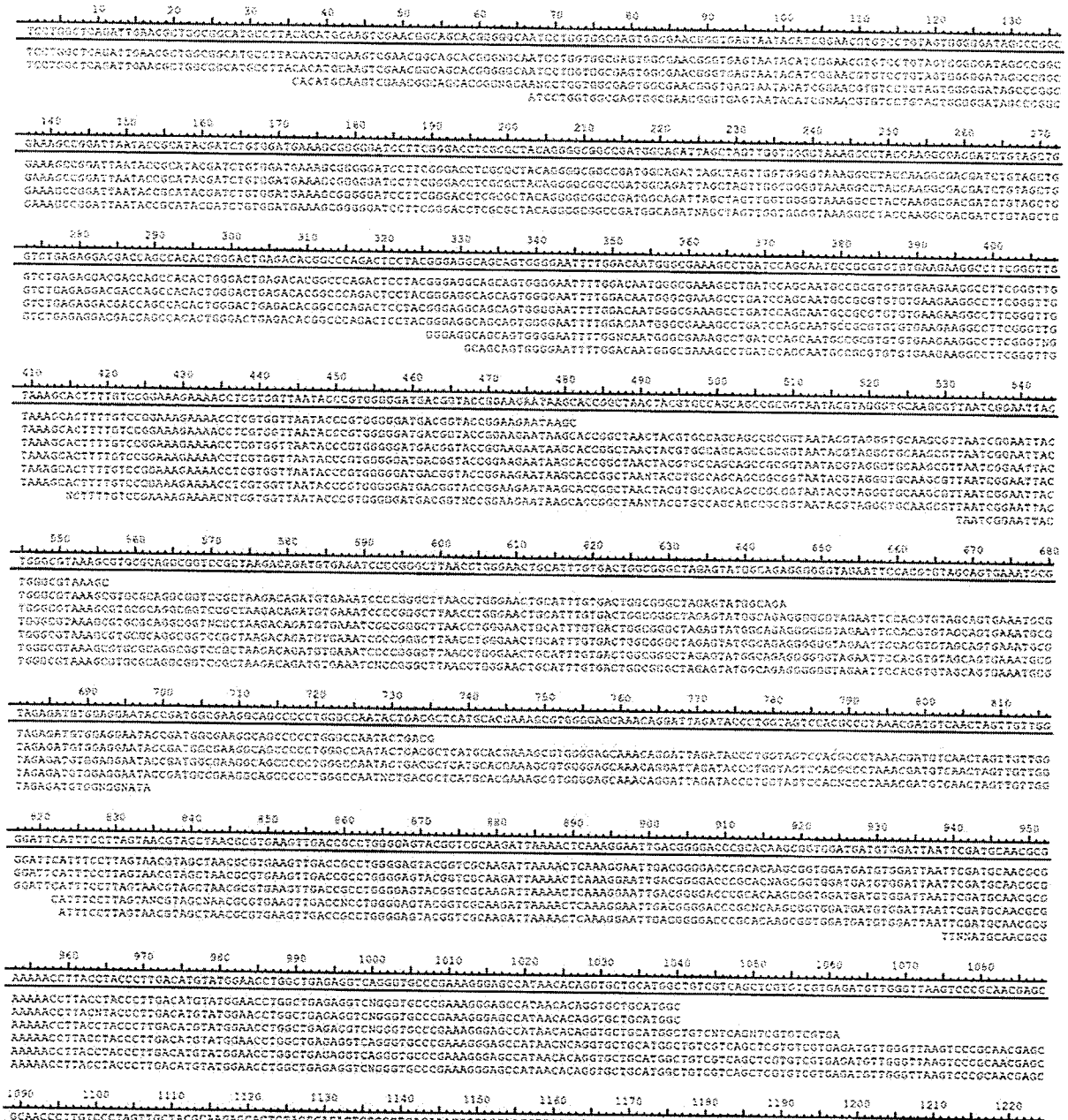


Figure 4-4. Alignment of sequences resulting from various primer reactions yielding a consensus seqeunce of the 16S rRNA gene of strain UW103.

Table 4-4. 16S rDNA sequence homology between the DCA-degrading strain UW103 and other *Burkholderia* species (Summary of NCBI Blast results for a 1454 bp portion of the 16S rRNA gene).

Strain	Genbank Assession #	Sequence homology (%)
Burkholderia sp. OY715	AJ300696	100.0
Uncultured eubacterium WD258	AJ292640	99.9
Burkholderia sp. H801	AB212238	99.9
Uncultured eubacterium WD266	AJ292642	99.8
Uncultured eubacterium WD227	AJ292639	99.7
Uncultured eubacterium WD289	AJ292645	99.7
Burkholderia sp. LMG 21262	AF452132	99.6
Burkholderia sp. FM-A	DQ118949	98.7
B. caledonica LMG 19076^{TS}	AF215704	98.5
B. phytofirmans PsJN^{TS}	AY497470	98.5
B. fungorum LMG 16225^{TS}	AF215705	98.0
B. terricola LMG 20594^{TS}	AY040362	97.7
B. graminis C4D1M^{TS}	U96939	97.5
B. phenoliruptrix AC1100^{TS}	AY435213	97.4
B. xenovorans LB400^{TS}	U86373	97.4
B. phymatum STM815^{TS}	AJ302312	97.2
B. multivorans DSM 13243^{TS}	Y18703	96.8
B. terrae KMY02^{TS}	AB201285	96.7
B. hospita LMG 20598^{TS}	AY040365	96.7
B. sordidicola S5-B^{TS}	AF512826	96.7
B. mallei ATCC 23344^{TS}	NC_006348	96.7
B. vandii LMG 16020^{TS}	U96932	96.7
B. pseudomallei ATCC 23343^{TS}	DQ108392	96.7
B. dolosa LMG 18941	AF175314	96.7

4.5.4 Phylogenetic Tree

Figure 4-5. Phylogenetic tree of the near complete 16S rRNA gene of *Burkholderia* spp., including strain UW103. Sequence alignments were performed using Clustal X (Jeanmougin et al. 1998) with parameters recommended by Hall (2004). The phylogenetic tree was created using MEGA 3.1 software (Kumar, Tamura, and Nei 2004) using the neighbor-joining method with bootstrap values from 1000 replicates, provided at nodes. The distance bar shown refers to substitutions per nucleotide. Genbank assession #s are provided in parentheses.

4.5.5 DCA Utilization Profiles and Toxicity

Strain UW103 was able to utilize DCA as a sole source of carbon and energy at pHs ranging from 5.5 to 7 with optimal growth occurring at pH 6.5 (Figure 4-6). Temperatures ranging from 15-30°C were able to support the growth of strain UW103 with optimal growth occurring at 24°C (Figure 4-6). Toxicity of DCA was observed at concentrations equal to or greater than 2 mM in YNBwoAA (Figure 4-7).

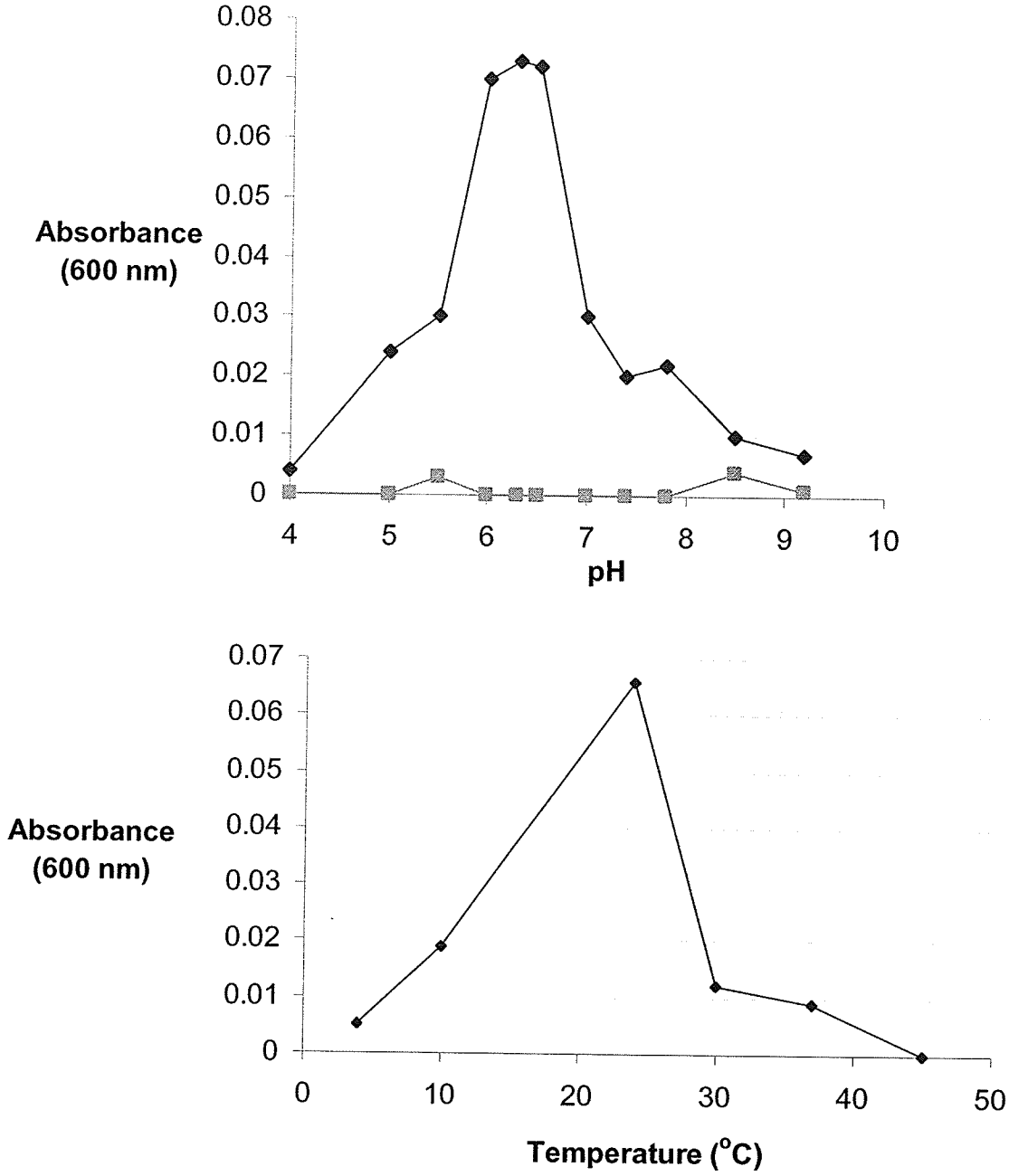


Figure 4-6. Growth of *Burkholderia* sp. UW103 using DCA as sole carbon and energy source. Top, growth in YNB range from pH 4 to pH 9.2: —◆—, 1 mM DCA-YNB; —■—, YNB no carbon source; Bottom, growth in YNB at temperatures from 4°C to 45°C. Absorbance measured after 4 days growth on 1mM DCA as sole carbon and energy source.

