This is to certify that the

dissertation entitled

BIOTRANSFORMATION OF NON-VOLATILE ORGANOFLUORINE COMPOUNDS

presented by

Blake Douglas Key

has been accepted towards fulfillment
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Craig Stiddle
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BIOTRANSFORMATION OF NON-VOLATILE ORGANOFLUORINE COMPOUNDS

By

Blake Douglas Key

A DISSERTATION

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ABSTRACT

BIOTRANSFORMATION OF NON-VOLATILE ORGANOFLUORINE COMPOUNDS

By

Blake Douglas Key

This research addresses the potential for biotransformation of non-volatile fluorinated organics, with a focus on sulfonates and carboxylates. Biodegradation of the following model sulfonates was evaluated: difluoromethane sulfonate (DFMS, CHF_2SO_3Na), trifluoromethane sulfonate (TFMS; CF_3SO_3Na), 2,2,2-trifluoroethane sulfonic acid (TES; $CF_3CH_2SO_3H$), perfluorooctane sulfonate (PFOSA; $C_8F_{17}SO_3K$), and 1H,1H,2H,2H-perfluorooctane sulfonic acid (H-PFOSA; $C_6F_{13}C_2H_4SO_3H$). Those compounds with hydrogen at the alpha-carbon degraded under aerobic and sulfur-limiting conditions. DFMS , TES, and H-PFOSA were transformed by a variety of bacteria, including *Pseudomonads*, *Bacillus subtilis*, and *Escherichia coli*. However, *E. coli* was not capable of transforming H-PFOSA. DFMS was completely defluorinated with a stoichiometric yield of fluoride. TFMS and H-PFOSA were only partially defluorinated with equimolar fluoride release from TES and 1-1.4 moles fluoride release from H-PFOSA. Eight volatile and fluorinated byproducts of H-PFOSA were detected by mass spectrometry and atomic emission detection.

Oxygen was required for growth on and transformation of DFMS. Complete shut down of transformation occurred with the removal of glucose or ammonium. Inhibition studies with other sulfur sources suggest that the sulfur-containing byproduct of DFMS transformation is assimilated by existing sulfur assimilating pathways.

Non-competitive inhibition was observed with sulfate, sulfite, methanesulfonate, cystine, and methionine. These results suggest that transformation of DFMS is linked to a sulfur-scavenging system in which DFMS is desulfonated then defluorinated.

Monofluoroacetate (MFA), difluoroacetate (DFA), and trifluoroacetate (TFA) were selected as model compounds for study of anaerobic degradation of fluorinated carboxylates. Denitrifying and sulfate-reducing enrichment cultures defluorinated MFA. A bacterial isolate, designated strain M7, was capable of growth on and defluorination of MFA under aerobic and denitrifying conditions. Strain M7 was unable to degrade DFA or TFA. Phylogenetic analysis of the 16S rRNA sequence shows a 96.0 to 97.5% similarity between strain M7 and the *Bradyrhizobium* species. Two other bacteria with the ability to utilize MFA under aerobic conditions were also capable of MFA degradation under denitrifying conditions, suggesting that this capability is widespread in nature.

Dedicated to my wonderful friend, lover, and lifetime companion Lynda Rae whom I cherish with all of my heart, mind, body, and soul.

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LIST OF SYMBOLS

U	specific substrate utilization coefficient (μmoles /mg protein/hr)
S	rate-limiting substrate concentration (μM)
I	inhibitor concentration (μM)
k	maximum specific substrate utilization coefficient (μmoles /mg protein/hr)
Ks	half-velocity coefficient (μM)
Ki	inhibition constant (μM)
X	total protein concentration (mg/L)
X_0	initial protein concentration (mg/L)
μ	specific growth rate (d^{-1})
μmax	maximum specific growth rate (d^{-1})
b	endogenous decay rate (d^{-1})
t	time in hours (hr)

CHAPTER 1

INTRODUCTION

Environmental Significance of Organofluorine Compounds

The commercial and domestic use of organofluorine compounds has dramatically increased in recent years. These compound are used as propellants, surfactants, agrochemicals, lamprey larvicide, insecticides, adhesives, refrigerants, fire retardants, and medicines [2, 3, 5, 7, 8, 9, 10, 17, 20, 21, 23, 27, 29]. Many of these compounds are used because they are chemically stable and are perceived as more inert biologically and therefore less likely to have an impact on human health or the environment. However, inert molecules tend to persist and accumulate, and they are more difficult to remediate. In addition, several fluorinated organics are subject to at least limited biotransformation under appropriate environmental conditions. Moreover, as discussed in Chapter 2, organofluorine molecules actually do exhibit significant biological effects, as inhibitors of enzymes, cell-cell communication, membrane transport, and processes for energy generation [3, 4, 6, 11, 22, 24, 28].

Choice of Model Compounds

Two categories of organofluorine compounds with particularly useful properties are the fluorinated sulfonates and the fluorinated carboxylates. Perfluorinated sulfonates and perfluorinated carboxylates are used as industrial surfactants and as catalysts in synthetic chemistry. Perfluorooctane sulfonate (PFOSA; $C_8F_{17}SO_3Na$) has excellent

chemical and thermal stability and is important commercially as a surfactant and as a precursor of other fluorinated surfactants and pesticides [1]. Shorter chained perfluorinated compounds, such as trifluoromethane sulfonate (TFMS; CF_3SO_3Na), are used as oligomerization or polymerization catalyst. TFMS is one of the strongest acids known, has great thermal stability, does not release fluoride in the presence of strong nucleophiles, and resists both oxidation and reduction [25]. Fluorinated carboxylates of industrial significance include perfluorooctanoic acid (PFOA), perfluorodecanoic acid (PFDA), and trifluoroacetic acid (TFA). In addition, monofluoroacetate (MFA) is used as a potent pesticide throughout the world.

PFOSA, TFMS and their partially fluorinated analogues difluoromethane sulfonate (DFMS; CHF_2SO_3Na), 1H,1H,2H,2H-perfluorooctane sulfonic acid (H-PFOSA; $C_6F_{13}C_2H_4SO_3H$), and 2,2,2-trifluoroethane sulfonic acid (TES; $CF_3CH_2SO_3H$) were selected as model compounds to represent the fluorinated sulfonates. MFA, difluoroacetate (DFA), and TFA were chosen as model compounds for the fluorinated carboxylates.

Hypotheses

The primary objective of this research was to study the biodegradability of non-volatile fluorinated compounds. Two major classes of fluorinated molecules were investigated: fluorinated sulfonates and fluorinated carboxylates. The following hypotheses were evaluated:

1. Perfluorinated sulfonates and carboxylates are refractory.
2. Hydrogen-substituted fluorinated sulfonates can be utilized as sulfur sources under aerobic and sulfur-limiting conditions.

3. Hydrogen-substituted fluorinated carboxylates can be utilized as carbon sources under aerobic and denitrifying conditions.
4. Biodegradation of hydrogen-substituted fluorinated sulfonates is linked to sulfur assimilation.
5. The capacity for transformation of hydrogen-substituted fluorinated sulfonates and carboxylates is widely dispersed in nature.

Organization of Thesis

Chapter 2 provides a comprehensive review of research on the fate and effects of organofluorine compounds. The review summarizes research on the fate of various fluorinated compounds and the effects that these compounds and their metabolic byproducts have on animals, plants, and microorganisms. In addition, the review identifies areas that need additional attention. Chapter 3 evaluates the biodegradability of representative fluorinated sulfonates using *Pseudomonas* sp. strain D2, an isolate capable of completely defluorinating DFMS under sulfur-limiting and aerobic conditions. Chapter 4 investigates the physiology of transformation of sulfur-containing organofluorine compounds in greater detail using *Pseudomonas* sp. strain D2. Results from Chapter 3 established that a structural or molecular limitation to transformation occurs when the alpha-carbon of fluorinated sulfonates is completely fluorinated (TFMS and PFOSA), while complete transformation (DFMS) or at least partial transformation occurs when the alpha-carbon has a hydrogen atom (TES and H-PFOSA). Chapter 4 provides additional insight into factors that affect transformation of these compounds. In whole cell experiments oxygen was required for growth and transformation of DFMS. While DFMS is not utilized as a source of carbon and energy, it is used as a source of sulfur under sulfur-limiting conditions. Defluorination of DFMS and other fluorinated sulfonates is linked to a sulfur-scavenging system that is

active when both a carbon source (glucose) and a nitrogen source (ammonium) are present in excess. Inhibition studies with other sulfur sources establish a non-competitive pattern of inhibition, suggesting that the sulfur released by DFMS transformation is assimilated through existing sulfur assimilation pathways.

To generalize the above findings, the defluorination ability of other bacteria and yeast were evaluated under aerobic and sulfur-limiting conditions (Chapter 5). Several phylogenetically related Pseudomonads, *Bacillus subtilis*, and *Escherichia coli* degraded at least one of the tested sulfur-containing organofluorine compounds. *Saccharomyces cerevisiae* was not able to transform any, suggesting that the mechanism may be specific to prokaryotes. In addition, H-PFOSA degraded in three different soil types, but not in aquatic samples.

MFA was selected as a model compound for this work. Pseudomonads and other bacteria, as well as some fungi, grow with MFA as a carbon source aerobically, but transformation under anaerobic conditions was not previously evaluated [12, 13, 14, 15, 16, 18, 19, 26, 30]. The first step in aerobic degradation of MFA is hydrolytic attack of the carbon-fluorine bond yielding glycolic acid [12]. Chapter 6 provides evidence that many of the same organisms are also capable of growth on and defluorination of MFA under denitrifying conditions. In addition, aerobic and anaerobic degradation of MFA is demonstrated for a new MFA-degrading isolate, *Bradyrhizobium* sp. strain M7. A hydrolytic mechanism is proposed for both aerobic and anaerobic conditions based upon production of glycolic acid intermediate.

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CHAPTER 2

FLUORINATED ORGANICS IN THE BIOSPHERE

INTRODUCTION

Research investigating the environmental fate of halogenated compounds has largely focused on brominated and chlorinated organics. Fluorinated organics have received less attention because fewer are regulated, their measurement in environmental samples is generally more difficult, and they are perceived as more inert biologically and therefore less likely to have an impact on human health or the environment. Of course, the perception of "inertness" and its environmental significance are debatable: inert molecules tend to persist and accumulate, and they are more difficult to remediate. In addition, several fluorinated organics are subject to at least limited biotransformation under appropriate environmental conditions. Moreover, organofluorine molecules actually do exhibit significant biological effects, as inhibitors of enzymes, cell-cell communication, membrane transport, and processes for energy generation [16, 19, 23, 37, 77, 85, 105].

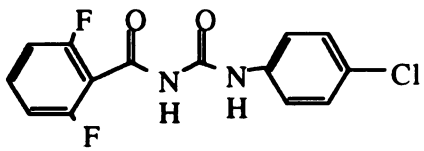
The chemistry of organofluorine molecules is unique because of the properties of fluorine. The fluorine atom has a van der Waals radius of 1.47 Å, a size more comparable to that of oxygen (1.52 Å) than to that of the other halogens (chlorine, 1.8 Å; bromine, 1.95 Å; and iodine, 2.15 Å). Fluorine was once thought to be similar in size to hydrogen (1.2 Å), but it is now considered isosterically similar to a hydroxyl group [90]. Compared to other halogens, fluorine is extremely electronegative having an electronegativity of 4.0 compared to an electronegativity of 3.0 for chlorine, 2.8 for

bromine, and 2.5 for carbon [76]. This high electronegativity confers a strong polarity to the carbon-fluorine bond [40]. The carbon-fluorine bond also has one of the largest bond energies in nature (105.4 kcal/mol); other carbon-halogen bond energies are similar to those of common metabolic intermediates [76]. The "unnatural" strength of carbon-fluorine bonds confers unusual stability. In fact, many fluorinated agrochemicals capable of enzyme inhibition are fluorine-stabilized analogues of the natural enzyme substrate. A dramatic illustration of the strength and stability of the C-F bond is monofluoroacetate, which can withstand boiling with 100% sulfuric acid without any defluorination [82]. For many man-made fluorinated organics, such as the perfluorinated organics, stability is probably related to the fact that the molecular structure is unlike anything currently known in nature.

Fluorine is the most abundant halogen in the earth's crust and ranks 13th in abundance among all elements [75]. This may explain instances of natural organofluorine production. The best known of these natural organofluorine compounds is monofluoroacetate (MFA). MFA is produced by plants in the genus *Dichapetalum*, as well as *Palicourea marcgravii, Acacia georginae, Gastrolobium grandiflorum*, and *Oxylobium* species [48]. The West African plant, *Dichapetalum toxicarium*, also produces ω-fluorooleic acid, ω-fluoropalmitic acid, and possibly ω-fluorocaprate and ω-fluoromyristate [93]. Certain fungi also produce fluorinated organics; *Streptomyces clavus* and *Streptomyces cattleya* produce the fluorine-containing antibiotic nucleocidin and 4-fluorothreonine respectively [48, 93, 94]. *Streptomyces cattleya* is also capable of producing monofluoroacetate [94]. Finally, production of CFC-11, CFC-12, CFC-113, HCFC-21, HCFC-22, tetrafluoroethylene, and chlorotrifluoroethylene has been reported in volcanic gases and drill wells [44, 51]. All of the known biologically-produced fluorinated organics contain only one carbon-

fluorine bond. This contrasts with many man-made fluorinated organics, which often contain many fluorine substituents and may even be fully fluorinated.

Because of their many useful properties, the number of man-made fluorinated organics has dramatically increased over the past few years. According to a report from Business Communications Company, Inc., sales of fluoropolymers (including surfactants, textile finishes, fluoroelastomers, and polymer resins) were expected to increase from $1.35 billion in 1994 to $1.76 billion by 1999 [12]. Tables 2.1 and 2.2 list several examples of aliphatic and aromatic fluorinated compounds. Representative structures of some aromatic fluorinated compounds are illustrated in Figure 2.1. These or related compounds have been used in aerosol propellants, surfactants, refrigerants, plastics, anesthetics, herbicides, insecticides, rodenticides, lampricides, plant growth regulators, medicines, adhesives, fire retardants, and even blood substitutes [4, 7, 15, 16, 26, 31, 32, 52, 54, 63, 67, 68, 69, 78, 92, 98, 99, 106, 115]. Synthesis is accomplished using classical chemical or electrochemical processes, although production of novel fluorochemicals using microbial pathways is also possible [79].



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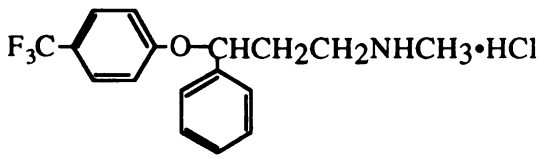

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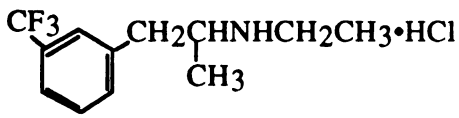

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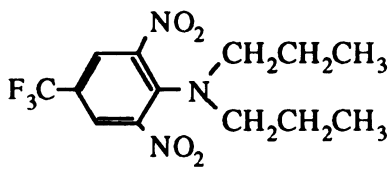
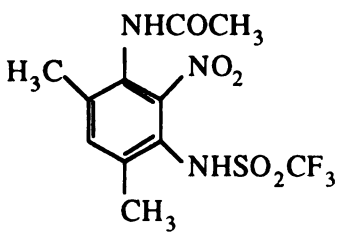
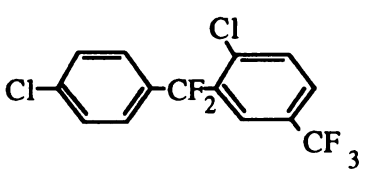


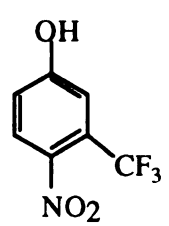
Trifluralin



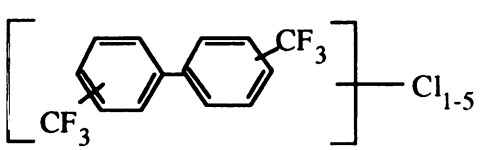
Mefluidide



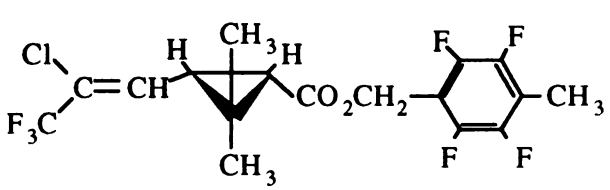
dichloro(trifluoromethyl)
difluorodiphenylmethane



3-Trifluoromethyl-4-nitrophenol



Series of chlorinated homologues
(1-5 chlorines)



Tefluthrin

Figure 2.1. Examples of aromatic fluorinated compounds.

The environmental fate of fluorinated organics depends upon the structure of the molecule. Although some generalizations are possible, research is needed before reliable predictions of fate will be possible for many compounds. Broadly speaking, fluorinated organics can be classified as either volatile or nonvolatile. Volatile fluorinated organics consist primarily of partially or completely fluorinated alkanes, ethers, or amines. Fully halogenated fluorinated organics have very long lifetimes in the atmosphere and can migrate to the stratosphere where they are destroyed by ozone and photolysis [1, 66, 80]. By contrast, volatile fluorinated organics containing one or more hydrogen atoms are susceptible to oxidation by hydroxyl radicals in the troposphere, yielding fluoride, chloride, and partially oxidized organic species, the most significant of which is trifluoroacetic acid (TFA) [100, 109]. TFA is also produced industrially, as are many other commercially important nonvolatile fluorinated organics.

Table 2.1. Examples of fluorinated aliphatic compounds and their applications.

Compound	Molecular Formula	CAS #	Application
Volatile			
CFC-11	$CFCl_3$	75-69-4	Refrigerant
CFC-12	CF_2Cl_3	75-71-8	Refrigerant
HFC-134a	CF_3CH_2F	811-97-2	Refrigerant
HCFC-22	CHF_2Cl	75-45-6	Refrigerant
Methoxyflurane	$CHCl_3CF_2OCH_3$	76-38-0	Anaesthetic
Halothane®	$CF_3CHClBr$	151-67-7	Anaesthetic
Perfluorotributyl-amine	$(C_4F_9)_3N$	311-89-7	Foam blowing agent
Nonvolatile			
Carboxylic Acid			
Monofluoroacetic acid	CH_2FCO_2H	62-74-8	Pesticide
Trifluoroacetic acid	CF_3CO_2H	76-05-1	Reagent
Perfluorooctanoic acid	$C_7F_{15}CO_2H$	335-67-1	Surfactant
Sulfonic Acid			
Trifluoromethane sulfonic acid	CF_3SO_3H	1493-13-6	Catalyist/Reagent
Perfluorooctane sulfonic acid	$C_8F_{17}SO_3H$	2795-39-3	Surfactant
1H,1H,2H,2H-perfluorooctane sulfonic acid	$C_6F_{13}CH_2CH_2SO_3H$	27619-97-2	Surfactant
Sulfonamide			
N-acetic-N-ethyl perfluorooctane sulfonamide	$(C_8F_{17}SO_2N(CH_2COOH)(CH_2CH_3))$	2991-50-6	Surfactant
Sulfluramid	$(C_8F_{17}SO_2NH(CH_2CH_3))$	4151-50-2	Insecticide
Miscellaneous			
Polytetrafluoroethylene	$(-(CF_2CF_2)_n-)$	9002-84-0	Teflon® polymer
Perfluoropolyether	$(-(CF(CF_3)CF_2O)_n-)$	60164-51-4	Lubricant
Zonyl® Alcohol	$(C_8F_{17}CH_2CH_2OH)$	678-39-7	Surfactant

Table 2.2. Examples of fluorinated aromatic compounds and their applications.

Compound	CAS #	Application
Acifluorfen	50594-66-6	Herbicide; CF_3-type
Diflubenzuron	35367-38-5	Insecticide
Fluometuron	2164-17-2	Herbicide; CF_3-type
Fluorobenzoates	N/A	Pharmaceutical and agricultural
Fluorouracil	51-21-8	Chemotherapeutic agents
Fluotrimazole	31251-03-3	Fungicide
Fluoxydine	671-35-2	Chemotherapeutic agents
Flurprimidol	56425-91-3	Plant growth regulator; OCF_3-type
Flutriafol	76674-21-0	Fungicide
Mefluidide	53780-34-0	Herbicide; CF_3SO_3-type
Nucleocidin	24751-69-7	Antibiotic
Tefluthrin	79538-32-2	Insecticide
Trifluralin	1582-09-8	Herbicide; CF_3-type
Trifluorobenzoates	N/A	Pharmaceutical and agricultural

Over the past 15 years, the number of fluorine containing agricultural chemicals have grown from 4% to approximately 9% of all agrochemicals and have increased in number faster than non-fluorinated agrochemicals [16]. These compounds are primarily used as herbicides (48%), insecticides (23%), and fungicides (18%) [16]. A summary of the fluorinated agrochemical usage for 1995 in the United States is provided for in Table 2.3.

Given the widespread production and use of fluorocarbons, it is perhaps not surprising that organofluorine has been detected in the blood of individuals from the general public as well as industrial workers [8, 38]. For workers handling fluoroorganics, organofluorine levels of 1.0 to 71 ppm have been reported in the blood serum [8]. Individuals who have not been exposed to industrial fluorochemicals have had organic fluorine concentrations from 0.0-0.13 ppm. However, it is unclear whether trace amounts of organic fluorine compounds found in human blood samples are from natural or industrial sources.

In the following sections volatile and nonvolatile fluorinated compounds are discussed and a current assessment of the fate and the effect of these molecules in the biosphere is provided.

Table 2.3. Fluorinated chemical application for crop protection in U.S., 1995. Calculated from the Agricultural Chemical Usage - 1995 Field Crops Summary, published by the USDA. (Note: this data only includes applications to corn, cotton, soybeans, and wheat for major producing states)

Fluorinated Agrochemical	Total Applied (1000 kg)	Type of Pesticide
Acifluorfen	675	Herbicide
Bifenthrin	43	Insecticide
Cyfluthrin	59	Insecticide
Diflubenzuron	56	Insecticide
Ethalfluralin	119	Herbicide
Fluazifop-P-butyl	200	Herbicide
Flumetsulam	48	Herbicide
Fluometuron	1273	Herbicide
Fomesafen	286	Herbicide
Lactofen	120	Herbicide
Norflurazon	470	Herbicide
Oxyfluorfen	20	Herbicide
Primisulfuron	19	Herbicide
Tefluthrin	130	Insecticide
Trifluralin	6570	Herbicide
Total	10,088	

FATE AND EFFECTS OF VOLATILE FLUORINATED ORGANICS

Chlorofluorocarbons

Volatile fluorinated organics include the chlorofluorocarbons (CFCs), the hydrochlorofluorocarbons (HCFCs), the hydrofluorocarbons (HFCs), halothane, fluorinated ethers, and fluorinated amines. Chemical formulas, atmospheric lifetime

estimates, and production values for some of these compounds are provided in Table 2.4. CFCs have long been used as refrigerants and aerosols in industrial processes and domestic products. In 1974, CFCs were implicated as agents of depletion of stratospheric ozone by Molina and Rowland [66] and more recently as contributors to global warming [33]. As a result, worldwide production of the CFCs is being phased out under the terms of the Montreal Protocol and its amendments. Nevertheless, CFCs continue to be released into the environment due to past production and continued use. The major sink for CFCs is expected to be stratospheric oxidation. In aerobic aquatic environments, CFCs are recalcitrant, but they are transformed in anaerobic soils and sediments, as well as anaerobic aquatic environments [22, 50, 55, 56, 57, 58, 88, 91]. The expected anaerobic degradation products are hydrochlorofluorocarbons (HCFCs) and hydrofluorocarbons (HFCs).

Hydrochlorofluorocarbons and hydrofluorocarbons

The phase out of CFC production and use has inspired a major research effort to assess the environmental fate of the CFC alternatives. The HCFC and HFC alternatives are one- and two-carbon aliphatics, similar in structure and physical properties to the CFCs, but containing one or more hydrogen atoms. Tropospheric oxidation is expected to be the most significant sink for the HCFCs and the HFCs. The presence of hydrogen makes HCFCs and HFCs more susceptible to tropospheric oxidation than the CFCs and therefore less likely to enter the stratosphere. In the troposphere, HCFCs and HFCs are oxidized by hydroxyl radicals, yielding HF, CO_2, and HCl (in the case of HCFCs) and in some cases trifluoroacetic acid (TFA) [1, 36]. Some volatile fluorinated organics (HCFC-134a, HFC-123, HFC 143a, and halothane) are oxidized by hydroxyl radicals to TFA [62, 109, 110]. It is likely that TFA is produced biochemically when these compounds are oxidized by monooxygenase activities, such as cytochrome P450 in the

liver and methane monooxygenase [17, 59, 70]. Rainfall is believed to be the primary mechanism for removal of TFA from the atmosphere [87, 100, 101, 110].

A minor sink for HCFCs and HFCs is biochemical reduction or oxidation in aquatic systems. Lesage et al. [56] reported reductive transformation of HCFC-123a to chlorotrifluoroethane (HCFC-133 and HCFC-133b) under methanogenic conditions. Oremland et al. [73] have also reported reductive dechlorination of HCFC-123 (CF_3CHCl_2) to chlorotrifluoroethane under anaerobic conditions and degradation of HCFC-21 ($CHFCl_2$) under both aerobic and anaerobic conditions. The products of HCFC-21 oxidation in these experiments were not investigated [73]. Oxidative transformations mediated by monooxygenases are known, but these reactions proceeded slowly compared to the reactions with analogous chlorinated compounds. Oxidative pathways are hypothesized for the transformation of HFC-134a (1,1,1,2-tetrafluoroethane) by rat liver microsomes [70]. DeFlaun et al. [21] reported oxidative transformation of HCFC-21 ($CHFCl_2$), HCFC-141b ($CFCl_2CH_3$), HCFC-131 ($CFCl_2CH_2Cl$), and HFC-143 (CHF_2CH_2F) by *Methylosinus trichosporium* OB3b. HCFC-123 (CF_3CHCl_2), HCFC-142b (CF_2ClCH_3), HFC-134a (CF_3CH_2F), HFC-134 (CHF_2CHF_2), and CFC-11 ($CFCl_3$) were not degraded. Chang and Criddle [17] observed slow and limited oxidation of HCFC-123, HFC-134a, and HCFC-142b by a defined methanotrophic consortium. They also reported oxidation of HCFC-22 (CHF_2Cl), with indirect evidence of product toxicity for HCFC-22 transformation and production of TFA from HFC-134a.

Table 2.4. Fluorocarbon production worldwide

Compound	Molecular Formula	Estimated Atmospheric Lifetime[a] (years)	Total Cumulative World Production (metric tons)[b]	Cumulative for years
HCFC-22	CHF_2Cl	6.7	3,602,365 ± 14,536	1970 to 1994
HFC-134a	CF_3CFH_2	14	85,717 ± 1,194	1990 to 1994
HCFC-141b	$CFCl_2CH_3$	7.1	139,382 ± 1,479	1990 to 1994
HCFC-142b	CF_2ClCH_3	17.8	193,324 ± 1,177	1981 to 1994
CFC-11	$CFCl_3$	60	8,583,266 ± 15,398	1931 to 1994
CFC-12	CF_2Cl_2	105	11,195,032 ± 19,542	1931 to 1994
CFC-113	$CF_2ClCFCl_2$	90	2,244,779 ± 8,797	1980 to 1994
CFC-114	CF_2ClCF_2Cl	185	185,111 ± 923	1980 to 1994
CFC-115	CF_3CF_2Cl	380	167,459 ± 684	1980 to 1994

a Wallington EST 28:320A-326A, and [26].
b AFEAS Report: Production, sales, and atmospheric release of fluorocarbons through 1994 [3].

Fluorinated Anesthetics

Methoxyflurane (2,2-dichloro-1,1-difluoroethyl methyl ether) is used very little in human medicine, but it is used in some veterinary applications [30]. The environmental fate of methoxyflurane is not known, but likely involves tropospheric oxidation by hydroxyl radicals. In rats, methoxyflurane is metabolized by two different pathways [61]. The first is an o-demethylation reaction with release of fluoride to form dichloroacetic acid. The second is hydroxylation at the ß-carbon with release of chloride and the formation of methoxydifluoroacetic acid.

