ISOLATION AND CHARACTERIZATION OF CARBOFURAN AND DICAMBA DEGRADING BACTERIA

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Abstract

This study was conducted to isolate and characterize bacteria that have the capacity to degrade both carbofuran and dicamba. The pathways of degradation for both pesticides were elucidated.

An aerobic, carbofuran-degrading bacterium was isolated from a high concentration carbofuran bioreactor. The isolate degrades carbofuran at the upper limit of carbofuran solubility (approximately 700 mg L^{-1}), to carbofuran phenol. In aqueous mineral salts medium with carbofuran as Furadan 4F (6 g L^{-1} a.i.), degradation of carbofuran to undetectable levels required approximately 100 days. Although carbofuran phenol was not completely degraded, the cells remained viable in the presence of unusally high concentrations of both surfactant and carbofuran phenol. Additional nutrient sources had little effect upon the rate of degradation of carbofuran in pure culture.

A dicamba-degrading consortium enriched from wetland soil, using the batch culture method, was used to elucidate the pathway of dicamba degradation under anaerobic conditions. The consortium consisted of one sulfate reducing bacterium, one fermenter, and three methanogens. The sulfate reducing bacterium was isolated from the consortium using sulfate as a terminal electron acceptor and 2-bromoethanesulfonic acid was added to inhibit the growth of the methanogens. Since the fermenter is dependent upon the methanogens, elimination of these organisms caused the elimination of the

fermenter. Three methanogens (*Methanothrix*, *Methanosarcina* and *Methanospirillum* sp) were isolated with acetate and headspace gas consisting of H_2-CO_2. Degradation of dicamba proceded through an initial demethylation reaction yielding 3,6-dichlorosalicylic acid, as determined by high performance liquid chromatography (HPLC) analysis of aqueous medium. This was followed by a reductive dehalogenation reaction at the *meta* position of 3,6-dichlorosalicylic acid forming 6-chlorosalicylic acid. The metabolites were isolated using thin layer chromatography. Confirmation of metabolite identity was achieved using HPLC, and mass spectrometry. It appears that the fermenter was responsible for mediating the demethylation reaction. The consortium was unable to mineralize the aromatic ring.

The substrate specificity of the dicamba-degrading consortium was investigated. The consortium was found to have the capacity to mediate the reductive dehalogenation of both 3-chlorosalicylic acid and 2,5-dichlorobenzoic acid at the *meta* position. The consortium was unable to dehalogenate either 3-chlorobenzoic acid, 4-chlorosalicylic acid, 5-chlorosalicylic acid, or 2,5-dichlorophenol. Addition of the reducing agent cysteine (0.025% and 0.050%) to a yeast extract amended (0.04%) mineral salts medium containing 3-chlorosalicylic acid reduced the rate of dehalogenation compared to medium containing sodium sulfide as the reducing agent. Only limited dehalogenation of 3-chlorosalicylic acid and 2,5-dichlorobenzoic acid was observed when the sulfate reducing bacterium was cultured alone in a yeast extract amended medium, suggesting that the mutualistic efforts of a mixed population of anaerobes were necessary to efficiently mediate reductive dehalogenation.

To my father, Ronald Henry Taraban

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Chapter I

Introduction

Successful employment of bioremediation technology requires, in many cases, the presence or use of microbes with unique capabilities and the understanding of how to manipulate such populations to achieve maximum biodegradation efficiency. Bioremediation can be carried out either in situ or in bioreactors. Regardless of how this technology is applied, much has yet to be learned. Bioreactor technology is currently being used in the development of an on site wastewater treatment process to cleanup rinsate. The procedure involves sorption of pesticides onto lignocellulosic material (peat), followed by solid state fermentation, and subsequent degradation of pesticides, in bioreactors.

I decided that bioreactors containing high concentration levels of carbofuran would serve as an ideal source from which to isolate a carbofuran-degdading bacterium, with unique or special qualities. Thus, the first objective of my research was to isolate a novel carbofuran-degrading bacterium from bioreactors containing high concentrations of Furadan 4F. At high carbofuran (as Furadan 4F) concentrations , bacteria contained within the bioreactors would be exposed to high concentration levels of surfactants, and the probable metabolite carbofuran phenol. Surfactants and phenolic compounds are both disruptive to bacterial cell membranes, so that any resistance to these compounds would be a most desirable quality.

In situ bioremediation of anaerobic sediments and soils contaminated with chlorinated pesticides will undoubtedly require knowledge of anaerobes. I was specifically

interested in the degradation of dicamba (3,6-dichloro-2-methoxy benzoic acid) under anaerobic conditions. Although the degradation of dicamba has been extensively studied under aerobic conditions, very few studies have examined the degradation of dicamba in anoxic environments.

The pathway of dicamba degradation under anaerobic conditions has been shown to involve an initial demethylation reaction forming 3,6-dichlorosalicylic acid. I speculated, that the next conceivable reaction in the degradative pathway might involve a reductive dehalogenation reaction, at the C-3 carbon position yielding 6-chlorosalicylic acid. Some of the most promising dehalogenating anaerobes appear to be sulfate reducing bacteria. I decided to try to enrich for such a consortium. Thus, the second objective of my research was to isolate dicamba-degrading anaerobes. A soil slurry inoculum was collected from a brackish wetland site. Such an environment would undoubtedly contain high amounts of sulfate, and therefore would most likely have high populations of sulfate reducing bacteria.

The third objective of my research was to characterize the dicamba-degrading consortium and isolates, with respect to substrate specificity. Since chlorobenzoate dehalogenating anaerobes exhibit substrate specificity, usually preferring to dechlorinate *meta* positioned halogenated sites. I evaluated the ability of the dicamba-degrading consortium to dehalogenate various *ortho*, *meta* and *para* positioned chlorobenzoates.

Chapter II

Literature Review

Agricultural production in the U.S. has increased dramatically over the past several decades. This increase in crop production is due in part to the widespread use of pesticides. At present there are approximately 600 different active pesticide ingredients registered for use in the U.S. It has been estimated that millions of kilograms are applied globally each year, with the annual world market at greater than 20 billion dollars (Racke, 1990). Application of pesticides directly to soil, providing residual pest control, has been a subject of much environmental concern. Among these concerns are: (1) the fate of residues once they have been applied; (2) the provision for appropriate disposal methods for unused, concentrated and dilute pesticide formulations and pesticide contaminated products; and (3) the development of methods for dealing with pesticide spills (Mullins et al., 1991). As a result, a great deal of interest from both the private and public sectors has been and will continue to focus on the problems associated with the use of pesticides in agriculture.

Pesticides applied to the soil enter an environment where sorption, degradative and transport processes operate in concert to determine the fate of these compounds. Movement of pesticide residues involve a variety of transport processes, including

volatilization, whereby pesticides with high vapor pressures can readily enter the atmosphere, and water transport, whereby pesticides can move through soil and into groundwater. The extent of leaching is governed by pesticide solubility and partitioning processes between sorbed and solution phase residues (Racke, 1990). Other governing environmental factors such as water flow, temperature and location of the pesticide will help to determine this process.

The mobility of pesticides is partially dependent upon the time period in which they are in the soil environment. Pesticides that are readily degraded do not have the opportunity to move to any significant extent. Those pesticides that are not rapidly degraded, however, may move readily through the soil and into groundwater, or enter runoff where they can end up in rivers, lakes and sediments.

Certain pesticides are more mobile than others. Dicamba for example, is highly mobile, primarily because it exists in a dissociated anionic form in soils. It is readily metabolized to 3,6-dichlorosalicylic acid, however, and this compound is sorbed by soils to a significant extent (Murray and Hall, 1989). When considering the mobility of pesticides not only must the structure of the parent compound be considered, but also the structure of the metabolites.

The degradation of pesticides in the soil has been the focus of a great deal of research. Abiotic transformations may result in the buildup of pesticide metabolites which could be further degraded by soil microorganisms. Several of the most heavily used pesticides appear to be highly resistant to mineralization. For example, two of the most widely used herbicides, atrazine and metolachlor, are quite resistant to mineralization although transformation of the aromatic ring readily occurs.

Atrazine (2-chloro-4-[ethylamino]-6[isopropylamino]- 1,3,5-triazine) belongs to a class of herbicides referred to as the s-triazines. The s-triazines are extensively used to control weeds in the production of corn and other crops. Because atrazine is one of the most heavily used pesticides in the U.S., a great deal of interest has focused on its potential as a soil and groundwater contaminant.

Atrazine generally has a half-life in soils ranging from 4 to 57 weeks (Mandelbaum et al., 1993). In the soil environment, atrazine can be transformed to several metabolites including deethylatrazine (2-chloro-4-[amino]-6-[isopropylamino]-1,3,5-triazine), deisopropylatrazine(2-chloro-4-[ethylamino]-6-[amino]-1,3,5-triazine),dealkylatrazine(2-chloro-4,6-[diamino]-1,3,5-triazine) and hydroxyatrazine (2-hydroxyl-4-[ethylamino]-6-[isopropylamino]-1,3,5-triazine) (Winkelmann and Klaine, 1991). Adams and Thurman (1991) suggest that dealkylation of atrazine is the most significant biotic degradation

pathway for atrazine in soil environments, whereas the most significant abiotic degradation pathway is via hydrolysis to hydroxyatrazine.

The persistence of the atrazine metabolites in the environment is a matter of some concern. Soil bioreactor studies have shown that less than 10% of [^{14}C] atrazine was converted to [$^{14}CO_2$] in 125 days (Nair and Schnoor, 1992). Recently, however, mineralization of atrazine has been shown with stable bacterial mixed cultures. Enrichment cultures were obtained from soils repeatedly exposed to atrazine, alachlor and metolachlor. After 12 subcultures, the half-life for the degradation of atrazine, at a concentration of 100 mg L^{-1} was 0.5 to 2 days (Mandelbaum et al., 1993).

Metolachlor [2-chloro-N-(2ethyl-6-methoxyphenyl)-N-(2-methoxyl-1-methylethyl) acetamide] is a selective herbicide used for the control of several annual grass weeds, yellow nutsedge, and certain broad-leafed weeds in corn, soybeans, peanuts and other crops. The degradation of metolachlor in soils has been studied extensively. Under aerobic sterile conditions, Ellgehaussen (1976 a,b) reported that 30% of the applied metolachlor was dechlorinated after 12 weeks. Under non-sterile conditions using [^{14}C] metolachlor, approximately 18% was oxidized at the acetyl group forming an oxalic acid derivative. Liu et al. (1988) studied the degradation of metolachlor in a soil perfusion system. They found that addition of [^{14}C] metolachlor to a soil that had received metolachlor (Dual) treatments for five years resulted in 18% mineralization. When soil

from the same field which had no history of metolachlor treatment was used, only 3.5% of the [^{14}C]metolachlor was libērated as $^{14}CO_2$. In subsequent experiments Liu et al. (1989) showed that absorption and transformation were the major fate of metolachlor in a liquid medium inoculated with a stable bacterial community. Approximately 80% of the [^{14}C] metolachlor (50μg/ml) disappeared from the medium and accumulated inside the cells. The remaining residue consisted primarily of two dechlorinated metabolites. When pure cultures were used, they were less efficient in metolachlor absorption and transformation. These results suggest that a mixture of microorganisms, rather than individual species, are more effective in the removal and transformation of metolachlor.

Methods to collect and treat pesticide-containing wastewater have been the topic of intense investigation. Recently, a pesticide wastewater treatment method with demulsification, sorption and filtration to remove pesticides from rinsate suspensions has been developed (Hetzel, et al., 1989).

In Hetzel's proposed system for rinsate cleanup, lignocellulosic materials such as peat moss and steam exploded wood (SEW) are used to remove pesticides from the aqueous phase. The sorption process is facilitated by the use of demulsification agents such as $Ca(OH)_2$. Once sorbed, from interactions with organic matter, the pesticide containing lignocellosic material is placed into bioreactors. The pesticide is then subjected to microbial degradation and interactions with the lignocellulosic and humic material.

In a recent study, Berry et al. (1993) evaluated the suitability of solid state fermentation as a means to dispose of atrazine and carbofuran (2,3-dihydro 2,2-dimethyl-7-benzofuranyl methylcarbamate). Pesticides were added to the bioreactors, containing either sphagnum peat moss or SEW as the lignocellulosic sorbent. In peat-filled bioreactors with atrazine at loading rates of 1.4, 2.0, and 7.2 g/kg (dry wt. basis), solvent-extractable atrazine decreased to less than 0.2% of starting levels within 480 days. The SEW-filled bioreactors containing atrazine at a loading rate of 2.0 g/kg showed a 20% decrease in solvent extractable atrazine within 320 days. For carbofuran loaded bioreactors (loading rate 5.0 g/kg) both peat-filled and SEW-filled bioreactors decreased to less than 0.05% of starting levels within 480 days. A bacterium was isolated from a peat-filled bioreactor capable of degrading carbofuran as Furadan 4F in aqueous medium at a concentration level of 6000 mg L^{-1} (Taraban et al., 1992). This bacterium is a Gram negative motile rod. Although carbofuran-degrading bacteria are not uncommon in soils, to the authors knowledge, none has been isolated and shown to degrade carbofuran at this high concentration. This isolate, however, does not appear to have the capacity to degrade carbofuran phenol. Carbon phenol concentrations reached maximum concentrations in excess of 1000 mg L^{-1} in studies conducted in liquid media with carbofuran as Furadan 4F. Under these high phenolic concentrations the cells remained viable exhibiting an unusually high resistance to phenolic toxicity. Bioassay studies were conducted to determine the toxicity of the sorbent following bioreactor shut down. The results of the

toxicity studies showed that the amount of pesticide in bioreactor peat could be reduced to non toxic levels with a prolonged incubation time period.

In contrast to microbial assisted pesticide disposal methods, non-assisted methods have also been developed. The most promising methods seem to involve UV irradiation and ozonation. Prolonged exposure causes fragmentation of pesticides, thus possibly detoxifying certain compounds or rendering the metabolite susceptible to further degradation.

Often a combination of assisted and non-assisted methods is most efficient. UV-ozonation of pesticide-laden wastewater has been shown to be an effective pretreatment for microbial metabolism (Kearney et al., 1983a, 1984b, 1984, 1985). In a recent study, Leeson et al. (1993) also was able to show the benefits of a binary scheme consisting of ozonation followed by microbial degradation. Exposure of atrazine to ozonation fragmented the herbicide into two products: 4-acetamido-6-amino-2-chloro-1,3,5-triazine and chlorodiamino-1,3,5-triazine. These degradate products were then biodegraded by *Klebsiella terragena* (strain DRS-I).

Bioreactor technologies may offer a viable and relatively inexpensive means to dispose of pesticides. The use of bioreactor systems for decontamination of large volumes

of pesticide contaminated soil is probably not feasible. In these cases, researchers must consider in situ bioremediation treatments.