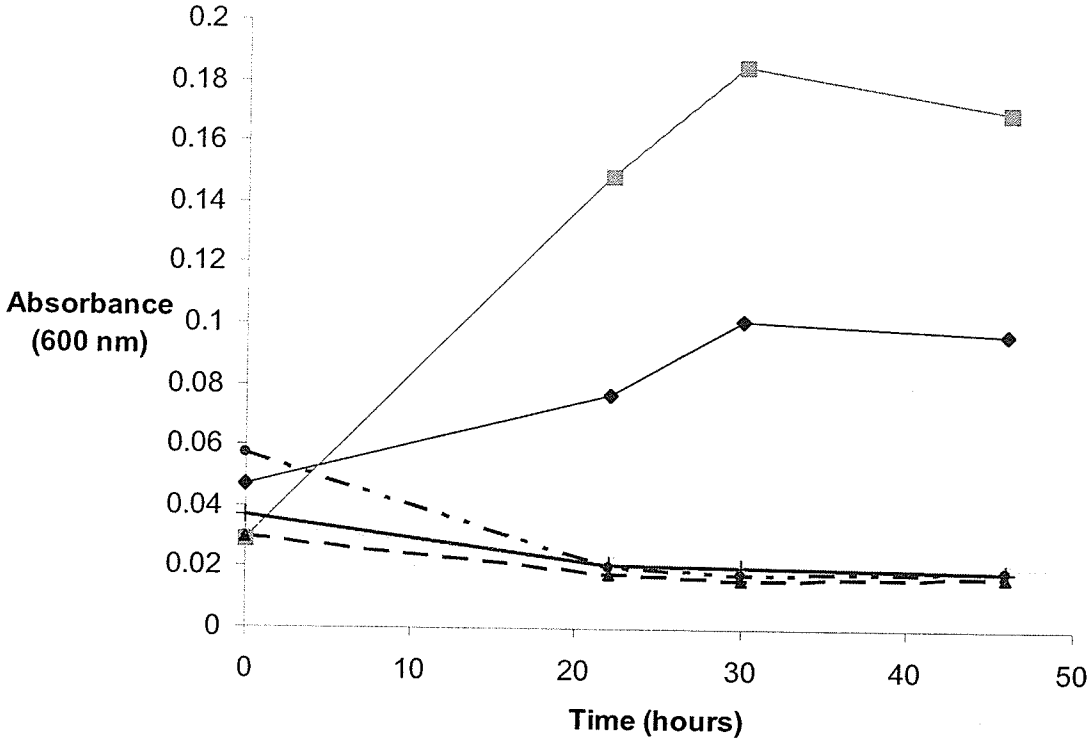


Figure 4-7. DCA toxicity towards *Burkholderia* sp. UW103: Growth measured by absorbance at 600 nm in YNB-broth containing 0.5-5 mM DCA. —◆—, 0.5 mM; —■—, 1 mM; —▲—, 2 mM; , 3 mM; —+—, 4 mM; —●—, 5 mM.

4.5.6 Utilization of Chlorinated Phenols and Benzoates

The growth of *Burkholderia* sp. strain UW103 on the CAM DCA would suggest that this strain may be able to utilize other chlorinated compounds. This is not the case, at least with the substrates tested. The DCA-degrader will not utilize a variety of chlorinated benzoic acids and phenols, even at concentrations as low as 0.1mM (Table 4-5). The herbicide dicamba (3,6-dichloro-2-methoxybenzoic acid), structurally similar to DCA, is the only chlorinated xenobiotic for which we could demonstrate utilization by strain UW103.

Table 4-5. Growth of DCA-degrader UW103 on chlorinated compounds at a concentration of 1 mM in phosphate buffered yeast nitrogen base without amino acids. Compounds negative for growth at 1 mM concentration were also tested at 0.1 mM concentrations.

Compound	Growth
3,5-dichloro-*para*-anisyl alcohol (DCA)	+
3-chlorophenol	-
2,4-dichlorophenol	-
2,6-dichlorophenol	-
3,5-dichlorophenol	-
2,6-dichloroanisole (2,6-dichloro methoxy benzene)	-
2,6-dichloro-*para*-nitroaniline	-
chloroform	-
3,6-dichloro-3-methoxybenzoic acid (dicamba)	+
pentachlorophenol	-

4.6 Discussion and Conclusions

The organisms and pathways responsible for the degradation of man-made chlorinated organic compounds in bioremediation projects are often well-studied. The capability of these organisms to degrade novel chlorinated compounds may be due to the presence of naturally chlorinated compounds present in the biosphere for millions of years. However, there are only a handful of studies that focus on the dechlorination and degradation of naturally chlorinated compounds. This study isolated and identified a microbe from compost, designated UW103, that was capable of using DCA as a sole source of carbon and energy.

Strain UW103 is a Gram negative, non-motile, non-fermentative, rod-shaped bacterium that produces catalase and is weakly oxidase positive. Phenotypic results, including Biolog®, and 16S rRNA gene sequencing suggest *Burkholderia* as the likely genus. The cells of *Burkholderia* gen. are Gram-negative, non-fermentative, straight or

curved rods (Palleroni 2001). Most are motile with a single polar flagellum or a tuft of polar flagella. To date a single species, *B. mallei*, is atrichous and non-motile. *Burkholderia* species produce catalase, yet oxidase activity is variable. Monosaccharides, disaccharides and polyalcohols are oxidized and assimilated as sole sources of carbon and energy. The genus is represented by plant and animal pathogens, and rhizospheric species (Viallard et al. 1998; Palleroni 2001).

The type species of *Burkholderia, B. cepacia*, has the ability to degrade a wide range of halogenated and other xenobiotic chemicals. The type strain, *B. cepacia* AC1100, has been well studied for its bioremediation potential (Danganan et al. 1995; Karns et al. 1983; Palleroni 2001; Wackett and Hershberger 2001). *B. cepacia* and related strains *B. ubonensis, B. dolosa, B. multivorans, B. ambifaria, B. pyrrocinia, B. stabilis, B. cenocepacia*, and *B. anthina*, represent the *B. cepacia* complex (Figure 4-5). Strains of the *B. cepacia* complex are opportunistic human pathogens, causing life-threatening infections in cystic fibrosis patients (Govan, Hughes and Vandamme 1996). *B. pseudomallei* and *B. mallei*, closely related to the *B. cepacia* complex, cause the human diseases glanders and meliodosis, respectively. A few *Burkholderia* species are also plant pathogens; *B. caryophylli, B. andropogonis,* and *B. solanacearum* cause disease of carnations, bacterial stripe of sorghum and maize, and bacterial wilt disease, respectively.

Within the *Burkholderia* genus two distinct clusters are evident based on 16S rDNA sequence data (Palleroni 2001; Figure 4-5). The first cluster includes the *B. cepacia* complex, *B. pseudomallei*, and *B. mallei*, and other species. The second cluster contains the species *B. phenazinium, B. caledonica, B. xenovorans, B. graminis* and *B. fungorum* and others. Most of the species in this cluster have been isolated from

environmental sources (Palleroni 2001; Sessitsch et al. 2005; Viallard et al. 1998). Many of these species are responsible for nitrogen fixation in rhizospheric relationships with plants; other species represent strains isolated from soils polluted with chlorinated organic compounds and characterized for their degradative capacity and bioremediation potential. *Burkholderia xenovorans* LB400 is a well-studied strain that degrades and dechlorinates polychlorinated biphenyls (Bopp 1986). *B* while *B. graminis* is found in the rhizosphere of grasses (Viallard et al. 1998). Neither *B. phenazinium* nor *B. graminis* are known to be pathogenic to humans (Fain and Haddock 2001). Phenotypic and phylogenetic comparison of the above described species and strain UW103 suggest that UW103 is more closely related to species in the second cluster.

An interesting phenomenon was observed of strain UW103 during the course of the project. The cellular morphology of this strain varied, depending on growth conditions. When grown on nutrient-rich media cells are longer slightly curved bacilli with an invagination in the center and dark staining ends. When grown on DCA as sole carbon and energy source for prolonged periods cellular morphology differs: cells are short or coccobacilli. It is understandable that under conditions of stress, as in the case of limited growth substrates, an organism may down-regulate unnecessary genes in order to conserve energy or building blocks. An organism in this altered genetic state may not morphologically resemble its counterpart grown under more optimal nutrient-rich conditions.