Halothane (1-bromo-1-chloro-2,2,2-trifluoroethane), a bromofluoroalkane is used as a human anesthetic and as a fire extinguishing agent. It is oxidized in the troposphere to

TFA. In fact, TFA currently detected in the environment is thought to have largely originated from tropospheric oxidation of halothane [62].

FATE AND EFFECTS OF NONVOLATILE FLUORINATED ORGANICS

Trifluoromethyl substituted aromatics

The majority of the fluorinated organics used in agricultural applications are trifluoromethyl-substituted aromatics (54.5%) [16]. Of these, trifluralin (1,1,1-trifluoro-2,6-dinitro-N,N-dipropyl-p-toluidine), a pre-emergent herbicide, is the most commonly used in the United States. Williams et al. [111] observed reduction of the nitro groups of trifluralin by rumen microflora, but no loss of the trifluoromethyl group and no cleavage of the ring was observed. Trifluralin had little effect on rumen microbe populations, as determined by volatile fatty acid production and endogenous gas evolution [111].

The pre-emergent herbicides norflurazon and fluridone, both trifluoromethyl-containing aromatics, inhibit carotenoid biosynthesis [16]. This inhibition causes overoxidation of chlorophyll and subsequently the loss of the ability to photosynthesize. Flurprimidol, a fluorine containing plant growth regulator, interferes with Gibberellin (plant growth hormone) biosynthesis [16]. Fluotrimazole and the structurally similar flutriafol, are fungicides that weaken the cell membrane by blocking the carbon-1,4 alpha demethylation step in ergosterol biosynthesis [114].

Several trifluoromethyl-substituted aromatics have been detected in sediments and in fish from the Niagara River and Lake Ontario [52]. Various trifluoromethyl-substituted polychlorinated biphenyls, dichloro(trifluoromethyl)-benzophenone, and

dichloro(trifluoromethyl)-difluorodiphenylmethane were found. These compounds originated at a dump site containing 55,000 tons of halogenated waste, of which 10% was from the production of 4-chloro-(trifluoromethyl)benzene [52]. Dichloro(trifluoromethyl)-difluorodiphenylmethane was present in fish at concentrations as high as 850 ng/g and in sediments from a creek near the dump site at concentrations as high as 35,000 ng/g. The trifluoromethyl-substituted PCBs are believed to bioaccumulate and partition into the sediment more effectively than non-fluorine containing PCBs based on octanol/water partition coefficients (log K_{OW} from 6.8 to 9.0) [52].

The lampricide, 3-trifluoromethyl-4-nitrophenol, has been used since 1958 to combat the sea lamprey problem in the Great Lakes basin. It is released into rivers and streams containing lamprey larvae. From 1991 through 1995 the five year average use of 3-trifluoromethyl-4-nitrophenol was 40,823 kg/year (active ingredient) [20]. Carey and Fox [14] observed defluorination of 3-trifluoromethyl-4-nitrophenol by photolysis, but reported that only 15% was degraded assuming that complete defluorination would yield 3 moles of fluoride.

Fluorinated aromatics

Diflubenzuron (1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl) urea), a urea-based larvicide, inhibits chitin synthesis and the molting process in a broad spectrum of insects. Four fungal isolates (*Fusarium* sp., *Penicillium* sp., *Rhodotoruia* sp., and *Cephalosporium* sp.) are capable of degrading diflubenzuron [89]. The proposed pathway for this transformation is through 4-chlorophenylurea and 2,6-difluorobenzoic acid. Although 4-chlorophenylurea is completely metabolized, 2,6-difluorobenzoic acid is persistent and lethal to soil microbes [89]. Several investigators have also shown that diflubenzuron affects non-target aquatic organisms [112].

Several bacterial isolates defluorinate fluorobenzoic acids [28, 29, 40, 71, 83, 84, 86]. The general pathway for degradation of fluorobenzoic acids is attack by a 1,2-dioxygenase followed by decarboxylation to yield fluorocatechols. These catechols are then subject to ring cleavage followed by defluorination.

Fluorinated sulfonamides

Sulfluramid (N-ethylperfluorooctane sulfonamide), a fluorinated insecticide used to control cockroaches and ants, is deethylated to perfluorooctane sulfonamide in rats, dogs, and rabbit renal mitochondria [5, 45, 60, 85]. Perfluorooctane sulfonamide has not been shown to undergo further transformation, but will most likely be converted to perfluorooctane sulfonic acid (PFOSA; $C_8F_{17}SO_3H$) which is believed to be highly recalcitrant. Schnellman et al. [85] demonstrated that perfluorooctane sulfonamide and sulfluramid are potent uncouplers of oxidative phosphorylation in rabbit renal mitochondria. They also reported that the metabolite perfluorooctane sulfonamide was three times more potent than sulfluramid at uncoupling oxidative phosphorylation. Other fluorinated sulfonamides have demonstrated delayed action toxicity in red imported fire ants [106]. This delayed action toxicity allows the insecticide to be applied in baits which are taken back to the colony by foraging members that distribute it throughout the ant colony, allowing for better control of ants.

Fluorinated sulfonic acid

Perfluorinated sulfonic acids are used as industrial surfactants and as catalysts depending on their chain length. Trifluoromethane sulfonic acid (triflic acid; CF_3SO_3H) is an excellent oligomerization/polymerization catalyst. Triflic acid is one of the strongest organic acids known, has great thermal stability, does not release

fluoride in the presence of strong nucleophiles, and resists both oxidation and reduction [92]. Perfluorooctane sulfonic acid (PFOSA) also has excellent chemical and thermal stability. PFOSA is important commercially as a surfactant and as a precursor of other fluorinated surfactants [2]. Unfortunately, production values for most of the perfluorinated compounds are considered proprietary information by manufacturers and are not disclosed to the public [34]. Perfluorooctane sulfonic acid and triflic acid are resistant to biological attack. However, a surfactant similar to PFOSA, 1H,1H,2H,2H-perfluorooctane sulfonic acid (H-PFOSA), was partially degraded by a Pseudomonad under aerobic and sulfur-limiting conditions yielding 1-2 moles fluoride per mole of H-PFOSA (Chapter 3). The degradation of H-PFOSA produced several volatile fluorinated compounds that have not yet been identified. 2,2,2-Trifluoroethane sulfonate (TES) was also partially degraded with equimolar release of fluoride (Chapter 3). Another fluorinated sulfonate, difluoromethane sulfonate (DFMS; CHF_2SO_3Na), was completely metabolized by this Pseudomonad yielding stoichiometric amounts of fluoride (Chapter 3). Transformation of DFMS, TES and H-PFOSA was subsequently observed with *Bacillus subtilus* and *Escherichia coli* (Chapter 5). However, *E. coli* was not capable of utilizing H-PFOSA. Evidence of H-PFOSA degradation was also observed in soil incubations. This and other work suggests that the transformation of fluorinated sulfonates requires the presence of hydrogen.

Although PFOSA is resistant to metabolism, it is not biologically inactive. For example, PFOSA was shown to inhibit gap junction intercellular communication (GJIC) in rat liver epithelial cells cultured in vitro [105]. In addition, Gadelhak [37] showed that perfluorooctane sulfonic acid was an uncoupler of phosphorylation in rat liver mitochondria. Although PFOSA alone was not as potent of an uncoupler as perfluorooctane sulfonamide, when PFOSA was ion-paired with various monoamines,

polyamines, and phospholipids, the effect of uncoupling was in some instances as high as that of perfluorooctane sulfonamide [37].

Fluorinated alcohols

While some of the perfluorinated organics undergo limited biotransformation, none undergo extensive defluorination. An example of a highly fluorinated molecule that has shown some limited defluorination is 1H,1H,2H,2H-perfluorodecanol. 1H,1H,2H,2H-perfluorodecanol was metabolized first to 2H,2H-perfluorodecanoic acid and then to perfluorooctanoic acid (PFOA) in adult male rats [47]. Hagen et al [47] suggest that the overall reaction is production of PFOA with the release of 2 moles of fluoride per mole of 1H,1H,2H,2H-perfluorodecanol. PFOA is metabolically stable in rats [72] and has been found in the blood serum of humans [47].

Fluorinated carboxylic acids

Monofluoroacetate (MFA) is one of the most toxic substances known, based on a lethal dose (LD_{50}) of 0.7-2.1 mg/kg for man [6]. Its toxicity is due to "lethal synthesis" of fluorocitrate which inhibits the aconitase enzyme of the Kreb's cycle [77] although recent investigations implicate fluorocitrate as a "suicide" substrate instead of a competitive inhibitor [19]. Given that certain plants can produce MFA, it is not surprising that several microorganisms can metabolize MFA. Pseudomonads and other bacteria, as well as some fungi, have been shown to grow with MFA and monofluoroacetamide (a systemic pesticide and rat poison) as a carbon source aerobically [39, 40, 41, 42, 53, 64, 65, 97, 108]. The first step in degradation of MFA has been shown to be a hydrolytic attack of the carbon-fluorine bond yielding glycolic acid [39]. Many of the same organisms are also capable of growth on and

defluorination of MFA under denitrifying conditions (Chapter 6). Gregg et al. [43] demonstrated defluorination of MFA by genetically modified rumen bacteria *Butyrivibrio fibrisolvens*. This was accomplished by transferring the plasmid responsible for MFA defluorination from *Moraxella sp.* strain B into *B. fibrisolvens.* We know of only one other report of anaerobic MFA degradation. Visscher et al. [107] reported reductive dehalogenation of MFA to acetate, under methanogenic conditions, but this transformation was not reproducible in subsequent investigations [74]. MFA has been shown to inhibit methanogenesis in anaerobic digestor sludge, rumen fluid, and freshwater mud [27]. Another fluorinated carboxylate, the herbicide flupropranate ($CHF_2CF_2CO_2H$), is persistent in soils [113].

Other fluorinated organic acids of industrial significance include perfluorooctanoic acid (PFOA), perfluorodecanoic acid (PFDA), and trifluoroacetic acid (TFA). No evidence of PFOA or PFDA transformation has been reported. Although, PFOA and PFDA are not metabolized they have been found to inhibit gap junction intercellular communication (GJIC) in rat liver epithelial cells and human kidney epithelial cells at concentrations of 100 and 250 μM respectively [23]. The inhibition of GJIC has been implicated in tumor promotion during carcinogenesis [49, 104, 116], teratogenesis [103], and reproductive dysfunction [9, 102, 117].

Significant hydrolytic defluorination has so far been observed only for monofluorinated molecules. Another biotransformation of importance is decarboxylation. Meyer and O'Hagan have demonstrated decarboxylation of 3-fluoropyruvate to MFA by cell-free extracts of *D. cymosum* [65]. In addition, Chauhan et al [18], have demonstrated decarboxylation of TFA by *Azoarcus tolulyticus* TOL-4. An intensive international effort is currently underway to explore the fate and effects of TFA, and some results of this effort are summarized in the next section.

A CASE HISTORY - TRIFLUOROACETIC ACID

Three of the chlorofluorocarbon (CFC) alternatives, HCFC-123, HCFC-124, and HFC-134a are expected to yield TFA as an atmospheric degradation product (Figure 2.2) [25, 100, 109, 110]. As a result, TFA is expected to become a ubiquitous low-level global contaminant. Estimates based on 100% conversion of HCFC-123, and HCFC-124 and 20% conversion of HFC-134a to TFA predict 0.1 μg/L TFA in rainwater [13] although it is possible that TFA levels in urban areas could reach levels as high as 2 to 20 μg/L [87]. Average TFA concentrations of 50 pg/m^3 in air, 100 ng/L in rain water, and 140 ng/L in surface waters have been reported [35]. TFA is concentrated in plant leaves and it has the potential to accumulate in ecosystems such as saline lakes, vernal and temporary rain pools, closed basin lakes, playa lakes, and aestival ponds and certain prairie lakes that exhibit high evaporative potential [100, 101]. Environmental factors such as climate, geology, topography, biota, and time are factors affecting evapoconcentration of solutes. TFA adsorbs weakly to most soils, and, therefore, soils are not expected to be a permanent sink for TFA. However, adsorption is favored in low pH soils which are enriched with iron and aluminum oxides [24]. Photo-oxidation of TFA-iron complexes by near-UV radiation in clouds and the ocean was dismissed because of the low concentrations of iron in these environments [13]. Sinks for TFA would include groundwater due to leaching from soils and vegetation due to bioaccumulation [13]. TFA can be degraded by the enzyme acetyl-CoA synthetase, but rates are low even with high concentrations of enzyme and substrate (> 10 mM) [27]. As a result, this pathway is unlikely in the environment where TFA is at nM levels [27]. Hydrolysis of TFA is also an unlikely mechanism for degradation due to the extreme stability of the carbon-fluorine bond and the shielding effect of three fluorine atoms on the carbon atom.

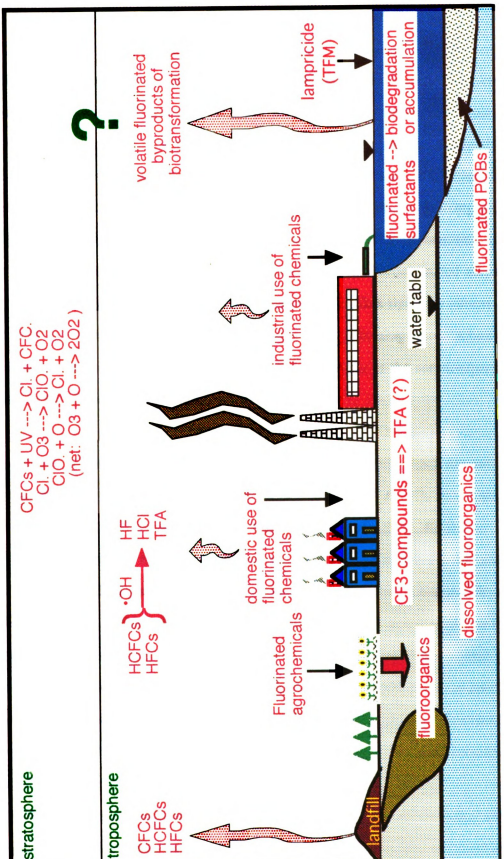
Even small quantities of TFA in solution can influence the pH of unbuffered solutions because TFA behaves like a strong inorganic acid at environmental pH [13]. Bioaccumulation in animals is thought to be unlikely due to a low octanol/water partition coefficient (log K_{ow} = -4.21) [13], but TFA can accumulate in plants through root uptake. Estimates of the concentration factor give approximately 10-32 times the soil concentration in plant leaves with virtually no degradation in the plant [13, 96]. Vascular plants seem to be affected by TFA when bioaccumulation through roots to leaves occurs, however, little toxicity was shown for seed germination [96].

Toxicological tests have been performed on five species of algae. Three species of microalgae (freshwater diatom *Navicula,* marine diatom, *Skeletonema*, and freshwater blue-green *Anabaena*) showed no toxic effects from TFA concentrations up to 1000 mg/L. The freshwater green alga *Chlorella sp.* also showed no toxicity up to 1200 mg/L. A second freshwater green alga, *Selenastrum capricornutum*, exhibited toxicity at concentrations as low as 0.36 mg/L [96]. Bott and Standley reported that TFA did not significantly affect the metabolism of acetate by microbial communities at environmentally expected concentrations [11]. However, Visscher et al. reported inhibitory effects on methanogenic activity at TFA concentrations $\geq$ 1μM [107]. TFA appears to be non-mutagenic in bacteria [10, 81].

The LD_{50} of TFA is reported to be between 200-400 mg/kg (oral exposure to rats), and sodium TFA is only slightly toxic when administered intraperitoneally to mice with no deaths at doses up to 5000 mg/kg [81]. Trifluoroacetic acid does not interfere with homeostasis of rat liver epithelial cell by inhibiting gap junction intercellular communication (GJIC) [105].

Although defluorination of monofluoroacetate by bacteria is well established, few reports of biodegradation of trifluoroacetic acid are available. Visscher et al. [107] reported reductive defluorination of TFA under methanogenic and sulfate-reducing conditions. They report that TFA is sequentially defluorinated to difluoroacetic acid (DFA), monofluoroacetic acid, and finally to acetic acid. Under aerobic conditions, Visscher et al [107] observed production of fluoroform (CHF_3). However, these results with TFA were not reproducible in subsequent experiments leading the investigators to conclude that TFA is widely recalcitrant to biodegradation [74]. Chauhan et al. [18] have demonstrated that *Azoarcus tolulyticus* TOL-4 is capable of decarboxylating TFA. Cells were grown under denitrifying conditions with toluene as the carbon source and then incubated aerobically without nitrate. No fluoride or volatile fluorinated products were detected [18]. The fate of the trifluoromethyl moiety of TFA is unknown. This is also true of most other trifluoromethyl-substituted compounds. Photolysis of the trifluoromethyl moiety has been reported for trifluoromethyl-benzoate and 3-trifluoromethyl-4-nitrophenol [14, 95]. Taylor et al. [95] observed equimolar concentrations of fluoride from trifluoromethyl-benzoate. Carey and Fox [14] observed defluorination of 3-trifluoromethyl-4-nitrophenol by photolysis, but reported that only 15% was degraded assuming that complete defluorination would yield 3 moles of fluoride. In both of these cases there appears to be only partial defluorination of the trifluoromethyl moiety. Of particular interest is the fate of these trifluoromethyl groups and their photo-degradation products and the role of photolysis as a sink for these compounds. In the absence of photolysis, it seems plausible that the trifluoromethyl group remains intact during biotransformation of trifluoromethyl-substituted aromatics, possibly contributing to the TFA load on the environment.

Figure 2.2. Biogeochemical cycling of organofluorine compounds.


stratosphere
CFCs + UV ---> Cl. + CFC.
Cl. + O3 ---> ClO. + O2
ClO. + O ---> Cl. + O2
(net: O3 + O ---> 2O2)
?
troposphere
CFCs
HCFCs
HFCs
HCFCs
HFCs
·OH
HF
HCl
TFA
volatile fluorinated byproducts of biotransformation
lampricide (TFM)
domestic use of fluorinated chemicals
industrial use of fluorinated chemicals
Fluorinated agrochemicals
landfill
CF3-compounds ==> TFA (?)
water table
fluorinated --> biodegradation surfactants or accumulation
fluoroorganics
dissolved fluoroorganics
fluorinated PCBs

SUMMARY AND CONCLUSIONS

It is clear that fluorinated molecules are unique with regard to their physical, chemical, and biological properties and they do not fit with the usual paradigm of chemicals of environmental concern. For example, the fluorinated surfactants have distinctly different characteristics to those of their hydrocarbon counterparts. Fluorinated surfactants are simultaneously oleophobic and hydrophobic, but solubilizing moieties such as carboxylic acids, sulfonic acids, phosphates, and quaternary ammonium groups can change their solubility in water [4, 54]. In addition, the perfluorinated alkyl chain is more "rigid" due to fluorine atoms on the molecule [46]. This rigidness almost certainly interferes with molecule/enzyme interactions, protecting fluorocarbon molecules from biological attack. Currently, little is known of the sorption/partitioning properties of fluorinated organics.

Instances in which the carbon-fluorine bond is ruptured by direct attack are only known for a rarely observed reductive defluorination of tri-, di-, and monofluoroacetate [107] and for hydrolytic defluorination of monofluorinated organics [39]. Reductive defluorination seems to require extreme and uncommon reducing conditions, and so far has been observed only under methanogenic conditions that proved impossible to replicate [74]. In addition, hydrolytic defluorination of carbon atoms with two or more fluorine substituents appears to be too slow to be of environmental significance [27]. More often, transformation of highly fluorinated organics requires attack at functional groups or bonds attached to the fluorinated moiety. Attack on adjacent functional groups can be hydrolytic, oxidative, or reductive, and can result in decarboxylation, desulfonation, deamination, and fluoride elimination.

Although there have been instances of partial defluorination of perfluoroalkyl chains, complete mineralization in the biosphere has not been reported. The products of these

partial transformations are of interest with regard to their lifetimes in the environment and their effects on the biosphere directly or indirectly (see Figure 2.2). For example, it has been shown that several nonvolatile compounds such as sulfluramid and 1H,1H,2H,2H-perfluorooctane sulfonic acid (Chapter 3 and 5) are transformed to volatile fluorinated compounds [5]. In addition, volatile compounds such as HCFC-123, HCFC-124, and HFC-134a are expected to yield TFA (a nonvolatile fluorinated compound) as a tropospheric degradation product [25, 100, 109, 110]. The CFCs are degraded in the stratosphere and are responsible for ozone depletion and global warming. While several of the fluorinated agrochemicals are reported to "dissipate" in soils, mineralization of these chemicals has not been demonstrated in most cases. Similarly, the environmental fate of the trifluoromethyl group, which is utilized in many fluorinated organic compounds, is largely unknown. The potential for TFA production from these trifluoromethyl-substituted aromatics should be investigated. The biogeochemical cycling of fluorinated compounds is just now being discovered and is not yet clearly understood.

The stability that makes fluorinated compounds desirable for commercial use also makes them potentially significant environmental contaminants due to their persistence. Very little research into the toxicological effects of these compounds and their byproducts on animals and plants has been done. Therefore, caution is warranted when evaluating these compounds because although they may not be chemically or even biochemically reactive, they may still be biologically active.

To successfully study the fate and the effects of fluorinated compounds in the environment, sufficiently sensitive analytical methods need to be developed and published. Currently, very few methods are available to measure or detect nonvolatile fluorinated organics in environmental samples. In addition, many of the perfluorinated

organics are produced as mixtures of straight and branched homologues making analysis even more challenging. Although traditional analytical methods for determining hydrocarbon analogues have been used, most environmental samples have co-eluting ions and metabolic products that confound analysis. Inorganic fluoride measurement, by ion-selective electrode or ion chromatography, has been an effective means of indirectly detecting transformation of these compounds, but this method requires defluorination which may not occur with many perfluorinated compounds. Destructive conversion of organofluorine compounds to inorganic fluoride ions can provide information on the ratio of fluorine in the samples, but this method cannot distinguish between different homologues. Another indirect method is monitoring of oxygen consumption during aerobic incubation. Methane and carbon dioxide production can be measured with anaerobic incubations. Again these methods fail to answer key questions about the fate of fluorinated compounds. Real progress on these fate issues may require synthesis of radiolabelled compounds.

It is likely that industrial, agricultural, domestic, and medical applications for fluorine containing compounds will continue to increase in the coming years. As we continue to use and manufacture molecules containing carbon-fluorine bonds, several questions arise pertaining to the fate of such compounds. Are they recalcitrant in the environment or do they possess substituents, such as the trifluoromethyl group, which persists as mobile, recalcitrant functional groups? In which environments do they accumulate? What are the products of biological or abiotic reactions? What is the fate of reaction products? What is the toxicity and mutagenicity of such reaction products? How can we accurately measure and detect these compounds in environmental samples? Clearly, considerable research is needed to better understand the fate and effects of fluorinated organics in the biosphere.

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CHAPTER 3

DEFLUORINATION OF ORGANOFLUORINE SULFUR COMPOUNDS BY *PSEUDOMONAS* SP. STRAIN D2

ABSTRACT

Little is known of the potential for biodegradation of sulfur containing fluorinated surfactants. Difluoromethane sulfonate (DFMS, CHF_2SO_3Na) was chosen as a representative model compound. A Pseudomonad (strain D2) was isolated that completely defluorinated DFMS under aerobic and sulfur-limiting conditions in minimal medium. Strain D2 was subsequently used to evaluate the potential for biotransformation of the following fluorinated sulfonates: trifluoromethane sulfonate (TFMS; CF_3SO_3Na), 2,2,2-trifluoroethane sulfonic acid (TES; $CF_3CH_2SO_3H$), perfluorooctane sulfonate (PFOSA; $C_8F_{17}SO_3K$), and 1H,1H,2H,2H-perfluorooctane sulfonic acid (H-PFOSA; $C_6F_{13}C_2H_4SO_3H$). Strain D2 was capable of utilizing those compounds containing hydrogen (TES and H-PFOSA), but only partial defluorination was observed. When TES was the sole sulfur source, one mole of fluoride was released per mole of TES transformed. Transformation of H-PFOSA yielded one mole of fluoride per mole of H-PFOSA when incubations were open to the atmosphere and 1.4 moles per mole when incubations were closed to the atmosphere. Eight volatile and fluorinated byproducts of H-PFOSA were detected by GC/mass spectrometry, but volatile transformation products were not detected for TES or DFMS. This is the first report of biotic transformation of fluorinated sulfonates resulting in defluorination. These findings suggest that the initial steps of transformation are linked to sulfur metabolism

with subsequent defluorination of the molecule. The results also suggest that hydrogen substitution is required for transformation.

INTRODUCTION

The commercial use of organofluorine compounds has dramatically increased in recent years. Many of these compound are used as propellants, surfactants, agrochemicals, adhesives, refrigerants, fire retardants, and medicines [2, 3, 4, 5, 6, 7, 11, 14, 15, 16, 22, 25]. One category with particularly useful properties is the fluorinated sulfonates. Perfluorinated sulfonates are used as industrial surfactants and as catalysts in synthetic chemistry. Perfluorooctane sulfonate (PFOSA; $C_8F_{17}SO_3Na$) has excellent chemical and thermal stability and is important commercially as a surfactant and as a precursor of other fluorinated surfactants and pesticides [1]. Shorter chained perfluorinated compounds, such as trifluoromethane sulfonate (TFMS; CF_3SO_3H), are used as oligomerization or polymerization catalyst. TFMS is one of the strongest acids known, has great thermal stability, does not release fluoride in the presence of strong nucleophiles, and resists both oxidation and reduction [19]. Difluoromethane sulfonate (DFMS; CHF_2SO_3Na), 1H,1H,2H,2H-perfluorooctane sulfonic acid (H-PFOSA; $C_6F_{13}C_2H_4SO_3H$), and 2,2,2-trifluoroethane sulfonic acid (TES; $CF_3CH_2SO_3H$) are partially fluorinated analogues of the perfluorinated sulfonates. Because of the apparent stability of these compounds and their potential for accumulation in the environment, it is important to understand their environmental fate and the mechanisms by which they are degraded. The biodegradability of TFMS, TES, PFOSA, and H-PFOSA was evaluated using a Pseudomonad that is capable of completely defluorinating DFMS under aerobic and sulfur-limiting conditions.

MATERIALS AND METHODS

Media and chemicals. A defined mineral medium containing (in grams per liter): glucose, 2.0; K_2HPO_4, 3.5; KH_2PO_4, 2.0; NH_4Cl, 1.0; $MgCl_2 \cdot 6H_20$, 0.5; 0.15M $CaCl_2 \cdot 2H_20$ stock, 1.0 ml/liter; trace elements stock I and II, 1.0 ml/liter. Trace elements stock I contained (in grams per liter): $FeCl_3$, 1.36; $CoCl_2 \cdot 6H_20$, 0.2; $MnCl_2 \cdot 4H_20$, 0.122; $ZnCl_2$, 0.07; $Na_2MoO_4 \cdot 2H_20$, 0.036; $NiCl_2 \cdot 6H_20$, 0.12; $B_3(OH)_3$, 0.062; $CuCl_2 \cdot 2H_20$, 0.017. Concentrated H_2SO_4 was added at 2.5 ml/L to the trace elements solution I. Trace elements solution II contained (in grams per liter): $Na_2SeO_3 \cdot 5H_20$, 0.006; $NaWO_4 \cdot 2H_20$, 0.033; $Na_2MoO_4 \cdot 2H_20$, 0.024. To this medium was added the appropriate organofluorine sulfonate as the sulfur source. Sodium difluoromethane sulfonate (DFMS) was provided by 3M, St Paul, MN. Potassium perfluorooctane sulfonate (PFOSA) and 1H,1H,2H,2H-perfluorooctane sulfonic acid (H-PFOSA) were obtained from ICN Pharmaceuticals Inc., Costa Mesa, CA. 2,2,2-Trifluoroethane sulfonyl chloride was obtained from Sigma Chemical, St. Louis, MO. Hydrolysis of 2,2,2-trifluoroethane sulfonyl chloride by addition to water and autoclaving yielded trifluoromethane sulfonic acid (TES). All other carbon sources and chemicals were obtained from Sigma Chemical.

Growth conditions for *Pseudomonas* sp. strain D2. Plate colonies of strain D2 were inoculated into 5 ml of nutrient broth (Difco, Detroit, MI) and incubated at 30° C for 24 hours. A 1% inoculum of this was used in above medium (pH of 6.9-7.0) with the specified organofluorine compound as the sole sulfur source. Cells were grown aerobically at 30°C and were shaken on a rotorary shaker at 160 rpm.