The soil environment is a dynamic and complex system. It should be looked upon as consisting of countless microenvironments, each with differences in oxygen concentration, pH, water availability, organic matter content and nutrients.

Soil microorganisms are as diversified as the environments in which they inhabit. Often times, attempts are made to predict the outcome of a specific microbial activity (i.e., pesticide degradation) in the natural environment based on laboratory studies. Caution is warranted when attempting to extend results from the laboratory to the field. Under optimal conditions, microorganisms display a predictable pattern of metabolism, growth and death. Optimal conditions for microorganisms rarely exist in soil environments. Morita (1990) has coined the term starvation-survival, and used it to describe the physiological state where sufficient energy is not present. Although this may be thought of as a dormant state, all that is necessary for resumed growth is the availability of an appropriate nutrient source. The possibility that certain pesticides can supply this appropriate energy source has been a subject of great speculation.

Microorganisms, in general, have a great ability to metabolize a wide variety of organic compounds. The range covers simple one carbon compounds to complex

polynuclear aromatic compounds. Microbes have evolved an ability to metabolize a variety of substrates under almost any condition.

Generally, pesticides applied to the soil remain in an aerobic environment for an extended period of time. It is during this period that they are first exposed to aerobes (i.e. aerobic microorganisms).

The most efficient pesticide degrading aerobes use a process known as respiratory catabolism, whereby reduced organic compounds serve as the initial electron donor and oxygen serves as the final electron acceptor.

In order to accomplish the breakdown of organic compounds, bacteria use various metabolic pathways including beta-oxidation, the hexose monophosphate pathway and the tricarboxylic acid cycle (TCA) or the Embden-Meyerhof-Parnas pathway coupled to the TCA cycle or the Entner-Doudoroff pathway and the TCA cycle. Using these catabolic processes, bacteria are able to completely oxidize organic substrates to carbon dioxide. These pathways, however only begin with relatively simple compounds such as five and six carbon sugars or fatty acids. Most organic pesticides must first be oxidized and transformed before they can ever reach these pathways.

Aerobes are capable of carrying out various types of reactions including, oxidations, hydroxylations, hydrogenations, demethylations, dehalogenations, deaminations and decarboxylations. In many instances transformations of pesticides in soils result from a combination of both biotic and abiotic reactions. Atrazine transformation and degradation for example, involves a wide variety of reactions. The dechlorination of atrazine to form hydroxyatrazine appears to be abiotic and its rate of dehalogenation is largely dependent upon pH. Erickson and Lee (1989) postulate that acid hydrolysis results from protonation of a ring or chain nitrogen atom followed by cleavage of the C-Cl bond. They suggested that protonation of the nitrogen atom would increase the electron deficiency of the carbon bonded to the chlorine and increase the tendency for nucleophilic displacement of chlorine by water. Dealkylation of atrazine on the other hand, is generally carried out by microbial oxidative enzymatic catalysis.

Many of the pesticides in use today are aromatic, and before they can be oxidized completely to carbon dioxide, the ring structure must first be cleaved. Ring cleavage is initiated by inserting oxygen onto the aromatic ring structure. In some instances, water serves as the source of oxygen. In most cases, benezenoid ring cleavage occurs via an oxygenase system, whereby molecular oxygen (O_2) is inserted into the aromatic substrate. There are two major dioxygenase cleavage pathways. The ortho-cleavage pathway requires that hydroxyl groups be present on adjacent ring carbons. Ring cleavage then occurs between these two carbons. The meta-cleavage pathway also requires hydroxyl

groups positioned adjacent to one another, however, ring cleavage occurs between the number two and three carbons.

While pesticides are generally applied to well aerated soils, they often end up in non-target environments such as streams and rivers, sediments and groundwater. Many of these non-target environments are anaerobic. Anaerobic environments exist when oxygen demand exceeds its supply. Under these conditions, facultative and anaerobic microorganisms predominate the microflora.

Fermentative bacteria metabolize substrates without utilizing a respiratory pathway. Energy is gained through substrate level phosphorylation by oxidizing organic substrates. The organic intermediates serve as both electron donors and electron acceptors.

There are also respiring bacteria that use an electron transport system with nitrate and sulfate as terminal electron acceptors. Because numerous microenvironments exist, each with its own dominant population, there are a wide variety of microorganisms including both respiring and non-respiring bacteria. Overall, however, the availability of electron donors and electron acceptors plays a significant role in determining the types of bacteria that will predominate any given area.

Methanogenic environments are those in which organic substrates are metabolized resulting in production of methane and carbon dioxide. The metabolic food chain in methanogenic environments, is composed of methanogens and fermenters. The consortium works as a single unit in the metabolism of organic compounds. Ferry and Wolfe (1976) were among the first researchers to characterize a methanogenic consortium that degraded benzoate to methane. The consortium consisted of three predominate organisms, a benzoate degrader (fermenter) and two methanogens. Benzoate degradation resulted in the production of the fermentation end products, acetate, formate and hydrogen. The methanogenic bacteria then converted the acetate and formate to methane and carbon dioxide. Without the presence of the methanogens intermediates would have accumulated. With the accumulation of intermediates, the reaction becomes thermodynamically unfavorable and benzoate degradation will cease. The characterization of benzoate degradation was an important step in understanding the potential for pesticide degradation under anaerobic conditions.

Denitrifying conditions are often produced during periods of flooding. Under these conditions oxygen is quickly consumed and nitrate becomes the electron acceptor. Nitrate is a thermodynamically favorable terminal electron acceptor, second only to oxygen. Nitrate reducers prefer oxygen as a terminal electron acceptor, but have the capacity to utilize nitrate when oxygen supplies diminish. The role that nitrate reducers play in anaerobic environments has been a topic of much interest. The interest is not only with

the possibilities of pesticide degradation, but also the capacity of these bacteria to remove excess nitrate from soil and subsurface thus preventing contamination of groundwater. A recent study has examined the potential for nitrate reduction in riparian soils, and the conclusions seem to agree that biological denitrification may significantly reduce nitrate and act as a buffering system to prevent discharge into groundwater (Ambus and Christensen, 1993). The potential for nitrate reducing bacteria to metabolize aromatic compounds in the presence of nitrate has been investigated. Braun and Gibson (1984) were able to show the degradation of 2-aminobenzoate by denitrifying bacteria.

Chlorinated aromatic compounds in the environment have generated a great deal of concern. Contamination of soil and groundwater have prompted the monitoring of chlorinated compounds in urban drinking water and in wells. Data collected by Hallberg (1986) indicate that, in Iowa alone, 25% of that state's population regularly consumes water contaminated with pesticides. Chlorinated pesticides are common contaminants, due to the heavy agricultural application of such pesticides such as atrazine, metolachlor and dicamba.

Often the dehalogenation of chlorinated aromatic compounds involves a variety of microorganisms, including both aerobes and anaerobes. The fact that dehalogenation occurs under anaerobic conditions was known since 1955 when Allen, (1955) was able

to demonstrate anaerobic pesticide dehalogenation. It is now widely accepted that the most promising detoxification (removal of chlorine substituents) occurs anaerobically.

Interest in chlorophenol degradation was generated by the extensive pentachlorophenol (PCP) usage as a herbicide and as a wood preservative. This highly toxic compound has been shown to be degraded under aerobic conditions (Chu and Kirsch, 1972; Saber and Crawford, 1985). Under anaerobic conditions, degradation was first observed by Ide et al. (1972). Since then PCP has been shown to be dehalogenated under a variety of anaerobic conditions, and complete mineralization was observed under methanogenic conditions (Guthrie et al., 1984; Mikesell and Boyd, 1986). The metabolites of PCP degradation under anaerobic conditions demonstrate the ability of certain anaerobes to dehalogenate this compound at either the *ortho*, *meta* or *para* positions (Ide et al., 1972; Weieiss et al., 1982; Mikesell and Boyd, 1986). It has been argued, however, that for PCP dehalogenation reactions, positions *ortho* to the phenolic hydroxy group are more easily dehalogenated, whereas *meta* positions are more difficult (Mikesell and Boyd, 1986). The various dehalogenations of PCP are usually due to several microorganisms, each most likely carrying out a specific function. While a PCP degrading bacterium has not yet been found, they most likely do exist.

Using sewage sludge as inoculum, Boyd et al. (1983) were among the first researchers to investigate the dehalogenation of monochlorinated phenols under anaerobic

conditions. Of the three chlorophenolic compounds tested (*o*-, *m*-, and *p*-chlorophenol), only *p*-chlorophenol was not significantly degraded after an eight week incubation period. Boyd and Shelton (1983) obtained similar results with monochlorinated phenols using fresh and acclimated sludge. They found the relative rates of chlorophenol disappearance to be in the following order: *ortho* > *meta* > *para*. More recently, Haggblom and Young (1990), observed degradation of 2-chlorophenol, 3-chlorophenol, 4-chlorophenol and 2,4-dichlorophenol under sulfate reducing conditions in microcosms containing estuarine sediment. Sulfate consumption corresponded to the stiochiometric values expected for complete oxidation of the chlorophenol compounds to CO_2. When molybdate was added, to inhibit sulfate reducers, dehalogenation of chlorophenol ceased. The relative rates of chlorophenol degradation, in decreasing order was 4-chlorophenol > 3-chlorophenol > 2-chlorophenol and 2,4-dichlorophenol, Haggblom and Young (1990) confirmed that substrate oxidation was indeed coupled to sulfate reduction.

There has been a great deal of research conducted concerning the dehalogenation of chlorobenzoates. A novel mechanism for the reaction was first reported by Suflita et al. (1982). They reported that the primary mechanism of chlorobenzoate dehalogenation involves the replacement of an aryl halide with an H atom. Since then, chlorobenzoate dehalogenation reactions have been shown in a variety of anaerobic environments including shallow aquifers. Gibson and Suflita (1986) were able to show that 3,4-dichlorobenzoic acid could be dehalogenated in either the *meta* or *para* position forming

4-chlorobenzoic acid or 3-chlorobenzoic acid. The 3-chlorobenzoic acid could also be dehalogenated, forming benzoic acid in microcosms containing aquifer material (Gibson and Suflita, 1986). Following dehalogenation, benzoic acid was readily mineralized by the consortia.

Most studies on chlorobenzoate dehalogenation have used undefined inoculum consisting of sludge, lake or estuarine sediment, or soil slurries. Shelton and Tiedje, (1984) enriched a methanogenic consortium from sewage sludge that grew on 3-chlorobenzoic acid as a carbon and energy source. From this consortium, they isolated a sulfate reducing bacterium (strain DCB-1, later *Desulfomonile tiedjei*) with the ability to dehalogenate 3-chlorobenzoic acid forming benzoate. *D. tiedjei* was the first sulfate reducer isolated capable of reductive dehalogenation of certain chlorobenzoates in pure culture. This bacterium has become the model dehalogenating system, and thus extensively studied.

D. tiedje has been characterized with respect to the nutritional requirements and substrate diversity. It grows optimally in a medium enriched with nicotinamide, 1,4-naphthoquinone, and thiamine. It can grow autotropically with H_2-CO_2 and with sulfate or thiosulfate as terminal electron acceptors (DeWeerd et al., 1990).

It appears that *D. tiedjei* can use 3-chlorobenzoic acid as an alternative terminal electron acceptor. It has been shown that that this bacterium is capable of coupling reductive dehalogenation of 3-chlorobenzoic acid to ATP production (Dolfing, 1990). In the presence of sulfate however, it prefers to use sulfate reduction as its mechanism of electron transport, thus effectively inhibiting reductive dehalogenation of 3-chlorobenzoate. Of the 55 different substrates tested with *D. tiedjei*, only pyruvate supported growth as the sole carbon source in a mineral medium (Stevens et al., 1989).

D. tiedjei appears somewhat unique in its ability to dehalogenate, preferring *meta* substituted chlorobenzoates. While *D. tiedjei* is capable of dehalogenating compounds in pure culture, this reaction is enhanced when the entire consortium is together. The entire consortium consists of *D. tiedjei*, a fermenter, and several methanogens.

Recently, an anaerobic consortium that degrades the herbicide, dicamba, was characterized (Taraban et al., 1993). This consortium consisted of a sulfate reducing bacterium, a fermenter and three methanogens. Degradation of dicamba proceeded through an initial demethylation reaction forming 3,6-dichlorosalicylic acid, followed by reductive dehalogenation to form 6-chlorosalicylic acid. The initial demethylation reaction appears to be mediated by the fermenter. The dehalogenation of 3,6-dichlorosalicylic acid to 6-chlorosalicylic acid is most likely carried out by the sulfate reducing bacterium. It

would appear that in anaerobic environments, pesticide degradation is most efficiently accomplished by a diverse population of microorganisms rather than an individual species.

The dicamba-degrading consortium was tested for it's ability to dehalogenate various chlorinated benzoates. While the dicamba-degrading consortium was unable to dehalogenate 3-chlorobenzoic acid, it was able to dehalogenate 3-chlorosalicylic acid, something *D. tiedjei* does not do. Both *D. tiedje* and the dicamba-degrading consortium can dehalogenate 2,5-dichlorobenzoic acid, forming 2-chlorobenzoic acid.

As expected, dehalogenating organisms appear to be substrate specific. Although *D. tiedjei* has been studied as a model organism, in the environment these dehalogenating organisms almost certainly mediate reductive dehalogenation in the presence of other anaerobes, which more than likely help to facilitate the reaction.

There are some interesting contrasts between chlorophenol and chlorobenzoate dehalogenation reactions. With chlorophenols displacement of Cl in the *ortho* position seems to be preferred. For chlorobenzoates, displacement of the Cl in the *meta* position is preferred. It is also interesting to note that the addition of sulfate to cultures that dehalogenate chlorobenzoates seems to block reductive dehalogenation, whereas with chlorophenol dehalogenation, there is some evidence that the addition of sulfate may actually enhance the reaction rate.

Transition metals such as Fe, Cr, Co, Ni, and Zn appear to be directly involved in dehalogenation reactions. These transition metals or metal complexes (having incompletely filled 3d orbitals), have been shown to be involved in dehalogenation reactions involving certain aliphatic and alicyclic compounds (Vogel et al., 1987). Transition metals are contained in many proteins found in methanogens and sulfate reducing bacteria. The active transition metal complexes (complexing the metal with a tetrapyrrole ring), have redox potentials less than 0 mV. The low redox of these electron donors allows for there reduction to be coupled with the dehalogenation of many aromatic compounds (Kuhn and Suflita, 1989).

Methanogens contain nickel metallo-enzymes and also possess F-430, used in the final step in methanogenesis. It has been shown that a macrocyclic Ni(I) complex can dehalogenate certain aliphatic compounds (Bakac and Espenson, 1986).