The toxicity of DCA at concentrations equal to or greater than 2 mM suggest that this concentration is higher than that normally experienced by this bacterium in its natural environment (Figure 4-7). The concentrations of DCA in natural environments are generally unknown. DCA is the most common CAM detected in environmental samples

from forested sites (de Jong et al. 1994). Concentrations range up to 180 mg per kg dry weight of forest litter colonized with Basidiomycete fungi. Although this concentration is likely on the high end of the range of DCA concentrations experienced by degraders in nature, it is difficult to estimate exactly what amount is readily available to degraders and what portion is adsorbed to organic material protecting organisms from potential toxicity.

CAM-producing basidiomycete fungi are capable of producing 0.5 to 108 mg DCA per liter liquid media under experimental conditions when grown on lignocellulosic substrate (Swarts et al. 1997). This corresponds to 0.002mM to 0.5mM. If these concentrations are indicative of those produced in nature, it is understandable that a concentration of 2mM is toxic to strain UW103. The concentrations of DCA in commercial compost, the source of this strain, are significantly lower, ranging from 4.8 to 44.6 μg/kg oven-dried material (Holloway and Reimer, unpublished data). Thus UW103 is seldom exposed naturally to more than 0.5 mM DCA.

The bacteriostatic or bactericidal properties of DCA on clinically or agriculturally relevant organisms has undergone limited testing. Given the toxicity of DCA on a DCA-degrader further research on the antibiotic or antifungal potential of this compound may be warranted.

It is surprising to note that *Burkholderia* sp. UW103 degrades only a very limited range of chlorinated compounds similar in structure to DCA (Table 4-2). A strain capable of utilizing 3,5-dichloroanisyl alcohol, presumably degraded through the corresponding carboxylic acid, as a sole source of carbon and energy would be expected to utilize at least some structurally related benzoic acids. All chlorinated benzoic acids and phenols tested were not utilized for growth by this strain. This suggests that the enzymes involved

in DCA degradation have a narrow substrate range and may be specific for CAM. The enzymes involved in DCA degradation are probably anciently evolved, specifically to degrade DCA, thereby having a very narrow substrate range. The presence of lignin-degrading fungi in the biosphere for millions of years has provided ample time for the evolution of bacterial strains with enzyme systems specific for CAM.

Biolog results indicated that strain UW103 was capable of utilizing a wide range of carbohydrates, amino acids, and organic acids. Therefore the catabolic capability of strain UW103 was diverse. This was in contrast to the catabolism of chlorinated compounds, which appeared to be highly specific for DCA. The catabolic pathway of DCA accommodated only compounds highly similar in structure to DCA or its catabolites.

The narrow substrate range of *Burkholderia* sp. UW103 for chlorinated compounds implies that this strain has not acquired genes for the breakdown of chlorinated xenobiotic chemicals. Other *Burkholderia* species, with 16S rDNA sequences highly homologous to *B. phenazinium*, *B. graminis*, *B. caledonica*, *B. fungorum*, and strain UW103 are capable of degrading highly chlorinated compounds. Uncultured eubacteria that share greater than 99% 16S rDNA homology to UW103 were detected in PCB-polluted soils (Nagales et al. 2001). *Burkholderia xenovorans* LB400, phenotypically and genotypically closely related to *B. phenazinium*, *B. caledonica*, *B. graminis* and strain UW103 is capable of growth on PCB as sole sources of carbon and energy and dechlorinates congeners containing six or less chlorine substituents (Fain and Haddock 2001). Other *Burkholderia* species, *B. phenoliruptrix*, and *B. fungorum*, have been isolated and studied for their ability to degrade man-made chlorinated phenols (Palleroni 2001; Coenye 2001*a*).

Strains of *B. caledonica,* a closely related species to strain UW103 by phenotypic tests and 16S rDNA sequencing, have been isolated in association with Basidiomycete fungi and suggested to be responsible for the degradation of aromatic acids produced by the fungi (Coenye et al. 2001*a*). *Burkholderia* sp. H801 and *Burkholderia* sp. OY715 share 100% and 99.9% homology with strain UW103, respectively. *Burkholderia* sp. H801 is associated with 2,4-D degradation while *Burkholderia* sp. OY715 is a nitrogen-fixing rhizospheric strain. Despite the apparent close relationship to these two isolates, strain UW103 is not capable of 2,4-D degradation and required an organic nitrogen source for growth.

Interestingly the chlorinated xenobiotic, dicamba, a structural isomer of DCAcid, was degraded by strain UW103 and utilized as a sole source of carbon and energy. It was not surprising that PCP was not utilized by this strain as aerobic organism are often not capable of metabolizing heavily halogenated compounds (Fetzner and Lingens 1994). Highly chlorinated compounds like PCP are rarely degraded in mechanisms other than reductive dehalogenation.

The dechlorination and mineralization of DCA by UW103 may be an example of dechlorination chemistry that existed in the natural environment, without the influence of man-made chlorinated chemicals. Further studies to elucidate the genes and enzymes involved in DCA-dechlorination may provide insight into dechlorination pathways not influenced by the introduction of xenobiotic chlorinated compounds into the environment in the last century.

CHAPTER 5

PRESENCE OF DCA IN AGRICULTURAL SOIL AND COMMERCIAL COMPOST

5.1 Abstract

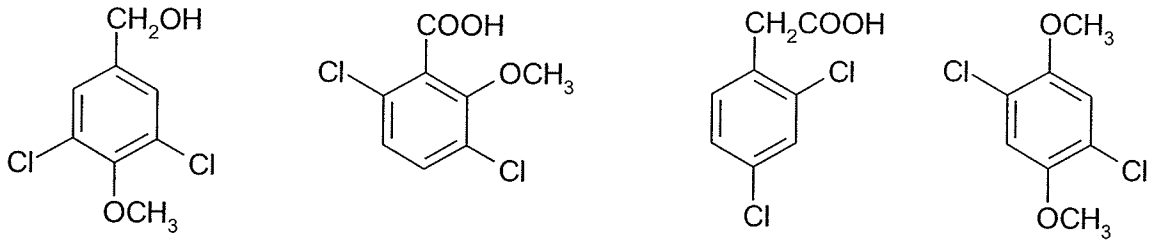
Chlorinated anisyl metabolites (CAM) are produced by basidiomycete fungi in high concentrations in nature. The relatively short half-lives of similarly structured chlorinated aromatic compounds in soil may be attributed to catabolic pathways that evolved to degrade natural chloroaromatics like DCA. The presence of basidiomycetes in agricultural soils and commercial composts also suggests CAM may be present. In this study we demonstrated the presence of DCA in 4 agricultural soils and 3 commercial composts in concentrations ranging from 4.8 to 147.6 μg per kg dry sample weight. Ethyl acetate extracts of soils and composts were analyzed for DCA using gas chromatography coupled to an electron capture detection system. In addition, DCA-degrading microbes were detected in all samples containing detectable levels of DCA using the metabolic indicator Resazurin®. DCA-degrading enrichment cultures were established from three of the five soils and composts that tested positive for DCA-degraders with the metabolic indicator.

5.2 Introduction

The chlorinated anisyl metabolite (CAM), 3,5-dichloro-para-anisyl alcohol (DCA), is synthesized by basidiomycete fungi and used as a substrate for aryl alcohol oxidases, generating H_2O_2 for lignin degradation (De Jong et al. 1994). Basidiomycetes are higher fungi that produce macroscopic fruiting bodies such as mushrooms and brackets (Alexpoulos and Mims 1979) present in the top 5 cm of soil. Basidiomycete fungi play an important role in a variety of ecosystems biodegrading lignin and soil humics which makes nutrients available for other microorganisms and plants. Basidiomycetes are also extremely valuable in agriculture, many species forming mycorrhizal relationships with cultivated plants. CAMs are synthesized by at least 18 genera of basidiomycete fungi belonging to 4 families from the orders Agaricales and Aphyllophorales (De Jong and Field 1997). DCA is produced at concentrations as high as 108 mg/l (0.5 mM) liquid culture *Hypholoma elongatum* (Swarts et al. 1997).

Environmental fates of CAM may include mineralization and biotransformations yielding dioxins or chlorohumus (De Jong et al. 1994). Chlorinated metabolites may be mineralized by dechlorinating soil microbes to chloride and CO_2. Selective microbial metabolism of chloroaromatic compounds, such as demethylation, yields chlorinated phenols that may also be mineralized by dechlorinating microbes. The pesticide Chloroneb is demethylated by bacteria and fungi in soil (Weise and Vargus 1973). DCA is demethylated by methanogenic sludge under anaerobic conditions (Verhagen et al. 1998*c*). Chlorinated phenols may undergo biotoxification, yielding dioxins that are extremely toxic and persistent. Chlorinated phenols may also polymerize to form non-toxic chlorohumus.

CAM are similar in structure to a variety of xenobiotic pesticides. The herbicides 2,4-D, dicamba and chloroneb® have a strong structural similarity to DCA (Figure 5-1).



1 2 3 4

Figure 5-1. Man-made compounds with chemical structures similar to DCA. Chlorinated anisyl metabolite, 3,5-dichloro-*para*-anisyl alcohol (1) and similarly structured pesticides; 2, dicamba; 3, 2,4-D; and 4, chloroneb ®.

These herbicides have only recently been introduced into the biosphere, less than half a century ago. This is a short time for microorganisms to have evolved metabolic pathways for their utilization; yet these pesticides do not persist in agricultural soils. The half-life of dicamba is 31 days under aerobic conditions in agricultural soil (Krueger, Butz and Cork 1991). The half-life of 2,4-D ranges from 3.4 to 35.9 days depending on soil texture and soil moisture (Cheah, Kirkwood and Lum 1998; Johnson, Lavy and Gbur 1995). Both dicamba and 2,4-D are completely mineralized to CO_2 and chloride under aerobic conditions. Studies have elucidated some of the catabolic pathways degrading these compounds but very little is known about why or how these pathways evolved.