Fluoride and DFMS analysis. Fluoride and DFMS were measured on a Dionex ion chromatography model 2000i/sp fitted with an IonPac AS4A ion exchange column and a

Dionex IonPac AG4A guard column. This system utilizes an anion micromembrane suppressor with a Dionex Conductivity Detector-II (CDM). The eluant was a carbonate buffer (1.8 mM Na_2CO_3 and 1.7 mM $NaHCO_3$) with a flow rate of 2 ml/min. Fluoride concentration was used as an indirect method of detecting biotransformation of the fluorinated sulfonates. Fluoride was also measured using an ion selective electrode (Orion 96-09 BN). A total ionic strength adjuster (TISA) was used to stabilize fluoride measurements in samples. TISA was prepared by adding 57 ml of glacial acetic acid and 58 g reagent grade sodium chloride into 500 ml of deionized water. This mixture was cooled on ice, adjusted to a pH of 5.0-5.5 with 5M sodium hydroxide, and diluted with water to a final volume of one liter. One part TISA to one part sample was used to measure fluoride.

Mass spectrometry and atomic emissions analysis. GC/MS data were obtained using a Hewlett-Packard 5995 series II GC/MS. A DB624 capillary column (30 m x 0.25 mm x 1.4 μm) was used for separation of volatile byproducts (J & W Scientific, Inc., Folsom, CA). Operating conditions were as follows: flow of 30 cm/sec linear velocity, initial temperature 40° C for 4 minutes followed by a 10° C/minute ramp to 200° C, injector temperature of 250° C, transfer line temperature of 225° C. Samples were taken by injecting a solid phase microextraction (SPME) fiber assembly with a 100 μM polydimethylsiloxane coating (Supelco Inc., Bellefonte, PA) in through the septum of the sample. This assembly was allowed to equilibrate for 30 minutes within the sample headspace before injecting onto the GC/MS.

A Hewlett-Packard 5890 series II GC with operating conditions identical to those above was used with a Hewlett-Packard 5921A atomic emission detector (AED) for elemental analysis of volatile products of H-PFOSA. Table 3.1 lists the emission wavelengths and plasma gases used for the various elements.

Table 3.1. Emission wavelengths and plasma gases used for atomic emission detection.

Element Monitored	Emission Wavelength (nm)	Plasma Reagent Gases
Sulfur	181	oxygen and hydrogen
Carbon	496	oxygen
Hydrogen	486	oxygen
Fluorine	690	hydrogen
Oxygen	777	hydrogen with auxillary gas (10% methane/90% nitrogen)

RESULTS

Enrichment and identification of bacteria. A Pseudomonad, designated as strain D2, was isolated from a mixed culture of bacteria that fortuitously contaminated a laboratory batch of carbon rich medium containing DFMS as the sole source of sulfur. Strain D2 was isolated by streaking on nutrient agar plates. This isolate is a motile, Gram-negative rod capable of using select fluorinated sulfonates as a source of sulfur for growth under aerobic and sulfur-limiting conditions. It is catalase positive and oxidase positive. Optimal growth was observed at 30° C. Fatty acid profiles for strain D2 were performed by Microbial ID, Inc. (MIDI), Newark, Delaware. This analysis gave a similarity index of 0.788 for *Pseudomonas chloroaphis* and a similarity index of 0.692 for *Pseudomonas fluorescens*. Biolog, Inc. (Hayward, CA), identified strain D2 as *Pseudomonas fluorescens*.

Biotransformation of difluoromethane sulfonate. Cells of strain D2 were grown for 24 hours in nutrient broth (Difco, Detroit, MI), and a 1% inoculum of this culture was added to medium containing DFMS. Fluoride release, disappearance of DFMS, and

optical density were monitored. Figure 3.1 shows complete defluorination of DFMS with stoichiometric yield of fluoride. Figure 3.2 demonstrates the corresponding growth associated with the utilization of DFMS as the sole sulfur source. Control samples (not shown) did not defluorinate DFMS and showed no change in optical density.

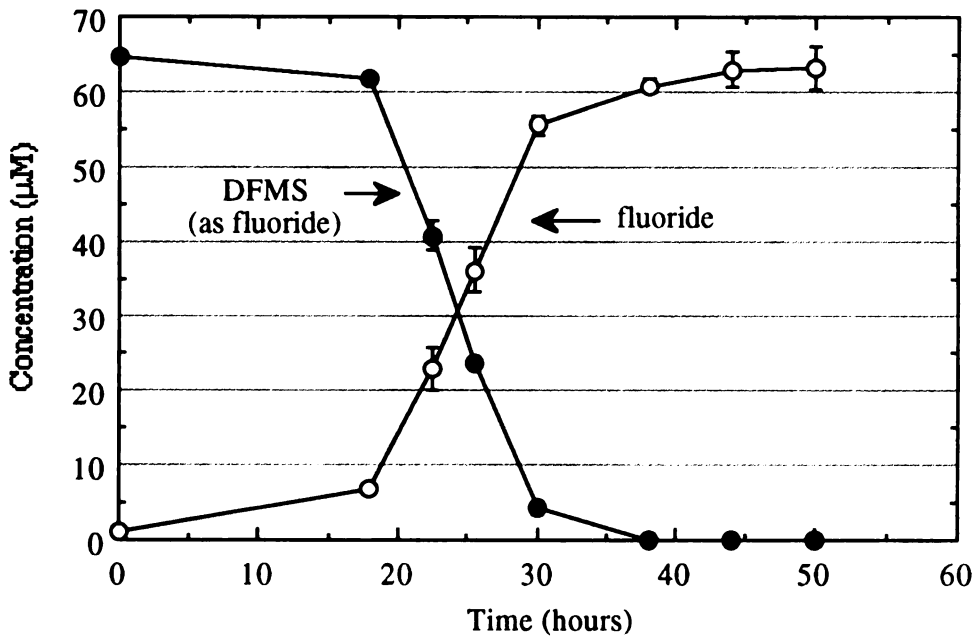

Figure 3.1. Defluorination of DFMS by *Pseudomonas* sp. strain D2. Error bars represent the standard deviation of triplicate samples.

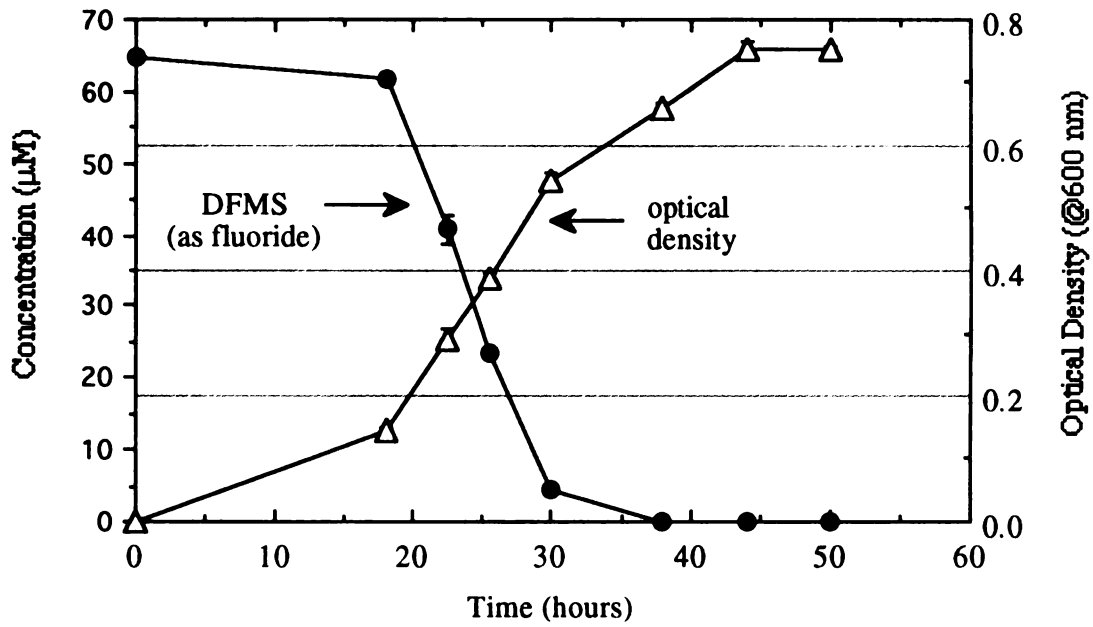

Figure 3.2. Growth of *Pseudomonas* sp. strain D2 on DFMS. Error bars represent the standard deviation of triplicate samples.

Biotransformation of other fluorinated sulfonates. *Pseudomonas* sp. strain D2 was used to evaluate the biotransformation of other sulfur containing fluorinated compounds such as trifluoromethane sulfonate (TFMS), 2,2,2-trifluoroethane sulfonic acid (TES), perfluorooctane sulfonate (PFOSA), and 1H,1H,2H,2H-perfluorooctane sulfonic acid (H-PFOSA). Biotransformation experiments were similar to those used for DFMS studies except that TES and H-PFOSA experiments were conducted in bottles sealed with teflon lined septa (1:6 liquid to gas volume). Strain D2 was only capable of utilizing the hydrogen-substituted sulfonates - DFMS, TES, and H-PFOSA .

Unlike the transformation of DFMS, TES and H-PFOSA were only partially defluorinated. When TES was the sole sulfur source for growth, one mole of fluoride was released per mole of TES degraded. Transformation of H-PFOSA yielded approximately one mole of fluoride per mole of H-PFOSA transformed when

incubations were open to the atmosphere and 1.4 moles per mole when incubations were closed to the atmosphere. Figures 3.3 and 3.4 show fluoride released from various concentrations of TES and H-PFOSA, respectively. As with biotransformation of DFMS, transformation of TES and H-PFOSA correlated with growth of strain D2 indicating that sulfur was released and assimilated. Figures 3.5 and 3.6 show growth of strain D2 with TES and H-PFOSA as the sole sources of sulfur.

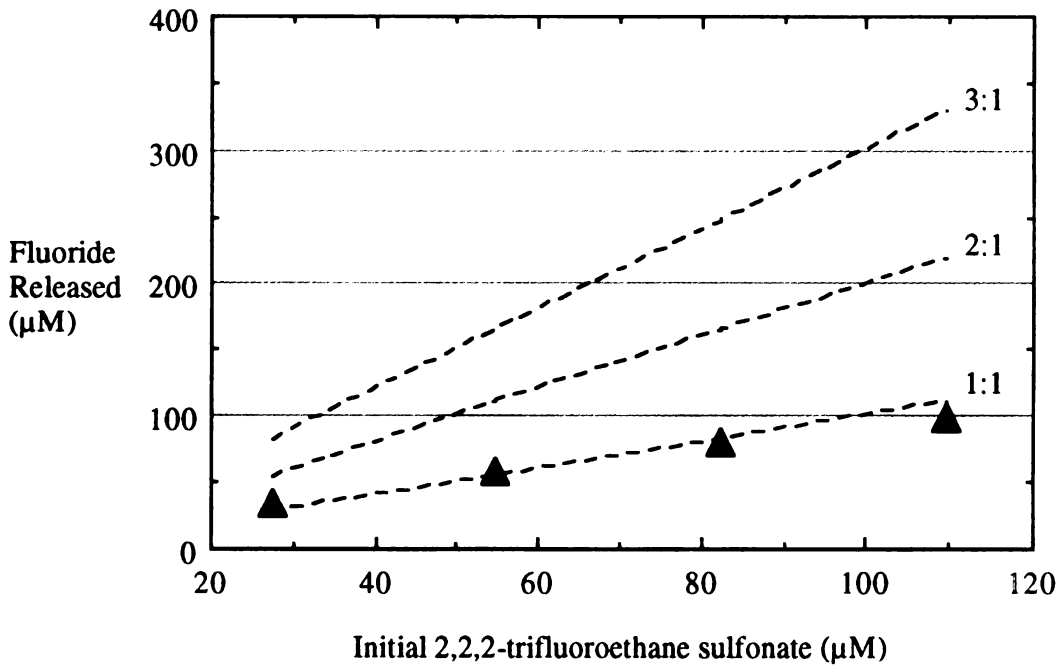

Figure 3.3. Fluoride release from TES. Numbers represent ratio of moles of fluoride to moles of TES. Triplicate samples were measured, but error bars are not visible due to scale.

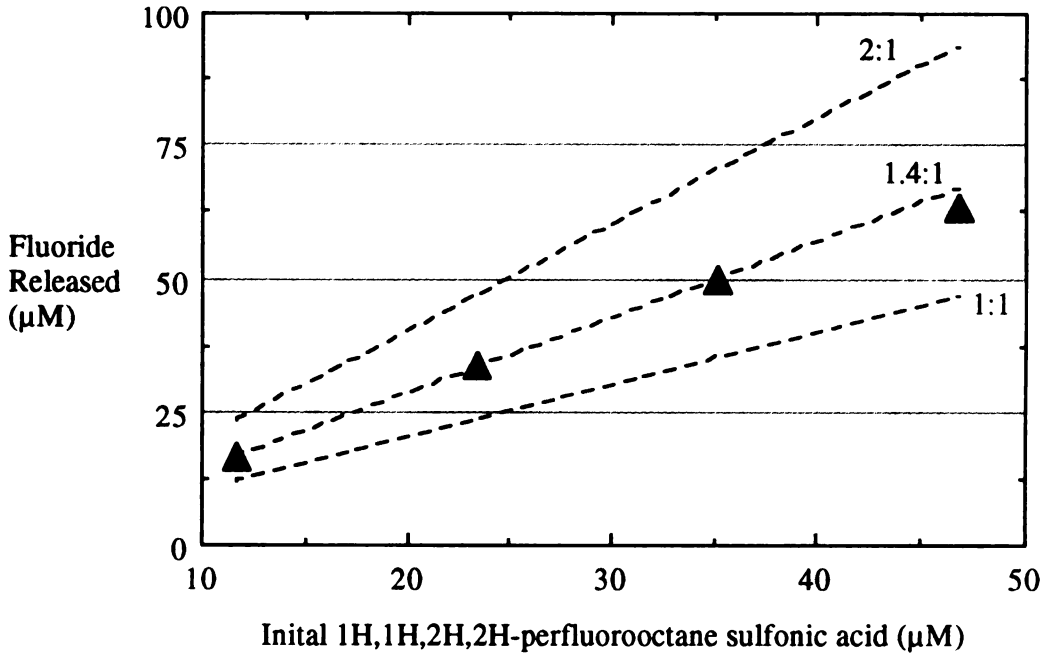

Figure 3.4. Fluoride release from H-PFOSA. Numbers represent ratio of moles of fluoride to moles of H-PFOSA. Triplicate samples were measured, but error bars are not visible due to scale.

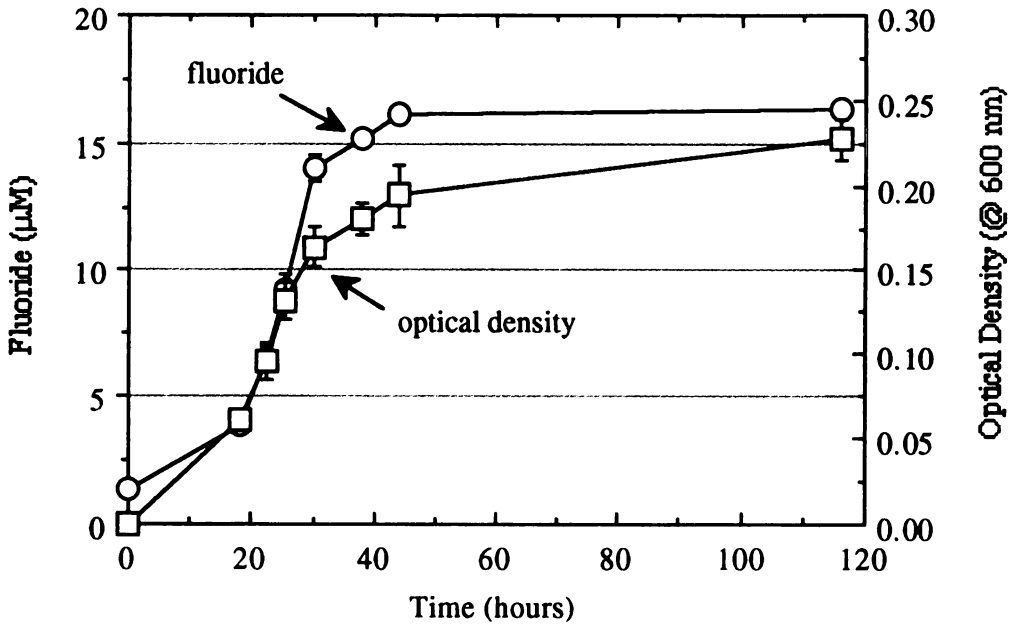

Figure 3.5. Growth of *Pseudomonas* sp. strain D2 on TES. Error bars represent the standard deviation of triplicate samples.

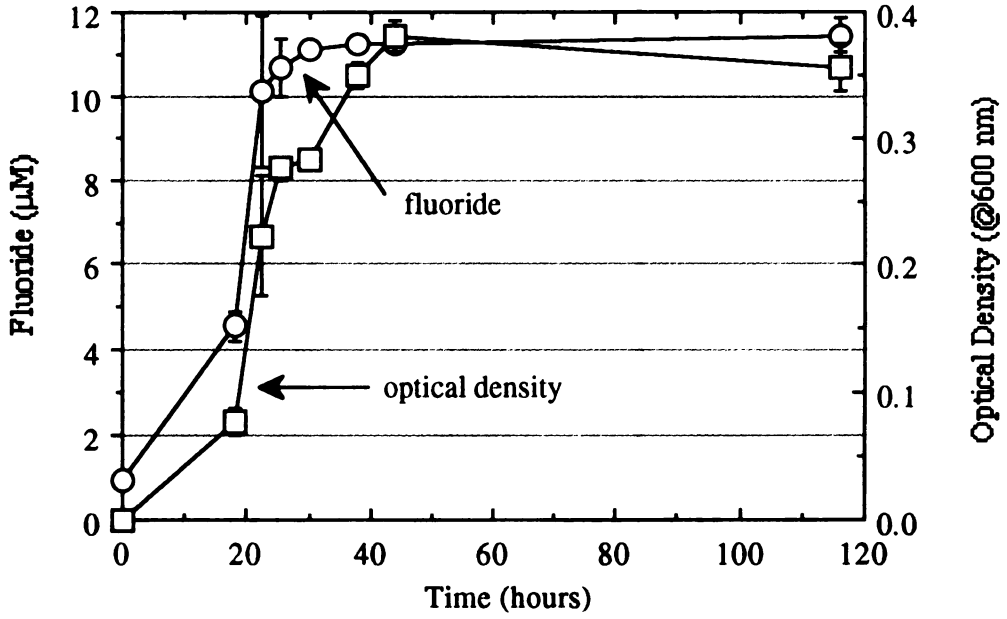

Figure 3.6. Growth of *Pseudomonas* sp. strain D2 on H-PFOSA. Error bars represent the standard deviation of triplicate samples.

Eight volatile and fluorinated byproducts of H-PFOSA were detected with GC/MS and AED. However, no transformation products were detected for TES. As shown in Figure 3.7, all of the volatile products of H-PFOSA contained carbon, oxygen, hydrogen, and fluorine. Sulfur was not detected in any volatile products of H-PFOSA. Complete GC/MS data and spectrums (both electron impact and chemical ionization) can be found in Appendices A and B. In addition, complete AED plots are also found in Appendix C.

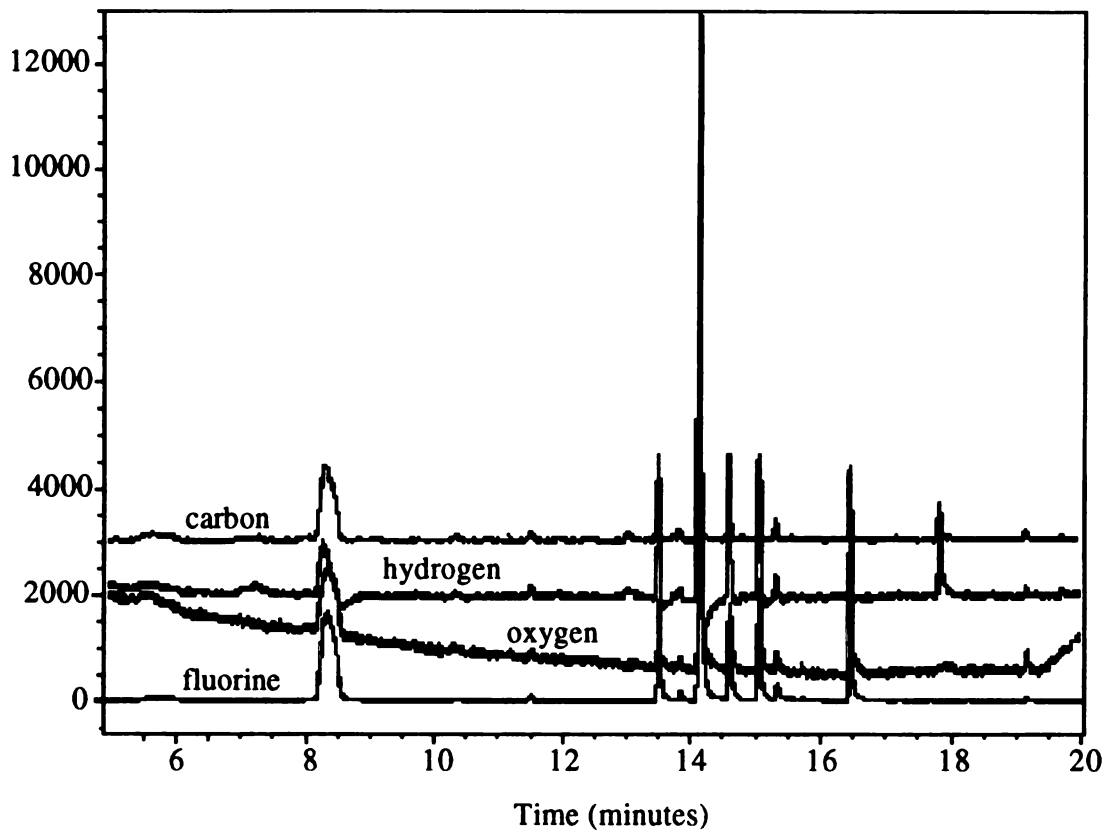

Figure 3.7. Atomic Emission Detection of volatile fluorinated byproducts of H-PFOSA biodegradation.

DISCUSSION

Several researchers have demonstrated the utilization of aliphatic sulfonates as sole sulfur source [9, 10, 12, 18, 20, 21, 23, 24], but there are no reports establishing that fluorinated sulfonates can serve as the sole source of sulfur for growth. In general, fluorinated organics are perceived as refractory in natural environments with the potential for accumulation. Therefore, it is important to understand the details of how these compounds are metabolized and/or degraded. It is demonstrated in this report that specific fluorinated sulfonates are at least partially degraded. Presumably, the growth associated with these transformations is due to the utilization of the sulfur in these

molecules (no growth was observed in their absence). As shown in Figures 3.5 and 3.6, growth continues even after the release of fluoride has stopped. A likely explanation is that the initial transformation and defluorination are rapid and not rate-limiting, while the assimilation of the sulfur byproduct into biomass is slower and rate-limiting. These findings suggest that the initial steps of transformation are linked to sulfur scavenging with subsequent defluorination of the molecule.

Kelly et al. [10] have shown that sulfite and formaldehyde are the products of degradation of methanesulfonic acid (MSA) by a methylotrophic bacterium. The methylotroph was not able to utilize sulfonates with more than three carbons when it was pregrown with MSA and grew poorly on compounds containing more than one carbon. An NADH-dependent monooxygenase was found to be responsible for the reaction. Based on these results and those of other investigators [21, 24], a possible mechanism for degradation of the fluorinated sulfonates is desulfonation of the molecule, with the production of sulfite or bisulfite and an aldehyde. The sulfite would then be subsequently used as a source of sulfur for growth (no free sulfite was detected in incubation of strain D2). An aldehyde formed from the degradation of H-PFOSA may or may not be stable and could decompose to other products. The discrepancy in fluorined stoichiometry between closed and open incubations of H-PFOSA with strain D2 could be explained by subsequent transformation of one or more of the volatile fluorinated products. Postulation of a mechanism that removes the sulfur moiety from the molecule is supported by atomic emissions spectroscopy data that failed to detect sulfur in any of the volatile fluorinated byproducts of H-PFOSA biodegradation. Table 3.2 illustrates possible structures for the volatile products from H-PFOSA transformation.

Table 3.2. Possible volatile fluorinated products of H-PFOSA transformation by strain D2.

Possible H-PFOSA products (-0 fluoride)	Molecular Weight
$C_6F_{13}CH_2CH_2$-O-CH_3	378
$C_6F_{13}CH_2$-O-CH_2CH_3	378
$C_6F_{13}CH_2\overset{\overset{O}{\|}}{C}CH_3$	376
$C_6F_{13}CH_2CH_2OH$	364

Possible H-PFOSA products (-1 fluoride)	Molecular Weight
$C_5F_{11}CF{=}CH{-}CH{=}O$	342
$C_5F_{11}CF{=}C{=}O$	328
$C_5F_{11}CF{=}C{=}C{=}O$	340
$C_5F_{11}\underset{\diagdown\,O\,\diagup}{CF\text{-}CH_2\text{-}CH}$	343

Possible H-PFOSA products (-2 fluoride)	Molecular Weight
$C_5F_{11}CH{=}\underset{\|}{C}\text{-}CH{=}O$ (OH)	340
$C_5F_{11}\overset{\overset{O}{\|}}{C}H\text{-}CH_2\text{-}CH_3$	327

The fate of volatile fluorinated compounds in the environment has been studied internationally during the past twenty years. The chlorofluorocarbons (CFCs) have been implicated in the destruction of the ozone layer and global warming [13, 17].

Replacements for the CFCs, hydrofluorocarbons (HFCs) and hydrochlorofluorocarbons (HCFCs), are expected to degrade before reaching the stratosphere and the ozone layer, but some of these can still contribute to global warming [26]. Although the identity of the volatile fluorinated products of H-PFOSA transformation is unknown, their production is important because it suggests an alternative route for the production of volatile fluorinated compounds. Until the identity of these compounds is determined, it is unclear what effect, if any, they might have on the environment or on global warming.

Another finding of this research was the observation that only fluorinated compounds with one or more hydrogen substituents are transformed by strain D2. It is likely that the enzyme responsible for the transformation of these compounds must interact at or near the carbon-sulfur bond. In the case of molecules with only fluorine at the alpha-carbon, the strong electronegativity of fluorine (4.0) may preclude attachment of the enzyme to the molecule and thus limit biotransformation. The fluorocarbon chain is also more "rigid" due to fluorine atoms on the molecule [8]. This rigidness almost certainly interferes with molecule/enzyme interactions, protecting fluorocarbon molecules from biological attack.

The results of this study clearly demonstrate that hydrogen-substituted fluorinated sulfonates are susceptible to biodegradation and defluorination. In addition, growth of bacteria may be supported by the sulfur byproduct of transformation under aerobic and sulfur-limiting conditions. By understanding the specific nature of the enzyme(s) or co-factor(s) involved in the transformation of these molecules, we may be able to gain insight into the mechanism of degradation of fluorinated sulfonates as well as non-fluorinated sulfonates. This insight may make it possible to construct fluorinated surfactants that are more readily biodegradable to harmless end products.

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CHAPTER 4

PHYSIOLOGY OF DIFLUOROMETHANE SULFONATE TRANSFORMATION BY *PSEUDOMONAS* SP. STRAIN D2

ABSTRACT

Difluoromethane sulfonate (DFMS; CHF_2SO_3Na), a fluorinated sulfonate, was chosen as a model compound to investigate the physiology of transformation of fluorinated sulfonates by *Pseudomonas* sp. strain D2. Previous research has established that a structural or molecular limitation to transformation is observed when the fluorinated sulfonates are completely fluorinated and do not possess hydrogen substituents. The present study investigates further the limitations on biotransformation of these compounds. In whole cell experiments, oxygen was required for growth and transformation of DFMS. It is also shown that DFMS cannot be utilized as a source of carbon and energy, but can be used as a sole source of sulfur under sulfur-limiting conditions with an added carbon source. It appears that actively metabolizing cells are required for the transformation of DFMS. This conclusion is based on the complete absence of transformation activity when glucose or ammonium were removed from the medium. It is hypothesized that transformation of DFMS and other fluorinated sulfonates is linked to a sulfur-scavenging system. Inhibition studies suggest that the sulfur-containing byproduct of DFMS transformation is assimilated through existing sulfur assimilation pathways. Non-competitive inhibition kinetics were observed with Ki values of 3-4 μM for sulfate, sulfite, methane sulfonate, cystine, and methionine.