In general, highly chlorinated compounds are dehalogenated more readily than compounds with less chlorine substituents. Kuhn and Suflita, (1989) explain this based on redox potential. They state that the redox potential of the chemical to be dehalogenated has to be higher than that of the electron donor. Highly halogenated compounds generally have higher redox potentials and can therefore react easier with an electron donor to yield a higher free energy and increased reaction rate.

Benzenoid metabolism may occur via two different pathways under anaerobic conditions. Dutton and Evans (1969) were the first to show the reductive pathway. The first step in this pathway is ring hydrogenation followed by a ring hydration then ring cleavage. The oxidative pathway, as shown by Vogel and Grbic-Galic (1986), involves an initial ring oxidation step rather than a ring reduction step. The oxidative pathway does not appear to be the primary means by which aromatic ring structures are metabolized in anaerobic environments.

Enhanced pesticide degradation in soils that have had repeated application of pesticides has been observed (Dick et al., 1990). Many different factors may account for the increased rates of pesticide degradation. Natural selection may allow for proliferation of pesticide degrading bacteria. Or, if an enzyme system exists in which to degrade the pesticide, then the enhanced degradation may be due to removal or lessening of a lag period, because of constant exposure to the pesticide and therefore constant enzyme production. Also, increased exposure to the pesticide may result in increases in pesticide-degrading populations and consequently increased rates of degradation. It should be noted, however, that not all pesticides respond to enhanced degradation. Some pesticides, such as metolachlor appear to be highly resistant to degradation even in soil with repeated exposure. Carbofuran serves as a good example of enhanced rates of degradation upon repeated exposure.

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Chapter III

Isolation and Characterization of a Carbofuran-degrading Bacterium

3.1 Abstract

An aerobe (designated C-1), capable of using carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate) as a sole carbon and energy source, was isolated from a peat-filled bioreactor used to dispose of Furadan 4F. The isolate, a Gram-negative, motile, rod shaped bacterium, degraded carbofuran and formed carbofuran phenol (2,3-dihydro-2,2-dimethyl-7-hydroxy benzofuran). Isolate C-1 appears to carry out only limited degradation of carbofuran phenol although cells remain viable in liquid media containing carbofuran phenol at concentrations in excess of one g L^{-1}. The carbofuran-degrading isolate is capable of degrading carbofuran as Furadan 4F even at concentrations approaching six g L^{-1}.

3.2 Introduction

Carbofuran is a broad spectrum insecticide that is usually applied at the time of planting to control corn rootworm (Karns and Tomasek, 1991). Because carbofuran is a cholinesterase inhibitor, it is also highly toxic to mammals. There is concern that improper handling of this potentially hazardous pesticide could result in surface water and groundwater contamination.

Carbofuran appears to be readily degraded by soil microorganisms. This is especially true in some agricultural soils that have received repeated applications of this insecticide. In fact, some soils exhibit an extremely rapid or enhanced rate of carbofuran degradation resulting in decreased efficacy (Karns et al., 1986; Racke and Coats, 1988).

Not surprisingly, the rate of carbofuran degradation in soil is influenced by several environmental factors including temperature, pH, and oxygen availability. Ou et al. (1982) were able to show that maximum rates of degradation occurred at temperature between 27 and 35 °C. Venkateswarlu et al. (1977) studied the degradation of carbofuran under flooded conditions and found degradation occurred more rapidly under flooded rather than non flooded conditions. This finding suggests that anaerobes and facultative anaerobes as well as aerobes can mediate carbofuran degradation.

The carbofuran degrading ability of aerobic bacteria has been well documented. Chaudhry and Ali (1988) isolated 15 bacteria capable of degrading carbofuran. Six of the isolates utilized carbofuran as a sole source of carbon. All of the isolates were capable of transforming carbofuran to carbofuran phenol while two of the isolates appeared to use an oxidative pathway to mineralize carbofuran to CO_2.

Carbofuran degrading bacteria appear to be widespread in soils. Generally, however, studies involving these organisms are conducted at carbofuran concentrations that would relate to field application rates. Limited information is available concerning the ability of microbes to degrade carbofuran at high concentration levels. Organisms that can not only survive in the presence of high level concentrations of carbofuran but also effectively degrade this insecticide could prove very useful in development of

bioremediation technology, where microorganisms may be exposed to high concentrations of pesticides for in situ or in vivo situations. The objective of this research was to isolate a carbofuran-degrading microbe that could withstand high concentration levels of carbofuran and the major metabolite carbofuran phenol.

3.3 Materials and Methods

3.3.1 Chemicals. Analytical grade carbofuran was purchased from Chem Service (West Chester, PA). The metabolite, carbofuran phenol and Furadan 4F (40% a.i.) were provided by F.M.C. Corp. Princeton, NJ.

3.3.2 Bioreactor Design. Bioreactors were constructed from 38-L Rubbermaid (Rubbermaid Co., Twinsburg, OH) waste containers fitted with locking lids. Reactors were housed in a large plywood incubator lined with 5.1-cm Styrofoam insulation, and maintained in a greenhouse that exhibited a temperature variation of between 18 and 32 °C. Aeration of the bioreactors was achieved by applying a vacuum to the headspace causing air to be drawn in through four stainless steel aeration tubes (60- by 0.6-i.d.) that extended from the lid surface of the bioreactor to within 3 cm of the bottom. Air flow was maintained between 10 and 15 ml min^{-1}. The bioreactors contained a sphagnum peat moss-cornmeal-crushed limestone mixture (67:22:11, dry wt. basis). A microbial inocula consisting of aged horse manure, agricultural soil, and peat material collected from 5 yr old bioreactors (50:25:25) was added to the bioreactors at a rate of 150 g kg^{-1} peat. The agricultural soil (a composite sample) was collected from several research plots that had a 25-yr pesticide application history. Carbofuran as Furadan 4F was applied to the peat mixture using a 6.7-L hand insecticide sprayer to give a final rate of 1.8 g kg^{-1}. (a.i., dry

wt. basis). Water was added to adjust the moisture content of the peat mixture to between 0.65 and 0.73 g kg^{-1} dry peat.

3.3.3 Enrichment Culture and Bacterial Isolation Techniques. Following a year long incubation period, a 5 g peat sample was taken from the bioreactor and mixed with 250 ml of mineral salts media containing 4.8 g K_2HPO_4, 1.2 g KH_2PO_4, 2.0 g NH_4NO_3, 0.2 g $MgSO_4(H_2O)_7$, 0.16 g $CaNO_3$, 0.04 g $FeSO_4$ (per liter). The pH was adjusted to 7 and then autoclaved for 20 min. Following sterilization, analytical grade carbofuran was added at a concentration of 200 mg L^{-1}. Serial dilutions were made from the third transfer culture. The agar streak technique using selective agar plates (2% agar containing mineral salts media and 200 mg L^{-1} analytical grade carbofuran) was used to isolate carbofuran-degrading bacteria. Within one week, small colonies appeared. Using aseptic techniques, selected colonies were transferred back to liquid culture media containing carbofuran. This process was repeated one additional time. Isolate C-1 was deemed pure by microscopic examination, staining techniques and repeated dilutions. High-performance liquid chromatography was used to determine aqueous phase concentrations of carbofuran and carbofuran phenol.

The tolerance of isolate C-1 towards high concentration levels of carbofuran was evaluated in liquid culture. Experiments were conducted using either mineral salts or brain heart infusion (0.02%) media. Following sterilization of the media, carbofuran as Furadan 4F was added to give final concentrations of either 2, 4 or 6 g L^{-1} (a.i.). Cultures were incubated in the dark at 25°C. Sterile controls served as a comparison to distinguish between abiotic chemical hydrolysis and biodegradation activity.

3.3.4 Analysis. Samples of culture media (0.5 mL) were collected periodically and mixed with methanol (1:1), centrifuged (14,000 x *g*) and then filtered thorough a Gelman (Ann Arbor, MI) 0.2 um-pore-size Acrodisc membrane. Following filtration, samples were chromatographed on an LDC Analytical HPLC system consisting of a CM 400 multiple-solvent delivery system with a model 3100 variable wavelength detector set at 270 nm. The pesticide and metabolite components were separated using a 25 cm Whatman C-18 Partisphere column and a mobile phase consisting of methanol and water (75:25).

3.4 Results and Discussion

Isolate C-1 is an aerobic, Gram negative, motile rod. When grown on agar plates, colonies were small, uniform and yellowish to white in appearance. The isolate degraded carbofuran (formulated or analytical grade) through a hydrolysis reaction yielding carbofuran phenol and methylamine (Fig. 3.1). The appearance of the metabolite, carbofuran phenol, was initially detected as an unidentified peak in HPLC chromatograms of culture filtrate. The HPLC retention time for the authentic carbofuran phenol standard was 4.62 min which compared favorably with the retention time of the metabolite (4.61min).

When isolate C-1 was grown in mineral salts media, containing 25 mg L^{-1} carbofuran, neither the parent compound nor the metabolite could be detected following 30 d of incubation. The fate of the carbofuran phenol was not determined. While it is possible that isolate C-1 further degraded this metabolite it is just as likely that carbofuran

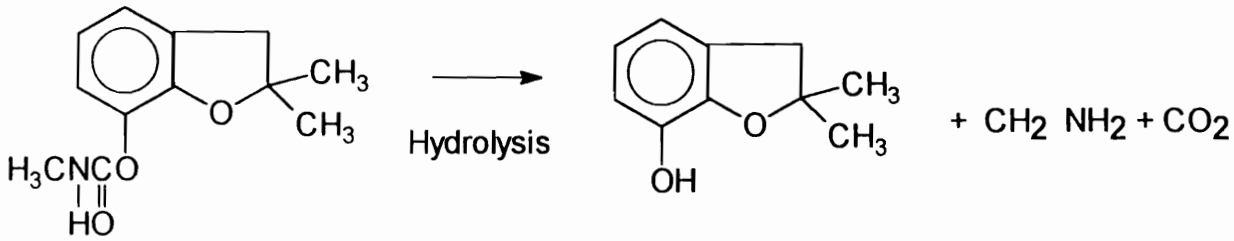

FIG. 3.1. Proposed pathway for transformation of carbofuran to carbofuran phenol as mediated by isolate C-1.

phenol underwent autooxidation or oxidative coupling reactions forming coupled products (Martin and Haider, 1971). Growth of bacteria on one-carbon compounds such as methylamine is quite common. Such bacteria are called methylotrophs many of which are members of well known genera of heterotrophs including *Pseudomonas*, *Bacillus*, and *Vibrio* (Brock and Madigan, 1988).

The isolate C-1 was tested for its ability to degrade high levels of carbofuran in a mineral salts media with and without a readily usable carbon and energy source (BHI). These studies were conducted using carbofuran as Furadan 4F (i.e., formulated pesticide containing surfactants) because the solubility of carbofuran in water is about 700 mg L^{-1} at 25°C. The culture media containing either 2, 4 or 6 g L^{-1} carbofuran were suspensions and not true solutions. Further, it is possible that surfactants served as a carbon and energy source as these compounds are, by federal law, required to be biodegradable.

The culture containing 2 g L^{-1} carbofuran exhibited a short lag period before the onset of rapid degradation (Fig. 3.2). The isolate when grown on mineral salts media only (CMS), degraded greater than 95% of the carbofuran in less than 100 d. When BHI was added to the culture media (CBHI) the isolate required less than 60 d to accomplish the same results (Fig. 3.2). The appearance of carbofuran phenol corresponded to the time at which maximum degradation of carbofuran was observed. The concentration of carbofuran phenol in cultures containing mineral salts media only (CPMS) and the BHI amended cultures (CPHBI) reached peak concentration levels, or about 500 mg L^{-1}, within 100 d. The sterile controls showed no significant loss of carbofuran (CSTER) or significant production of carbofuran phenol (CPSTER). Figures 3.3 and 3.4 show the degradation profile of carbofuran at starting concentrations of 4 and 6 g L^{-1}, respectively. The addition

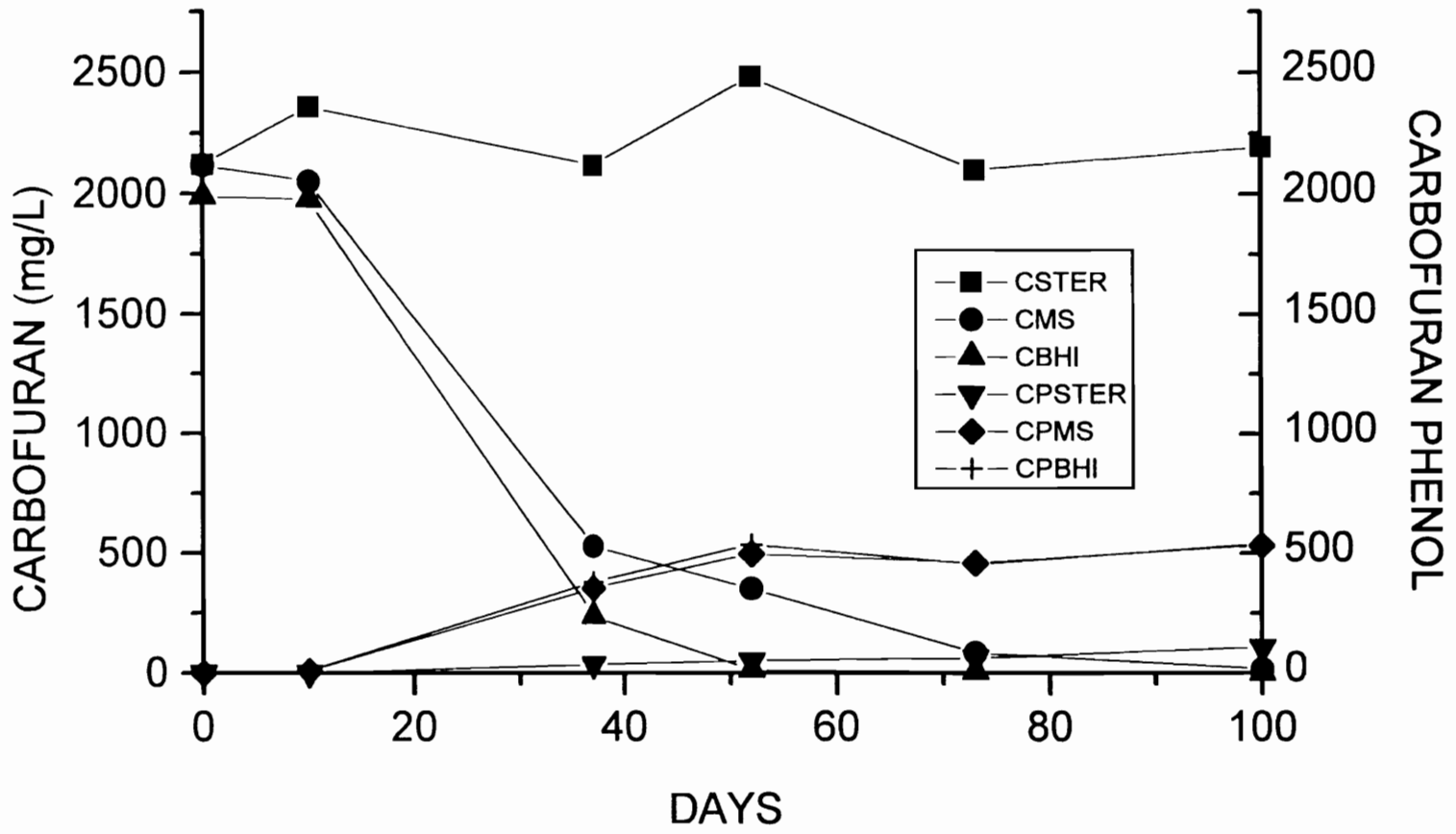

FIG. 3.2. C-1 degradation profile of carbofuran at 2000 mg/L.