In an oxidative degradation pathway of 2,4-D, dichlorocatechol 1,2-dioxygenase dechlorinates dichlorocatechol to chloromaleylacetic acid (Dorn et al. 1974; Schmidt and Knackmuss 1980). In the reductive dechlorination of 2,4-D by *Azotobacter chroococcum* MSB-1 and *Alcaligenes eutrophus* JMP134, 2,4-D is metabolized via reductive *ortho*-

dechlorination to 4-chlorophenoxyacetic acid (Figure 2-10; Balajee and Mahadevan 1990; Vollmer, Stadler-Fritzsche, and Schlomann 1993).

We presume that CAM, cosubstrates in lignin depolymerization, have been present in the biosphere for as long as basidiomycete fungi. After all it was the evolution of the ability to utilize lignin as a carbon and energy source that began the basidiomycete lineage approximately 300 million years ago (Berbee and Taylor 1993). The presence of CAM in the environment for millions of years should have been sufficient for microorganisms to evolve specific catabolic pathways for their utilization. The relative abundance of CAM, including DCA, in the environment would have provided an advantage to any organism with the ability to dechlorinate and utilize it as a carbon and/or energy source. Could these enzymes be responsible for the short half-lives of pesticides similar in structure to DCA?

Detection of CAM in agricultural soil and commercial compost is an important step in identifying a possible source of catabolic pathways responsible for pesticide degradation. The ubiquity of CAM-producing basidiomycetes suggests that CAM may be widespread in the environment (Verhagen et al. 1996). De Jong *et al.* (1994) detected DCA in all random samples of moldy litter and rotting logs taken from several forested sites. Environmental samples contained up to 180 mg DCA per kg dry sample weight. DCA was not detected in fresh forest litter or intact wood. Although forest soil has never been specifically tested for the presence of DCA, samples of forest soil collected worldwide contained adsorbable organic halogen concentrations between 20 mg and 360 mg/kg dry weight soil (De Jong and Field 1997). Adsorbable organic halogen, commonly known as AOX, is the water soluble organic halogenated component (Muller 2003). There are no reports of agricultural soil specifically tested for the presence of

DCA; however, at least 35 of species of basidiomycetes from the orders *Agaricales* and *Aphyllophorales* are common to grassland soils (Bisby 1938). The presence of basidiomycetes in grassland ecosystems suggests that CAM may be present in agricultural soil. There are no reports of CAM detection or quantification in agricultural soil.

Compost is a source of lignin and lignin-degrading microorganisms for agricultural soil and therefore may also be a source of CAM and CAM-degraders. Commercial and in farm compost is added during agricultural practices to improve soil structure and increase organic matter content. Compost contains a variety of plant tissues, abundant in the macromolecules cellulose and lignin, degraded by basidiomycete and actinomycete fungi in the final stages of composting. There are no reports of the presence of CAM, the most common chlorinated organic compounds synthesized by basidiomycete fungi, in commercial compost.

The detection and isolation of DCA-degraders from compost and agricultural soil would provide support for the theory that DCA and DCA-degradation are ubiquitous.

5.3 Objective of Study

The objective of this study was to determine the presence and quantity of the chlorinated fungal metabolite, 3,5-dichloro-para-anisyl alcohol (DCA), in four agricultural soils and three commercial composts. We also tested each soil and compost sample for the presence of DCA-degrading microorganisms.

5.4 Materials and Methods

5.4.1 Source of Soil and Compost Samples

One kg soil samples were taken from agricultural fields near Stony Mountain, Oak Bluff, Kane and Minnedosa Manitoba. All soil samples were taken from the top 10 cm of the A horizon.

The soil sample from Stony Mountain, Manitoba consisted of Dencress clay and is classified as a Gleyed Rego. It is a black soil that is imperfectly drained and strongly calcareous beneath the surface. It is estimated that is field may have received between 10-20 tonnes of composted manure per acre on an annual basis for 80 years. Manure was from cattle fed a mixture of alfalfa and barley when not grazing. A broad-spectrum herbicide was used, but not recorded, and Ultom®, a broadcast herbicide was used for controlling weeds.

Soil from Oak Bluff, Manitoba contains Osborne heavy clay, is imperfectly drained and is classified as a Rego Humic Gleysol. Crop rotation includes wheat and soya beans. Audicy and Round up® herbicides were used. Neither manure nor compost had been applied to this field.

The sample site at Kane, Manitoba is imperfectly to poorly drained, often resulting in excessive moisture that limits agricultural use at times. Kane soils contain Red River and Osborne clays and are classified as Gleyed Rego Black Chernozems. Crops planted include red spring wheat, canola and barley. Herbicides used include Horizon®, Curtail M, Round up®, and Buctril M. The fungicide Tilt® was applied during the sampling year. Neither manure nor compost has been applied to this field.

The study site just north of Minnedosa, Manitoba is located on till in aspen parkland. The soil is Black Chernozem and occurs on till with loam texture, representative of 1/3 cultivated soils in Manitoba. Crops planted included wheat, barley, oats, canola and flax. The soil had been fertilized at a rate of 80-90 and 110 kg N ha^{-1} for cereals and canola, respectively, and 30-40 kg P ha^{-1} and 20 kg S ha^{-1} for both crops.

Three commercial composts were purchased commercially. President's Choice™ Bovine Manure contained 0.5% total nitrogen, 0.5% available phosphoric acid (P_2O_5) and 0.5% soluble potash (0.5-0.5-0.5). Envirogro™ Composted Mushroom Manure plus Compost 1-1-1 contained 40% composted mushroom manure, 30% animal manures and 30% food processing by-products. Bell's Soil Booster contained composted kelp, bone meal, peat moss and manure.

5.4.2 Preparation and Extraction of Soil and Compost Samples

Soil samples were air-dried and sieved through a pore diameter of 2 mm. Twenty gram samples of soil were oven-dried at 110°C to determine the gravimetric moisture content. Four-50 gram samples of each air-dried soil were extracted with 50 ml ethyl acetate. The extractions were performed on a rotary shaker overnight and then filtered through Whatman filter paper under reduced pressure. The extracted soil sample and filter were rinsed with five two ml portions of fresh ethyl acetate and combined with the fitrate. The filtered extract was evaporated to dryness under reduced pressure. The remaining residue was dissolved in 2.0 ml of ethyl acetate. Ten µl of 0.1 M 4-bromoanisole was added as an internal standard. Extracts were analyzed with an HP6890 Series Gas Chromatograph System equipped with a fused-silica capillary column (J&W Scientific DB-5ms; 30 m by 0.25 mm internal diameter; film thickness, 0.25 µm). The

injector temperature was 250°C, the electron capture detector (ECD) temperature was 300°C and the temperature program began at 50°C, held for 2 minutes, increased by 25°C per minute to 300°C and then held for 30 seconds. Helium was used as the carrier gas. Volume of 1 µl were injected automatically.

A soil sample was prepared in duplicate, and one of these was spiked with 1 mM DCA. The samples were extracted as described above and analyzed by GC-ECD. The chromatograms were compared and used to determine the retention time of DCA, which was used to identify peaks representing DCA in the remaining soil extractions.

5.4.3 Detection of DCA-Degraders

DCA-degrading microorganisms were detected with the Bushnell Haas Microbial Activity Assay using resazurin as an indicator and 1 mM DCA as the sole carbon and energy source. Carbon dioxide production from respiration is detected by a decrease in pH which changes the color of the pH indicator, resazurin. An inoculated tube containing no carbon source acts as a negative control. A change in color in the experimental tube but not in the control tube indicates that the inoculum contained organisms capable of respiration utilizing the sole carbon source. Bushnell Haas mineral salts medium and resazurin were purchased from Sigma-Aldrich (St. Louis MI). Buffer dilutions of soil samples were added to sterile 20 ml volumes of DCA-Bushnell Haas broth and to a Bushnell Haas broth control containing no carbon source. A colour change within 1 week of inoculation in the experimental tube and not the control tube was taken to indicate the presence of DCA-degraders.

DCA-degraders were also detected by enrichment culture. Growth (absorbance greater than 0.1 at 600 nm) in yeast nitrogen base without amino acids, containing 50 mM

phosphate buffer, pH 6.3 and 1 mM DCA after three or four 100-fold dilution serial subcultures was taken to be indicative of the presence of DCA-degraders. The lack of growth in control flasks, containing no carbon source, indicated that the organisms in the enrichment culture were utilizing DCA as their sole carbon source.

5.4.4 Organic Matter Measurement

Minnedosa samples contained 2.8% organic carbon, measured by loss on ignition at 550°C. Assuming organic matter contains 58% C, the Minnedosa soil sample contained 4.9% organic matter (2.8/0.58) (Czurak-Dainard, unpublished data). Wet oxidation by a modified Walkley-Black method was used to measure organic matter content from Stony Mountain, Kane and Oak Bluff soil samples (Black 1965; Carter 1993).

5.5 Results

DCA was detected in concentrations as high as 148 μg per kg oven-dry material for Steer compost (Table 5-1). The lowest levels of DCA detected were from Minnedosa soil (<4 μg DCA/kg soil) and Bells compost (4.8 μg DCA/kg soil). DCA was detected in commercial composts at concentrations of 4.8, 44.6, and 148 μg per kg oven-dry material. The range of DCA in agricultural soils was from less than 4.2 μg per kg oven-dry material, the limit of detection, to 24.5 μg per kg oven-dry material.

Microorganisms capable DCA degradation were detected in all samples except those containing the lowest levels of DCA (Table 5-1). DCA-degraders were detected in three of the four agricultural soils tested by the Bruchnell Hass Microbial Activity Assay. Interestingly these are the same three of four soil samples that contained measurable amounts of DCA. DCA-degraders were also detected by enrichment culture from two of

these samples. DCA-degraders were not detected in the Minnedosa soil sample by the Bruchnell Hass Microbial Activity Assay nor by enrichment culture. Either DCA is not present in this soil or the level of DCA is below the limit of detection of 4.2 μg per kg oven-dry material.