INTRODUCTION

Although fluorinated compounds are used more frequently today, the environmental fate of many of these compounds is largely unknown. Reductive defluorination has been observed for tri-, di-, and monofluoroacetate [22]. However, Oremland et al. [15] were not able to replicate this work during further studies and concluded that trifluoroacetic acid was "generally refractory to microbial degradation." Hydrolytic defluorination of monofluoroacetate by bacteria has been reported [3, 4, 5, 6, 10, 13, 14, 19, 23]. More often, the transformation of fluorinated organics appears to require attack on ring structures and functional moieties such as sulfonic, carboxylic, and amine groups.

Defluorination of and growth on sulfur-containing organofluorine compounds under aerobic and sulfur-limiting conditions by *Pseudomonas* sp. strain D2 (chapter 3) and other bacteria (chapter 5) has been demonstrated. Among the compounds degraded were difluoromethane sulfonate (DFMS; CHF_2SO_3Na), 1H,1H,2H,2H-perfluorooctane sulfonic acid (H-PFOSA; $C_6F_{13}C_2H_4SO_3H$), and 2,2,2-trifluoroethane sulfonic acid (TES; $CF_3CH_2SO_3H$). Trifluoromethane sulfonate (TFMS; CF_3SO_3Na) and perfluorooctane sulfonate (PFOSA; $C_8F_{17}SO_3K$) were not degraded. This work suggested that the transformation of these compounds is restricted to molecules containing hydrogen. In this report other limitations to the transformation of sulfur-containing organofluorine compounds are examined using DFMS as a model compound. It is hypothesized that the transformation of DFMS is linked to sulfur metabolism or sulfur assimilation. Sulfur inhibition studies are used to evaluate whether utilization of DFMS as a sulfur source shares some intermediates or characteristics of sulfur assimilation pathways.

MATERIALS AND METHODS

Media and chemicals. A defined mineral medium containing (in grams per liter): glucose, 2.0; K_2HPO_4, 3.5; KH_2PO_4, 2.0; NH_4Cl, 1.0; $MgCl_2 \cdot 6H_2O$, 0.5; 0.15M $CaCl_2 \cdot 2H_2O$ stock, 1.0 ml/liter; trace elements stock I and II, 1.0 ml/liter. Trace elements stock I contained (in grams per liter): $FeCl_3$, 1.36; $CoCl_2 \cdot 6H_2O$, 0.2; $MnCl_2 \cdot 4H_2O$, 0.122; $ZnCl_2$, 0.07; $Na_2MoO_4 \cdot 2H_2O$, 0.036; $NiCl_2 \cdot 6H_2O$, 0.12; $B_3(OH)_3$, 0.062; $CuCl_2 \cdot 2H_2O$, 0.017. Concentrated H_2SO_4 was added at 2.5 ml/L to the trace elements solution I. Trace elements solution II contained (in grams per liter): $Na_2SeO_3 \cdot 5H_2O$, 0.006; $NaWO_4 \cdot 2H_2O$, 0.033; $Na_2MoO_4 \cdot 2H_2O$, 0.024. Sodium difluoromethane sulfonate (DFMS) was provided by 3M, St Paul, MN. All other carbon sources and chemicals were obtained from Sigma Chemical.

Fluoride and DFMS analysis. Fluoride and DFMS were measured on a Dionex ion chromatography model 2000i/sp fitted with an IonPac AS4A ion exchange column and a Dionex IonPac AG4A guard column. This system utilizes an anion micromembrane suppressor with a Dionex Conductivity Detector-II (CDM). The eluant was a carbonate buffer (1.8 mM Na_2CO_3 and 1.7 mM $NaHCO_3$) with a flow rate of 2 ml/min. Fluoride concentration was used as an indirect method of detecting biotransformation of the fluorinated sulfonates. Fluoride was also measured using an ion selective electrode (Orion 96-09 BN). A total ionic strength adjuster (TISA) was used to stabilize fluoride measurements in samples. TISA was prepared by adding 57 ml of glacial acetic acid and 58 g reagent grade sodium chloride into 500 ml of deionized water. This mixture was cooled on ice, adjusted to a pH of 5.0-5.5 with 5M sodium hydroxide, and diluted with water to a final volume of one liter. One part TISA to one part sample was used to measure fluoride.

Growth conditions for *Pseudomonas* sp. strain D2. Plate colonies of strain D2 were inoculated into 5 ml of nutrient broth (Difco, Detroit, MI) and incubated at 30° C for 24 hours. A 1% inoculum prepared in this manner was introduced into the defined mineral medium (pH of 6.9-7.0) with DFMS as the sole sulfur source. Cells were typically grown aerobically at 30°C and were shaken on a rotary shaker at 160 rpm. To test whether strain D2 could grow under denitrifying conditions, growth medium was amended with 10 mM sodium nitrate, degassed with a 98% N_2 and 2% H_2 gas mixture, and capped with teflon-faced butyl rubber septa. Culture manipulations were performed in a Coy anaerobic glove box (Coy Laboratories, Ann Arbor, MI). Glucose or acetate provided the carbon and energy source for these incubations. To test whether DFMS could simultaneously serve as carbon source and sulfur source, medium (without glucose) was amended with 5 or 10 mM DFMS. To test whether DFMS could serve as carbon source, medium (without glucose) was amended with 50 μM sulfate and 5 or 10 mM DFMS. A 1% inoculum of nutrient broth grown cells was used for these experiments.

Growth conditions and preparation of cells for inhibition experiments. Because fluoride concentration correlated with growth of strain D2, both fluoride and optical density measurements were used to determine stationary phase. Stationary phase cells were harvested by centrifugation (15 minutes at 12,100 X g in a Beckman SS-34 rotor at 4°C), washed in medium without a sulfur source, and resuspended to one tenth the original culture volume in the same medium to a cell density of approximately 1000-1500 mg protein/L. One milliliter of the resulting 10X concentrated cell suspension was added to 4 ml of medium. Specific substrate utilization curves were determined by varying the concentration of DFMS as well as the concentration of the tested sulfur sources. Fluoride was measured as an indirect measurement of transformation over a 20 minute time period and total cell protein was determined using the modified Lowry

method, with bovine serum albumin as the standard [12]. Specific rates of substrate utilization were computed as the mass degraded over a given time period divided by the total mass of protein used in the assay.

Modeling of Kinetic parameters. Difluoromethane sulfonate transformation rate coefficients were determined using a model for specific substrate utilization rate:

$$U = (k*S) / (Ks + S) = -(dS/dt)/X$$

where U is the specific substrate utilization coefficient (μmoles DFMS/mg protein/hr), k is maximum specific substrate utilization coefficient (μmoles DFMS/mg protein/hr), S is the rate-limiting substrate concentration (μM), Ks is the half-velocity coefficient (μM), t is time in hours (hr), and X is the total protein concentration (mg protein/L). Values for k and Ks were estimated using a nonlinear curve fit obtained from Systat version 5.2.1, based on initial DFMS degradation rates at a fixed cell protein concentration.

Two models were used to determine the nature of the inhibition by other sulfur sources. The first model was the competitive inhibition model:

$$U = (k * S) / (Ks*(1 + I/Ki) + S) = -(dS/dt)/X$$

where I is the inhibitor concentration (μM) and Ki is the inhibition constant (μM). The second model used was the non-competitive inhibition model:

$$U = (k*S) / ((I/Ki + 1)*(Ks + S)) = -(dS/dt)/X$$

The inhibition rate coefficients were determined using a nonlinear fit of the initial DFMS degradation rates at a fixed cell protein concentration. Rates of transformation were determined at concentrations of DFMS varying from 1.3 μM to 65 μM and inhibitor concentrations from 2 μM to 200 μM.

Sonicated crude cell extract preparation. Cultures of strain D2 were grown in 1L batches aerobically in 2L Erlenmeyer flasks for approximately 36 hours from a 1% inoculum. *Pseudomonas* strain D2 was screened for defluorination activity by measuring transformation rates prior to preparation of cell extracts. Actively transforming cultures were transferred to 250 ml centrifuge tubes and centrifuged at 12,100 X g for 15 minutes at 4°C. Cells were then washed twice and resuspended in 50 ml of buffered growth medium without DFMS. Thirty milliliters of this concentrated cell suspension was sonicated on ice, for 10 minutes at 1 second bursts at stage 5 (50% time on and 50% time off). The effective sonication time was 5 minutes. The sonicate was centrifuged at 12,100 X g for one hour at 4°C and divided into a supernatant and cell pellet. Whole cells, whole sonicate, supernatant, and cell pellet (resuspended in 30 ml medium) were used to assay DFMS transformation. DFMS was added to these samples at 32 μM and 20 μM ß-NADH was added to a subset of these samples.

French press crude cell extract preparation. Cells grown and harvested as described above were resuspended in 5 ml 20 mM Tris-buffer (pH 7.0) with 1 mM EDTA, placed on ice, and supplemented with the following protease inhibitors: 1 μl/ml of leupeptin solution and 5μl/ml aprotinin. The cells were then passed three times through a chilled French pressure cell at 1000-1200 psi. The cell extract was diluted to 20 ml. Three reaction mixtures were used to evaluate cell free activity. The first consisted of growth medium, as described above, amended with glucose and 1 mM DFMS. The second was a mixture containing 50 μM ascorbic acid, 50 μM $FeCl_2$, and 10 mM imidazole buffer (pH 6.75) amended with 1 mM DFMS. The third was 20 mM Tris-buffer (pH 7.0) amended with 1 mM DFMS. Three different cofactors, ATP, ß-NADH, and α-ketoglutarate were chosen to evaluate biotransformation requirements. A portion of the cell extract was centrifuged at 27,200 X g for 20 minutes and 0.5 ml of the supernatant was added to 1.5 ml of reaction mixture with one of the three cofactors. Controls

consisted of samples with no cofactor, samples with no cell extract, and cell extract that was not centrifuged. The 2 ml samples were placed into 12 ml screw-capped tubes and shaken for 20 minutes to one hour at 21 °C.

RESULTS

Although *Pseudomonas* sp. strain D2 can grow under denitrifying conditions with sulfate as the sole sulfur source, it was not capable of growth under denitrifying conditions when the sole sulfur source was difluoromethane sulfonate (Figure 4.1).

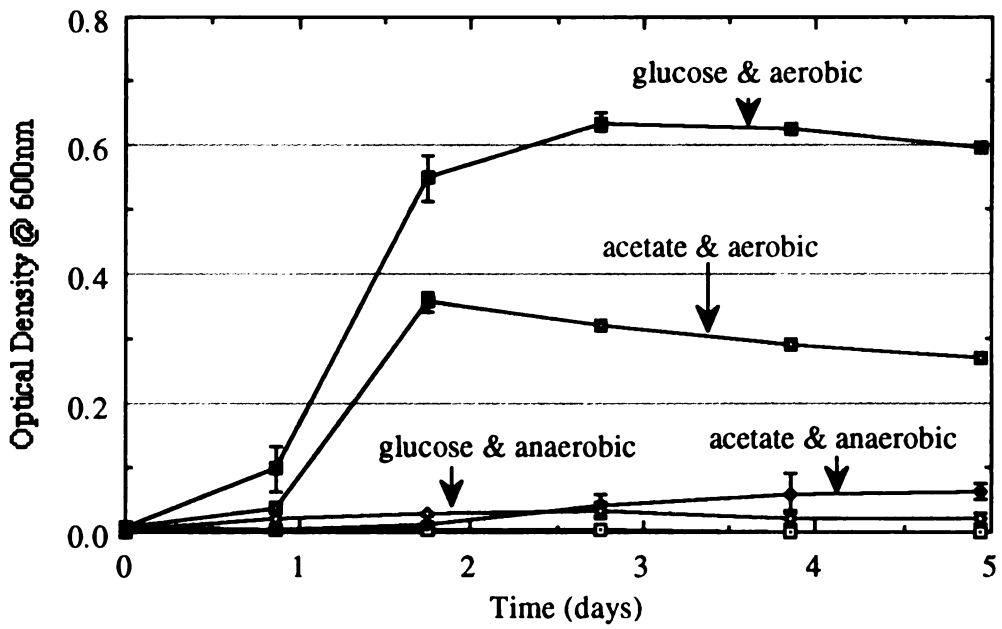

Figure 4.1. Growth of *Pseudomonas* sp. strain D2 with glucose and acetate under aerobic conditions. No growth was observed under anaerobic conditions. Error bars represent the standard deviation of triplicate samples.

As shown in Figure 4.2, *Pseudomonas* sp. strain D2 was not capable of using DFMS as a source of both carbon and sulfur nor was it capable of utilizing DFMS as a source of carbon when sulfur was provided in the form of sulfate.

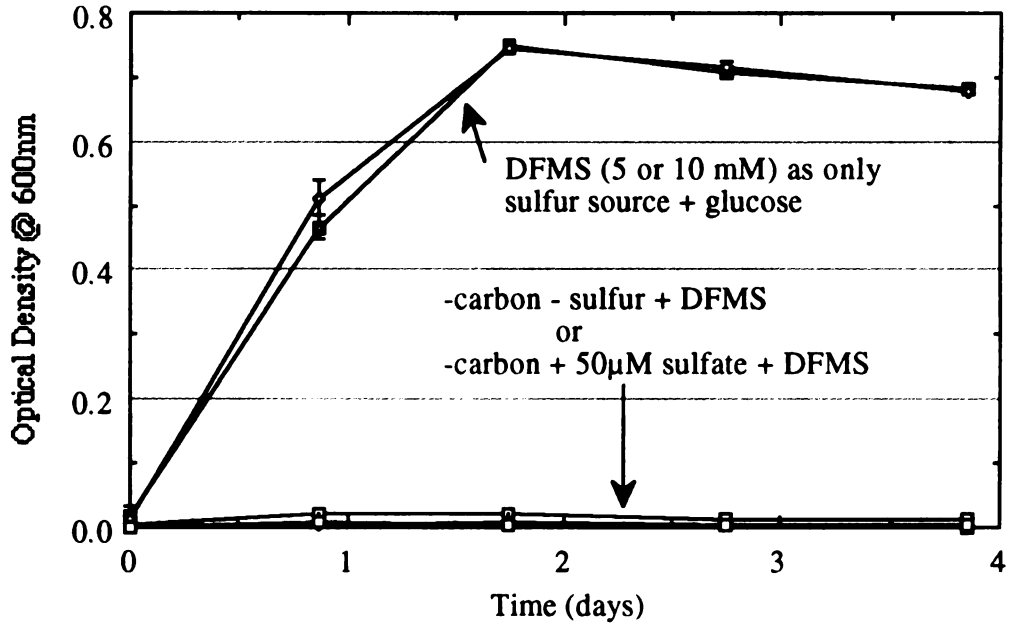

Figure 4.2. Growth of *Pseudomonas* sp. strain D2 with DFMS as sole source of sulfur. DFMS cannot be used as carbon source. Error bars represent the standard deviation of triplicate samples.

As shown in Figure 4.3, transformation did not occur when cells that had been grown to stationary phase with DFMS as sole sulfur source were incubated without a source of carbon and energy (no glucose). In addition, transformation of DFMS did not occur when ammonium, the nitrogen source, was removed from the medium.

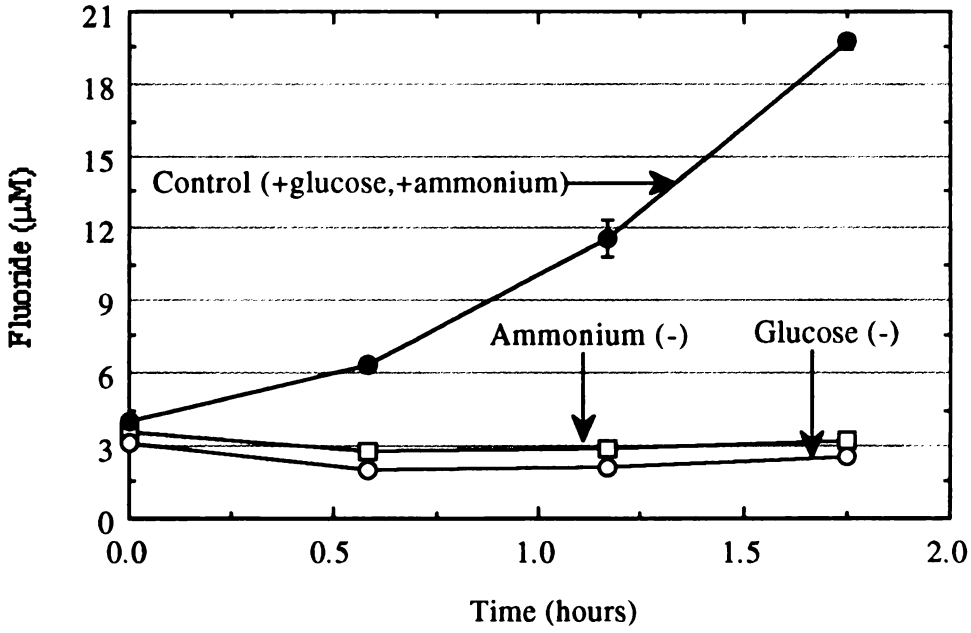

Figure 4.3. Effects of removal of glucose or ammonium, from growth medium, on the transformation of DFMS in stationary phase cells. Error bars represent the standard deviation of triplicate samples.

To determine whether transformation of DFMS was liked to sulfur metabolism or sulfur assimilation, sulfur inhibition studies were initiated to further elucidate the physiology of DFMS utilization. Sulfate, sulfite, methane sulfonate (a structural analogue of DFMS), cystine, and methionine were used as inhibitors of transformation. Non-competitive inhibition kinetics were observed. Figure 4.4 illustrates non-competitive inhibition of DFMS transformation for varying concentrations of sulfate. Figures 4.5, 4.6, and 4.7 show the effects of sulfite, methane sulfonate, and cystine respectively on DFMS transformation. Methionine had a similar effect on the transformation of DFMS.

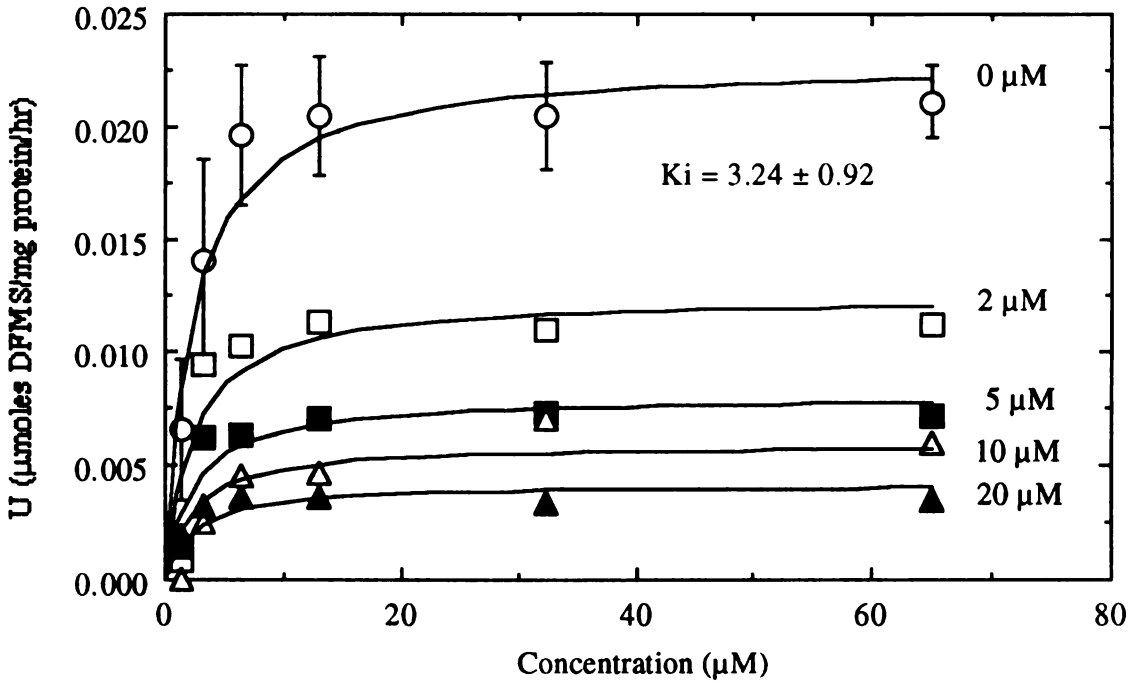

Figure 4.4. Non-competitive inhibition of DFMS transformation rates by sodium sulfate. Error bars represent the standard deviation of triplicate samples.

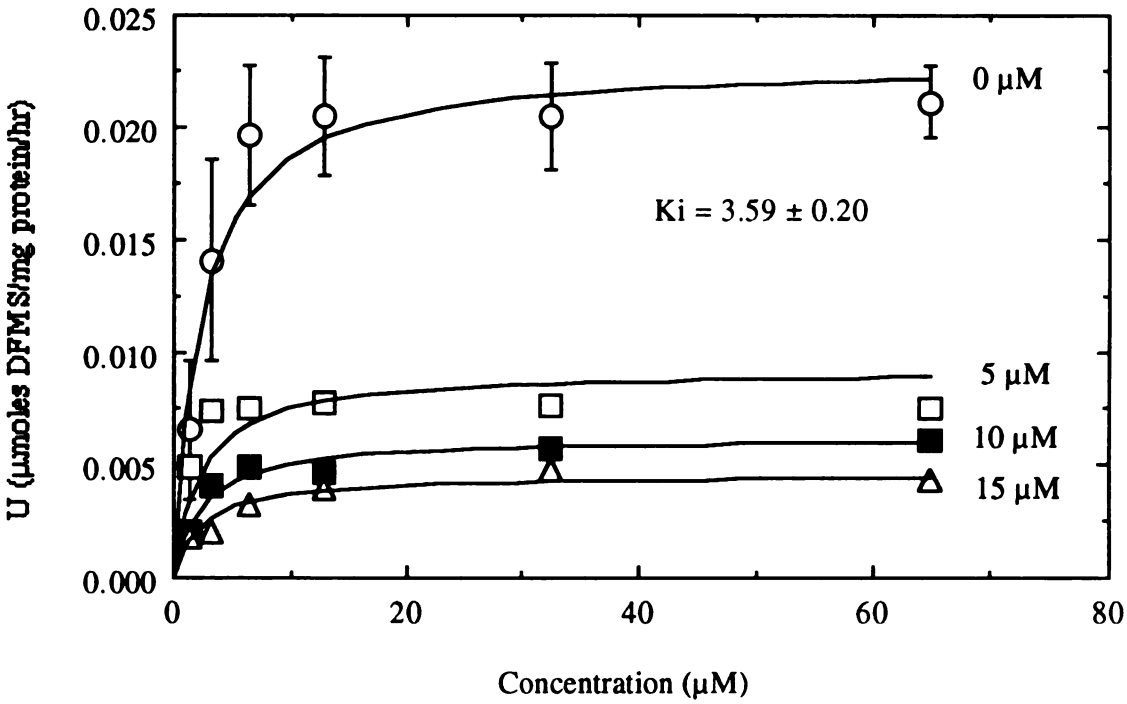

Figure 4.5. Non-competitive inhibition of DFMS transformation rates by sodium sulfite. Error bars represent the standard deviation of triplicate samples.

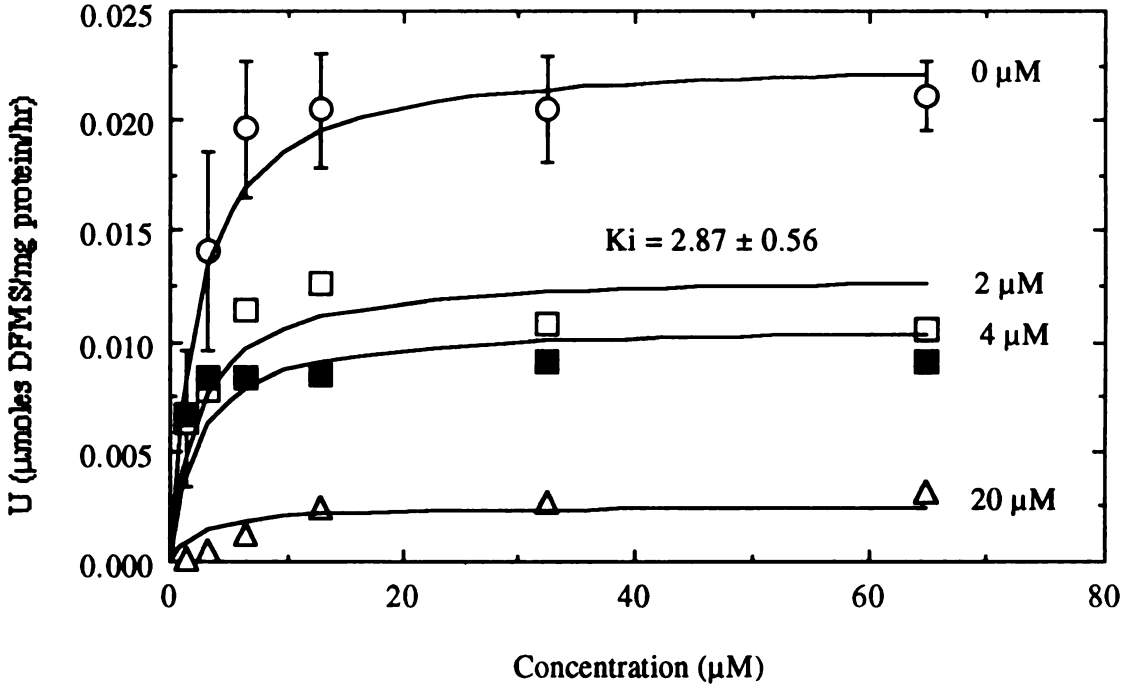

Figure 4.6. Non-competitive inhibition of DFMS transformation rates by sodium methane sulfonate. Error bars represent the standard deviation of triplicate samples.

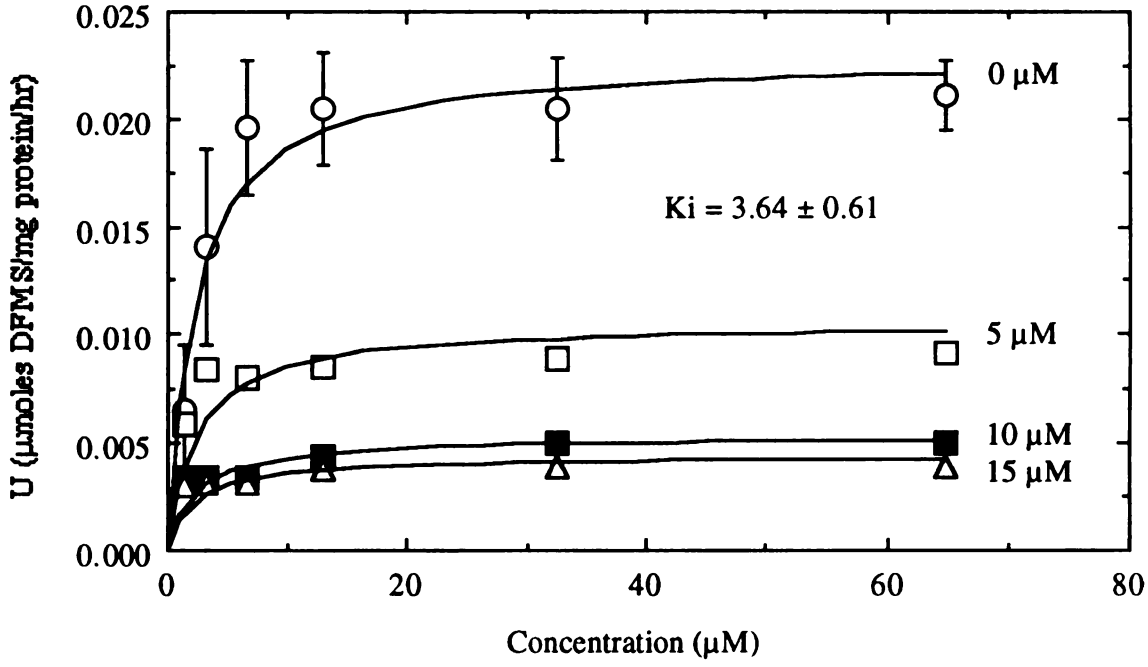

Figure 4.7. Non-competitive inhibition of DFMS transformation rates by cystine. Error bars represent the standard deviation of triplicate samples.