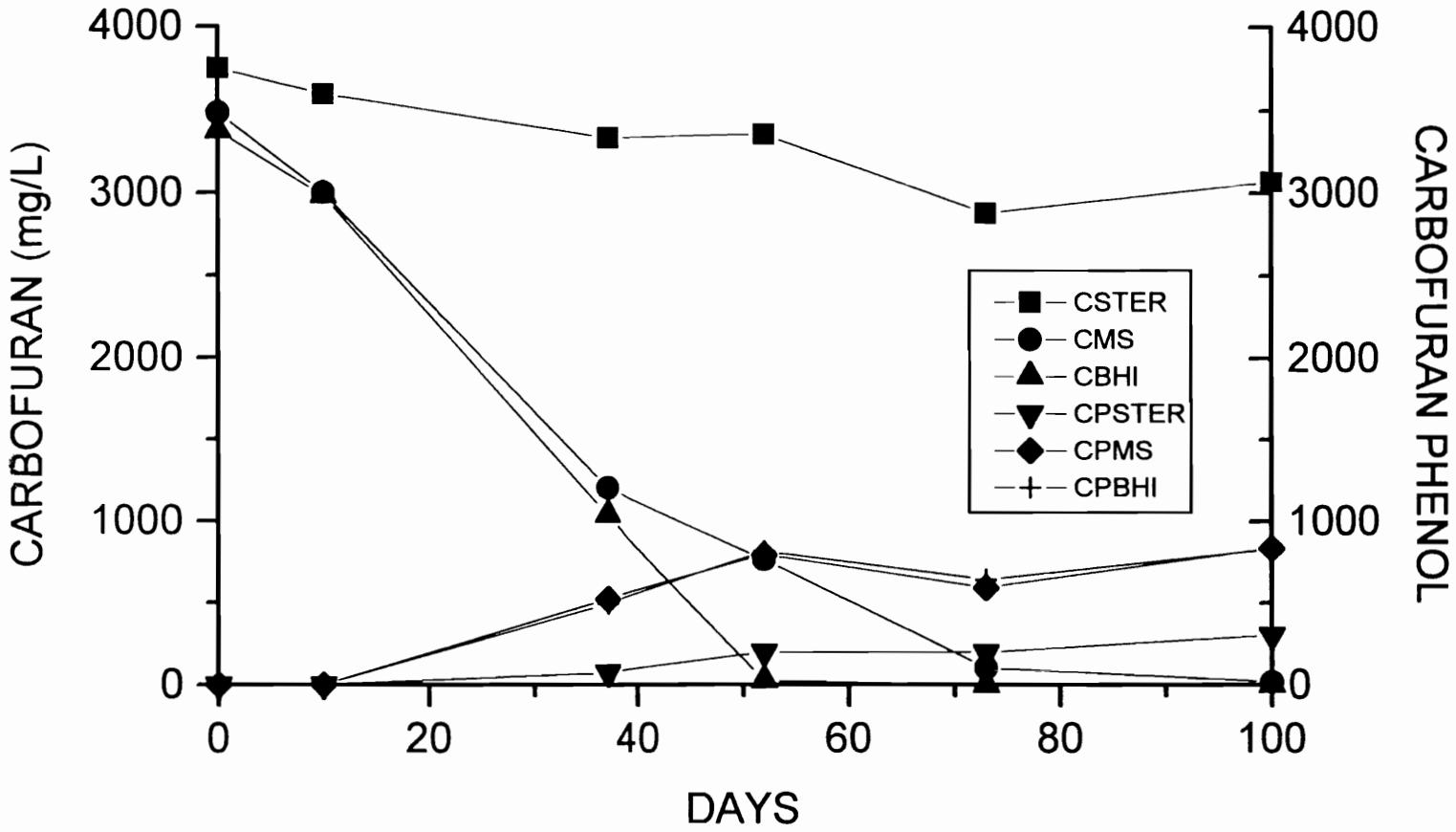

FIG. 3.3. C-1 degradation profile of carbofuran at 4000 mg/L.

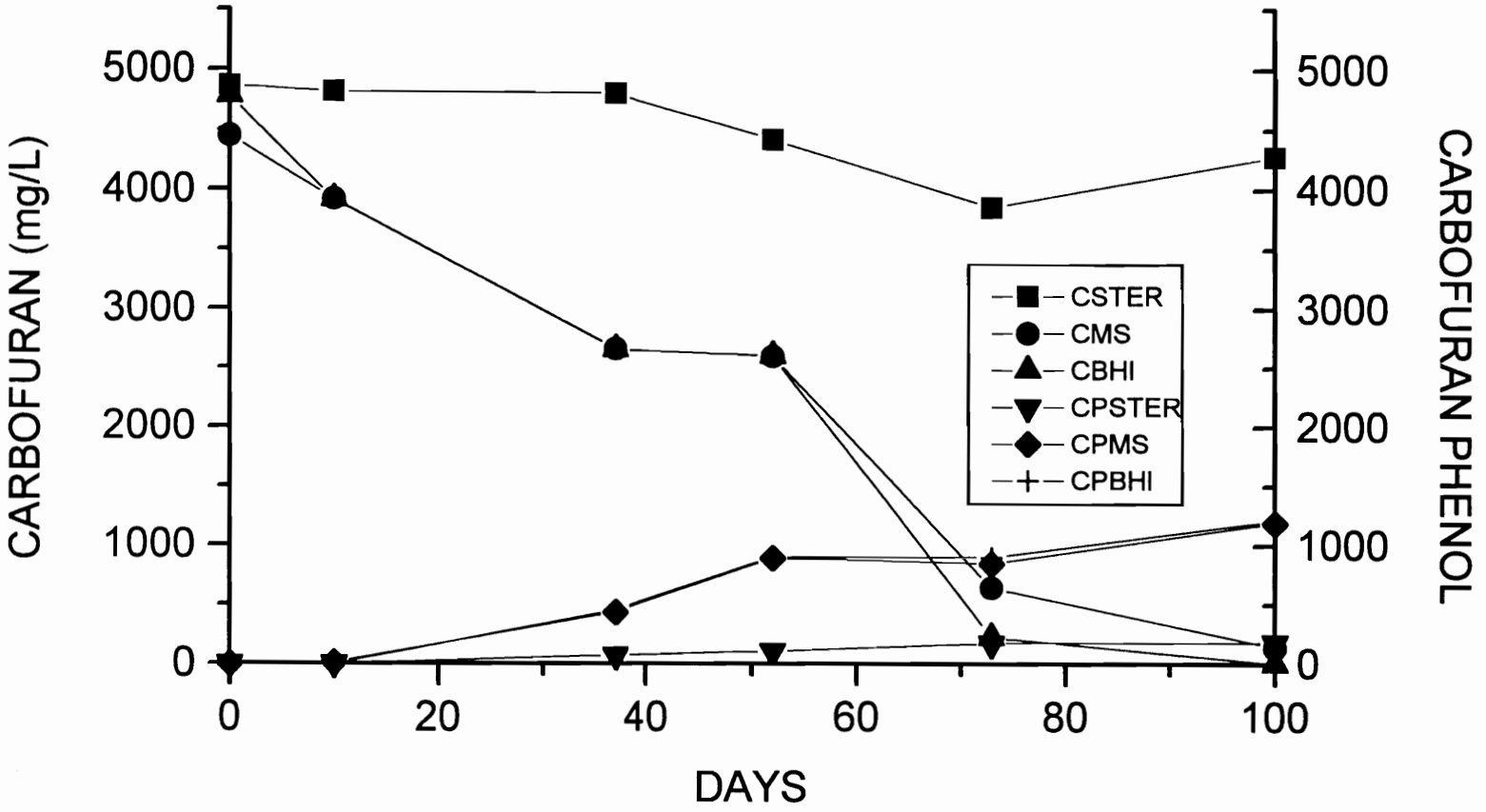

FIG. 3.4. C-1 degradation profile of carbofuran at 6000 mg/L.

of BHI appeared to have little effect on these treatments which indicates that additional nutrient amendments are not required for isolate C-1 to transform carbofuran to carbofuran phenol. By day 100, carbofuran phenol concentrations at the two highest carbofuran concentrations tested reached a plateau at about 1 g L^{-1}. The fact that isolate C-1 survived in the presence of such a high concentration of a phenolic compound is quite surprising in light of the fact that phenols are generally toxic to bacteria. In the presence of high phenol concentrations, membranes are disrupted resulting in cell death. In all treatments, however, cells remained viable, suggesting a high tolerance to carbofuran phenol.

3.5 Conclusion

Isolate C-1 was able to transform carbofuran as Furadan 4F at the upper limits of carbofuran solubility. The isolate, however, appears to have only limited ability to degrade carbofuran phenol. Additional carbon sources do not appear to be required , to enhance degradation, nor do they appear to inhibit carbofuran degradation. The carbofuran degrading ability of C-1 along with the resistance exhibited toward high concentration levels of surfactant and phenolic compounds make this isolate an excellent candidate for use in bioremediation efforts.

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Chapter IV

Degradation of Dicamba by an Anaerobic Consortium Enriched From Wetland Soil

4.1 Abstract

The biodegradability of dicamba was investigated under anaerobic conditions. A consortium consisting of a sulfate-reducer, three methanogens, and a fermenter (identified as a *Clostridium* sp.) was enriched from wetland soil using dicamba as a carbon and energy source. Degradation proceeded through an initial demethylation reaction, forming 3,6-dichlorosalicylic acid, followed by reductive dechlorination, forming 6-chlorosalicylic acid. The finding that the sulfate-reducer in pure culture could not transform dicamba coupled with the observation that the dicamba-degrading consortium could not demethylate dicamba in the presence of high H_2 concentration levels, provided evidence that the fermenter was responsible for demethylation. The consortium was unable to degrade or mineralize the aromatic ring.

4.2 Introduction

Dicamba (3,6-dichloro-2-methoxybenzoic acid) is commonly used as a post emergence herbicide to control weeds in cereal grain crops and to control broadleaf weeds in pastures and rangelands. Because it exists primarily in the dissociated anionic form in soils, dicamba is highly mobile and susceptible to dispersal as a result of both runoff and leaching (3, 5, 13). This chemically stable compound enters non-target environments such

as streams and rivers, sediments, and groundwater. Many of these non-target environments are anaerobic.

The fate of dicamba under aerobic conditions has been extensively investigated. Dicamba is probably degraded in moist soils through biologically mediated processes (16, 17,18). Studies by Smith (16, 17) have shown that dicamba is readily transformed to 3,6-dichlorosalicylic acid (3,6-DCSA) in soil. Krueger et al. (8) established that dicamba could be degraded by pure cultures of bacteria under aerobic conditions. They isolated eight species of bacteria, from soil and water samples with a long history of dicamba exposure, capable of using dicamba as the sole carbon source. They also found that the primary metabolite of dicamba in soil was 3,6-DCSA.

Little is known about the fate of dicamba under anaerobic conditions. Gu et al. (6) was able to show the probable demethylation of dicamba, forming 3,6-DCSA, under methanogenic conditions. Low levels of methane production in wetland soil serum bottle microcosms indicated that the aromatic ring of dicamba was not mineralized. Based on the number of metabolite peaks in HPLC chromatograms of culture medium, these authors suspected that dicamba was undergoing reductive dehalogenation.

Microbial mediated reductive dehalogenation of chlorinated aromatic compounds is now widely recognized as a process that plays a crucial role in restoration of contaminated anoxic environments (9, 12). Over the past 10 years, several organic chemicals including the halobenzoates, (17, 19) chlorinated phenols, (1, 2) and the polychlorinated biphenyls (14) have been studied with respect to reductive dehalogenation. Kuhn and Suflita (9) have reported that aryl reductive dehalogenation has been observed for a few pesticides.

Biodegradation of the phenoxyalkanoic acid herbicide, 2,4-dichlorophenoxy acetic acid (2,4-D), which is similar in herbicidal activity to dicamba and equally as mobile, has been evaluated under methanogenic conditions in environmental samples including pond sediment aquifer material (4), and sewage sludge (11).

Under methanogenic conditions, 2,4-D is known to undergo reductive dehalogenation forming products such as 2-, and 4-chlorophenoxyacetate (9). While most studies have examined degradation of dicamba under aerobic conditions, to the authors knowledge, none has investigated the degradative pathway under strict anaerobic conditions. This study was conducted to elucidate the degradative pathway of dicamba by a methanogenic dicamba-degrading consortium enriched from wetland soil.

4.3 Materials and Methods

4.3.1 Chemicals. Analytical grade dicamba (purity 98.7%) was purchased from Chem Services, Inc. West Chester, PA while 3,6-dichlorosalicylic acid, 3-chlorosalicylic acid (3-CSA), and 6-chlorosalicylic acid (6-CSA) with a purity between 97-99% were provided by Berry & Associates, Inc. Dexter, MI. 3-Chlorobenzoic acid was purchased from Aldrich Chemical Co., Milwaukee, WI (purity 99%). All chemicals were used without further purification.

4.3.2 Culture conditions and microorganisms. A dicamba-degrading consortium was enriched from wetland soil using dicamba as a carbon and energy source. The soil inoculum consisted of a Lawns sandy loam (fine-loamy, mixed, non acid, thermic family of Typic Sulfaquents) collected from a site located in Surry County, VA. Forty ml

of soil slurry (containing 1.4 g solids) was added to a 160-ml serum bottle along with 60 ml of deoxygenated mineral salts medium and 9.3 μmol dicamba. The mineral salts medium, containing 1 ml of a 0.1% resazurin solution, was prepared as described by Boyd et al. (2). The medium was autoclaved for 15 min and maintained under N_2 that was previously passed through hot (300°C) copper fillings to remove traces of O_2. During transfer of the soil slurry inoculum and medium, serum bottles were maintained under a positive pressure of O_2-free N_2. Thick butyl rubber stoppers and aluminum crimp seals were used to close the serum bottles. The dicamba-degrading consortium was transferred every 6 to 8 weeks (over a two year period) based on substrate depletion. Five ml of dicamba-depleted culture was transferred to 160-ml serum bottles along with 95 ml of fresh mineral salts medium (without resazurin) containing 0.1 mmol dicamba and 0.04% yeast extract (filter sterilized). Transfer cultures (incubated at 25°C up to the third transfer and 35°C thereafter) were maintained under a gas phase consisting of 80% N_2 and 20% CO_2.