In two of the three commercial composts analyzed, DCA-degrading microorganisms were detected by the Bruchnell Hass Microbial Activity Assay (Table 5-1). These two composts also contained the highest levels of DCA detected in this experiment. DCA-degrading microorganisms were also enriched from Steer compost, the sample containing the highest level of DCA. In the compost containing the lowest detectable level of DCA, Bells compost, DCA-degraders were not detected by either assay.

Table 5-1. Concentrations of the fungal metabolite, dichloro-*para*-anisyl alcohol (DCA), in agricultural soils and commercial composts and presence of DCA-degraders.

Source	µg DCA / kg oven-dried soil	Standard deviation (µg DCA / kg)	Detection of DCAlc-degraders: Bushnell Haas Microbial Activity Assay	Detection of DCAlc-degraders: Enrichment Culture	pH	Organic matter (%)
Agricultural Soils						
Oak Bluff	18.2	16.4	+	+	7.5	5.7
Kane	13.3	4.4	+	-	7.5	3.2
Stony Mountain	24.5	15.9	+	+	7.8	8.5
Minnedosa	<4.2		-	-	6.8	4.9
Commercial Composts						
Bells	4.8	3.1	-	-		
Mushroom	44.6	32.6	+	-		
Steer	147.6	63.7	+	+		

5.6 Discussion and Conclusions

DCA was detected at three of four soil sampling sites and all three commercial composts tested (Table 5-1). DCA degrading microorganisms were detected in all samples where DCA was detected except Bells compost which contained the lowest level of DCA detected in this study.

Six of the seven samples we analyzed contained detectable concentrations of DCA, with values ranging from 4.8 to 148 µg DCA per kg dry sample weight (Table 5-1). Concentrations of DCA in the compost and soil samples tested were generally 1000 fold lower than those concentrations previously found in forest litter and rotting wood, which ranged from 7 to 180 mg per kg dry sample weight (De Jong et al. 1994). Thus it would appear that CAM production in composts, and especially agricultural soils, is much less

than in forests. This makes sense considering the relative amount of lignin in each of those ecosystems.

The lower concentration of DCA in composts and agricultural soils compared to samples from forested sites could be explained by relative lignin concentrations in the respective environments. Forest soil ranges from 10-35% lignin with an average greater than 25% lignin (Finzi et al. 2001; Wright and Coleman 2000) whereas grassland soils range from 3-15% lignin and lignin concentration of soil and surface material is directly proportional to plant height (Murphy et al. 2002). Wood and forest litter are composed mainly of lignin whereas cellulose and hemicelluloses generally predominate in grasses and composts (Tuomela et al. 2000). DCA's role lies in lignin depolymerization therefore we would expect DCA to be more abundant in the forest ecosystem compared to grasslands or composts.

It is possible that CAM are produced in soils and composts in the same amounts as found in forest litter but that the rate of CAM degradation in soil and compost is also high and close to the production rate of CAM that the steady-state concentration of DCA is very low. CAM have been detected in the close vicinity of fruiting bodies of basidiomycetes; however just outside CAM-producing colonies CAM were no longer detected (De Jong and Field 1997). This suggests that CAM could be biodegraded or biotransformed quickly as they diffuse away from the basidiomycete colony. Therefore, the rate of synthesis of DCA in grassland soils may be higher than suggested by the low concentrations detected. Two of the three composts tested contained higher concentrations of DCA that all four soils tested. Soil is a very different environment for bacteria than forest litter and compost. Soil is rich in organic nitrogen, contains a diverse array of species, and contains an average of 10^8 microorganisms per gram soil at depths

between 3 and 8 cm (Tortora et al. 2001). Forest litter tends to be deficient in organic nitrogen, a limiting factor to the biodiversity (Kimmins 1997; Magill and Aber 2000; Widmer et al. 1999). The higher number of species and populations in soil probably make a better source of DCA-degrading microorganisms than forest soil or litter. Higher rates of metabolism in soil compared to forest litter could explain the relatively low levels of DCA found in agricultural soil. A large amount of research would be needed to determine if the rate of DCA metabolism is higher in soil than in forest litter.

Basidiomycete colonies likely do not occur uniformly throughout the soil. If the distribution of DCA is sporadic in the field, greater numbers of samples per site are required to ensure that the value of DCA is a true reflection of the mean DCA concentration. The samples tested in this study included soil from the top 10 cm. It would be interesting to obtain a vertical profile of DCA concentration in agricultural soil by sampling at varying depths.

Lower DCA-concentrations observed in this study compared to those found in rotting wood and forest litter may be due to differences in basidiomycete colonization. Fewer species of basidiomycete fungi are observed on grassland soils than forest ecosystems. Greater than 90% of basidiomycete species in Manitoba occur in or directly adjacent to forested areas (Bisby 1938). However, at least 35 species of basidiomycete fungi from 6 families in the orders Agaricales and Aphyllophorales have been observed on Manitoba grassland soils. *Hypholoma incertum* is commonly observed on Manitoba lawns (Bisby 1938). *Hypholoma* species have demonstrated the highest capacity for CAM production of all basidiomycete fungi (Verhagen et al. 1996). It is possible that Basidiomycete species that synthesize DCA may not be the dominant lignin degraders in our samples. Future work looking at the distribution of Basidiomycetes in Manitoba

grasslands and agricultural soils could explain the presence of CAM in these environments.

The time of year of sampling may also play a role in the amount of detectable DCA in soil samples. There would be higher levels of nutrients available in the spring and as the season progressed they would be consumed. The first nutrients to be consumed in spring would be those most easily metabolized such as carbohydrates, and the organic and amino acids. As those nutrients are quickly consumed, microbes would look to those nutrients that are difficult for most organisms to degrade, and therefore would experience less competition. At this point strains capable of CAM degradation may utilize these chlorinated aromatic compounds as sources of carbon and energy. Our samples were taken in early to mid-summer and may still contain CAM, including DCA, if present.

The densities of soil, forest litter and compost are not consistent. One kilogram forest litter covers a much larger area than one kilogram soil to a 10 cm depth. Therefore comparing amount of DCA detected per unit weight soil or forest litter may not be relevant. Forest soils, in contrast to forest litter, have never been specifically tested for CAM; however they have been analyzed for the presence of chlorinated dioxins, which result from the peroxidase-catalyzed oxidation of chlorophenols, biotranformation products of CAM (De Jong et al. 1994). Chlorinated dioxins are 13 times more concentrated in forest soils than grassland soils possibly reflecting higher CAM concentrations in forest environments (Rotard et al. 1994).

DCA concentrations were highest in the steer compost. Steer manure is a good substrate for basidiomycete fungi, which scavenge undigested lignocellulose, the principle component of cattle dung from grasslands (Carroll and Wicklow 1992). The

Stony Mountain soil sample contained the highest level of DCA of all soil samples analyzed (24.5 μg/kg dry soil). This soil has received composted cattle manure every year for the last 80 years. The mushroom compost contained a moderate amount of DCA (44.6 μg/kg dry sample) compared to other analytes. We expected this sample to have the highest concentration of DCA of all samples tested as it contains 40% composted mushroom (basidiomycete) manure. However, that was not the case.

The presence of DCA-degraders in our samples coincides with the presence of DCA as detected by analytical techniques. DCA-degraders were detected in all compost and soil samples containing detectable levels of DCA using the Bushnell Haas Microbial Activity Assay. We were also able to isolate DCA enrichment cultures from 2 of the 3 soil samples that tested positive for DCA degraders with the Bushnell Haas assay. An enrichment culture was also isolated from Bovine compost, which contained the highest DCA concentration (147 μg/kg dry compost) of all samples analyzed. Interestingly DCA-degrading microorganisms were not detected in samples that contained the lowest levels of DCA (Table 5-1).

Further work could include the isolation and identification of the organisms in the enrichment cultures from the various soils and composts tested here. Characterization of DCA-degrading pathways in these organisms may provide evidence that strain UW103 is not alone in its capability to dechlorinate and mineralize DCA. Assessing the members of the enrichment cultures' abilities to degrade man-made compounds could support or refute the involvement of CAM-catabolic pathways in the evolution of pathways to degrade xenobiotic chlorinated aromatic compounds.

It was concluded that the CAM DCA is present in some Manitoba agricultural soils and commercial composts, but at lower concentrations than those found in forest litter colonized by basidiomycete fungi. Analysis of biotransformation processes of CAM in soil and compost may elucidate the relevant environmental fates of DCA in these ecosystems.

CHAPTER 6

GENERAL DISCUSSION

The mechanisms of biodegradation, including dehalogenation, are well-studied, but there has been little exploration into the origins of microbial dechlorination especially as chlorinated compounds are widely considered anthropogenic in origin. There has been a growing recognition that halogenated and chlorinated compounds are synthesized and excreted by living organisms; not all halogenated compounds detected in the environment are man-made. Recent compilations have documented greater than 2,500 halogenated compounds of natural origin (Gribble 1994 and 1996). Some of the most important of these compounds are the chlorinated anisyl metabolites (CAM) produced by many species of lignin-degrading Basidiomycete fungi. CAM are found in various environments, including forest and agricultural soils, at concentrations high enough to be a resource for bacteria or fungi. Given that the Basidiomycetes evolved approximately 300 million years ago, and lignin-bearing trees even longer, secretion of naturally produced chlorinated aromatic compounds may have been taking place for an evolutionary significant amount of time.