DISCUSSION

We have previously shown that *Pseudomonas* sp. strain D2 is capable of growth with difluoromethane sulfonate (DFMS), 2,2,2-trifluoroethane sulfonic acid (TES), and 1H,1H,2H,2H-perfluorooctane sulfonic acid (H-PFOSA) as sole sulfur source (Chapter 3). DFMS was completely defluorinated while TES and H-PFOSA were only partially defluorinated. Molecules that were completely fluorinated and did not contain hydrogen (trifluoromethane sulfonate; TFMS and perfluorooctane sulfonic acid; PFOSA) were not utilized as a sulfur source and were not defluorinated. These results suggest that hydrogen-substitution is required for transformation.

Most reports on the utilization of sulfonates by bacteria have been restricted to aerobic conditions [7, 9, 11, 16, 17, 18, 20, 21]. However, Chien et al [1] have shown that a fermenting *Clostridium* isolate can grow with taurine and isethionate as sulfur source, and a fermenting *Klebsiella* isolate can grow with cysteate as a sulfur source. *Pseudomonas* sp. strain D2 was not able to grow on or transform DFMS under anaerobic conditions even though it was capable of growth under denitrifying conditions with sulfate as the sulfur source. Evidently, whole cells of strain D2 require molecular oxygen to grow with fluorinated sulfonates as the sole sulfur source. Additional evidence is needed to establish whether molecular oxygen is required for the transformation.

These results show that DFMS could not be used as a carbon and energy source. In most of these experiments, glucose served as the source of carbon and energy. When glucose was removed from the medium, cells did not grow or transform DFMS. When pregrown cells were used, removal of glucose prevented transformation (Figure 4.3). This suggests that energy from glucose metabolism or some metabolic product of glucose utilization may be required for transformation. In all experiments, ammonium was the source of nitrogen. Removal of ammonium from the medium also prevented transformation (Figure 4.3). It is unclear what role ammonium has on the transformation activity of pregrown cells. Perhaps it is related to enzymatic activation, energy production, or some transport mechanism.

Presumably, the transformation of DFMS, as well as the other fluorinated sulfonates, is related to sulfur scavenging. A plausible hypothesis is that the first step in transformation of DFMS is desulfonation yielding sulfite (or another sulfur byproduct) and a fluorinated intermediate that spontaneously decomposes releasing fluoride. The sulfur byproduct is then assimilated into biomass through normal sulfur assimilation pathways. To evaluate this hypothesis, the effects of other sulfur sources on the transformation of DFMS were

investigated. Sulfate, sulfite, methane sulfonate, cystine, and methionine were used as possible inhibitors of DFMS transformation. As is shown in Figures 4.4 through 4.7, all of these sulfur sources were inhibitory to transformation (methionine not shown). The data obtained from these experiments did not fit the competitive inhibition model. However, the non-competitive model fit well, with an average correlation coefficient of 0.98 ± 0.013. Of interest is the similarity in Ki values (3-4 μM) for these different sulfur sources. This suggests some commonality in the mechanism of inhibition. One possibility is the inhibition of sulfur transport mechanism(s). This inhibition could affect binding proteins in the periplasm or cell membrane complexes that are used for sulfur uptake. Perhaps the high electronegativity of the carbon-fluorine bond interferes with proper binding to transport proteins or other sulfur sources compete for binding proteins in the periplasm but not within the cell. The overall effect of competition outside of the cell combined with abscence of competition on the inside could result in non-competitive pattern of inhibition. It is important to note that the Michaelis-Menten model used to model non-competitive inhibition is based on the assumption of one enzyme interacting with one substrate and a inhibitor molecule. In the case of whole cells experiments, a one step reaction is unlikely to explain the observed non-competitive inhibition. In any case, these results strongly suggest that the transformation of DFMS is related to sulfur-scavenging activities of the cell and that the sulfur byproduct(s) enter via existing sulfur assimilation pathways.

In an attempt to clarify the mechanism of transformation of DFMS, crude cell extract experiments were conducted. These experiments investigated the hypothesis that an NADH-dependent oxygenase was responsible for the initial attack on the molecule. This is similar to what has been shown by others [7, 8, 9, 18]. In addition, the possibility of an energy requirement was evaluated and ATP was used as a cofactor for these experiments. Finally, α-ketoglutarate was used to determine if a dioxygenase similar to

that encoded by the tfdA gene of *Alcaligenes eutrophus* (responsible for the first step in 2,4-dichlorophenoxyacetic acid degradation) was present [2]. No transformation activity was observed in cell extracts, even with added cofactors. Perhaps additional cofactors are required or critical membrane or cellular components were destroyed during lysis of cells. Because inhibition was observed with various forms of sulfur found in intact cells (such as cysteine) the negative results might be partially explained by sulfur release during cell lysis.

This work establishes several limitations on the biotransformation of sulfur-containing organofluorine compounds and establishes the importance of sulfur assimilation processes in the transformation of DFMS. Structural or molecular limitations evidently prevent transformation when the molecule is completely fluorinated and the presence of hydrogen facilitates transformation under aerobic and sulfur-limiting conditions.

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CHAPTER 5

BIOTRANSFORMATION OF SULFUR-CONTAINING ORGANOFLUORINE COMPOUNDS BY DIVERSE ORGANISMS

ABSTRACT

Previous work (Chapters 3 and 4) established that difluoromethane sulfonate (DFMS, CHF_2SO_3Na), 2,2,2-trifluoroethane sulfonic acid (TES; $CF_3CH_2SO_3H$), and 1H,1H,2H,2H-perfluorooctane sulfonic acid (H-PFOSA; $C_6F_{13}C_2H_4SO_3H$) are degraded by a Pseudomonad under aerobic and sulfur-limiting conditions in minimal medium. The present study focuses on generalizing this observation to other bacteria and yeast. The phylogenetically related *Pseudomonas fluorescens*, *Pseudomonas chloroaphis*, and *Pseudomonas stutzeri* KC were tested. *Bacillus subtilis*, *Escherichia coli*, and *Saccharomyces cerevisiae* were also evaluated. The bacteria were capable of transforming sulfur-containing organofluorine compounds. However, *E. coli* was not able to degrade H-PFOSA. Yeast was unable to utilize any of the organofluorine compounds as a source of sulfur. Transformation was also evaluated in aquatic samples and soil. No transformation was observed in the aquatic samples, but transformation was detected in the soil samples. It is concluded that the ability to degrade fluorinated sulfonates is not unique to strain D2 and in fact is widely distributed in nature. However, the results also suggest that transformation in the environment requires aerobic and sulfur-limiting conditions.

INTRODUCTION

Sulfur-containing organofluorine compounds are commonly used as catalysts, reagents, surfactants, and pesticides [1, 2, 3, 4, 5, 6, 7, 9, 10]. Several of these are perfluorinated compound such as perfluorooctane sulfonate (PFOSA; $C_8F_{17}SO_3Na$), perfluorooctane sulfonamide ($C_8F_{17}SO_2NH_2$), and trifluoromethane sulfonic acid (TFMS; CF_3SO_3H). TFMS is one of the strongest organic acids known, has great thermal stability, does not release fluoride in the presence of strong nucleophiles and resists both oxidation and reduction [6]. These compounds generally have excellent chemical and thermal stability. Partially fluorinated analogues of the perfluorinated sulfonates include difluoromethane sulfonate (DFMS; CHF_2SO_3Na), 1H,1H,2H,2H-perfluorooctane sulfonic acid (H-PFOSA; $C_6F_{13}C_2H_4SO_3H$), and 2,2,2-trifluoroethane sulfonic acid (TES; $CF_3CH_2SO_3^-$).

A previous report (Chapter 3) describes experiments to assess biodegradation of TFMS, DFMS, TES, H-PFOSA, and PFOSA by *Pseudomonas* strain D2. Under aerobic and sulfur-limiting conditions, strain D2 utilized compounds with hydrogen substituents (DFMS, TES, and H-PFOSA) as sulfur sources. D2 completely defluorinated DFMS and partially defluorinated TES and H-PFOSA. The present study generalizes these earlier findings to various other bacteria, yeast, and complex environmental samples (activated sludge, soil, and water).

MATERIALS AND METHODS

Media and chemicals. A defined mineral medium containing (in grams per liter): glucose, 2.0; K_2HPO_4, 3.5; KH_2PO_4, 2.0; NH_4Cl, 1.0; $MgCl_2 \cdot 6H_2O$, 0.5; 0.15M $CaCl_2 \cdot 2H_2O$ stock, 1.0 ml/liter; trace elements stock I and II, 1.0 ml/liter. Trace elements

stock I contained (in grams per liter): $FeCl_3$, 1.36; $CoCl_2 \cdot 6H_20$, 0.2; $MnCl_2 \cdot 4H_20$, 0.122; $ZnCl_2$, 0.07; $Na_2MoO_4 \cdot 2H_20$, 0.036; $NiCl_2 \cdot 6H_20$, 0.12; $B_3(OH)_3$, 0.062; $CuCl_2 \cdot 2H_20$, 0.017. Concentrated H_2SO_4 was added at 2.5 ml/L to the trace elements solution I. Trace elements solution II contained (in grams per liter): $Na_2SeO_3 \cdot 5H_20$, 0.006; $NaWO_4 \cdot 2H_20$, 0.033; $Na_2MoO_4 \cdot 2H_20$, 0.024. To this medium was added the appropriate organofluorine sulfonate as the sulfur source. Fermenting cultures of *E. coli* were prepared similar to that described above except that 5.5 g/L of K_2HPO_4 and 3.0 g/L KH_2PO_4 were used. Nitrate-respiring cultures were prepared as described above with the addition of 3.88 g/L $NaNO_3$. Both the fermenting medium and nitrate-respiring media were degassed under vacuum. Glucose (1 g/L) was used as a carbon source for fermenting cultures and glycerol (0.5 g/L) was used as carbon source in aerobic and nitrate-respiring cultures of *E. coli.* For experiments with yeast, 10 ml/L of a vitamin stock and 5 ml/L of a amino acid stock were added. The vitamin stock contained (in grams per liter): folate, 0.021; pyridoxine (B_6), 0.21; nicotinic acid, 2.1; riboflavin; 0.18; pantothenic acid, 0.53; p-aminobenzoic acid 0.025; and B_{12}, 0.025. The amino acid stock solution contained 4.2 g/L glycine and 4.2 g/L of the 21 essential L-amino acids except methionine, cystine, and cysteine. Sodium difluoromethane sulfonate (DFMS) was provided by 3M, St Paul, MN. Potassium perfluorooctane sulfonate (PFOSA) and 1H,1H,2H,2H-perfluorooctane sulfonic acid (H-PFOSA) were obtained from ICN Pharmaceuticals Inc., Costa Mesa, CA. 2,2,2-Trifluoroethane sulfonyl chloride was obtained from Sigma Chemical (St. Louis, MO). Hydrolysis of 2,2,2-trifluoroethane sulfonyl chloride by addition to water and autoclaving yielded trifluoromethane sulfonate (TES). All other chemicals were obtained from Sigma Chemical.

Experiments with diverse cell types. Strain D2 was identified as *Pseudomonas fluorescens* previously (Chapter 3). *Pseudomonas fluorescens* (ATCC deposit no. 17400) and *Pseudomonas chloroaphis* (ATCC deposit no. 9446) were obtained from

American Type Culture Collection (ATCC), Rockville, MD. *Bacillus subtilis* (ATCC deposit no. 6051) and *Escherichia coli* (ATCC deposit no. 10798) were obtained from the culture collection of the Microbiology Department at Michigan State University. *Pseudomonas stutzeri* KC (ATCC deposit no. 55595) is routinely cultured in our laboratory. Plate colonies of bacteria were inoculated into 5 ml of nutrient broth (Difco, Detroit, MI) and incubated at 30° C for 24 hours. A 1% inoculum of bacteria was used in the above medium (pH of 6.9-7.0) with the specified organofluorine compound as the sole sulfur source. Cells were grown aerobically at 30 °C and were shaken on a rotary shaker at 160 rpm. *Saccharomyces cerevisiae* (American Ale, type no. 1056) was obtained from Wyeast Laboratories, Inc., Mt Hood, OR. *Saccharomyces cerevisiae* was grown in malt extract for 36 hours and then inoculated at 1% of medium. Yeast were grown aerobically 21 °C and were shaken on a rotary shaker at 160 rpm. *E. coli* was used to determine if degradation of DFMS would occur under anaerobic conditions. A 1% inoculum of nutrient broth grown cell of *E. coli* were added to fermenting medium and nitrate-respiring medium. Control cultures were prepared in medium that was amended with sulfate as the source of sulfur.

Experiments with activated sludge, river water, and soil. To evaluate the fate of fluorinated sulfonates in the environmental samples, samples of activated sludge were obtained from the East Lansing Wastewater Treatment Facility, East Lansing, MI and river water was obtained from the Red Cedar River, on the campus of Michigan State University, East Lansing, MI. In addition three soils samples were also obtained from various locations on campus including a sample from a woodland swamp (natural area), a sample from a botanical garden (agricultural area), and a sandy soil from the banks of the Red Cedar River. The river and activated sludge samples were prepared by placing nine milliliters of the sample into sterile flasks (50 ml). One subset was amended with 1 ml sterile water (no added carbon source), another subset was amended with 1 ml of 20

g/L stock of glucose, and another was amended with 1 ml of 10 times concentrated growth medium with glucose (20 g/L). Triplicate samples were prepared with 32 µM DFMS as a source of sulfur and incubated at 21 °C. These samples were shaken on a rotary shaker at 160 rpm. Soil samples were prepared by adding 2 g of soil to sterile balch tubes (28 ml) and amending with 100 µl of 0.1 µg/µl of H-PFOSA stock solution (+ H-PFOSA) or 100 µl sterile water (- H-PFOSA). In addition subsets of these samples were amended with 200 µl of 10 mM glucose stock (+carbon) or 200 µl sterile water (-carbon). For all tested conditions and soil types, killed controls were prepared by autoclaving at 121°C for 30 minutes.

Analysis of biotransformation. Fluoride was measured using an ion selective electrode (Orion 96-09 BN). A total ionic strength adjuster (TISA) was used to stabilize fluoride measurements in samples. TISA was prepared by adding 57 ml of glacial acetic acid and 58 g reagent grade sodium chloride into 500 ml of deionized water. This mixture was placed on ice for cooling, and the pH was adjusted to between 5.0-5.5 with 5M sodium hydroxide. This mixture was then diluted with 500 ml of water to a final volume of one liter. One part TISA to one part sample was used to measure fluoride.

Mass spectrometry, GC/ECD, and GC/AED analysis. GC/MS data were obtained using a Perkin-Elmer GC with a Finnigan Ion Trap Mass Spectrometer. A DB624 capillary column (30 m x 0.25 mm x 1.4 µm) was used for separation of volatile byproducts (J & W Scientific, Inc., Folsom, CA). Operating conditions were as follows: flow of 30 cm/sec linear velocity, initial temperature 40° C for 4 minutes followed by a 10° C/minute ramp to 200° C, injector temperature of 250° C, transfer line temperature of 225° C. Samples were taken by injecting a solid phase microextraction (SPME) fiber assembly with a 100 µM polydimethylsiloxane coating (Supelco Inc., Bellefonte, PA) in through the septum of the sample. This assembly was allowed to equilibrate for 30

minutes within the sample headspace before injecting onto the GC/MS. Identical conditions were used for GC/ECD analysis with a Hewlett-Packard 5890 series II GC/ECD and a DB624 capillary column. A Hewlett-Packard 5890 series II GC with operating conditions identical to those above was used with a Hewlett-Packard 5921A atomic emission detector (AED) for elemental analysis of volatile products of H-PFOSA. Emission wavelengths and plasma gases used for the various elements were previously detailed (Chapter 3).

Sulfate and fluoride measurement. Sulfate was measured on a Dionex ion chromatograph model 2000i/sp fitted with an IonPac AS4A ion exchange column and a Dionex IonPac AG4A guard column. This system utilizes an anion micromembrane suppressor with a Dionex Conductivity Detector-II (CDM). The eluant was a carbonate buffer (1.8 mM Na_2CO_3 and 1.7 mM $NaHCO_3$) with a flow rate of 2 ml/min. Fluoride was measured using an ion selective electrode (Orion 96-09 BN).

RESULTS

Biotransformation of fluorinated sulfonates by diverse cell types. In previous studies, *Pseudomonas* sp. strain D2 completely defluorinated DFMS and partially defluorinated TES and H-PFOSA. In the present study, the phylogenetically related Gram-negative and oxidase-positive bacteria, *P. fluorescens* , *P. chloroaphis*, and *P. stutzeri* KC were all capable of complete defluorination of DFMS (Figure 5.1, *P. chloroaphis* and *P. stutzeri* KC are not shown) with stoichiometric yield of fluoride. These organisms showed partial defluorination of TES and H-PFOSA (Figure 5.2 and Figure 5.3, *P. chloroaphis* and *P. stutzeri* KC are not shown). None of the tested Pseudomonad strains were capable of growth on or transformation of the completely fluorinated sulfonates TFMS and PFOSA.

Bacillus subtilis was chosen as a representative of the Gram-positive bacteria. This organism was capable of defluorination of DFMS as shown in Figure 5.1. However, it was not capable of degrading as much DFMS as the *Pseudomonas* strains in this medium. Defluorination was also observed with TES (Figure 5.2) and H-PFOSA (Figure 5.3), but again a smaller amount was degraded, as compared to the *Pseudomonas* strains. *B. subtilis* was not capable of growth on or transformation of TFMS or PFOSA.

Escherichia coli K-12 was selected as a representative Gram-negative, oxidase-negative, facultative anaerobe. As shown in Figure 5.1, when grown aerobically, *E. coli* K-12 was capable of defluorinating DFMS, but like *B. subtilis*, it did not degrade as much DFMS as the *Pseudomonas* strains. *E. coli* K-12 also showed a similar partial defluorination of TES like that of the *Pseudomonas* strains and *B. subtilis*. However, *E. coli* K-12 was not capable of growth on or transformation of H-PFOSA. In addition, K-12 was not capable of growth on or transformation of TFMS or PFOSA, when these compounds were added as the sole source of sulfur. Furthermore, *E. coli* was not capable of growth or transformation of DFMS under anaerobic conditions. Both fermenting and nitrate-respiring conditions were evaluated with DFMS as the sole source of sulfur. When sulfate was added to these anaerobic cultures, growth was observed yet no transformation of DFMS occurred.

Yeast were not capable of degrading any of the tested fluorinated sulfonates even when supplemented with amino acids and vitamins.

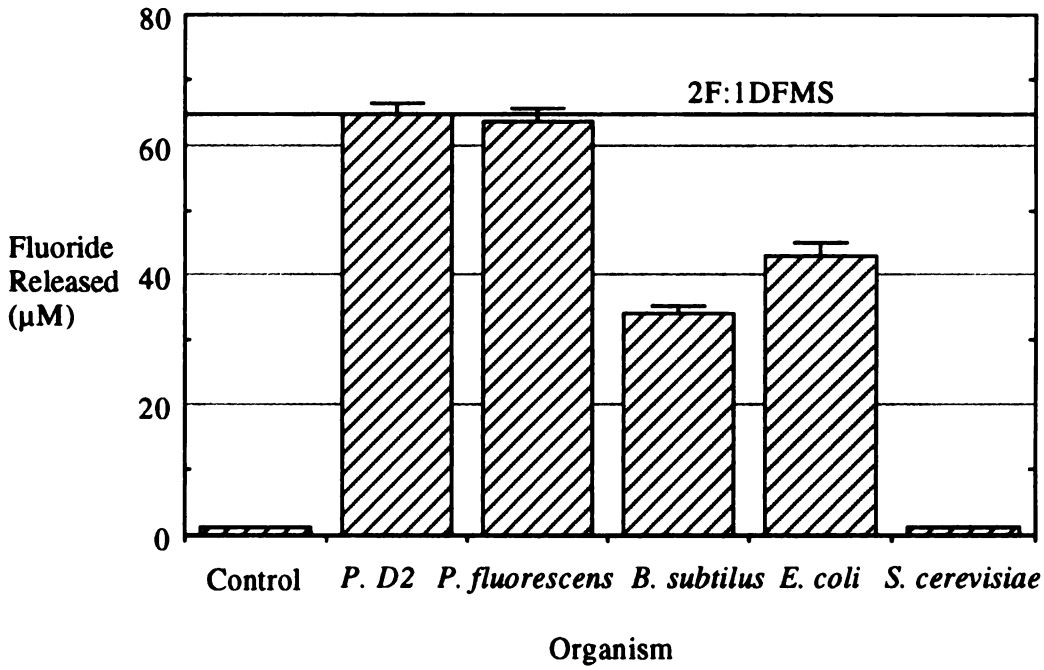

Figure 5.1. Defluorination of DFMS (32 μM) by various bacteria and yeast under aerobic and sulfur-limiting conditions. Error bars represent the standard deviation of triplicate samples.

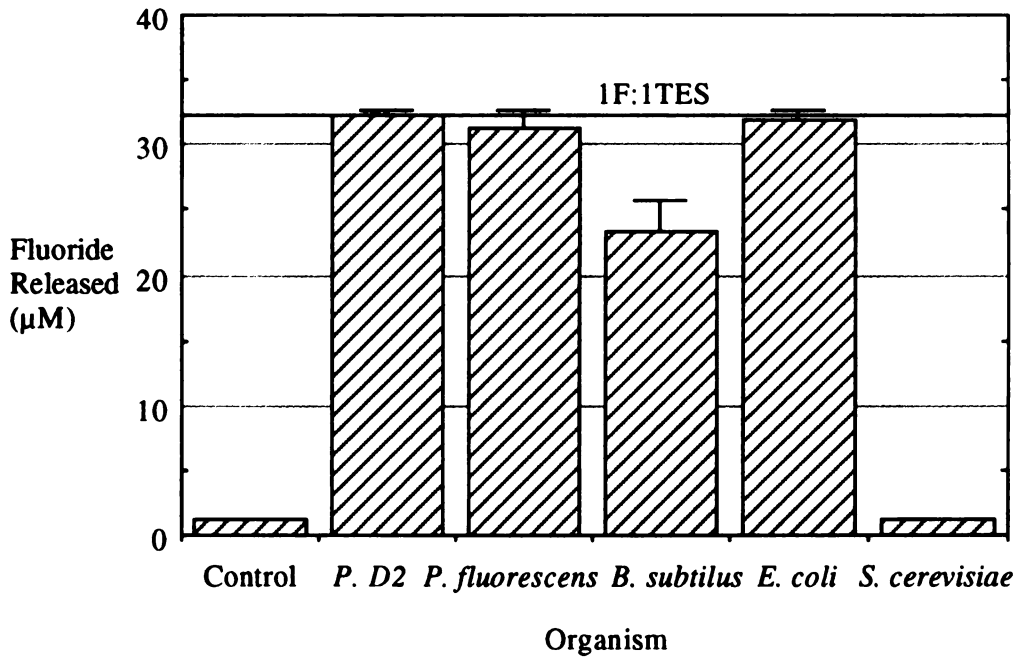

Figure 5.2. Defluorination of 2,2,2-trifluoroethane sulfonic acid (32 μM) by various bacteria and yeast under aerobic and sulfur-limiting conditions. Error bars represent the standard deviation of triplicate samples.

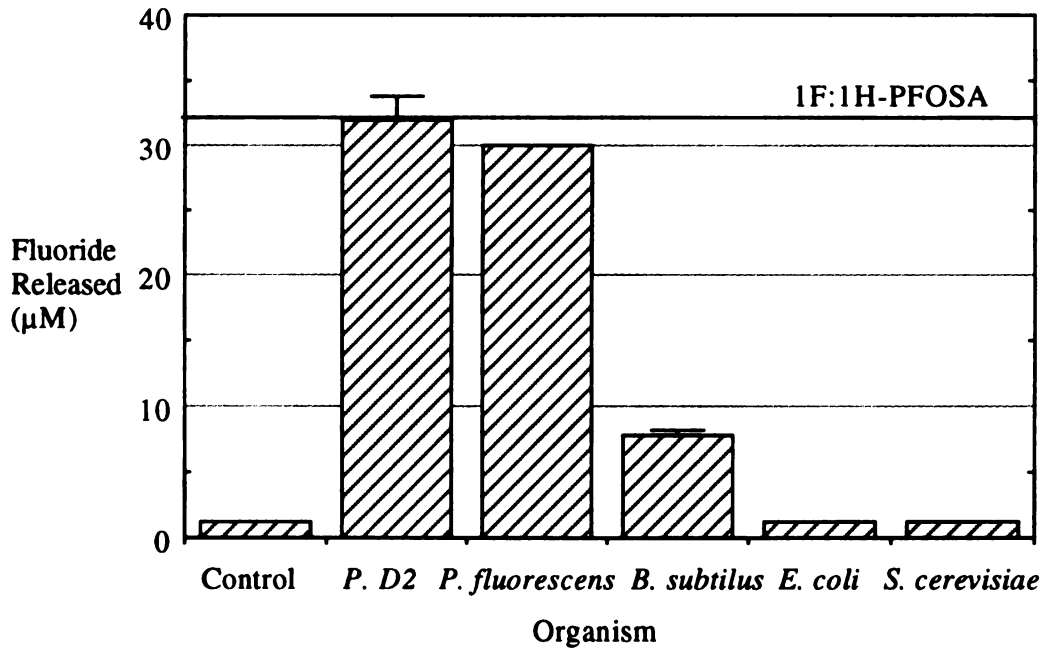

Figure 5.3. Defluorination of 1H,1H,2H,2H-perfluorooctane sulfonic acid (32 μM) by various bacteria and yeast under aerobic and sulfur-limiting conditions. Error bars represent the standard deviation of triplicate samples.

Biotransformations in aqueous samples. Two different aquatic samples were used to determine the fate of fluorinated sulfonates in natural microbial communities: activated sludge from a wastewater treatment facility and river water. To insure that these samples were not carbon limited 2 g/L glucose was added to all but the no carbon control. It was shown previously (Chapter 4) that ammonium is also required for the transformation of DFMS, so complete growth medium was added to the carbon plus ammonium replicates. No transformation was observed in any of the activated sludge and river samples. Sulfate concentration in these samples was 1.1 mM for the activated sludge and 0.90 mM for the river water.

Biotransformations in soils. A volatile transformation product was detected by gas chromatography with an electron capture detector (Figure 5.4) and GC/MS ion trap (Figures 5.6 and 5.7) in all soil samples with and without added carbon. This peak was not observed in the autoclaved controls nor in the samples without H-PFOSA. This peak was identical to a peak found as a transformation product of H-PFOSA by *Pseudomonas* sp. strain D2 (Figures 5.5, 5.8 and 5.9). Atomic emission analysis of these peaks indicates that they are fluorinated and do not contain sulfur.

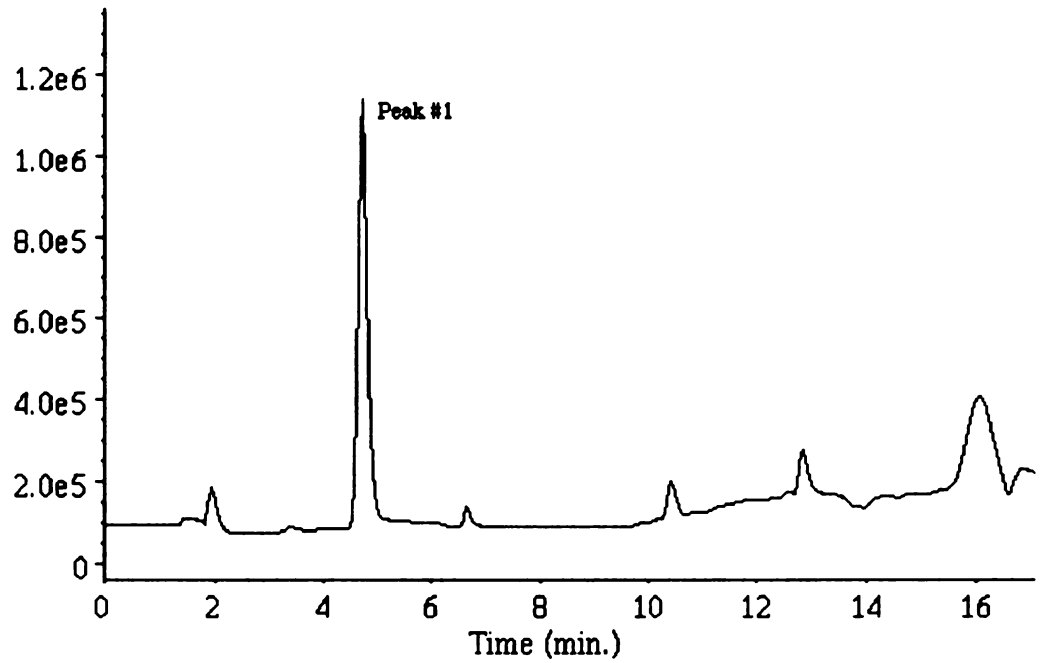

Figure 5.4. GC/ECD chromatogram of the natural area incubation with glucose and H-PFOSA.