The enrichment culture technique was used to isolate the sulfate-reducer from the dicamba-degrading consortium. The enrichment medium was a mineral salts medium containing bromoethanesulfonic acid (BES, 20 mM), sulfate (20 mM), and 0.04% yeast extract. Slant agar tubes (25 x 50 mm Bellco anaerobic culture tubes, Bellco Glass Inc., Vineland, NJ) were inoculated with the enrichment culture following the third transfer. The gas phase in the culture tubes consisted of 80% N_2 and 20% CO_2. Following three weeks of incubation at 35°C, selected colonies were transferred to serum bottle cultures.

Degradation experiments, involving the dicamba-degrading consortium, were initiated by transferring 5 ml of substrate-depleted enrichment culture and 5 ml of yeast

extract maintained culture to 160-ml serum bottles (three) containing 90 ml freshly prepared minerals salts medium (without sulfate) plus yeast extract (0.04%) and 0.1 mmol dicamba. Two of the three serum bottle cultures were used for metabolite isolation purposes. A sterile control was prepared by adding 10 ml of sterilized inoculum (autoclaved three times over four days) to 90 ml of mineral salts medium prepared as mentioned above. The cultures were incubated in the dark at 35°C. The headspace gas in microcosm experiments consisted of either 80% N_2 and 20% CO_2 or 80% H_2 and 20% CO_2.

Samples of culture medium (0.5 ml) were periodically collected and stored frozen in glass vials. Dicamba and metabolite concentrations in the culture medium were determined by high-performance liquid chromatography (HPLC).

4.3.3 Metabolite isolation and identification. Serum bottle cultures were filtered (no. 1 filter; Whatman, Inc., Clifton, NJ), acidified (pH 2.0), and extracted three times with 10 ml of ether. The combined extracts were evaporated to 10 ml, dried by passage through Na_2SO_4, and evaporated to 0.2 ml under N_2. Thin-layer chromatography using 0.25-mm precoated silica gel plates (Fisher Scientific, Fairlawn, NJ) was used to separate components. The mobile phase used to separate 3,6-DCSA (R_f = 0.44) consisted of CH_2Cl_2-acetic acid (30:1 [vol/vol]). The mobile phase used to separate 6-CSA (R_f = 0.59) was $CHCl_3$-acetic acid (30:1 [vol/vol]). A UV-lamp was used to visualize 3,6-DCSA and 6-CSA. Metabolites were extracted from silica gel using methanol. A portion of the methanol extract was evaporated under N_2 to dryness in preparation for mass spectrometry (6-CSA only) while the remainder was used to determine the UV spectrum and HPLC retention time. Prior to mass spectral analysis, 6-CSA was derivatized using

diazomethane, forming methyl 6-chloro-2-methoxy benzoate. A Beckman DU-7 scanning spectrophotometer (Beckman Instrument, Inc., Irvine, CA) was used to determine UV spectra. Mass spectral data used to confirm the identity of the methylated derivative of 6-CSA was obtained using a 7070E-HF high-resolution mass spectrometer (VG Analytical, Manchester United Kingdom) with a direct insertion probe at an electron energy of 70 eV.

4.3.4 Analysis. In preparation for HPLC analysis culture samples were thawed, mixed with methanol (1:1), centrifuged (14,000 x *g*), and filtered through Gelman (Ann Arbor, MI) 0.2μm Acrodisc membrane filters. Samples were chromatographed on an LDC analytical HPLC system consisting of a CM 400 multiple solvent delivery system with a 3100 variable wavelength detector set at 271 nm. A 25-cm Whatman C-18 Partisphere column was used to separate components. The mobile phase was 31.7:7.5:58.4:2.4 acetronitrile:methanol:water:acetic acid.

4.4 Results and Discussion

Our dicamba-degrading enrichment culture took only about 30 days to degrade dicamba (Fig. 4.1) even though the starting concentration was ten times greater than that reported by Gu et al. (6). Increased rate of degradation in the enrichment culture was undoubtedly related to factors such as incubation temperature, nutrient availability, and perhaps the time required to acclimate or build up the dicamba-degrading consortium.

Appearance of the demethylated metabolite, 3,6-DCSA, initially detected as an unidentified peak in HPLC chromatograms of culture filtrate, corresponded with the time in which was observed the maximum rate of dicamba disappearance (Fig. 4.1).

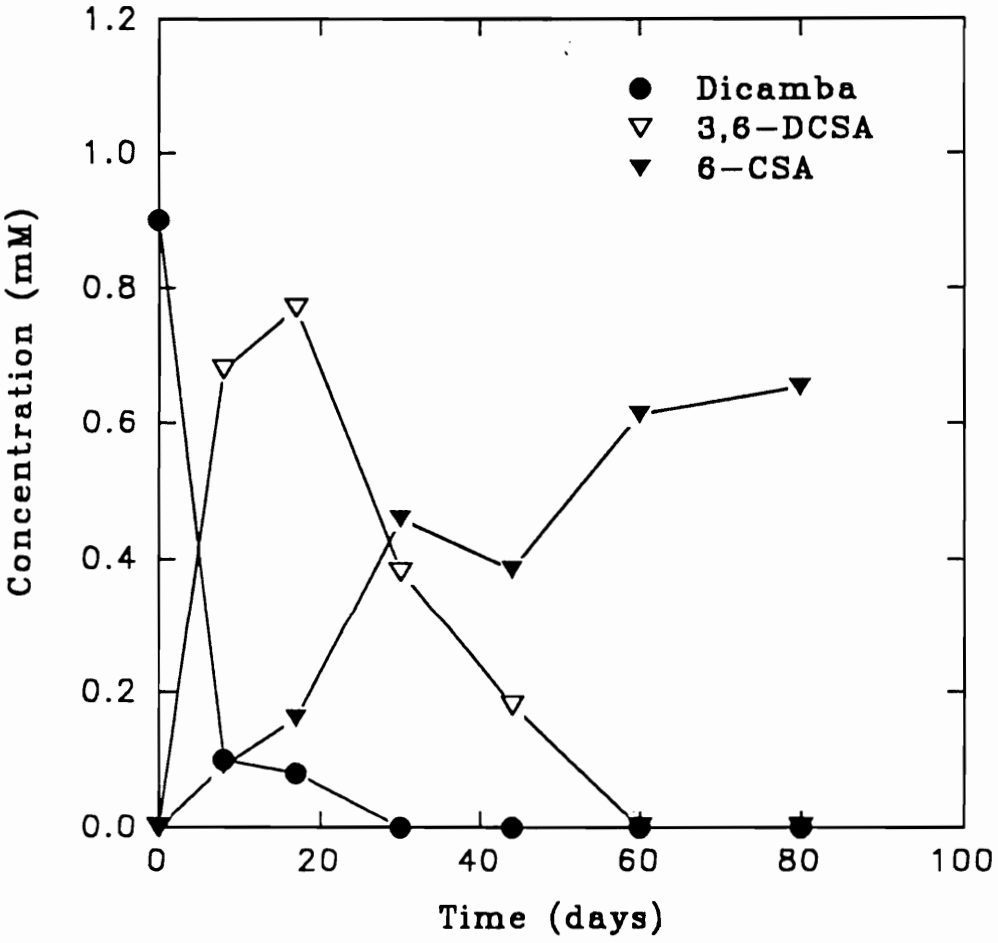


FIG. 4.1. Degradation of dicamba with subsequent production of 3,6-dichlorosalicylic acid (3,6-DCSA) and 6-chlorosalicylic acid (6-CSA).

Demethylation of dicamba was rapid occurring without any apparent lag period. By day 20, 3,6-DCSA production had reached its maximum level while by day 60 the concentration had decreased to undetectable levels. The disappearance of 3,6-DCSA was positively correlated with the appearance of another metabolite, first detected as an unidentified peak in HPLC chromatograms, which was subsequently identified as 6-CSA. While the dicamba-degrading consortium was able to reductively dehalogenate 3,6-DCSA, it was unable to dechlorinate 6-CSA resulting in accumulation of this metabolite in the culture medium. We did not detect a loss of dicamba or production of the metabolites 3,6-DCSA and 6-CSA in the autoclaved sterile control.

The identity of the metabolite 3,6-DCSA was confirmed by comparison with an authentic standard using TLC, HPLC retention time (5.7 min.), and UV absorbance ($\lambda_{max,}$ 221 nm). The second metabolite was believed to be either 3-CSA or 6-CSA. Authentic 6-CSA and 3-CSA standards were analyzed by HPLC. The HPLC retention time recorded for 6-CSA was 4.8 min which corresponded with the retention time observed for the metabolite. The HPLC retention time observed for 3-CSA was 10.3 min. The identity of the second metabolite, 6-CSA was confirmed on the basis of mass spectral analysis (m/z, 200), corresponding to the mass of the methylated derivative methyl 6-chloro-2-methoxy benzoate (Fig. 4.2). The UV absorbance (λ_{max}, 215 nm) and TLC also corresponded with the authentic 6-CSA standard. The proposed pathway for dicamba degradation by the enrichment consortium is shown in Figure 4.3.

Aryl reductive dehalogenation in natural samples often requires long acclimation periods (12). The acclimation period may also be a response to the growth of dehalogenating populations (12). The two year enrichment process that this consortium

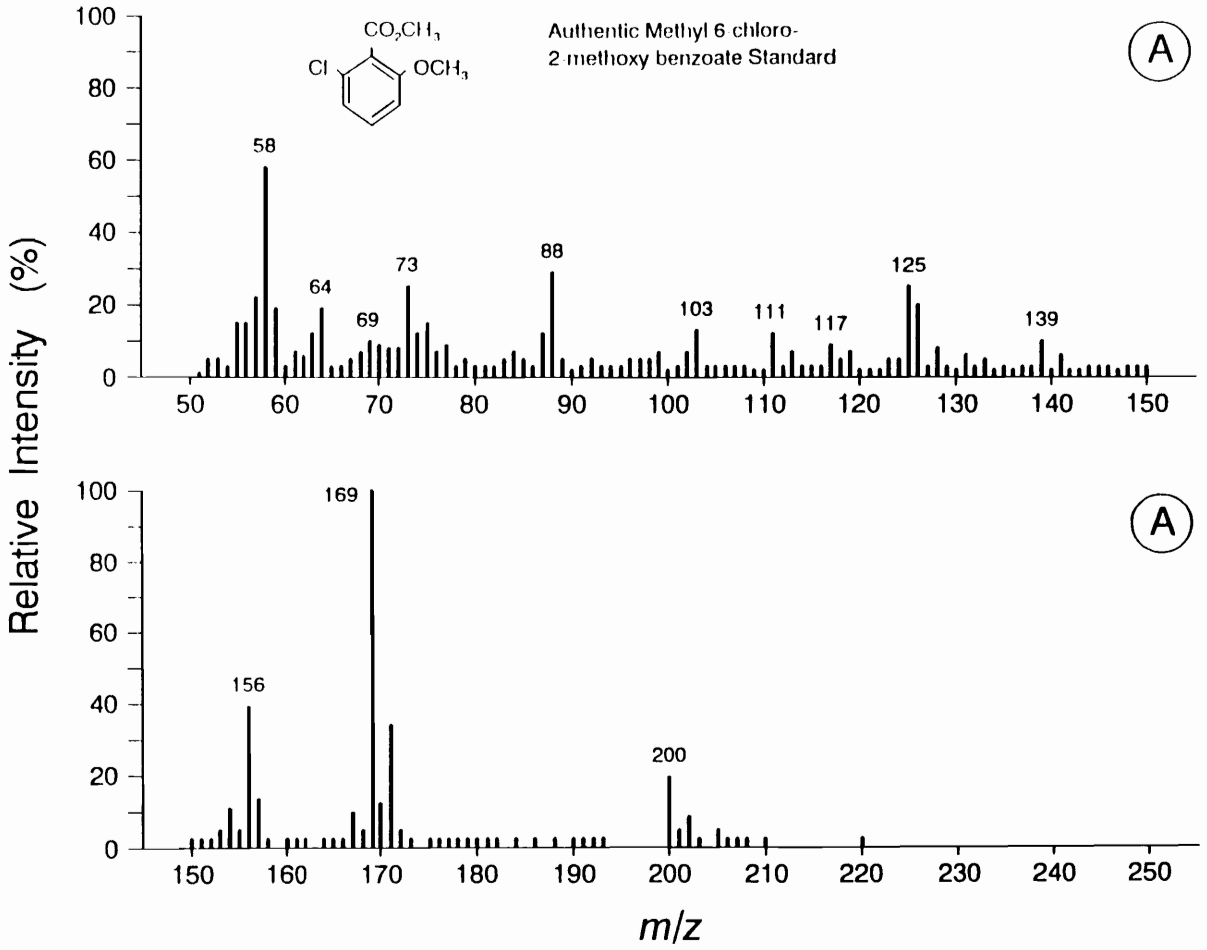
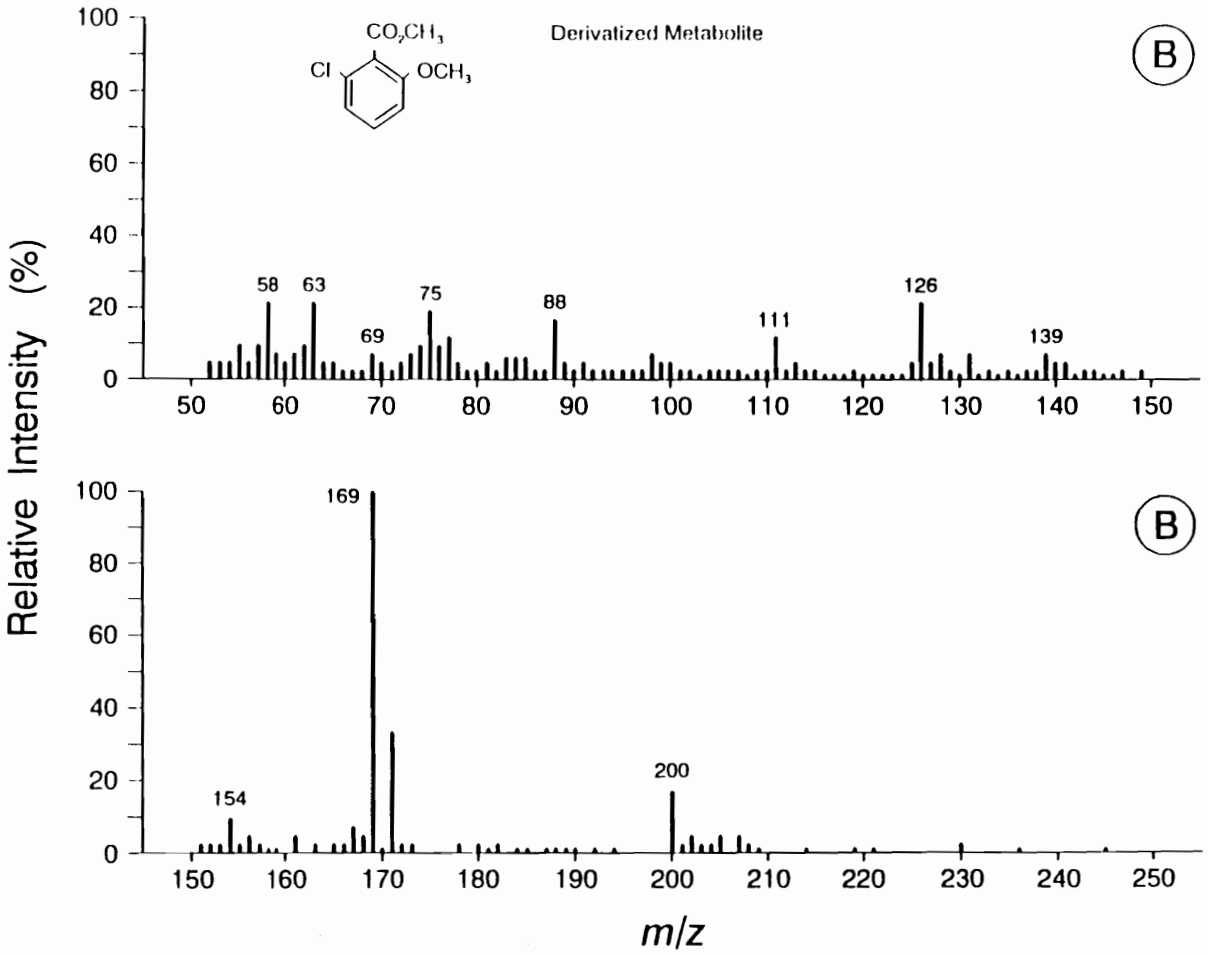