The ubiquity of DCA is suggested by the ubiquity of Basidiomycete fungi on Earth and the capacity of the majority of genera to produce CAM (De Jong and Field

1997; Bisby 1938). The presence of DCA in Manitoba agricultural soils and two commercial composts, demonstrated in this study, and the presence of DCA in pristine forest soils (De Jong et al. 1994) support this theory. Analysis of a wide range of soil types from various geographic regions across the globe would confirm the ubiquity of DCA on earth. With the presence of this naturally produced compound in soil, we would also expect the presence of DCA-degraders. This study detected and isolated DCA-degraders from most of the soils and composts where DCA was detected. Degradation of DCA in methanogenic sludge was demonstrated by Verhagen et al. (1998*c*); however, the specific organisms responsible were not identified.

In our studies a strain, designated UW103, was isolated from an enrichment culture of commercial compost and was capable of growth on DCA as sole carbon and energy source. This strain was characterized for its DCA utilization and degradation, as detected by UV spectrophotometry and gas chromatography. The disappearance of DCA from DCA-YNB inoculated with UW103 was confirmed by GC-FID analysis and decreased absorbance in the UV range suggests cleavage of the aromatic ring. Mineralization of DCA by UW103 was demonstrated by CO_2 liberation and stoichiometric dechlorination.

If DCA-degraders and DCA are ubiquitous, then dechlorination of DCA by *Burkholderia* sp. UW103 may be representative of a large group of organisms that make up an important component of the natural chlorine cycle on earth. Chloride removal from DCA was reproducibly demonstrated in cultures of UW103. UW103 cultures grown on DCA showed an increase in moles of chloride equivalent to 2.1 times the moles of DCA added. This ratio suggests that UW103 has the ability to dechlorinate DCA at both the 3 and 5 positions. No change in chloride concentration of a control inoculated with

nonviable cells was observed, suggesting that DCA dechlorination is resulting from a microbiological process. Furthermore, growth of UW103, monitored by absorbance at 600 nm, and DCA disappearance from the medium, monitored by GC-FID, paralleled DCA dechlorination .

The chlorine cycle, until recently, has been overlooked as a component of the biogeochemical cycles taking place on earth (Winterton 2000; Oberg 1998; Oberg 2002). Chlorine is one of the most abundant elements in the biosphere and is a major constituent of organic matter. As part of a complex biogeochemical cycle, chlorine atoms are cycled between inorganic chloride and substituents on organic compounds. The natural synthesis of chlorinated compounds has been well studied recently; a variety of organisms, including Basidiomycete fungi, and natural chemical events such as forest fires play a large role in the incorporation of inorganic chloride into organic molecules (Gribble 1994 and 1996). Subsequent studies regarding the degradation of naturally chlorinated compounds are few in number. The anaerobic bacterial degradation and dechlorination of chlorinated hydroquinone fungal metabolites (Milliken et al. 2004*b*) and the biotransformation of DCA by methanogenic sludge (Verhagen et al. 1998*c*) are two examples. The degradation and dechlorination of DCA by *Burkholderia* sp. UW103 in this study, to our knowledge, is the first elucidation of a CAM catabolic pathway by a pure aerobic culture.

The molar ratio of chloride liberated to available DCA suggests the "di"-dechlorination of DCA by UW103. However, the mechanism by which each chloride is removed may differ. The product of the initial dechlorination is 3-chloro-anisic acid, detected by mass spectrometry of DCA-fed cultures, suggesting that the chlorine substituent in position five is replaced by a hydrogen atom upon dechlorination.

Replacement of chloride by hydrogen suggests dechlorination by a reductive mechanism (Slater, Bull, and Hardman 1995; Copley 1997).

Reductive dehalogenation can occur during specific reactions involved in carbon metabolism or coupled to energy conservation by anaerobes that use organohalides as terminal electron acceptors in a respiratory process (Tiedje 1992; Mohn and Tiedje 1992). Alternatively, reductive dechlorination may be co-metabolic, occurring with no apparent benefit to the organism. The mineralization and utilization of DCA as sole carbon and energy source demonstrates the utility of dechlorination of DCA to this organism. The use of DCA as a terminal electron acceptor by UW103 is also not likely as this isolate is strictly aerobic by definition and by observation. The removal of the chlorine atom in position five is one step involved the utilization of DCA during carbon metabolism eventually leading to mineralization as demonstrated by a greater than 85% CO_2 yield from DCA. Interestingly reductive dehalogenation is more often observed in the initial catabolic steps of highly chlorinated compounds as this mechanism is thermodynamically more favourable when there are multiple electron-withdrawing substituents on the aromatic ring (Copley 1997). In addition, few examples exist of reductive dechlorination by pure cultures. One example is the reductive *ortho*-dechlorination of the herbicide 2,4-D by the obligate aerobes *Azotobacter chroococcum* MSB-1 and *Alcaligenes eutrophus* JMP134 (Balajee and Mahadevan 1990; Vollmer, Stadler-Fritzsche, and Schlomann 1993). A second example is the reductive dechlorination of ethyl 2-chloroacetoacetate by the aerobic fungus *Saccharomyces cerevisiae* (Jorg and Bertau 2004).

For now we can only speculate on the dechlorination mechanism and resulting product after removal of the chloride in position three. The liberation of protons in a DCA-utilizing culture containing UW103, demonstrated in a previous study (Olson

2000), is indicative that at least one dechlorination mechanism is hydrolytic, whereby the chloride substituent is replaced by a hydroxyl group derived from water and the remaining proton is liberated into solution with chloride as HCl (Slater, Bull, and Hardman 1995; Copley 1997). Since two adjacent hydroxyl groups are routinely required for cleavage of the aromatic component and protocatechuate is a common precursor to aromatic ring cleavage, a mechanism replacing the remaining chloride with a hydroxyl group is likely. Strain UW103 grows luxuriously on protocatechuate as a sole carbon and energy source.

Dechlorination of mono- and dechlorinated aromatic compounds in aerobic metabolism is most often carried out by oxygenases (Copley 1997; Fetzner 1998). Dioxygenases from aerobic bacteria and their ability to dechlorinate a range of chlorinated benzoates, are well-characterized. A dioxygenase from *Pseudomonas aeruginosa* catalyzes the (fortuituous) *ortho*-dechlorination of both 2-chloro and 2,4-dichlorobenzoate (Romanov and Hausinger 1996) while another from *Alcaligenes* sp. strain BR60 attacks 3- and 4- chlorobenzoates (Nakatsu and Wyndham 1993). Both reactions yield protocatechuate which subsequently undergoes normal *meta*-aromatic ring cleavage and enters common pathways of non-halogenated compounds.

The dechlorination of DCA by UW103 is significant because the presence of chloride substituents on an organic molecule are the most limiting factor preventing the survival of biodegraders and the utilization of the carbon atoms within a chlorinated organic compound (Slater, Bull, and Hardman 1995; Copley 1997). The increased electronegativity and size of the halide substituent relative to a hydrogen atom interfere with enzymatic breakdown of chlorinated compounds. The presence of chlorinated compounds in nature has allowed the evolution of pathways that accomidate the

catabolism of chlorinated organic compounds, releasing chloride into the environment completing the natural cycle of chlorine.

In the last half century the natural chlorine cycle has been presented with anthropogenic chlorinated compounds. Halogenated pesticides, coolants, dielectrics, flame-retardants, plasticizers, and preservatives have been synthesized by human beings and seeped into the biosphere. Organisms in the environment, even those involved in the natural chlorine cycle, were not previously exposed to these novel chlorinated compounds. *Burkholderia* sp. UW103, as component of the natural chlorine cycle degrades and dechlorinates DCA, but is not capable of utilizing most similarly structured chlorinated benzoates nor phenols. This strain, clearly, has not evolved nor acquired genes capable of the dechlorination of representative anthropogenic chlorinated aromatic compounds. Interestingly other *Burkholderia* species, a few with highly homologous 16S rRNA gene sequences to UW103, have been isolated and studied based on their ability to utilize chlorinated anthropogenic compounds. Other *Burkholderia* species, with 16S rDNA sequences highly homologous to *B. caledonica*, *B. phenazinium*, *B. graminis*, and strain UW103 are capable of degrading highly chlorinated compounds. Uncultured eubacteria with 16S rDNA 99.7-99.9% homologous to that of UW103 were detected in PCB-polluted soils (Nagales et al. 2001). *Burkholderia xenovorans*. LB400, closely related to *B. phenazinium*, *B. caledonica, B. caribiensis*, and *B. graminis*, is capable of growth on PCBs as sole source of carbon and energy and dechlorinates congeners containing six or less chlorine substituents (Fain and Haddock 2001).

The enzymes involved in DCA degradation by UW103 must have anciently evolved, specifically to degrade DCA, thereby having a very narrow substrate range. This implies that strain UW103 has not evolved the capability to breakdown chlorinated

xenobiotic chemicals, nor acquired the necessary genes from other microbes. Dicamba, or 3,6-dichloro-*ortho*-anisic acid, an isomer of 3,5-dichloro-*para*-anisic acid (DCAcid), an early catabolite of DCA, is the only chlorinated anthropogenic compound known to be utilized by UW103. Other chlorinated benzoates and phenols, including 2,4-D, structurally similar to DCA, are not utilized by UW103. The degradation of such toxic and alien chemicals as PCP or PCBs, recently introduced into the biosphere and once considered non-biodegradable, is proof that the capability of microorganisms to evolve to utilize chlorinated compounds does exist.

The degradation of anthropogenic organohalides is a modification of biochemistry that evolved for the utilization of naturally produced similarly-structured compounds (Milliken et al. 2004B). Previously demonstrated in the anaerobic metabolism of chlorinated hydroquinone metabolites of Basidiomycete fungi, the acquisition of novel capabilities of natural enzymes to accommodate xenobiotic chemicals is the only logical source for such pathways. Additional support for the use of pathways evolved for the degradation of natural chlorinated compounds in the degradation of anthropogenic chlorinated organic comounds was demonstrated with the removal of high levels of 2,4,6-trichlorophenol from, previously unexposed, forest soil microbial community (Sanchez, Vasquez, and Gonzalez 2004).