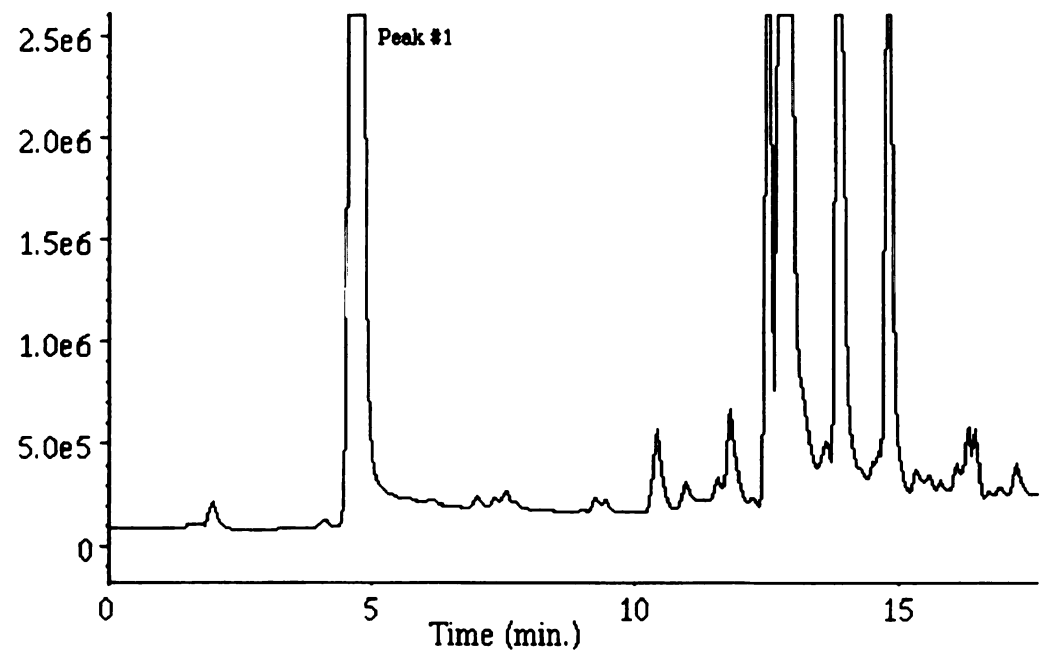

Figure 5.5. GC/ECD chromatogram of strain D2 incubated with glucose and H-PFOSA.

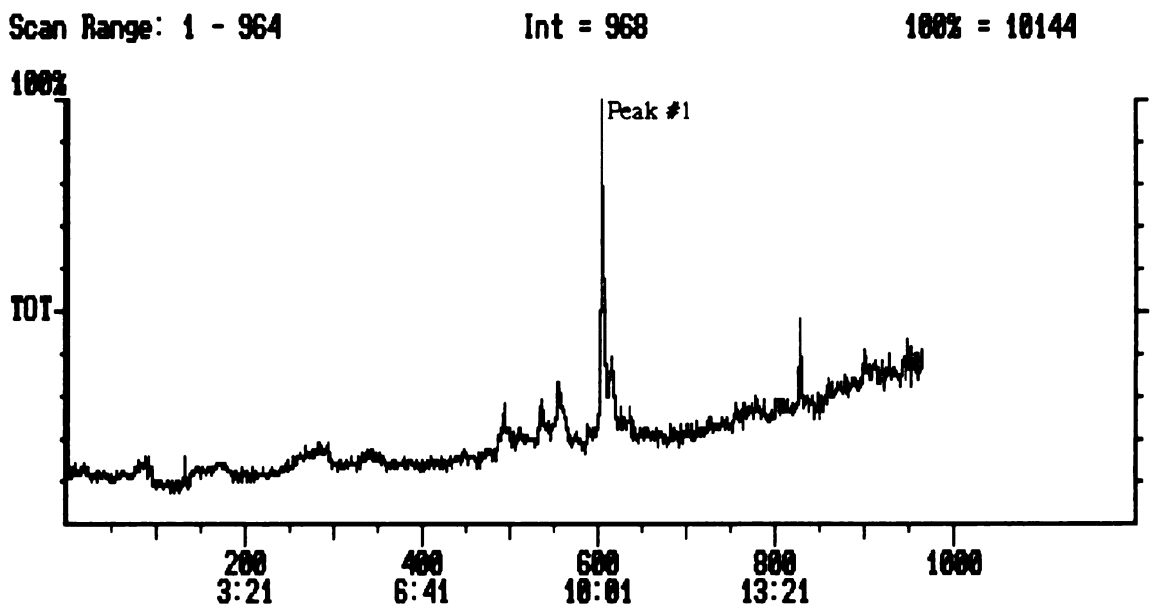

Figure 5.6. GC/MS chromatogram of the natural area incubation with glucose and H-PFOSA.

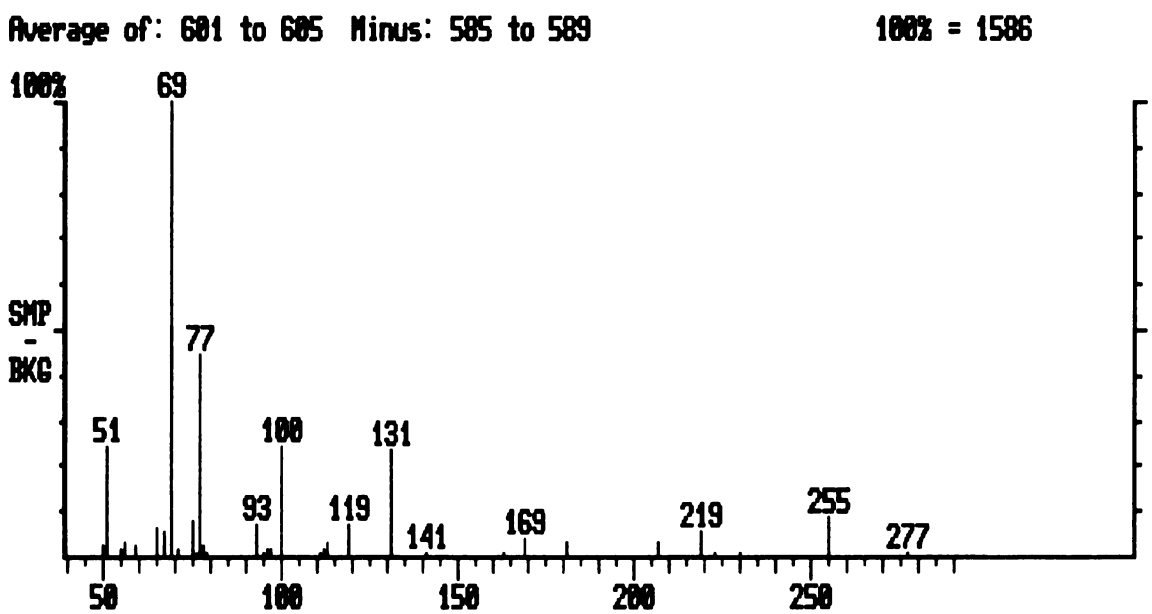

Figure 5.7. GC/MS spectrum number 604 of the natural area incubation with glucose and H-PFOSA.

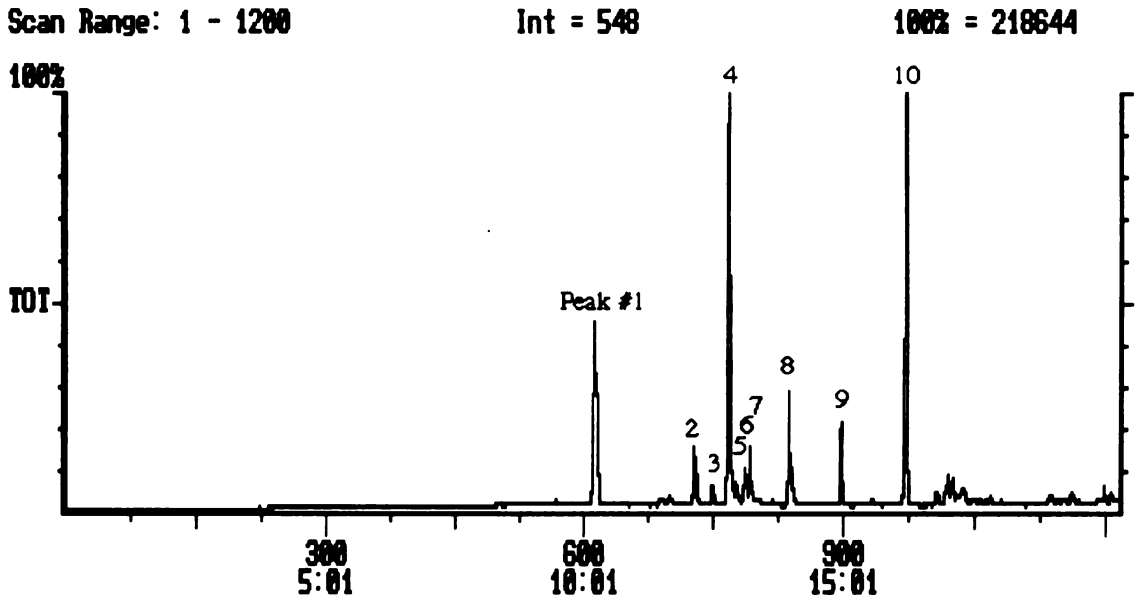

Figure 5.8. GC/MS chromatogram of strain D2 incubated with glucose and H-PFOSA.

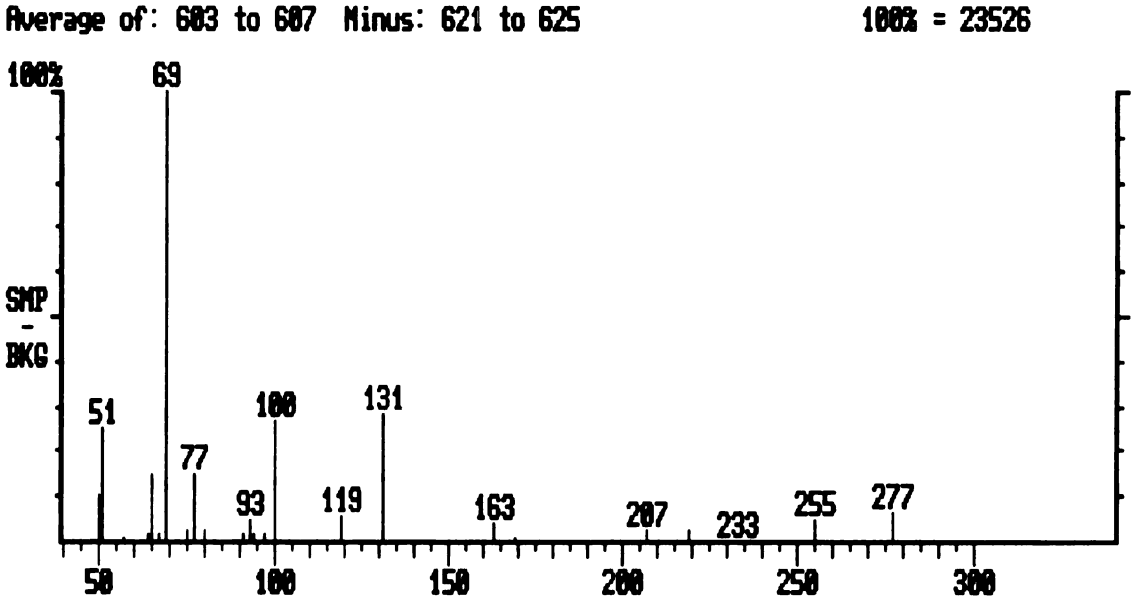

Figure 5.9. GC/MS spectrum number 604 of strain D2 incubated with glucose and H-PFOSA.

DISCUSSION

As is shown in Figures 5.1 through 5.3, Pseudomonads related to strain D2 showed transformation patterns that were similar to those of strain D2 with respect to DFMS, TES, and H-PFOSA. *B. subtilis* was not as effective at transforming all of the fluorinated sulfonate present. A minimal medium was used for all of these experiments, so that the inability of *B. subtilis* to degrade all of the sulfonate present may be associated with depletion of some essential nutrient or carbon source. This limited transformation may also be the result of production of a toxic byproduct. A similar result was observed with *E. coli* when DFMS was the sole sulfur source but not when TES was the sole sulfur source. Interestingly, *E. coli* was not capable of growth on or transformation of H-PFOSA. *E. coli* may lack the necessary transport mechanism to transport H-PFOSA across the cell membrane or a different mechanism(s) of transformation may be present in *E. coli*. Uria-Nickelsen et al. [8] demonstrated growth of *S. cerevisiae* on various sulfonates such as taurine, isothionate, and cysteate. However, *S. cerevisiae* was not capable of growth on any of the fluorinated sulfonates tested. The electronegativity of the carbon-fluorine bond may inhibit the uptake of organofluorine sulfur compound or it may inhibit the transformation of these compounds.

No transformation was observed in the river water or activated sludge samples. This is most likely due to high background sulfate concentrations (0.9-1.1 mM sulfate) that inhibits transformation of DFMS. However, in soil incubations with H-PFOSA a volatile peak was observed. This product had a mass spectrum identical to one of the peaks generated by incubations of *Pseudomonas* sp. strain D2 with H-PFOSA. The mass/charge ratio for many of the fragments from this product are typical of fluorinated compounds (i.e. 69, 100, 119, 131, 169, and 219). Figures 5.4 and 5.6 illustrate that this product had the same run time and response to GC/ECD. In addition to these results,

atomic emission analysis of this peak indicates that it is fluorinated and does not contain sulfur. These observations support the conclusion that H-PFOSA is transformed in soils with the production of a volatile fluorinated compound that is identical to one of the products of H-PFOSA degradation by *Pseudomonas* sp. strain D2. Further characterization of this product may provide clues to the mechanism(s) of transformation.

The results of this work demonstrate that fluorinated sulfonates can be degraded by a wide range of bacteria as well as within complex communities of microorganisms. In addition, this data suggest that if the environmental restrictions of sulfur limitation and aerobic conditions are satisfied then degradation of these compounds is possible. Future work is needed to identify the mechanism(s) of transformation and to explain why transformation is apparently limited to bacteria, with differences in the extent of transformation and types of sulfonates susceptible to transformation.

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CHAPTER 6

BIODEGRADATION OF MONOFLUOROACETATE UNDER DENITRIFYING CONDITIONS

ABSTRACT

This research investigated the potential for biodegradation of monofluoroacetate (MFA), a fluorinated pesticide, under anaerobic conditions. Enrichment cultures were evaluated for MFA degradation under denitrifying and sulfate-reducing conditions. Defluorination was detected under both conditions. Although no isolates were obtained from the sulfate-reducing enrichments, a bacterium, designated strain M7, was isolated from the denitrifying enrichment. The isolate was capable of growth on MFA and defluorination under denitrifying conditions. Phylogenetic analysis of the 16S rRNA sequence for the isolate indicated a 96.0 to 97.5% match for the *Bradyrhizobium* genus. When incubated with MFA, crude cell extracts of aerobically or anaerobically grown strain M7 released glycolate and fluoride indicating hydrolytic defluorination. Strain M7 was not capable of defluorinating molecules with more than one fluorine substituent, but was capable of dechlorinating monochloroacetate, dichloroacetate, and trichloroacetate. Other bacteria with the ability to utilize MFA under aerobic conditions were evaluated under denitrifying conditions. Two of five isolates were capable of growth on MFA under denitrifying conditions. These findings demonstrate that defluorination of MFA is not limited to aerobic conditions and is likely widespread in the biosphere.

INTRODUCTION

Monofluoroacetate (MFA) is one of the most toxic substances known, based on a lethal dose (LD_{50}) of 0.7-2.1 mg/kg for man [1]. Its toxicity is due to "lethal synthesis" of fluorocitrate which inhibits the aconitase enzyme of the Kreb's cycle [22] although recent investigations implicate fluorocitrate as a "suicide" substrate instead of a competitive inhibitor [3]. MFA is produced naturally by plants in the genus *Dichapetalum*, as well as *Palicourea marcgravii, Acacia georginae, Gastrolobium grandiflorum*, and *Oxylobium* species [11, 12, 23]. The West African plant, *Dichapetalum toxicarium*, produces ω-fluorooleic acid, ω-fluoropalmitic acid, and possibly ω-fluorocaprate and ω-fluoromyristate [24]. Certain fungi are known to produce fluorinated organics: *Streptomyces clavus* and *Streptomyces cattleya* produce the fluorine containing antibiotics, nucleocidin and 4-fluorothreonine, respectively [12, 24, 25]. *Streptomyces cattleya* is also capable of producing MFA [25].

Given that certain plants can produce MFA, it is not surprising that several microorganisms can metabolize MFA. While many investigators have reported aerobic defluorination of MFA by bacteria (mainly *Pseudomonads*) and fungi [6, 7, 8, 9, 15, 18, 19, 26, 29], few reports of MFA biodegradation under anaerobic environments are available. Gregg et al. [10] demonstrated defluorination of MFA by genetically modified rumen bacteria *Butyrivibrio fibrisolvens*. The engineered strain contained a plasmid encoding MFA defluorination genes derived from *Moraxella* sp. strain B. The goal of this research was to protect cattle from MFA poisoning by accidental ingestion of MFA-containing plants. Evidently, there is only one other report of anaerobic MFA degradation. Visscher et al. [28] reported reductive dehalogenation of MFA to acetate under methanogenic conditions, but this transformation was not reproducible in subsequent investigations [21].

The present report documents isolation and characterization of *Bradyrhizobium sp.* strain M7, a facultative aerobe that is capable of growth on and defluorination of MFA under denitrifying conditions. The defluorination reaction is shown to proceed by a hydrolytic pathway like that utilized by aerobic organisms. The observation of MFA degradation is then generalized to other bacterial species.

MATERIALS AND METHODS

Chemicals. Sodium monofluoroacetate, 95 %, was obtained from Aldrich Chemical (Milwaukee, WI). Sodium difluoromethane sulfonate, sodium difluoromalonate, and sodium tetrafluorosuccinate were provided for by 3M, St Paul, MN. All other carbon sources and chemicals were obtained from Sigma Chemical (St. Louis, MO). Nutrient agar and nutrient broth were purchased from Difco (Detroit, MI)

Denitrifying and sulfate-reducing enrichments. Primary effluent from the East Lansing Wastewater Treatment Facility, East Lansing, Michigan was used as a source of organisms for enrichment culture. Denitrifying enrichments were prepared by amending this effluent with 2.35 mM sodium nitrate as electron acceptor and 5 mM sodium monofluoroacetate as a carbon source. Sulfate-reducing enrichments were initiated with 2.80 mM sodium sulfate as an electron acceptor. Enrichments were sealed with teflon lined stoppers, and incubated at 20° C for approximately four weeks. Subsequent enrichments and subculturing were performed in defined mineral medium containing (in grams per liter 18 Mohm water): K_2HPO_4, 0.7; KH_2PO_4, 0.4; $NaNO_3$, 0.4; NH_4SO_4, 0.2; $MgSO_4 \cdot 7H_20$, 0.1; 0.15M $CaCl_2 \cdot 2H_20$ stock, 0.2 ml/liter; trace elements stock, 0.2 ml/liter; yeast extract, 0.05. Trace elements stock contained (in grams per 500 ml of 18 Mohm water): $FeSO_4$, 0.68; $Na_2MoO_4 \cdot 2H_20$, 0.12;

$CuSO_4 \cdot 5H_20$, 0.125; $ZnSO_4 \cdot 7H_20$, 0.29; $Co(NO_3)_2 \cdot 6H_20$, 0.145; $NiSO_4 \cdot 6H_20$, 0.11; $NaSeO_3$, 0.018; $B_3(OH)_3$, 0.031; NH_4VO_3, 0.06; and $MnSO_4 \cdot H_20$, 0.505. Concentrated H_2SO_4 was added at 1ml/L to the trace metals solution. After initial subculturing, yeast extract was removed from the medium.

Bacterial strains and growth conditions. Strain M7 was grown to stationary phase aerobically in medium containing 10 mM MFA, unless otherwise noted. Cells were typically grown at 30 °C and shaken at 150 rpm on a rotary shaker. For MFA degradation experiments, cells were harvested by centrifugation (15 minutes at 12,100 X g in a Beckman SS-34 rotor at 4°C), washed in buffered medium then resuspended in fresh medium. For anaerobic incubations, bottles with cells and medium were degassed with nitrogen, amended with MFA, and capped with teflon stoppers. Protein was assayed using the modified Lowry method, with bovine serum albumin as the standard [17]. Five isolates capable of aerobic growth on MFA were provided by M. Emptage (DuPont Central Research and Development, Wilmington, DE). All of these isolates were Gram-negative rods. Strain WS26b-12-Z and strain CFR1-16-BO have not been identified, strain WS3-12-Z has been identified as a Pseudomonad by Vitek and Biolog, strain NZ14-5a has been identified as *Ancylobacter aquaticus* by Biolog, and strain DW1-9-G has been identified as *Agrobacterium tumefaciens* by MIDI (M. Emptage, personal communication). These isolates were grown in the same medium used for strain M7.

Chemical analysis. MFA, nitrate, nitrite, and fluoride were measured on a Dionex ion chromatography model 2000i/sp fitted with an IonPac AS4A ion exchange column and a Dionex IonPac AG4A guard column. This system utilizes an anion micromembrane suppressor with a Dionex Conductivity Detector-II (CDM). The eluant was a carbonate buffer (1.8 mM Na_2CO_3 and 1.7 mM $NaHCO_3$) with a flow rate of 2 ml/min. Fluoride

was also measured using an ion selective electrode (Orion 96-09 BN). In addition, ion exclusion chromatography was used to determine fluoride and MFA using a Dionex IonPac ICE-AS1 column. The eluant for ion exclusion chromatography was 1.0 mM octanesulfonic acid / 2% 2-propanol at a flow of 0.8 ml/min. Nitrous oxide was detected using a thermal conductivity detector (TCD). A Hewlett-Packard 5890 GC fitted with a molecular sieve column (13x, 80/100 mesh, Alltech Associates, Inc., Deerfield, IL) was operated isothermally at 150 C°. Headspace samples (100 µl) were injected with a 1 ml gas-tight syringe.

Metabolite analysis. Strain M7 was grown in 500 ml batches in 1L Erlenmeyer flasks for 5 days from a 1% inoculum. Actively transforming cultures were centrifuged at 12,100 X g for 15 minutes in a Beckman SS-34 rotor at 4°C. Cells were then washed twice and resuspended in 8 ml of buffered growth medium without MFA. Six milliliters of this concentrated cell suspension was placed on ice and was disrupted by a 550 Sonic Dismembrator (Fisher Scientific, Pittsburgh, PA) for 10 minutes at 1 second bursts at stage 5 (50% time on and 50% time off). The effective sonication time was 5 minutes. The sonicate was centrifuged for 14,000 X g for 30 minutes at 4°C and divided into a supernatant and cell pellet. The supernatant (final protein concentration of 35 µg/ml) from this crude cell extract was then incubated with 1 mM MFA in growth medium and shaken at 150 rpm at 30 °C. One fraction was degassed with nitrogen, and the other fraction remained aerobic. As a control for glycolate accumulation, whole cells were also incubated with 1 mM MFA aerobically and anaerobically. Samples were withdrawn periodically from both fractions and assayed for glycolate. Glycolate was measured by ion exclusion chromatography as described above and by the colorimetric method of Calkins [2].

Kinetic characterization. Growth kinetic parameters were determined using van Uden's modified version of Monod's microbial growth model which includes endogenous decay of microorganisms :

$$\mu = \mu_{max} * (S / (Ks + S)) - b = (dX/dt)/X$$

where μ is specific growth rate (d^{-1}), μ_{max} is the maximum specific growth rate (d^{-1}), S is the rate-limiting substrate concentration (mg/L), Ks is the half-velocity coefficient (mg/L), b is the endogenous decay rate (d^{-1}), t is time in days (d), and X is the concentration of microorganisms (mg/L) [27]. The model used for substrate consumption was:

$$U = (k * S) / (Ks + S) = -(dS/dt)/X$$

where U is the specific substrate utilization coefficient (d^{-1}) and k is maximum specific substrate utilization coefficient (d^{-1}). Values for k and Ks were estimated using a nonlinear curve fit obtained from Systat version 5.2.1 based on initial MFA degradation rates at a fixed cell protein concentration. Yield (Y; mg protein/mg MFA) was determined by dividing the change in total cell protein by the change in MFA concentration. Maximum specific growth rate was calculated by taking the slope of the equation:

$$\ln(X/X_0) = \mu_{max} * t$$

where X_0 is the initial cell concentration (mg/L).

The endogenous decay rate was calculated by taking the slope of the equation:

$$\ln(X) = b*t.$$

Molecular characterization. To establish the phylogenetic relationship for M7, 16S rRNA gene-based molecular analyses were performed. The total genomic DNA from strain M7 was isolated using the SDS-based lysis method [30]. The 16S rRNA gene was amplified as described previously [30]. The PCR amplified products were purified and directly used as the templates for automated fluorescent sequencing [31]. The DNA sequence was determined using 10 primers from both directions [30].

Sequences were assembled using assembling programs in the Genetic Computer Group (GCG) software package [4], and a preliminary analysis was done by searching the current databases (GenBank release 91.0 and EMBL release 44.0) using the program FASTA. Sequences were then aligned manually to the 16S rDNA sequences of the species, which showed high similarity scores in the outputs of FASTA, in the previously aligned 16S rDNA sequence database, RDP (Ribosomal Database Project) [16] using the GDE multiple sequence editor program from RDP. Initial phylogenetic screening was constructed using the DNA distance program, Neighbor-Joining, in the PHYLIP package [5] based on all 16S rDNA sequences from alpha subdivision. Based on the initial phylogenetic results, appropriate subsets of 16S rDNA sequences were selected and subjected to final phylogenetic analyses through maximum parsimony method with a bootstrap procedure of 500 replicates, DNA distance matrix method, Neighbor-Joining, and maximum-likelihood method. The SEQBOOT program was used to obtain confidence levels.

RESULTS

Evaluation of initial enrichments and isolation of denitrifying bacteria. Both the denitrifying and sulfate-reducing enrichments demonstrated defluorination of MFA (Figure 6.1). The sulfate-reducing enrichments turned black as evidence of sulfate reduction with removal of sulfate as measured by ion chromatography. Nitrate and nitrite were completely depleted in the denitrifying enrichments. No isolates were obtained from the sulfate-reducing enrichments. However, an isolate designated strain M7 was isolated aerobically from the denitrifying enrichment by streaking on 1.5 % (wt/vol) nutrient agar plates. Colonies were small (approximately 1 mm) and appeared only after a lengthy incubation period. Colonies were opaque specks and did not spread.

Figure 6.2 illustrates defluorination of MFA, with the corresponding reduction of nitrate and accumulation of fluoride ion. A minor accumulation of nitrite during log growth phase was observed followed by subsequent utilization. Nitrous oxide was detected in the headspace of anaerobically grown cultures. Growth kinetic data for this strain M7 under both aerobic and anaerobic conditions are provided in Table 6.1.