FIG. 4.2. Mass spectra of authentic methyl 6-chlorobenzoate (A) and a derivitized metabolite (B) isolated from serum bottle cultures containing dicamba and the consortium.


Derivatized Metabolite
B
B
Relative Intensity (%)
m/z
58
63
69
75
88
111
126
139
154
169
200

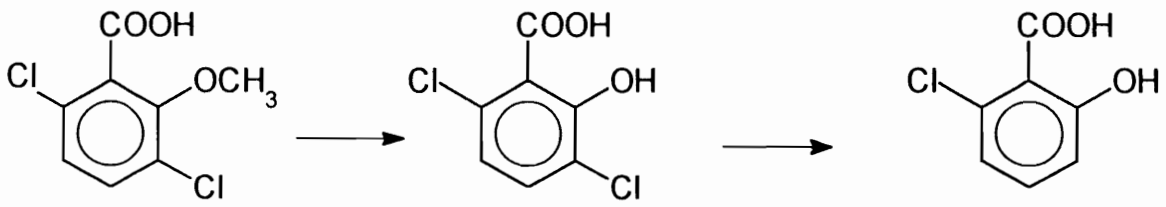


FIG. 4.3. Proposed pathway for transformation of dicamba to 3,6-DCSA and subsequently to 6-CSA.

underwent presumably provided a substrate specific environment in which the acclimation period was reduced. In our enrichment culture system, yeast extract was added to ensure that required growth factors were provided and that a high population of microorganisms would be maintained. It also served as a source of nutrients for the consortium.

Pure cultures capable of aryl reductive dehalogenation are rare (12). Shelton and Tiedje (15) isolated a novel sulfidogen, DCB-1 from a methanogenic 3-chlorobenzoate-degrading consortium, enriched from sewage sludge, capable of carrying out reductive dehalogenation. The reductive dehalogenation capability of (*Desulfomonile tiedjei*) DCB-1 appears restricted to meta substituted benzoates (10). The finding that a sulfate-reducer was responsible for dehalogenation of a chlorinated benzoate in a methanogenic consortium prompted us to investigate the roles of anaerobes in our methanogenic dicamba-degrading consortium.

We were able to isolate a sulfate-reducing Gram negative, motile rod from the enrichment consortium. Hydrogen sulfide was produced, as evidenced by the odor of spent culture medium, when the cells were grown in mineral salts medium containing sulfate, yeast extract, and a headspace gas consisting of H_2 and CO_2. The isolate was able to oxidize lactate, however, acetate was not utilized. Three methanogens were also isolated from the consortium using H_2, CO_2, and acetate as substrates. The methanogens were tentatively identified, by microscopic examination, as belonging to the genera *Methanothrix*, *Methanospirillum*, and *Methanosarcina*. The *Methanospirillum* and *Methanosarcina* species were capable of H_2 utilization with the *Methanothrix* and *Methanosarcina* species capable of acetate utilization. Initial attempts to isolate the fermenter were not successful. The fermenter, tentatively identified as a Clostridium from

microscopic examination, appears to be syntrophically associated with the methanogens, possibly dependent upon these organisms for H_2 removal.

The sulfate-reducer was tested for its ability to degrade dicamba. The isolate was cultured in minerals salts medium with yeast extract and a headspace gas of either N_2-CO_2 or H_2-CO_2, both with and without sulfate. By omitting sulfate from the medium, we attempted to force the electron flow towards reductive dehalogenation of dicamba. Under the conditions specified above, the isolate was unable to demethylate or dechlorinate dicamba over a 60 d incubation period. We also found that the methanogens were unable to transform dicamba.

The initial demethylation reaction yielding acetate, carried out by the dicamba-degrading consortium, appears to be mediated by the fermenter. Dicamba was not demethylated when the consortium was cultured (60 d incubation period) in medium containing yeast extract with a headspace gas consisting of H_2 and CO_2, supporting our contention that the fermenter was responsible for the demethylation reaction. Not surprisingly, hydrogen must be maintained at low concentration levels to allow for demethylation activity. Apparently, demethylation of dicamba is necessary before dechlorination can occur, possibly because the methyl group sterically hinders the dehalogenation reaction. Based on the fact that 3-chlorobenzoate is not dechlorinated by the dicamba-degrading consortium, it is reasonable to conclude that reductive dehalogenation is facilitated by the presence of a hydroxyl group positioned *ortho* to the reaction site.

4.5 Conclusion

The dicamba-degrading consortium transforms dicamba via a demethylation and *meta* positioned reductive dehalogenation reaction to produce 3,6-DCSA and 6-CSA, respectively. The initial demethylation reaction appears to be mediated by the fermenter. Neither the sulfate reducing bacterium or the methanogens were able to transform dicamba when grown in pure culture.

Reductive dehalogenation is a biologically mediated process that undoubtedly plays an important role in determining the fate of herbicides such as dicamba in anaerobic soils and sediments. While it is important that we continue to study dehalogenation using pure cultures to gain insight as to how microbes catalyze reductive dehalogenation, and to elucidate the reaction mechanism, it is also clear that much is to be learned from studying defined enrichment cultures that rely on microbial interactions to bring about reductive dehalogenation. It is likely that aryl reductive dehalogenation in many natural anaerobic environments results from a mutualistic effort involving a diverse microbial population.

4.6 References

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Chapter V

Substrate Specificity of a Dicamba-Degrading Consortium

5.1 Abstract

The substrate specificity of a dicamba-degrading methanogenic consortium was investigated. The consortium, originally enriched from wetland soil using dicamba as a carbon and energy source, is capable of carrying out ayrl reductive dehalogenation. The consortium, consisting of one sulfate reducing bacterium, one fermenter and three methanogens, was able to dehalogenate 3-chlorosalicylic acid (3-CSA) and 2,5-dichlorobenzoic acid (2,5-DCBA) at the C-5 position. Addition of the reducing agent cysteine to a yeast extract amended (0.04%) mineral salts medium containing 3-CSA reduced the rate of dehalogenation compared to medium containing sodium sulfide as the reducing agent. This consortium was unable to dehalogenate either 4-chlorosalicylic acid, 5-chlorosalicylic acid, or 2,5-dichlorophenol. Only limited dehalogenation of 3-CSA and 2,5-DCBA was observed when the sulfate reducing bacterium was cultured alone in yeast extract amended medium, suggesting that in this consortium a mutualistic effort involving a mixed population of bacteria was necessary to efficiently carry out reductive dehalogenation. It appears that this consortium dehalogenates chlorinated benzoates at the *meta* position and requires the presence of at least one other substituent group.

5.2 Introduction

Chlorinated aromatic compounds can enter the environment from a variety of industrial and agricultural sources. The fate of chlorinated aromatics is of concern due to the toxicity and relative persistance of these compounds in the environment. Generally, as the number of chlorine substituents are removed from a chlorinated compound, the less toxic it becomes. It appears that many chlorinated aromatics are only dehalogenated under anaerobic conditions by a process known as reductive dehalogenation. Until recently this process was poorly understood and the organisms responsible for this reaction even less understood. Using sludge as the inoculum, Boyd et al. (1983), were able to show the reductive dehalogenation of such compounds as 2-chlorophenol and 3-chlorophenol.

Madsen and Aamand, (1991) observed the dehalogenation of 2,4,6-trichlorophenol to 4-chlorophenol using a stable bacterial culture enriched from sludge. There results suggest that spore-forming anaerobes were responsible for these reactions. Using pond sediment, Van Dort and Bedard, (1991) were able to show reductive *ortho* and *meta* dehalogenation of 2,3,5,6-tetrachlorobiphenyl (350 μM) to 2,5-dichlorobiphenyl (21 %), 2,6-dichlorobiphenyl (63 %) and 2,3,6-trichlorobiphenyl (16 %) in 37 weeks.

Relatively few studies however have examined defined anaerobic consortia and there role in reductive dehalogenation (Mohn and Tiedje, 1992). Shelton and Tiedje

(1984) isolated a sulfate reducing bacterium (DCB-1) from a consortium enriched from sewage sludge, capable of carrying out reductive dehalogenation. The reductive dehalogenation of strain DCB-1 (*Desulfomonile tiedjei*) appears restricted to *meta*-substituted benzoates (Linkfield and Tiedje, 1990).

Using *D. tiedjei*, DeWeerd and Suflita (1990) examined cell extracts and showed that the rate of dehalogenation was 10-fold greater in *D. tiedjei* extracts prepared from cells grown in the presence of 3-chlorobenzoic acid, suggesting that the enzyme was inducible. While the enzyme was not actually isolated, it was found to be membrane associated.

The mechanism of reductive dehalogenation has been the subject of some controversy. It appears that bacteria can use various halogenated aromatic compounds as electron acceptors. Monn and Tiedje (1990) were able to show that *D. tiedjei* actually conserves energy for growth from reductive dehalogenation coupled to formate oxidation. It would appear that instead of being simply a fortuitous reaction, at least in some dehalogenating bacteria, it is actually an energy yielding reaction.

Recently Griffith et al. (1992) investigated the mechanism of chlorine displacement by *D. tiedjei*. They incubated cell suspensions in D_2O in the presence of 2,5-DCBA. They found that the deuterium exclusively displaced Cl at the C-5 position. These results favor a dehalogenation mechanism that would not allow for proton exchange at other

positions, as would occur if partial ring reduction occured as an initial step. The authors suggest that the dehalogenation mechanism might involve a direct electron addition.

While several chlorinated aromatic compounds can be completely dehalogenated, others are only dehalogenated at certain positions, and still others appear to be resistant to reductive dehalogenation entirely. Dolfing (1992) looked at the Gibbs free energy values and redox potentials for the reductive dehalogenation of various halogenated aromatic compounds with H_2 serving as an electron donor. They showed that redox potentials for the couples of Ar-X/Ar-H for chlorobenzenes, chlorobenzoates and chlorophenols , are in the range of 266-478 mV. Based on these calculations, it would appear that microorganisms can benefit energetically by routing electrons from anaerobic environments to halogenated aromatic compounds as electron acceptors. The objectives of this study were to characterize the substrate specificity in respect to reductive dehalogenation, and to further characterize the relationships within the consortium.

5.3 Materials and Methods

5.3.1 Chemicals. Analytical grade salicylic acid, 3-chlorosalicylic acid, 4-chlorosalicylic acid and 5-chlorosalicylic acid (purity 97-99%) were purchased from Chem Services, Inc. West Chester, PA while 2,5-dichlorobenzoic acid , 2-chlorobenzoic acid and 2,5-dichlorophenol with a purity between 97-99% were provided by Berry & Associates, Inc. Dexter, MI. All chemicals were used without further purification.

5.3.2 Culture Conditions and Microorganisms. A dicamba-degrading consortium, as previously described (Taraban et al., 1993) was enriched from wetland soil using dicamba as a carbon and energy source. The soil inoculum consisted of a Lawnes sandy loam (fine-loamy, mixed, non-acid, thermic family of Typic Sulfaquents) collected from a site in Surry County VA. Fourty ml of soil slurry (containing 40 g solids) was added to a 160-ml serum bottle along with 60 ml of deoxygenated mineral salts medium and 9.3 μmol of dicamba. The mineral salts medium, containing 1 ml of a 0.1% resazurin solution, was prepared as described by Boyd et al. (1983). The medium was autoclaved for 15 min and maintained under N_2 that was previously passed through hot (300°C) copper fillings to remove traces of O_2. During transfer of the soil slurry inoculum, and medium, serum bottles were maintained under a positive pressure of O_2-free N_2. Thick butyl rubber stoppers and aluminum crimp seals were used to close the serum bottles. The dicamba-degrading consortium was transferred every 6 to 8 weeks (over a two year time period) based on substrate depletion. The consortium was maintained in yeast extract amended medium and also in dicamba amended medium plus yeast extract until the current experiments were initiated. Five ml of dicamba-depleted culture and 5 ml of yeast extract maintained culture were transferred to 160-ml serum bottles along with 90 ml of fresh mineral salts medium (without resazurin) containing 0.1 mmol dicamba and 0.04% filter sterilized yeast extract. The sulfate reducer and methanogens were isolated as described earlier (Taraban et al., 1993). Transfer cultures (incubated at 25°C up to the third transfer and 35°C thereafter) were maintained under a gas phase consisting of 80% N_2 and 20% CO_2.