Evolution of microbes to utilize anthropogenic chlorinated compounds must begin with existing enzymes serving new functions in the biodegradation of chlorinated pollutants. Since the enzymes involved are relatively recently recruited for such purposes we would expect them not to be ideally suited for these roles, as is the case. Often only low concentrations of halogenated pollutants are removed from polluted environments and the transformation and mineralization rates are extremely slow in most cases.

A pristine environment, harbouring microbes with natural dehalogenases and introduced to a anthropogenic chlorinated compound, mutational events increasing substrate affinities and ranges of dehalogenases will be selected for. Over time, a series of selected mutations results in improved dehalogenation efficiencies and substrate range. Similarly, the modified *ortho*-pathway, involved in the mineralization of chlorinated catechols, may have evolved from the *ortho*-pathway in response to the introduction of naturally produced chlorinated aromatic compounds into the environment by Basidiomycete fungi and other organisms over the last 300 million years.

Chlorinated substrates often interfere with aromatic catabolism through the inhibition of ring-cleaving oxygenases or conversion to dead-end metabolites. *Meta*-cleavage of chloroaromatic compounds is often unproductive due to suicide inactivation of catechol-2,3-dioxygenase by 3-chlorocatechols (Bartels, Knackmuss and Reineke 1984). Chlorocatechols, common products of chloroaromatic metabolism, inhibit ring-cleaving catechol dioxygenases. Some bacteria have evolved a "modified" *ortho*-cleavage pathway that allows them to cleave chlorocatechols, via *ortho*-cleavage, yielding chloromuconic acids, which cycloisomerize releasing chloride spontaneously (Schlömann 1994). Other bacteria have recruited dechlorinating enzymes at the start of the catabolic pathway; nonchlorinated products can then enter the cell's existing catabolic pathways for nonchlorinated aromatic compounds. The first step of 3- and 4-chlorobenzoate degradation by *Pseudomonas* sp. CBS3 is dechlorination yielding 3- and 4-hydroxybenzoate, respectively (Johnston, Briggs, and Alexander 1972; Keil, Klages, and Lingens 1981).

But not all organisms are required to undergo the selective pressure and years of evolution to acquire the genes necessary for catabolism of chlorinated aromatic

compounds. Once one strain has evolved the ability to utilize an anthropogenic compound, this gene may be shared with other organisms in its environment. Acquisition of genes required to degrade synthetic compounds in a microorganism's environment may occur through integration into plasmids or transposons. In addition, chromosomal gene mobilization, transduction, conjugation or transformation provide means of gene sharing among organisms.

Horizontal transfer of catabolic genes via plasmids and transposons is a common mechanism of bacterial evolution. RFLP patterns of 464 isolates from an industrial landfill revealed *cba* gene (responsible for 3-chlorobenzoate degradation) conservation in all isolates suggesting recent genetic spread by horizontal transfer of Tn5271 (Peel and Wyndham 1999). Extensive interspecies transfer of homologous degradative genes is responsible for the evolution of 2,4-D-degrading bacteria (Fulthorpe et al. 1995 and McGowan et al. 1998).

Genes encoding xenobiotic-catabolizing enzymes are often found on plasmids. A number of plasmids encoding the degradation of chlorobenzoates and chlorinated pesticides have been studied. Plasmids such as pAC27 (Ghosal and You 1989) and pP51 (van der Meer et al. 1991) encode the modified *ortho* pathway, catalyzing the dechlorination of 3-chlorobenzoate. Plasmid pJP4 encodes the modified *ortho* pathway in addition to enzymes for the degradation of the herbicide 2,4-dichlorophenoxyacetate (2,4-D; Goris et al. 2002). Plasmids pP51 (van der Meer et al. 1991), pEMT1 (Goris et al. 2002), pBAH1 (Haak, Fetzner, and Lingens1995), and pSS70 (Layton et al. 1992) carry genes for the degradation and dechlorination of 1,2,4-trichlorobenzene, 2,4-D, 2-halobenzoates, and 4-chlorobenzoate, respectively. Plasmids carrying dechlorinating

enzymes are known to be shared amongst microbes from *Alcaligenes*, *Ralstonia*, *Burkholderia*, and *Pseudomonas* genera.

Genetic rearrangment may also contribute to rapid bacterial evolution; rearrangements that inactivate or deactivate specific pathways create new genetic combinations that are subject to environmental selective pressure (Barbieri et al. 2001).

CHAPTER 7

SUMMARY AND CONCLUSIONS

Despite the presence of chlorinated anthropogenic compounds in the biosphere for less than a century, a variety of microorganisms have demonstrated a remarkable ability to utilize and degrade them. The synthesis of natural chlorinated compounds in terrestrial environments and their degradation to complete the natural chlorine cycle may provide clues to the fate of chlorinated xenobiotics. Only a few organisms have been described that degrade naturally-produced chlorinated compounds.

In this study, a bacterium was isolated from commercial compost that is capable of degrading the chlorinated anisyl metabolite, DCA, as a sole carbon and energy source in pure culture. The bacterium was identified by phenotypic and genetic methods as belonging to the *Burkholderia* genus, *B. graminis* the closest related validly described species. Characterization of this organism's utilization of the fungal metabolite reveals that complete mineralization takes place, including the removal of both chlorine atoms. Interestingly, this organism is able utilize the similarly structured anthropogenic compound dicamba, but does not appear capable to degrade a variety of xenobiotic chlorinated aromatic compounds, particularly chlorinated phenols.

The presence of DCA and DCA-degrading organisms in agricultural soil, demonstrated in this study, may provide the source of natural dehalogenating enzymes

that evolved to accommodate the chlorinated herbicide dicamba. In general, the compounds utilized by *Burkholderia* sp. UW103 are highly similar in structure (dichlorinated, aromatic compounds with additional small substituents) to the natural compound, DCA, which is known to be mineralized in pure culture. Compounds that deviate only slightly from the natural compound studied here, mono-, di-, and polychlorinated phenols and even some chlorinated benzoates are not utilized by *Burkholderia* sp. UW103. This supports the hypothesis that the natural pathway for chlorinated anisyl utilization is responsible for the ability of UW103 to utilize the anthropogenic compound dicamba. Further evidence lies in the detection of an early degradation product of DCA, 3,5-dichloro-*para*-anisic acid, an isomer of dicamba.

The presence of anthropogenic compounds in the biosphere for the last century has provided selective pressure for the evolution of new metabolic functions. Recruitment of enzymes from natural catabolic pathways may provide a source of genes for the evolution of catabolic pathways to degrade chlorinated xenobiotic compounds. Upon the evolution of novel capabilities genes may be shared among other organisms in their environment, a few may harbour the capacity to amplify the capability set forth in the original evolution. The study of the enzymes and genetics of chloroaromatic degradation by *Burkholderia* sp. UW103, as it occurs in the natural environment, may lead to clues involving the evolution of dehalogenative and degradative capacities of potential bioremediative *Burkholderia* strains.

Further study of the natural catabolic pathways, including dechlorination, involved in DCA degradation by *Burkholderia* sp. UW103 will be used to compare natural chloroaromatic degradation to the dechlorination of xenobiotic compounds.

CHAPTER 8

CONTRIBUTION TO KNOWLEDGE

Knowledge of the fundamental pathways of the degradation of halogenated compounds is an under-appreciated prerequisite to biotechnical applications. This knowledge is valuable in developing biotechnological solutions for removal of environmental contaminants from soil and water. Effective bioremediation strategies and systems rely on biochemistry that evolved for the utilization of natural halogenated compounds. Construction of bacterial strains with more efficient or novel capabilities requires knowledge of natural enzymes and pathways that could be used to funnel chlorinated xenobiotics into "normal" metabolism. This information could also be used in the design of novel enzymatic technologies for waste management.

An understanding of the fate of natural compounds in the environment is also important to define the limits of remedial intervention of polluted soils. Knowledge of natural pathways for organochlorine cycling provides insight into the fate of anthropogenic halogenated pollutants in the presence of existing microorganisms from a previously pristine environment. Knowledge of the innate capabilities of microbes, and definition of the limits to their abilities, not previously exposed to anthropogenic compounds may reduce the necessity for remedial intervention of polluted soils.

The focus of most organohalogen degradation studies has been the elucidation of catabolic pathways breaking down halogenated pollutants without consideration for the origin of the catalytic enzymes involved. Further study of the biochemistry and genetics of DCA-degrading strains will provide clues to the evolution of natural pathways to accommodate anthropogenic halogenated compounds.