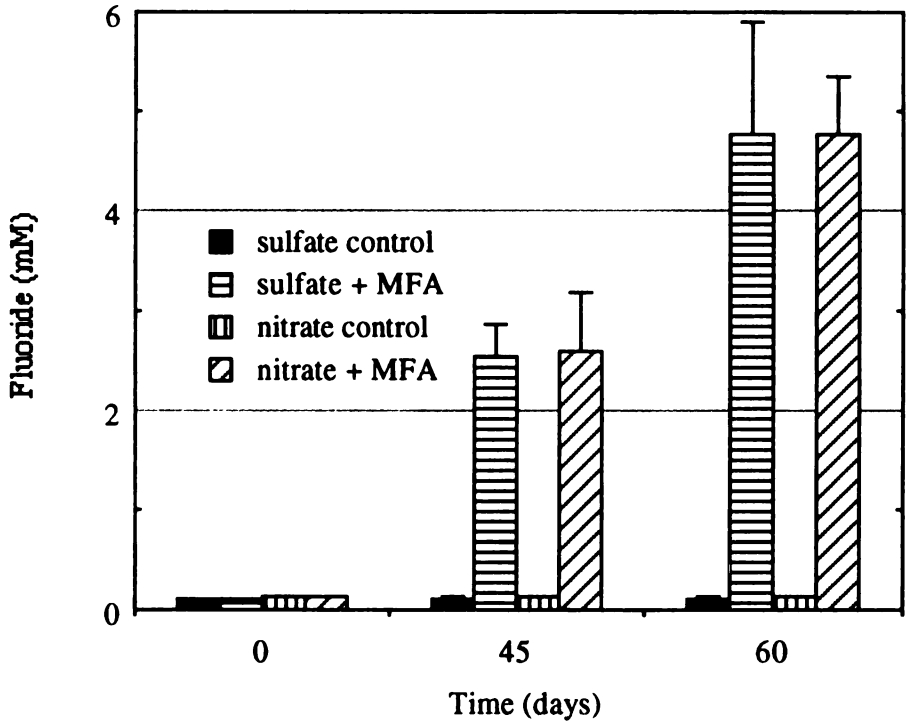

Figure 6.1. Defluorination of MFA by denitrifying and sulfate-reducing enrichments. Error bars represent the standard deviation of triplicate samples.

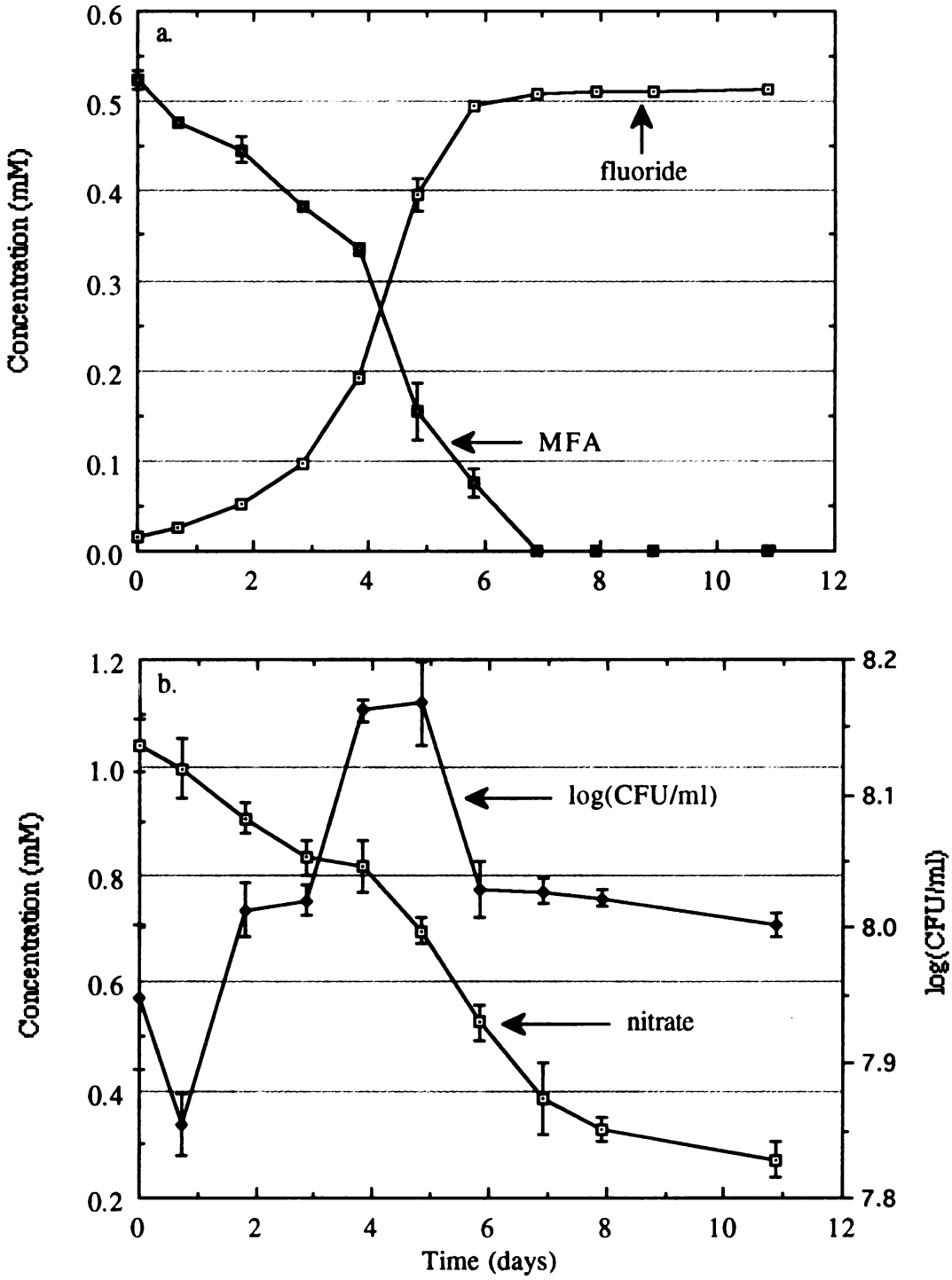

Figure 6.2. Growth of *Bradyrhizobium sp.* strain M7 on MFA under denitrifying conditions: (a) consumption of MFA and production of fluoride, (b) consumption of nitrate and growth of cells. Error bars represent the standard deviation of triplicate samples.

Table 6.1. Kinetic parameters for *Bradyrhizobium sp.* strain M7

Kinetic parameter	Denitrifying conditions	Aerobic conditions
k (d^{-1})	3.0 ± 0.30	4.9 ± 0.40
Ks (mg MFA/L)	4.9 ± 0.20	26 ± 7.0
b1 (d^{-1})	0.14 ± 0.02	0.14 ± 0.02
b2 (d^{-1})	0.013 ± 0.002	N/A
Y (mg protein/mg MFA)	0.13 ± 0.01	0.15 ± 0.01
μ_{max} (d^{-1})	0.29 ± 0.02	0.52 ± 0.03

Physiological characterization . Strain M7 is a motile, Gram-negative rod (2.5-4 by 0.8 μM) that utilizes MFA as its sole source of carbon under aerobic and denitrifying conditions. Liquid cultures of strain M7 grow slowly, requiring 2-3 days to enter log phase with a 1% inoculum in nutrient broth. It is both catalase positive and oxidase positive. Optimal growth is observed at 30° C. Strain M7 was classified as a denitrifier based on a growth yield that was proportional to the amount of nitrate reduced, use of both nitrate and nitrite as electron acceptors for MFA degradation, and detection of N_2O during anaerobic growth. Fatty acid profiles were performed by Microbial ID, Inc. (MIDI), Newark, Delaware. Although no match to the MIDI library was obtained there were some unique fatty acids present: 48% of the fatty acids were present as cis-11-octadecenoic acid, 18.5% as octadecenoic acid, and 14.3% as cis-9-hexadecenoic acid.

Phylogenetic analysis. Phylogenetic analyses established by bootstrap parsimony method showed that strain M7 is affiliated with the *Rhizobium-Agrobacterium* group of the alpha subdivision of the *Proteobacteria*, and very closely related to *Bradyrhizobium* species (Figure 6.3). Similar trees were also obtained by DNA distance matrix methods

and maximum likelihood methods. Strain M7 has 96.0 to 97.5% similarities to *Bradyrhizobium* species. Since the similarities of 16S rRNA genes from most *Bradyrhizobium* species range from 95.5 to 99.9%, it is proposed that strain M7 is a new unidentified species of the genus *Bradyrhizobium*.

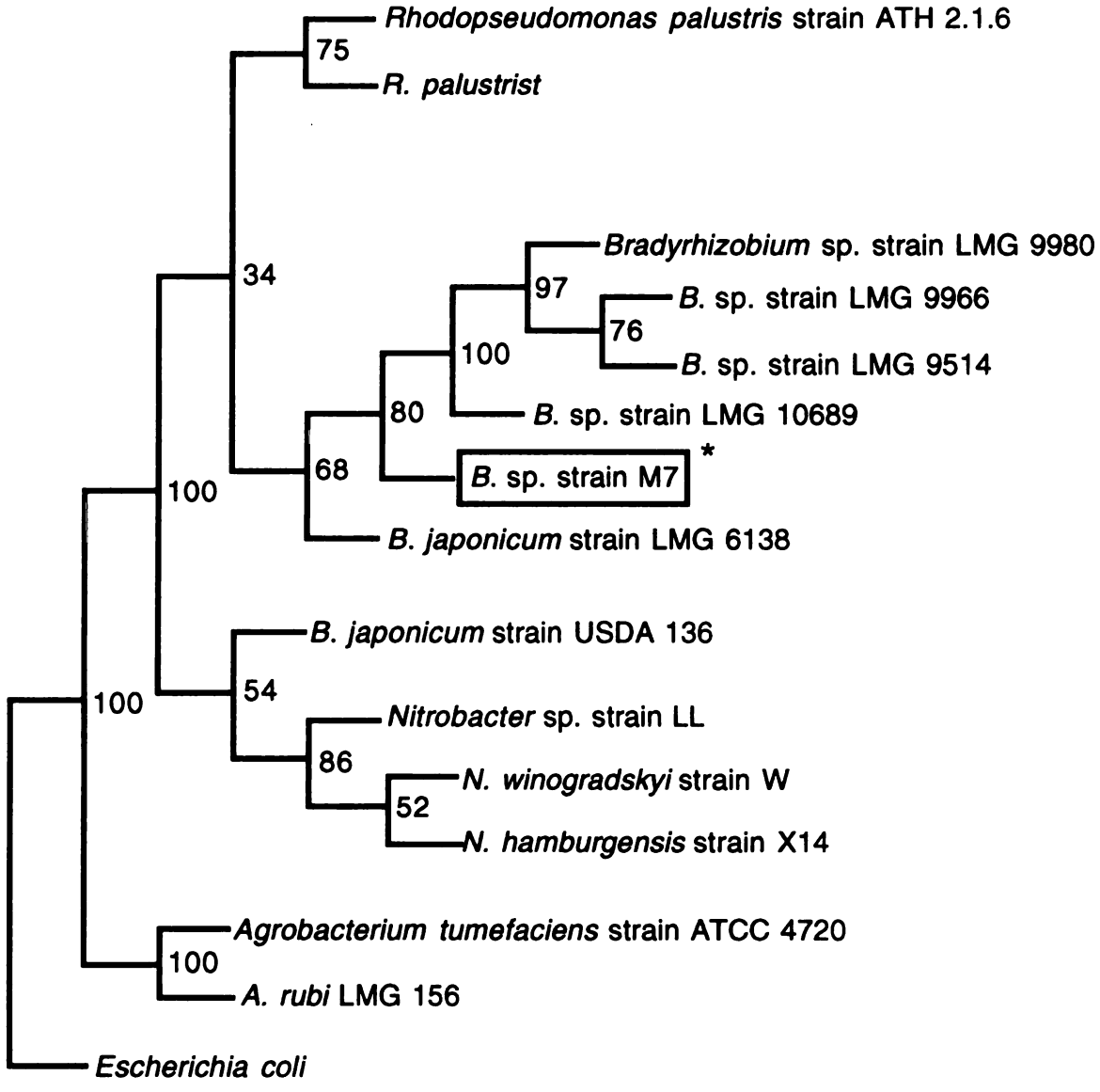

Figure 6.3 Phylogenetic tree showing the location of *Bradyrhizobium* sp. strain M7 in relation to other *Bradyrhizobium* species.

MFA degradation. When aerobically grown cells were converted to oxygen-free conditions, specific substrate utilization rates were reduced by a sixth (k, 0.83 ± 0.17 d^{-1}; Ks, 11 ± 2 mg/L) as compared to cells that were incubated aerobically (see Figure 6.4). When anaerobically grown cells were converted to aerobic conditions however, there was no detectable inhibition of rates as compared to cells that were incubated anaerobically (data not shown). Cells that were grown anaerobically and then incubated anaerobically with MFA had a lower specific substrate utilization rate (k) and a lower half-velocity coefficient as compared to aerobically grown and aerobically incubated cells.

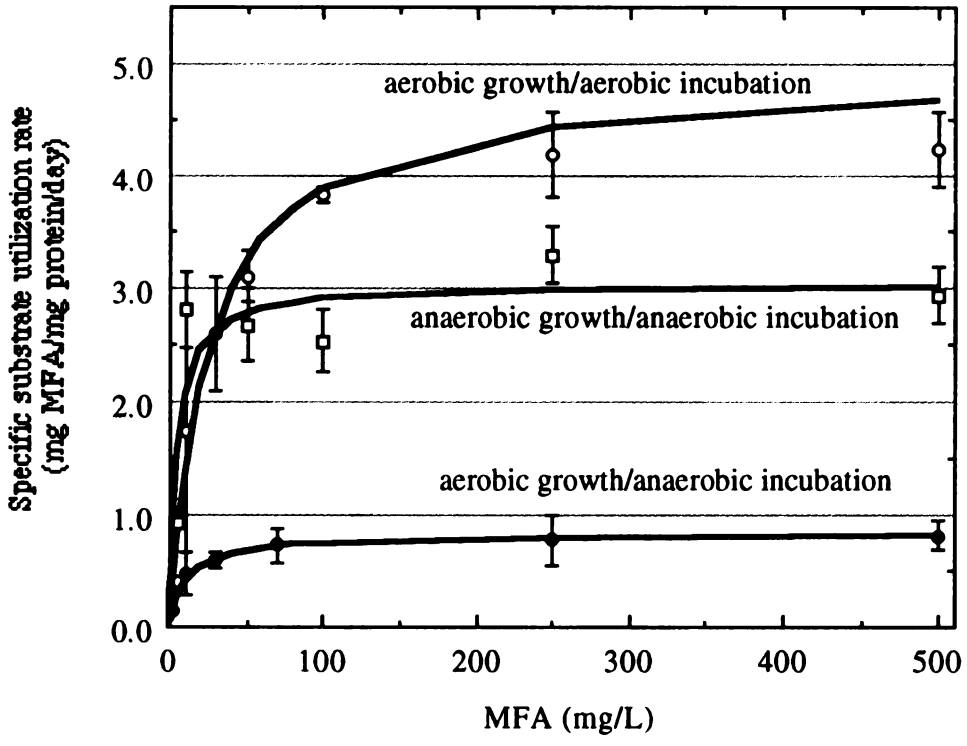

Figure 6.4. Comparison of specific substrate utilization rates for aerobically and anaerobically grown *Bradyrhizobium sp.* strain M7. Error bars represent the standard deviation of triplicate samples.

Identification of glycolate as a metabolite of MFA degradation. Several investigators have demonstrated that the first step in degradation of MFA is hydrolytic attack of the carbon-fluorine bond resulting in glycolic acid as an intermediate [6, 7, 26]. Aerobic and anaerobic cell extracts and whole cells of strain M7 were incubated with MFA to assay glycolate production. No accumulation of glycolate was observed for aerobic or anaerobic whole cells. However, supernatant from crude cell extracts from aerobically grown cells, incubated both anaerobically and aerobically, showed stoichiometric accumulation of glycolate and fluoride (see Table 6.2)

Table 6.2. Glycolate determination in cell free extract of strain M7. Values were obtained by ion exclusion chromatography. Glycolate levels were confirmed by colorimetric assay.

	Glycolate (μM) produced	MFA (μM) converted
Anaerobic whole cells	0	1215
Anaerobic supernatant	276	282
Aerobic whole cells	0	1200
Aerobic supernatant	305	326

Degradation of other halogenated compounds. As shown in Table 6.3, strain M7 was unable to defluorinate compounds with more than one fluorine substituent. However, chlorinated analogues of MFA monochloroacetate, dichloroacetate, and trichloroacetate degraded both aerobically and anaerobically using cells that had been grown on MFA. *Bradyrhizobium sp.* strain M7 was not capable of growth on the chlorinated analogues under aerobic or anaerobic conditions.

MFA degradation by other bacteria under denitrifying conditions. Five strains capable of MFA degradation aerobically were evaluated for MFA degradation under denitrifying conditions. Two of the five tested strains, strains DW1-9-G and WS3-12-Z, were capable of growth on MFA under denitrifying conditions. Strain WS3-12-Z showed some accumulation of nitrite with MFA degradation. None of the other strains were capable of growth under denitrifying conditions with MFA as the sole carbon source.

Table 6.3. Substrate degradation and growth by *Bradyrhizobium sp*. strain M7

	Denitrifying conditions		Aerobic conditions	
Substrate	substrate degradation	growth	substrate degradation	growth
monofluoroacetate	+	+	+	+
difluoroacetate	-	-	-	-
trifluoroacetate	-	-	-	-
difluoromalonate	-	-	-	-
tetrafluorosuccinate	-	-	-	-
trifluoromethane sulfonate	-	-	-	-
difluoromethane sulfonate	-	-	-	-
monochloroacetate	+	-	+	-
dichloroacetate	+	-	+	-
trichloroacetate	+	-	+	-
glycolate	+	+	+	+
glyoxylate	+	+	+	+
oxalate	+	+	+	+

DISCUSSION

Although many bacteria are known to use MFA as a growth substrate under aerobic conditions [6, 7, 8, 9, 15, 18, 19, 26, 29], this is the first report of a naturally occurring bacterium using MFA as a growth substrate under anaerobic conditions. It is also the first report of a *Bradyrhizobium* species possessing this trait. The results with other

MFA-degrading bacteria demonstrate that the ability to degrade MFA under anaerobic conditions is not unique to strain M7. For example, strains DW1-9-G and WS3-12-Z were also able to grow on MFA under denitrifying conditions. Although no isolate was obtained from the sulfate-reducing enrichments, defluorination of MFA was observed under this condition. Therefore, it may be possible to obtain a sulfate-reducing isolate that is capable of degrading MFA. Clearly, the ability to degrade MFA under anaerobic conditions is not restricted to the *Bradyrhizobium* and in fact several other bacteria can degrade MFA under anaerobic conditions. These findings suggest that this trait is widely distributed in nature.

Defluorination of MFA, by strain M7 under denitrifying conditions appears to proceed by the same hydrolytic mechanism and pathway as aerobic MFA degradation although, it remains to be seen whether a single enzyme system is responsible for the transformation under both conditions. MFA was defluorinated hydrolytically with production of glycolate as an intermediate under both aerobic and anaerobic conditions. The results of this study suggest that the same or a similar halidohydrolase produced by aerobes capable of MFA degradation is also produced by strain M7 and other denitrifying bacteria. The pattern of hydrolytic defluorination, with the production of glycolate, and degradation of the chlorinated analogues , is similar to results obtained by others [6, 9, 29]. The existence of a transmittable plasmid could explain the broad distribution of MFA-degrading capabilities among different genera of bacteria. Kawasaki et al.[13] demonstrated that the halidohydrolase responsible for defluorination of MFA is plasmid encoded in *Moraxella sp.* strain B. Kawasaki et al. and others have cloned this plasmid into *Escherichia coli* [14] and *Butyrivibrio fibrisolvens*. [10]. In addition, Meyer and van Rooyen [20] have transferred the plasmid responsible for MFA defluorination from *Pseudomonas cepacia* into *Bacillus subtilis*

and have shown that the genetically engineered *B. subtilis* was also capable of defluorinating MFA.

As shown in Table 6.1, the maximum specific growth rate (μ_{max}), the maximum specific substrate utilization rate (k), and the half-velocity coefficient constant (Ks) are different for aerobically grown cells as compared to the anaerobically grown cells. Another difference between aerobic and anaerobic degradation of MFA can be seen in Figure 6.4. When aerobically grown cells are incubated under anaerobic conditions, transformation of MFA is inhibited. However, the contrary is not true when anaerobic cells are incubated aerobically. The inhibition of aerobically grown cells incubated under anaerobic conditions is dramatic, with a one sixth reduction of maximum specific utilization rates. These findings are not readily explained. It is possible that some transport system that functions under aerobic conditions is affected under anaerobic conditions or perhaps different enzyme systems are involved under aerobic and anaerobic conditions.

The results of this study demonstrate defluorination of MFA and growth on MFA as sole carbon and energy source under aerobic and anaerobic conditions by *Bradyrhizobium* sp. strain M7, a genus previously unknown to possess dehalogenating activity. In addition this work establishes defluorination of MFA by other MFA-degrading organisms and by a sulfate-reducing enrichment. The mechanism for degradation of MFA by strain M7 is hydrolytic attack yielding glycolate and fluoride under both aerobic and anaerobic conditions. As observed in aerobic systems the hydrolytic activity is not observed with more highly fluorinated analogues, but is observed with chlorinated analogues.

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CHAPTER 7

CONCLUSIONS AND FUTURE WORK RECOMMENDATIONS

CONCLUSIONS

1. The ability to transform fluorinated sulfonates is restricted at a molecular or structural level. Completely fluorinated sulfonates were not degraded by the organisms evaluated in this work. However, fluorinated sulfonates containing hydrogen (DFMS, TES, and H-PFOSA) were defluorinated and used as sources of sulfur for growth.

2. The ability to transform fluorinated sulfonates was restricted by physiological conditions. No transformation of fluorinated sulfonates was observed under anaerobic conditions. Transformation was inhibited by other sulfur sources and was completely inhibited when other sulfur sources were present at levels sufficient to support growth. DFMS was not utilized as a carbon source. A carbon and nitrogen source were required for transformation by whole cells.

3. Transformation of fluorinated sulfonates under aerobic and sulfur-limiting conditions was linked to a sulfur-scavenging system. Based upon inhibition studies with other sulfur sources it is suggested that desulfonation of the molecule is followed by sulfur assimilation through existing sulfur assimilation pathways.

4. Although DFMS was completely defluorinated, with stoichiometric yield of fluoride, TES and H-PFOSA were only partial defluorinated. Transformation of H-PFOSA by *Pseudomonas* sp strain D2 generated several volatile and fluorinated products. None of these products contained sulfur.

5. In addition to strain D2, several other *Pseudomonas* species could utilize DFMS, TES and H-PFOSA as sulfur sources for growth. The gram positive species *Bacillus subtilis* was also capable of transformation of DFMS, TES, and H-PFOSA. *Escherichia coli* was capable of degrading DFMS and TES, but did not grow on or transform H-PFOSA. *Saccharomyces cerevisiae* was not able to utilize fluorinated sulfonates as a sulfur source.

6. Samples of river water and activated sludge showed no transformation of DFMS. However, soil samples incubated with H-PFOSA generated a volatile product that is most likely fluorinated. It appears that the ability to degrade partially fluorinated sulfonates is widely distributed among bacteria, but this ability may not be expressed if environmental conditions are not suitable.

7. The bacterium *Bradyrhizobium* sp. strain M7 was shown to have MFA-degrading abilities. Strain M7 used MFA as a carbon source under both aerobic and anaerobic conditions.

8. A few bacteria that use MFA as a carbon source under aerobic conditions can also use it as the carbon source under denitrifying conditions. In addition, defluorination was observed under sulfate-reducing conditions. These findings suggest that the ability to defluorinate MFA is most likely widespread throughout nature including some anaerobic environments.

9. The pathway for MFA degradation under denitrifying conditions was hydrolytic attack, with release of fluoride and production of glycolate. This pathway is similar under both aerobic and anaerobic conditions.

FUTURE RESEARCH

1. The volatile byproducts of H-PFOSA transformation should be identified.

2. Improved analytical methods for direct detection and quantification of fluorinated sulfonates in environmental samples are needed. This should include improved methods for sample preparation, separation of mixtures, and detection.

3. The enzyme(s) or cofactors responsible for the transformation of the fluorinated sulfonates should be characterized. This could provide insight into the reasons for inhibition by other sulfur sources and will likely provide clues into the nature of the reaction mechanism (i.e. oxidative, hydrolytic, nucleophilic, etc.). It will also help to explain why some organisms are capable of the transformation (bacteria) while others are not (yeast).

4. Further evaluation of the fate of these compounds in environmental samples is needed. In addition, the fate of fluorinated sulfonates in anaerobic environments should be evaluated.

5. Further work is needed to determine the fate and identity of the byproduct(s) of TES transformation.

APPENDIX A

APPENDIX A

GC/MS DATA USING ELECTRON IMPACT WITH ION TRAP DETECTOR

GC/MS (Ion Trap) analysis:

GC:	Perkin Elmer Autosystem GC
MS:	Perkin Elmer Ion Trap Detector
Column:	DB624 capillary column (30 m x 0.25 mm x1.4 μm) from J & W Scientific (Folsom, CA)
Operation:	40°C for 4 minutes, 10°C/minute ramp to 200°C
Injector:	250°C
Transfer Line:	225°C
Sample:	Headspace for 15-30 minutes with a solid phase microextraction (SPME) fiber assembly with a 100 μM polydimethylsiloxane coating (Supelco, Inc., Bellefonte, PA)

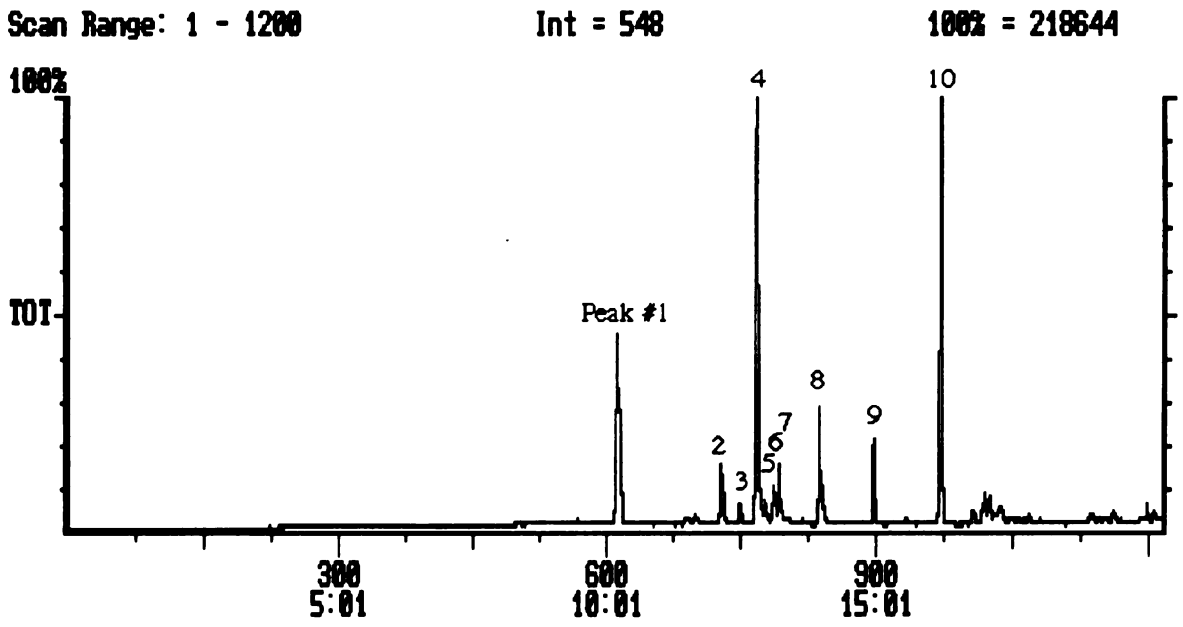

Figure A.1. GC/MS chromatogram of strain D2 incubated with H-PFOSA and glucose.