Substrate specificity experiments involving the dicamba-degrading consortium were initiated by transferring 3 ml of culture to 97 ml of fresh mineral salts medium, plus yeast extract (0.04%) and 0.1 mmol of either 3-chlorosalicylic acid, 4-chlorosalicylic acid, 5-chlorosalicylic acid, 2,5-dichlorobenzoic acid, or 2,5-dichlorophenol. Additional cultures were set up for the purpose of metabolite isolation and identification.

5.3.3 Manipulation of consortium. Experiments were conducted with the dicamba-degrading consortium using both 3-chlorosalicylic acid and 2,5-dichlorobenzoic acid along with mineral salts medium plus 0.04% yeast extract. The consortium was exposed separately to additions of 20 mM 2-broroethanosulfonic acid (BES), 20 mM Na_2SO_4 and a vitamin solution consisting of the following in $\mu g\ L^{-1}$: nicotinamide, 500; napthoquienone, 1.4; hematin, 50; nicatimamide, 50; lipoic acid, 50; and thiamine, 50 (DeWeerd et al.,1990). The methanogens (all three) were collectively examined for their possible role in reductive dehalogenation, using 3-chlorosalicylic acid with mineral salts medium with and without yeast extract.

Experiments were also conducted with the dicamba-degrading consortium with mineral salts medium, 0.04% yeast extract, 3-chlorosalicylic acid and reduced with either 0.025% sodium sulfide nanohydrate, 0.025% cysteine or 0.050% cysteine. The headspace gas in microcosm experiments contained either 80% N_2- 20% CO_2 or 80% H_2 - 20% CO_2. All microcosms were incubated at 35^{o}C and maintained in the dark.

Culture medium (0.5 ml) were periodically collected and stored frozen in glass vials until analyzed. Substrate and metabolite concentration in the culture medium was determined by high-performance liquid chromatography (HPLC).

5.3.4 Metabolite isolation and identification. Serum bottle cultures were filtered (no. 1 filter; Whatman, Inc., Clifton,NJ), acidified (pH 2.0), and extracted three times with ether. The combined extracts were evaporated to 10 ml, dried by passage through Na_2SO_4, and evaporated to 0.2 ml under N_2. Thin-layer chromatography using 0.25 mm-thick precoated silica gel plates (Fisher Scientific, Fairlawn, NJ) was used to separate components. The mobile phase used to separate 2-chlorobenzoic acid and salicylic acid (R_f = 0.269) and (R_f = 0.692) consisted of CH_2Cl_2-acetic acid (30:1 [vol/vol]). A UV lamp was used to visualize 2-chlorosalicylic acid and 3-chlorosalicylic acid. The metabolite was extracted from silica gel with methanol. A portion of the methanol extract was evaporated to dryness under N_2 in preparation for mass spectrometry, while the remainder was used to determine the UV spectrum and HPLC retention time. Prior to mass spectral analysis, 2-chlorobenzoic acid was derivatized using diazomethane forming methyl 2-chlorobenzoate. A Beckman DU-7 scanning spectrophotometer (Beckman Instrument, Inc., Irvine,CA) was used to determine the UV spectra. Mass spectral data used to confirm the identity of the methylated derivative of 2-chlorobenzoic acid was obtained using a 7070E-HF high resolution mass spectrometer (VG Analytical, Manchester United Kingdom) with a direct insertion probe at an electron energy of 70 eV.

5.3.5 Analysis. In preparation for HPLC analysis, culture samples were thawed, mixed with methanol (1:1), and centrifuged (14000 x *g*) and filtered through Gelman (Ann Arbor, MI) 0.2 μm Acrodisc membrane filters. Samples were chromatographed on an LDC analytical HPLC system consisting of a CM 400 multiple solvent delivery system with a 3100 variable wavelength detector set at 271 nm. A 25-cm Whatman C-18 partisphere column was used to separate components. The mobile phase was 31.7:7.5:58.4:2.4 acetronitrile:methanol:water:acetic acid.

5.4 Results and Discussion

The dicamba-degrading methanogenic consortium was able to completely dehalogenate 3-CSA forming SA within 50 days (Fig. 5.1). Within an additional 40 days, SA could no longer be detected in the medium. This treatment included 0.04% yeast extract with a gas phase consisting of N_2-CO_2. The consortium was also able to dehalogenate 2,5-DCBA at the C-5 position to 2-CBA under the same treatment conditions within 42 days (Fig. 5.2). Figures 5.3 and 5.4 show the proposed pathway for the reductive dehalogenation of 3-CSA and 2,5-DCBA respectively.

The identity of the metabolite 2-CBA was confirmed by comparison with an authentic standard using TLC (R_f = 0.269), HPLC retention time (6.64 min), UV

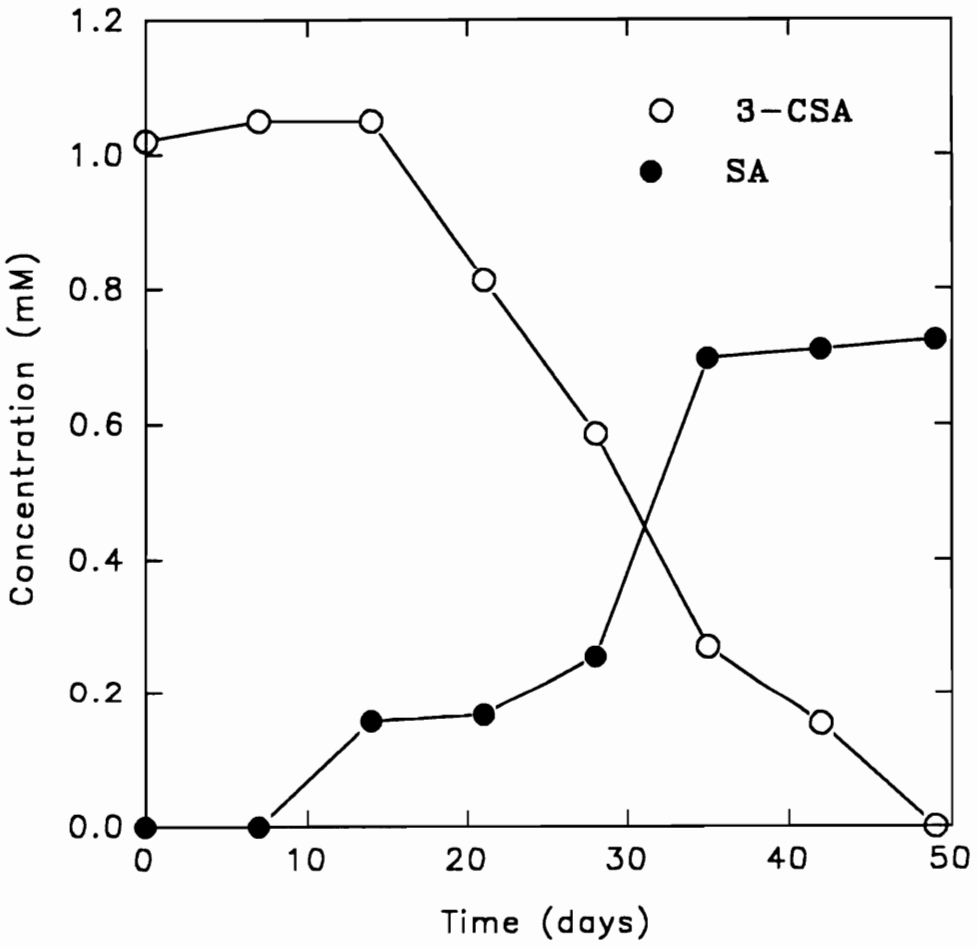

FIG. 5.1. Degradation of 3-CSA with subsequent production of SA.

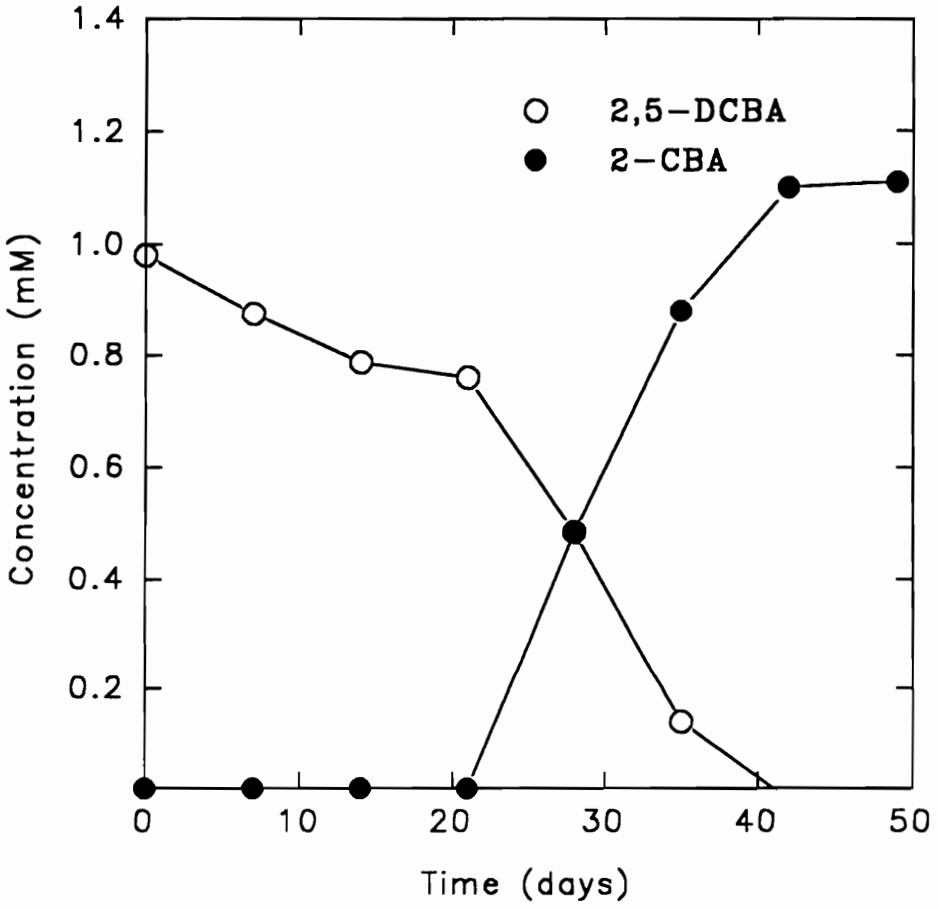

FIG. 5.2. Degradation of 2,5-DCBA with subsequent production of 2-CBA.

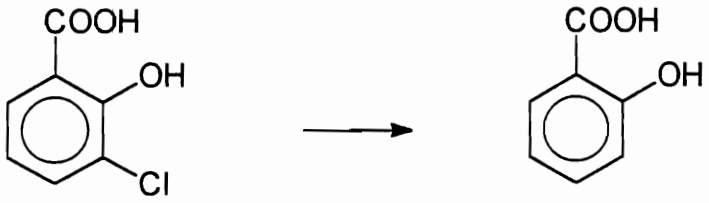

FIG. 5.3. Proposed pathway for the reductive dehalogenation of 3-CSA

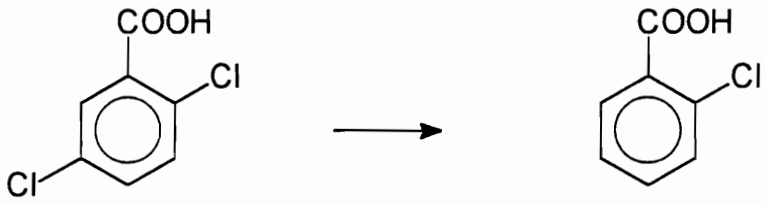

FIG. 5.4. Proposed pathway for the reductive dehalogenation of 2,5-DCBA

absorbance (λ_{max}, 249 nm) and on the basis of mass spectral analysis (*m/z* 271), corresponding to the mass of the methylated derivative methyl 2-chlorobenzoate. The identity of the metabolite SA was confirmed by comparison with an authentic standard using HPLC retention time (6.62 min), UV absorbance (λ_{max}, 270 nm) and with TLC, (R_f = 0.692). The consortium was unable to dehalogenate 4-CSA, 5-CSA, or 2,5-dichlorophenol, and 3-chlorobenzoate (Taraban et al., 1993) within a 60 d incubation period which clearly shows that the dehalogenation reaction was substrate specific. The dependency of structure on dehalogenation activity is not suprising considering that aryl reductive dehalogenation is likely mediated by an enzyme. The fact that the consortium was able to dehalogenate 3-CSA and 2,5-DCBA but not 4- or 5-CSA, or 3-chlorobenzoate suggests an important role for the -OH and -Cl groups. The -Cl substituent group, when attached to benzoic acid, is generally considered electron withdrawing regardless of its position (*meta or para*). The affects of the -OH substituent group on the electronics of the aromatic ring are dependent upon positioning. If the -OH group is *meta* to the -COOH than it is expected to be electron donating. On the other hand if the -OH group is *para* to the -COOH it would be strongly electron withdrawing.

Based on the postulated mechanism that electron addition is directly at the C-Cl position (Griffith et al., 1992), it is reasonable to conclude that dehalogenation occurs at an electron deficient carbon atom. In the case of 3-chlorobenzoic acid, the carbon atom in the 3 position is probably highly electron deficient because of the electron withdrawing capability of the -Cl and the -COOH groups. Based on this reasoning, 3-chlorobenzoic acid should be readily dehalogenated when in fact the dicamba-degrading consortium was unable to effectively dechlorinate this compound. Although the dicamba-degrading

consortium was unable to dehalogenate this compound DCB-1 is perfectly capable of accomplishing this task. While it is likely that electronics of the ring play an important role in terms of aryl reductive dehalogenation, it remains unclear as to the exact electronics of the aromatic ring required for a successful enzymatic mediated aryl dehalogenation reaction.

The consortium was also exposed to 2,5-DCP. 2,5-DCP is similar to 2,5-DCBA with a -OH group at the C-1 position instead of a -COOH group. The consortium did not dehalogenate 2,5-DCP (60 day incubation time), confirming the importance of the carboxyl group at the C-1 position (Fig. 5.5).

Overall the results of the substrate specificity study indicate that the necessary structural elements required for dechlorination mediated by the dicamba-degrading consortium are a -Cl group *meta* to an aryl -COOH group with the accompanying ring carbon rendered even more electron deficient as a result of an *ortho* -OH or *para* -Cl electron-withdrawing group. In *o*-hydroxy benzoic acids, the activation of the -Cl group by a -OH group alone is not sufficient to render it susceptible towards dechlorination nor is the presence of a -Cl substituent group *para* to the 5-chloro position in 2,5-dichlorophenol.