CHAPTER 9

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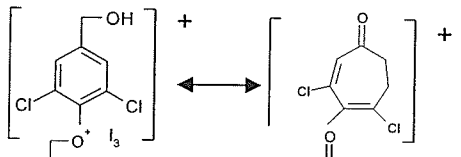
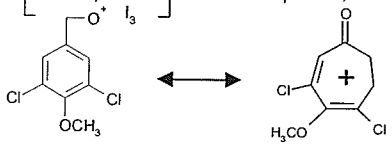
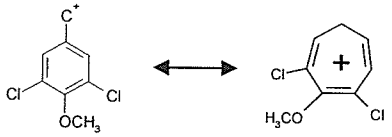
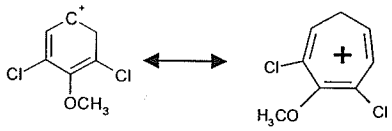
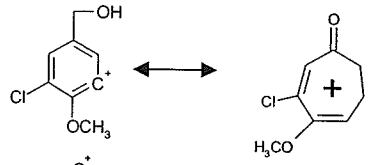
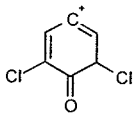
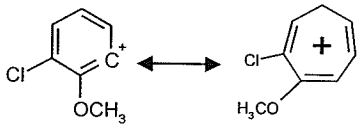
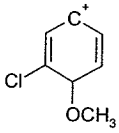
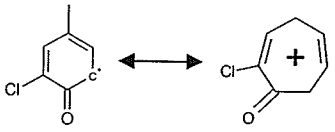
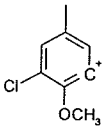
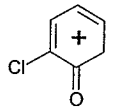
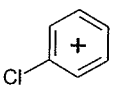
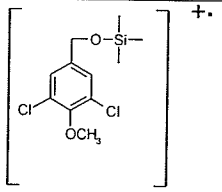
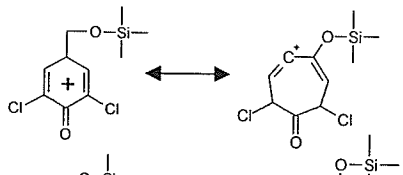
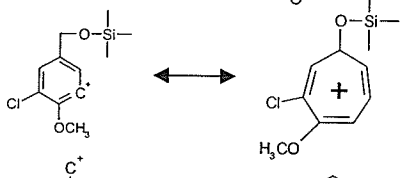
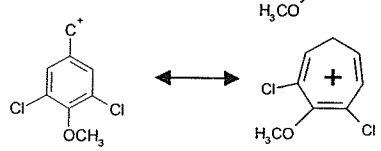
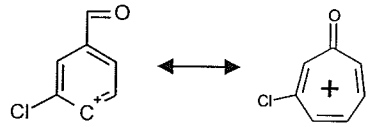
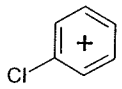
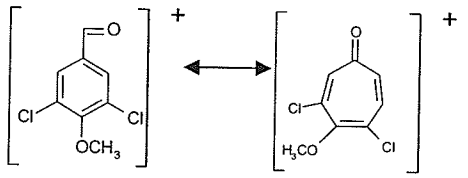
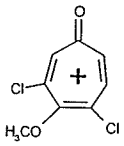
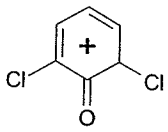
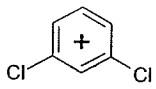
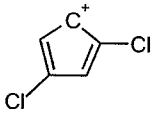
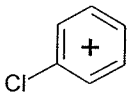
Appendix I. Proposed ion fragments in mass spectrum of DCA.

Mass (Proportion)	Lost fragment(s)	Ion Structure(s)
206, 208, 210 (9:6:1)	molecular ion	
205, 207, 209 (9:6:1)	H	
189, 191, 193 (9:6:1)	OH	
177, 179, 181 (9:6:1)	CHO Cl	
171, 173 (3:1)	Cl	
162, 164, 166 (9:6:1)	CHO CH_3	
155, 157 (3:1)	O Cl	
143, 145 (3:1)	CO Cl	
142, 144 (3:1)	Cl COH	
141, 143 (3:1)	HCl COH	
128, 130 (3:1)	CO Cl CH_3	
111, 113 (3:1)	CHO Cl OCH_3	

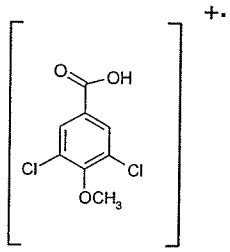
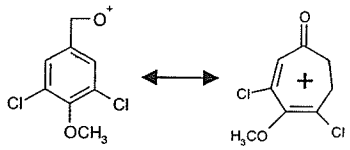
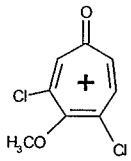
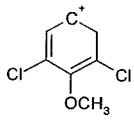
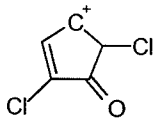
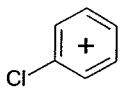
Appendix II. Proposed ion fragments in mass spectrum of BSA-derivatized DCA.

Mass (Proportion)	Lost Fragment(s)	Ion Structure(s)
278, 280, 282 (9:6:1)	molecular ion	
263, 265, 267 (9:6:1)	CH_3	
243, 245 (3:1)	Cl	
189, 191, 193 (9:6:1)	$OSi(CH_3)_3$	
139 (3:1)	$Si(CH_3)_3$ $O\ CH_3$ Cl	
111, 113 (3:1)	$Si(CH_3)_3$ $O\ CH_3$ Cl CO	

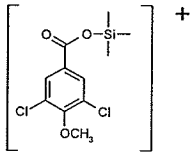
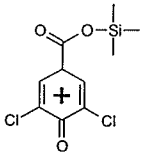
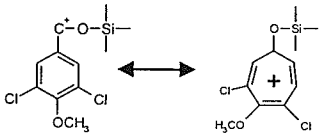
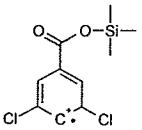
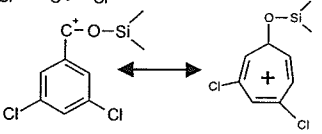
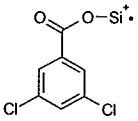
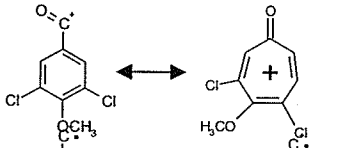
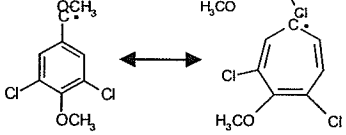
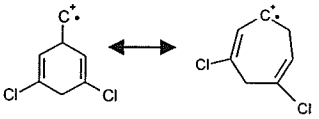
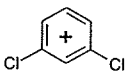
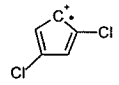
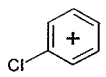
Appendix III. Proposed ion fragments in mass spectrum of DCAld.

Mass (Proportion)	Lost fragment(s)	Ion Structure(s)
204, 206, 208 (9:6:1)	molecular ion	
203, 205, 207 (9:6:1)	H	
161, 163, 165 (9:6:1)	CO CH_3	
145, 147, 149 (9:6:1)	CHO CHO H	
133, 135, 137 (9:6:1)	CH_2CO CHO	
111, 113 (3:1)	CHO CHO Cl	

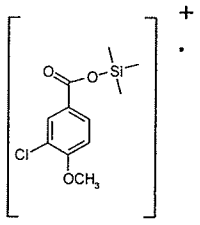
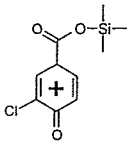
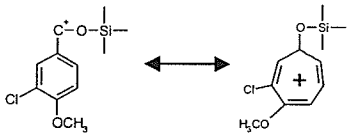
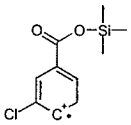
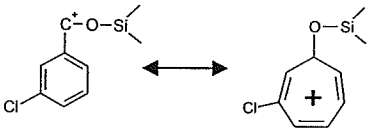
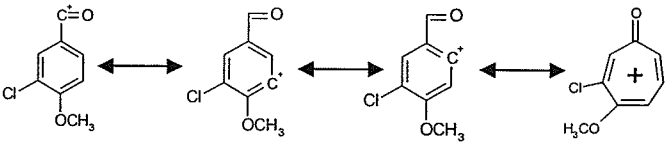
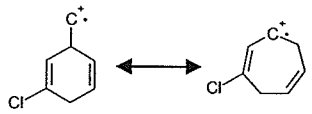
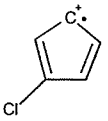
Appendix IV. Proposed ion fragments in mass spectrum of DCAcid.

Mass (Proportion)	Lost fragment(s)	Ion Structure(s)
220, 222, 224 (9:6:1)	molecular ion	
205, 207, 209 (9:6:1)	CH_3	
203, 205, 207	CHO	
177, 179, 181 (9:6:1)	CH_3CO	
149, 151, 153	CO CO CH_3	
111, 113 (3:1)	CO_2 HCl CHO	

Appendix V. Proposed ion fragments in mass spectrum of BSA-derivatized DCAcid.

Mass (Proportion)	Lost fragment(s)		Ion Structure(s)
292, 294, 296 (9:6:1)	molecular ion		
277, 279, 281 (9:6:1)	CH_3		
276, 278, 280 (9:6:1)	O		
262, 264, 266 (9:6:1)	CHO H		
233, 235, 237 (9:6:1)	OCH_3 CO		
218, 220, 222 (9:6:1)	CHO CH_3 x 3		
203, 205, 207 (9:6:1)	$OSi(CH_3)_3$		
188, 190, 192 (9:6:1)	$Si(CH_3)_2$ CH_3 O_2		
160, 162, 164 (9:6:1)	OCH OCH $OSI(CH_3)_2$		
145, 147, 149 (9:6:1)	OCH $Si(CH_3)_3$	H CO_2	
132, 134, 126 (9:6:1)	$Si(CH_3)_3$ CO	CO_2 CH_3	
111, 113 (3:1)	$Si(CH_3)_3$ CHO Cl CO2		

Appendix VI. Proposed ion fragments in mass spectrum of BSA-derivatized MCA.

Mass (Proportion)	Lost fragment(s)	Ion Structure(s)
258, 260 (3:1)	molecular ion	
243, 245 (3:1)	CH_3	
244, 246 (3:1)	O	
228, 230	OCH H	
199, 201 (3:1)	CHO CHO H	
169, 171 (3:1)	$Si(CH_3)_3$ H_2O	
126, 128 (3:1)	$Si(CH_3)_2$ CHO CHO O	
98, 100 (3:1)	CH_3 CO_2 CO $Si(CH_3)_3$	
111, 113 (3:1)	CHO H $Si(CH_3)_3$ CO_2