The methanogens were isolated and collectively exposed to 3-CSA and 2,5-DCBA, with and without 0.04 % yeast extract. The headspace gas consisted of both N_2-CO_2 and H_2-CO_2. The methanogens were unable to dehalogenate either 3-CSA or 2,5-DCBA

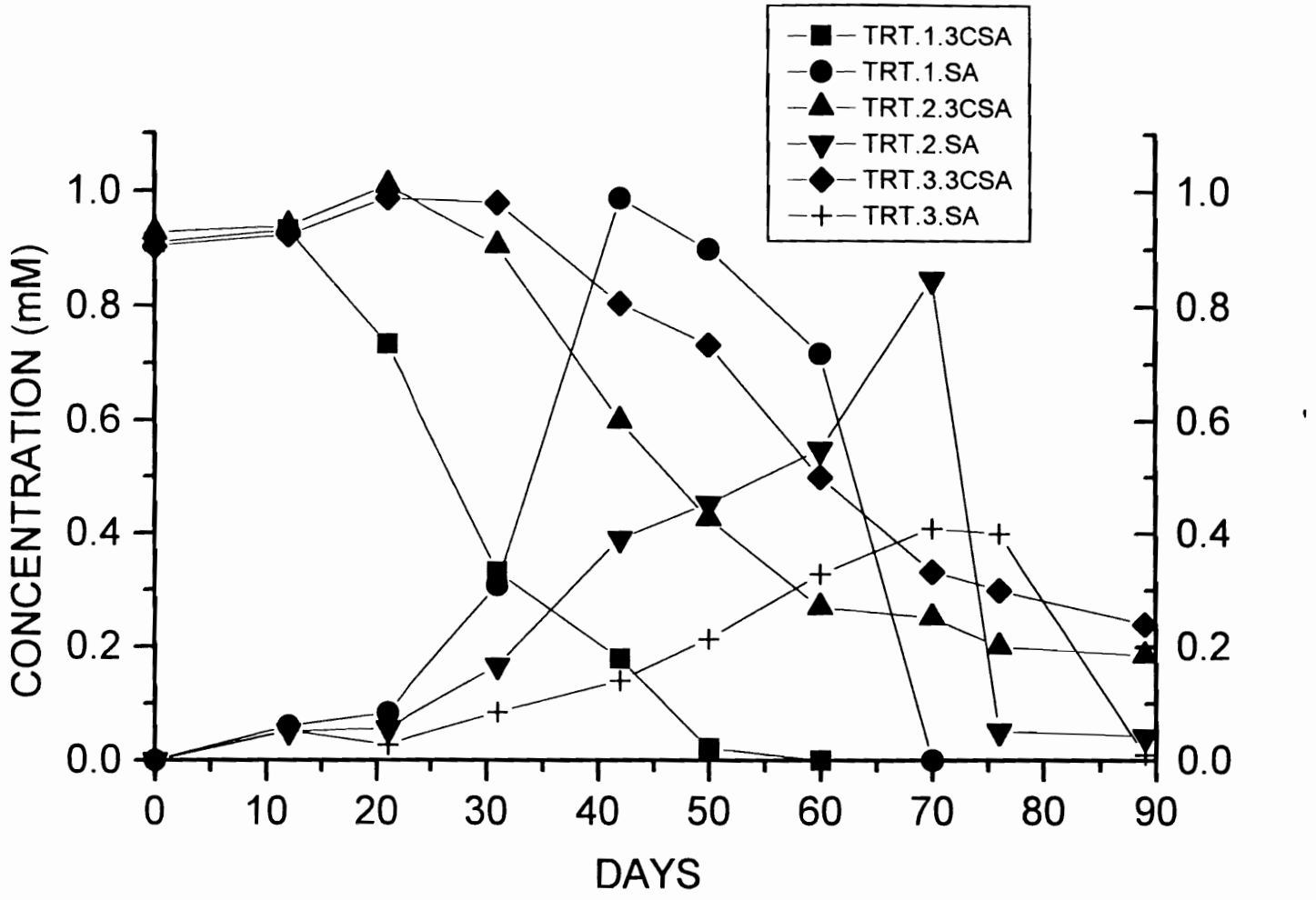

FIG. 5.5. Dehalogenation of 3-CSA in response to eh variation.

under any of the treatment conditions. These results strongly suggest that the methanogens were not solely responsible for the consortium's ability to reductively dehalogenate these substrates.

The sulfate reducer was tested under several treatment conditions to determine it's role in reductive dehalogenation. When cultured in yeast extract amended medium with headspace gas consisting of N_2-CO_2, only limited dehalogenation of 3-CSA and 2,5-DCBA was observed. Approximately 5% of both 3-CSA and 2,5-DCBA was dehalogenated at the *meta* position forming SA and 2-CBA, respectively. In an attempt to increase the efficiency of dehalogenation of these substrates, a vitamin solution was used. The isolate did not respond to the vitamin additions and still only dehalogenated approximately 5% of the substrates.

Sodium sulfate was added, with and without 0.04% yeast extract, and headspace gas consisting of both N_2-CO_2 and H_2-CO_2. Sulfate was used to determine the role of alternative electron acceptors in relationship to reductive dehalogenation. The sulfate reducing bacterium did not dehalogenate 3-CSA or 2,5-DCBA under any treatment condition when the medium was amended with sulfate. It would appear that sulfate is a preferred electron acceptor for the sulfate reducer. When sulfate is present, electrons are transferred through sulfate reduction and away from reductive dehalogenation.

The sulfate reducing bacterium is most likely responsible for mediating the dehalogenation reactions, although it is clear that the entire consortium is required for

efficient dehalogenation. There may be some essential vitamin or co-factor that is being provided by some member of the consortium that is missing when the sulfate reducer is cultured alone.

The consortium was manipulated by additions of BES, sulfate and molybdate to inhibit various members of the consortium. The role of the methanogens was evaluated by adding 20 mM BES , 3-CSA and headspace gas of N_2-CO_2, Methanogenesis is effectively shut down by addition of BES to the medium. Dehalogenation did not occur when BES was added, suggesting that methanogens played a role in carrying out the dehalogenation, possibly by supplying required co factors. It may be that the required reducing conditions are not met unless the methanogens are present and functioning normally. The importance of the methanogens in this consortium with respect to reductive dehalogenation is not well understood. With dicamba as the substrate, the first step involves a demethylation reaction, most likely producing acetate (Taraban et al., 1993). Two of the methanogens in this consortium (*Methanothrix* and *Methanosarcina* species) are acetate utilizers. It may be that in this pathway, the methanogens function to keep the reaction thermodynamically favorable by maintaining acetate at low concentrations. With 3-CSA and 2,5-DCBA there are no intermediates produced that can be utilized by the methanogens. This would not allow for a theory involving metabolite removal for these substrates as may be the case for dicamba degradation.

The consortium was also given 3-CSA, 20 mM Na_2 SO_4 with and without 0.04% yeast extract and a headspace gas of both N_2-CO_2 and H_2-CO_2. It was shown previously

that when the sulfate reducing bacterium was cultured alone with sulfate, it preferred to route electrons toward sulfate reduction and away from reductive dehalogenation. This was also the case when the entire consortium was tested. Dehalogenation of 3-CSA did not occur. These results support the theory that in this consortium, reductive dehalogenation is mediated by the sulfate reducer and not by the fermenter or the methanogens.

Madsen and Aamand (1991) conducted a similar experiment with a methanogenic enrichment culture. Their enrichment culture did reductively dehalogenate pentachlorophenol under methanogenic conditions. When sulfate, thiosulfate and sulfite were added however dehalogenation ceased. Linkfield and Tiedje (1989) also found that the dehalogenation of 3-CBA to benzoic acid was inhibited when *D. tiedjei* was cultured with sulfate.

The consortium was also tested with 3-CSA, 0.04% yeast extract, 10 mM molybdate and headspace gas of both N_2-CO_2 and H_2-CO_2. Molybdate was used to efficiently inhibit the sulfate reducing bacterium by blocking it's respiratory pathway. The fermenter and methanogens were not inhibited by molybdate and should have functioned normally. Dehalogenation of 3-CSA did not occur, again providing evidence that reductive dehalogenation in this consortium is mediated by the sulfate reducing bacterium.

Although evidence is in support of the sulfate reducer being responsible for reductive dehalogenation, it remains unclear the roles played by the fermenter and methanogens. An experiment was conducted in which the medium was reduced using different reductants. Figure 6 shows the effect of initial redox potential variation on reductive dehalogenation of 3-CSA. Using sodium sulfide as the reductant alone, the consortium required 50 days to convert 3-CSA to SA. When cysteine (0.025%) was used to reduce the medium, only 60% of the substrate was dehalogenated at day 50. The third treatment involved cysteine at 0.050% and approximately 25% of the substrate was dehalogenated at day 50.

The standard redox potentials (E_o') for sodium sulfide nanohydrate (0.025%) and cysteine (0.025%) are -225 and -340 mV, respectively. The increased rate of dehalogenation when 0.025% sodium sulfide nanohydrate was used as the reductant may be in response to the close proximity of the redox potential for 3-CSA dehalogenation. As the redox potential is significantly lowered by the addition of cysteine (0.025%), the dehalogenation rate is substantially decreased. This may be due to the increased difference between the redox potential in the medium as compared to the redox potential for 3-CSA dehalogenation. As cysteine is increased to 0.050%, the redox potential is only slightly lowered, however the rate of dehalogenation is significantly slowed. In this case redox potential may not be solely responsible. As the concentration of cysteine was increased, the methanogens in the consortium may have become inhibited. Populations of methanogens could have been affected resulting in an "unbalanced" consortium. Also the effect of cysteine as an energy source for anaerobes has not been fully investigated. Thus,

there is a possibility that some energy is gained from the metabolism of cysteine and thus the rate of reductive dehalogenation was slowed.

5.5 Conclusion

Reductive dehalogenation among anaerobes is quite diverse with respect to the substrates that can be dehalogenated. With stable, defined cultures and with pure cultures, such as *D. tiedjei* dehalogenation appears to be substrate specific. Although the dicamba-degrading consortium efficiently dehalogenates 3-CSA and 2,5-DCBA at the *meta* position, only limited dehalogenation was observed with these compounds when the sulfate reducing bacterium was cultured alone. The consortium appears to require the presence of a carboxyl group at the C-1 position, along with the additional electron withdrawing energies provided by an *ortho* positioned hydroxyl group, or an additional chlorine substituent group positioned *para* to the site of dehalogenation. In this defined consortium, the presence of all members is required to efficiently mediate aryl reductive dehalogenation.

5.6 References

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Chapter VI

Summary

The ability of the carbofuran-degrading isolate (C-1) to remain viable while in the presence of high surfactant and high carbofuran phenol concentration levels is a unique attribute. Although the ability to degrade carbofuran to carbofuran phenol is not novel, the isolates ability to degrade carbofuran at the upper limits of carbofuran solubility (approximately 700 mg L^{-1}) may be. The resistance exhibited by C-1 to carbofuran phenol and surfactants may prove a useful quality. The possibility of transferring resistance to phenol toxicity, to a carbofuran phenol degrading organism, or transferring carbofuran phenol degradative abilities to C-1 would create a complete biosystem for which carbofuran at high concentrations could be effectively degraded.

The potential for in situ bioremediation for carbofuran contaminated aerobic soils is quite probable. Isolate C-1 is easily cultured and mass quantities could be produced quickly and inexpensively. Once applied to the contaminated site, most likely by spray application, C-1 would almost certainly survive. Generally, alternative carbon sources in the environment are a concern for bioremediation efforts. Often the target compound is not degraded because of preference for an alternative substrates, a phenomena referred to as catabolite repression. For isolate C-1, however, carbofuran is degraded even with the addition of alternative carbon sources, such as Brain Heart Infusion, which is an extremely rich nutrient source.

Bioremediation efforts in anaerobic environments presents a difficult challenge. Since anaerobes are highly susceptible to oxygen toxicity, this makes mass production and transport of the microbes to the specified site and inoculation a monumental task. Alternatively, in situ bioremediation with organisms naturally present at the site is possible. The key to in situ bioremediation would be to manipulate the site to achieve maximum degradation efficiency.

Our dicamba-degrading consortium has the ability to demethylate and dehalogenate dicamba at the C-2 and C-3 positions, respectively. Dicamba is transformed to 6-chlorosalicylic acid, which is not further degraded. Much in the same way as 3,6-dichlorosalicylic acid (the first metabolite of dicamba degradation), 6-chlorosalicylic acid would have limited mobility in most soils. The toxicity of 6-chlorosalicylic acid needs to be investigated, to determine the significance of the dehalogenation reaction with respect to 3,6-dichlorosalicylic acid toxicity.

Further research might involve the isolation of a 6-chlorosalicylic acid degrading anaerobe. Although *ortho* positioned halogenations are difficult to remove, if accomplished, the resulting metabolite would be readily degraded in an anaerobic environment.

The dicamba-degrading consortium is significant for reasons other than its ability to degrade dicamba. Defined consortia present a unique opportunity to study the relationship between various bacterial species. The consortium exhibits at least two other *meta* positioned dehalogenation reactions, offering an alternative view of substrate specificity among anaerobic consortia.

It would be interesting to further evaluate the performance of the sulfate reducing bacterium with respect to its apparent ability to dehalogenate certain chlorobenzoates. Although the ability of the sulfate reducer that I isolated is limited in pure culture, with respect to the dehalogenation reactions, it may be possible to enhance the rate by manipulating specific biotic or abiotic factors. Information regarding the manipulation of isolated dehalogenating cultures could provide useful information in terms of successful attempts to bioremediate contaminated soils in situ.

The interactions of the dicamba-degrading consortium were investigated and the results suggest that a complex syntropic relationship exists. Undoubtedly, H_2 transfer between the fermenter and the methanogens is necessary for the initial demethylation reaction to occur. Also it appears that the methanogens may be involved in maintaining thermodynamically favorable conditions, by metabolite removal.

The study of anaerobic consortia is of vital importance to our understanding of reductive dehalogenation in the natural environment. If bioremediation efforts are to be successful in anaerobic environments, then the relationship between anaerobes must be well defined.

Vita

Ronald H. Taraban was born on August 14, 1963 in Wyandotte, Michigan. He attended Taylor Center High School in Taylor, Michigan, where he graduated in 1981. He then attended Eastern Michigan University where he first became interested in microbiology. In 1986 he earned a B. Sc. degree in the department of Biology. He decided to continue his education at Eastern Michigan University, studying general microbiology and fungal pathogens of both plants and animals. In 1990, he received a M. S. degree. Also in 1990, he accepted an assistantship at Virginia Polytechnic Institute and State University, where he studied bacterial degradation of pesticides under both aerobic and anaerobic conditions. In 1993 he received a pH. D. degree from the department of Crop and Soil Environmental Sciences.