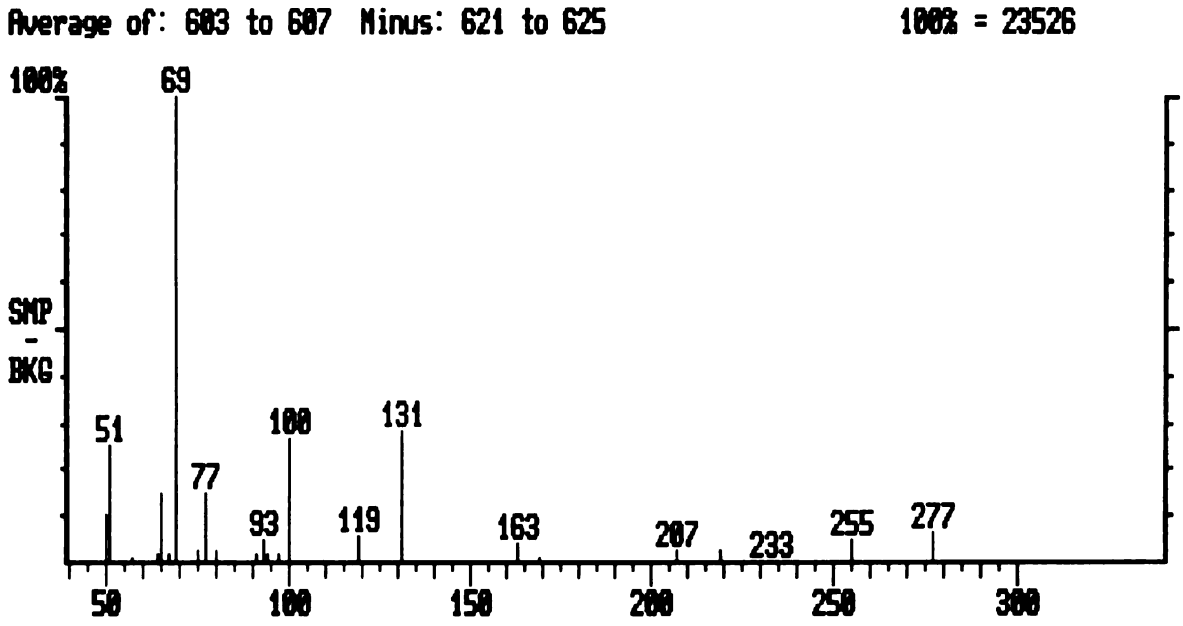

Figure A.2. GC/MS spectrum for peak #1 of strain D2 incubated with glucose and H-PFOSA.

Mass	Intensity	% Base
50	4,564	13.13
51	8,426	24.24
65	4,213	12.12
69	34,758	100.00
75	3,159	9.09
77	4,915	14.14
80	2,808	8.08
93	3,510	10.10
94	2,106	6.06
100	9,479	27.27
131	8,777	25.25
163	2,808	8.08
207	2,808	8.08
255	3,159	9.09
277	4,564	13.13

Table A.1. GC/MS listing for peak #1 of strain D2 incubated with glucose and H-PFOSA.

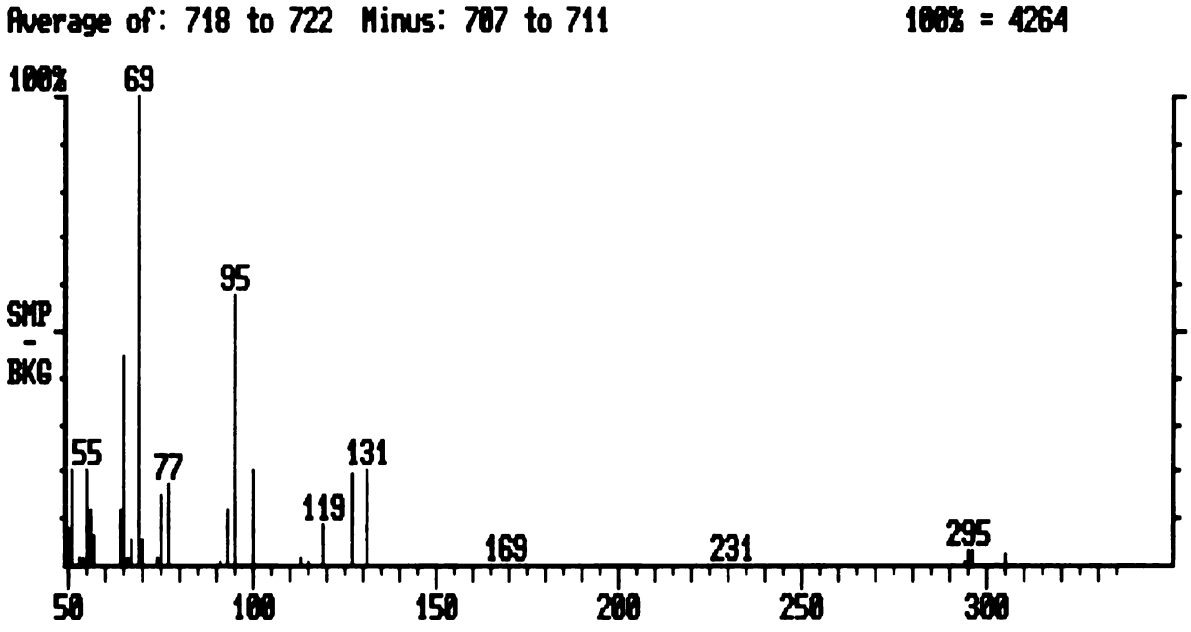

Figure A.3. GC/MS spectrum for peak #2 of strain D2 incubated with glucose and H-PFOSA.

Mass	Intensity	% Base
50	454	4.71
51	1,565	16.23
54	404	4.19
55	1,212	12.57
56	707	7.33
57	555	5.76
64	858	8.90
65	3,283	34.03
67	505	5.24
69	9,648	100.00
70	353	3.66
74	404	4.19
75	1,010	10.47
77	1,111	11.52
93	1,212	12.57
95	4,192	43.46

Mass	Intensity	% Base
100	1,565	16.23
119	909	9.42
127	1,616	16.75
131	2,020	20.94
181	353	3.66
295	303	3.14
305	303	3.14

Table A.2. GC/MS listing for peak #2 of strain D2 incubated with glucose and H-PFOSA.

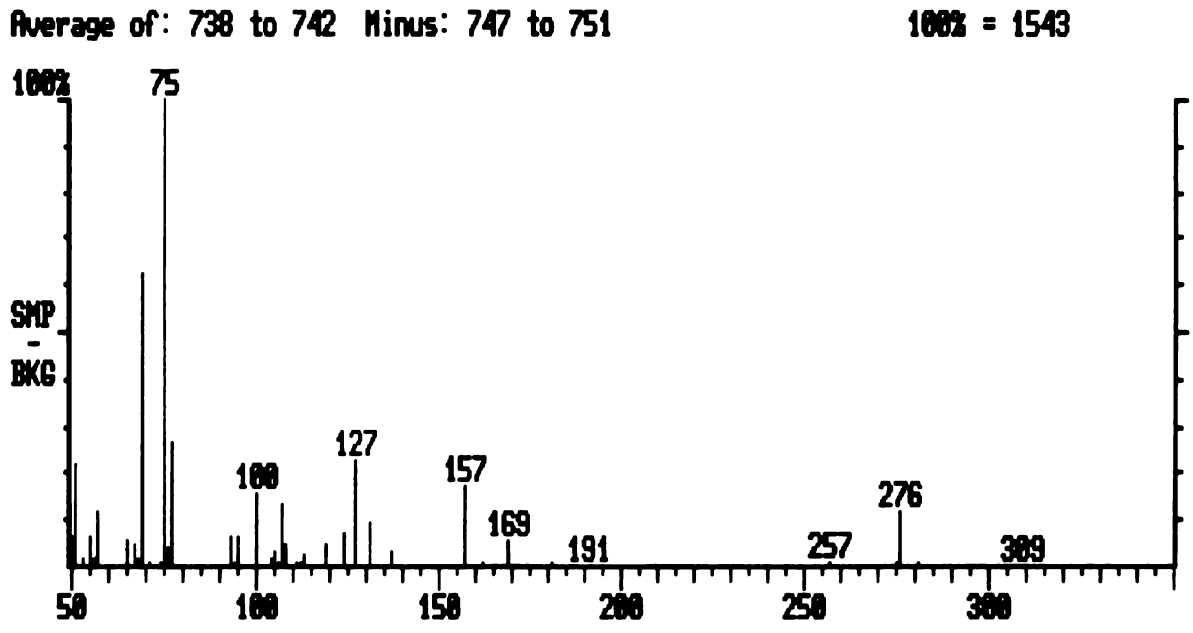

Figure A.4. GC/MS spectrum for peak #3 of strain D2 incubated with glucose and H-PFOSA.

Mass	Intensity	% Base	Mass	Intensity	% Base
50	180	5.53	112	120	3.69
51	587	17.97	119	256	7.83
57	256	7.83	124	165	5.07
65	150	4.61	127	602	18.43
67	120	3.69	131	873	26.73
69	3,268	100.00	137	135	4.15
71	120	3.69	157	361	11.06
75	2,801	85.71	169	210	6.45
76	150	4.61	181	451	13.82
77	813	24.88	219	105	3.23
93	286	8.76	257	120	3.69
95	225	6.91	276	195	5.99
100	497	15.21	281	225	6.91
107	331	10.14			
108	165	5.07			
111	75	2.30			

Table A.3. GC/MS listing for peak #3 of strain D2 incubated with glucose and H-PFOSA.

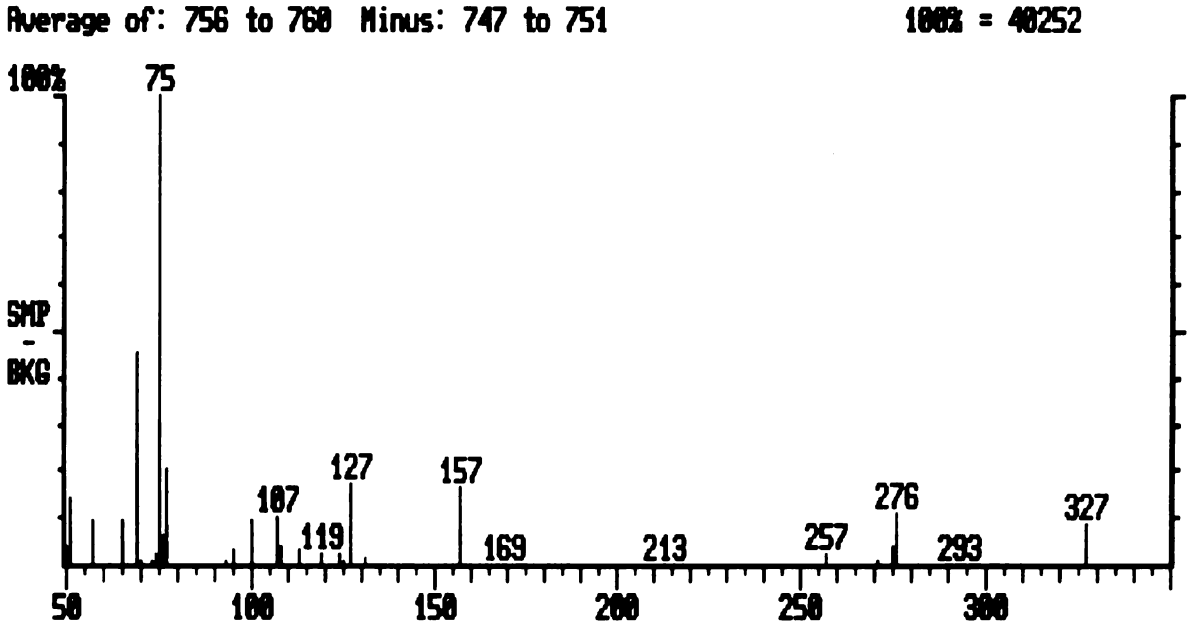

Figure A.5. GC/MS spectrum for peak #4 of strain D2 incubated with glucose and H-PFOSA.

Mass	Intensity	% Base
50	2,808	4.32
51	7,372	11.35
57	6,319	9.73
65	6,319	9.73
69	29,140	44.86
70	2,106	3.24
75	64,952	100.00
76	3,510	5.41
77	13,341	20.54
100	6,670	10.27
107	6,319	9.73
108	3,159	4.86
113	2,106	3.24
124	3,862	5.95
127	11,235	17.30
131	2,808	4.32

Mass	Intensity	% Base
157	10,181	15.68
257	2,457	3.78
275	2,457	3.78
276	6,319	9.73
327	8,075	12.43

Table A.4. GC/MS listing for peak #4 of strain D2 incubated with glucose and H-PFOSA.

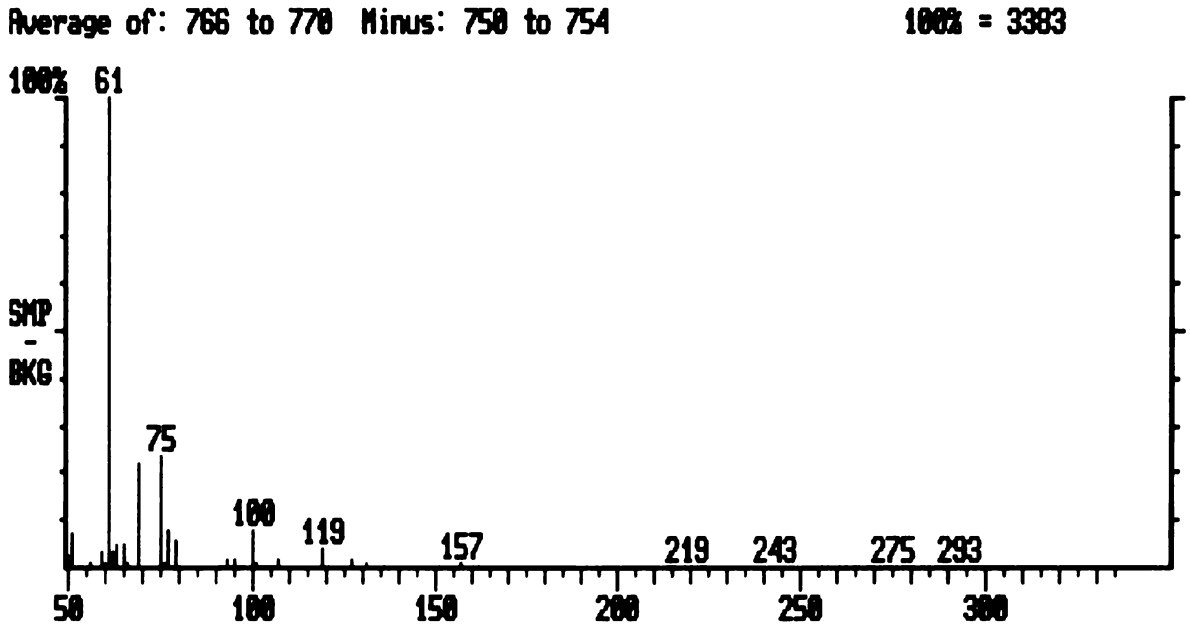

Figure A.6. GC/MS spectrum for peak #5 of strain D2 incubated with glucose and H-PFOSA.

Mass	Intensity	% Base	Mass	Intensity	% Base
50	144	2.55	101	144	2.55
51	372	6.57	107	186	3.28
56	124	2.19	119	310	5.47
59	248	4.38	131	579	10.22
60	186	3.28	181	331	5.84
61	5,672	100.00	207	144	2.55
62	165	2.92			
63	372	6.57			
65	227	4.01			
69	3,208	56.57			
70	124	2.19			
75	869	15.33			
77	269	4.74			
79	393	6.93			
93	310	5.47			
100	496	8.76			

Table A.5. GC/MS listing for peak #5 of strain D2 incubated with glucose and H-PFOSA.

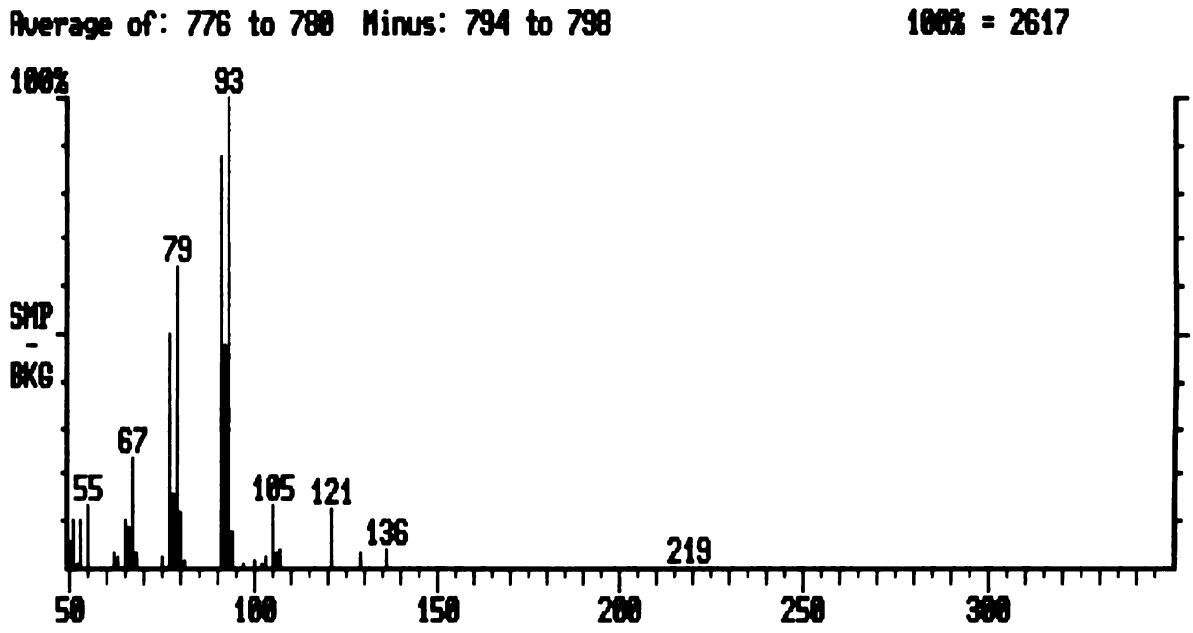

Figure A.7. GC/MS spectrum for peak #6 of strain D2 incubated with glucose and H-PFOSA.

Mass	Intensity	% Base	Mass	Intensity	% Base
50	304	7.79	81	228	5.84
51	532	13.64	91	3,142	80.52
53	354	9.09	92	1,799	46.10
55	633	16.23	93	3,902	100.00
62	152	3.90	94	304	7.79
63	177	4.55	100	228	5.84
65	329	8.44	105	456	11.69
66	481	12.34	106	202	5.19
67	887	22.73	107	202	5.19
68	177	4.55	121	532	13.64
69	1,495	38.31	131	532	13.64
70	126	3.25	136	228	5.84
77	1,875	48.05			
78	760	19.48			
79	2,230	57.14			
80	557	14.29			

Table A.6. GC/MS listing for peak #6 of strain D2 incubated with glucose and H-PFOSA.

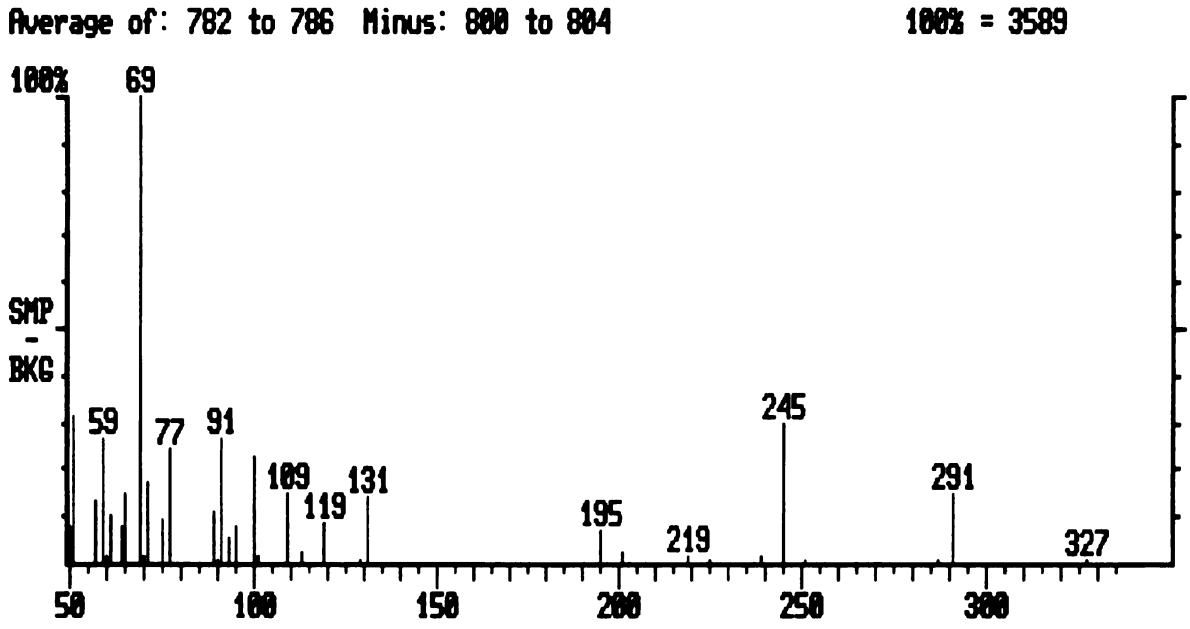

Figure A.8. GC/MS spectrum for peak #7 of strain D2 incubated with glucose and H-PFOSA.

Mass	Intensity	% Base
50	518	6.67
51	1,743	22.42
57	659	8.48
59	1,508	19.39
61	706	9.09
64	518	6.67
65	942	12.12
69	7,775	100.00
70	329	4.24
71	1,036	13.33
75	706	9.09
77	1,460	18.79
89	754	9.70
91	1,508	19.39
93	471	6.06
95	565	7.27

Mass	Intensity	% Base
100	1,178	15.15
109	1,036	13.33
119	612	7.88
131	1,319	16.97
195	518	6.67
219	424	5.45
245	1,602	20.61
291	1,036	13.33

Table A.7. GC/MS listing for peak #7 of strain D2 incubated with glucose and H-PFOSA.

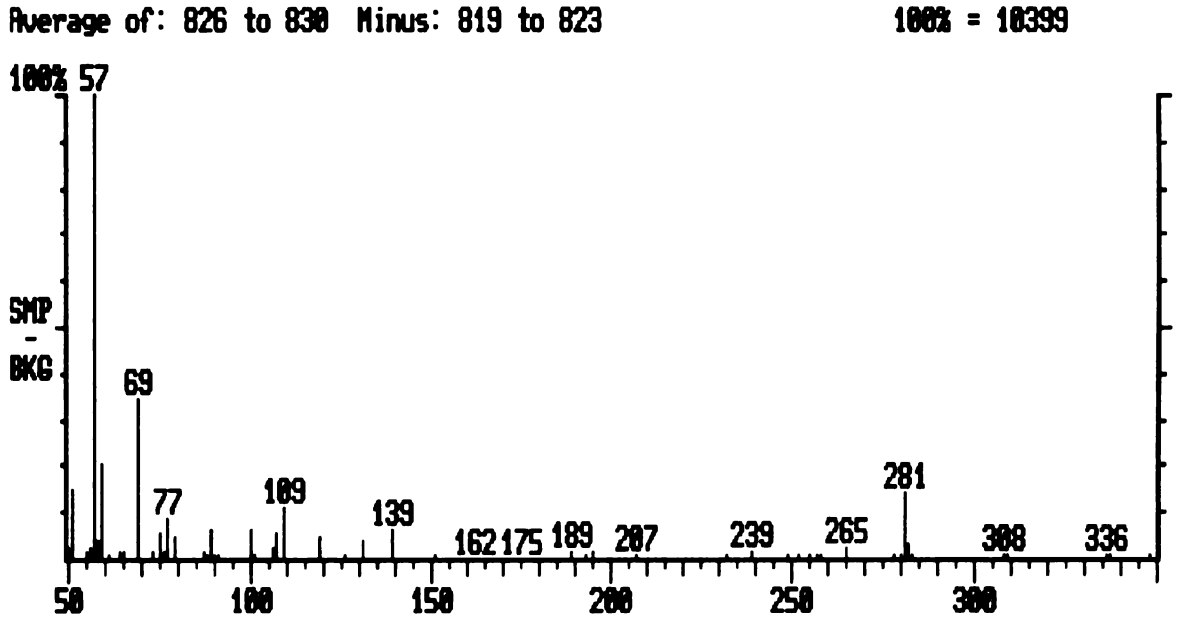

Figure A.9. GC/MS spectrum for peak #8 of strain D2 incubated with glucose and H-PFOSA.

Mass	Intensity	% Base
50	672	3.49
51	3,062	15.89
56	746	3.88
57	19,269	100.00
58	970	5.04
59	3,809	19.77
64	896	4.65
65	896	4.65
66	373	1.94
69	8,365	43.41
75	1,344	6.98
76	746	3.88
77	1,792	9.30
79	1,045	5.43
87	597	3.10
88	672	3.49

Mass	Intensity	% Base
89	1,195	6.20
90	672	3.49
91	597	3.10
100	1,269	6.59
107	1,419	7.36
109	1,941	10.08
119	1,045	5.43
126	448	2.33
131	970	5.04
139	1,568	8.14
181	597	3.10
195	821	4.26
207	522	2.71
252	522	2.71
255	522	2.71
257	746	3.88

Mass	Intensity	% Base
308	672	3.49
309	448	2.33
336	522	2.71
337	522	2.71
348	448	2.33

Table A.8. GC/MS listing for peak #8 of strain D2 incubated with glucose and H-PFOSA.

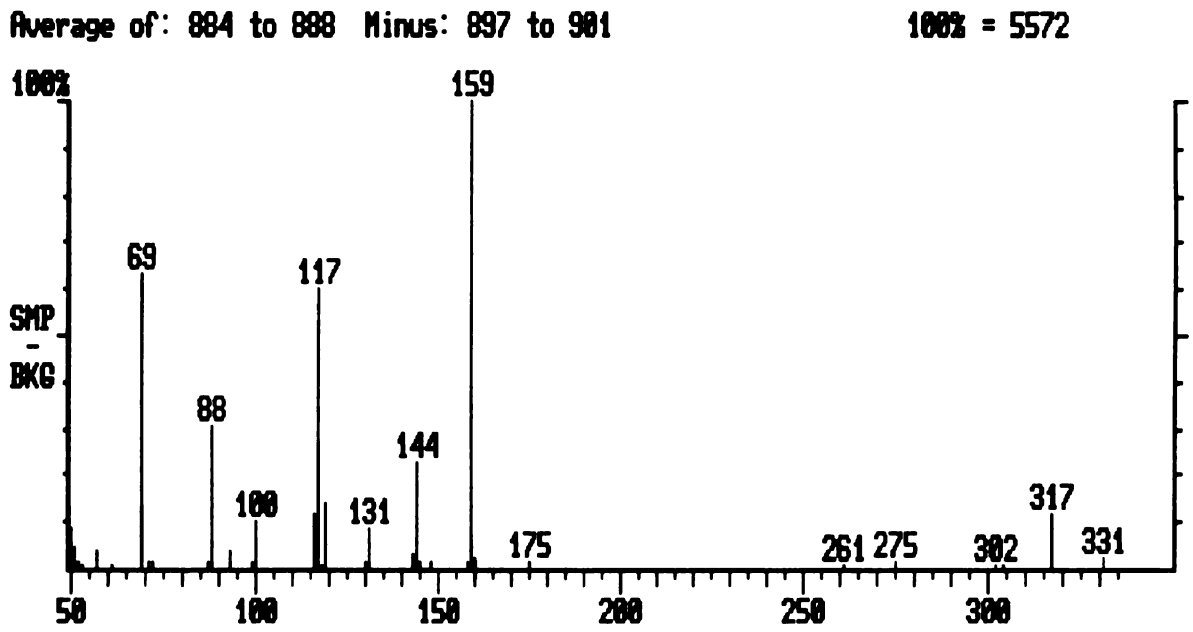

Figure A.10. GC/MS spectrum for peak #9 of strain D2 incubated with glucose and H-PFOSA.

Mass	Intensity	% Base
50	794	7.74
51	927	9.03
57	463	4.52
69	8,080	78.71
88	3,377	32.90
93	662	6.45
99	529	5.16
100	993	9.68
116	1,125	10.97
117	6,490	63.23
119	1,788	17.42
131	1,457	14.19
143	397	3.87
144	2,649	25.81
145	529	5.16
159	10,266	100.00
275	463	4.52
317	1,192	11.61
331	529	5.16

Table A.9. GC/MS listing for.peak #9 of strain D2 incubated with glucose and H-PFOSA.

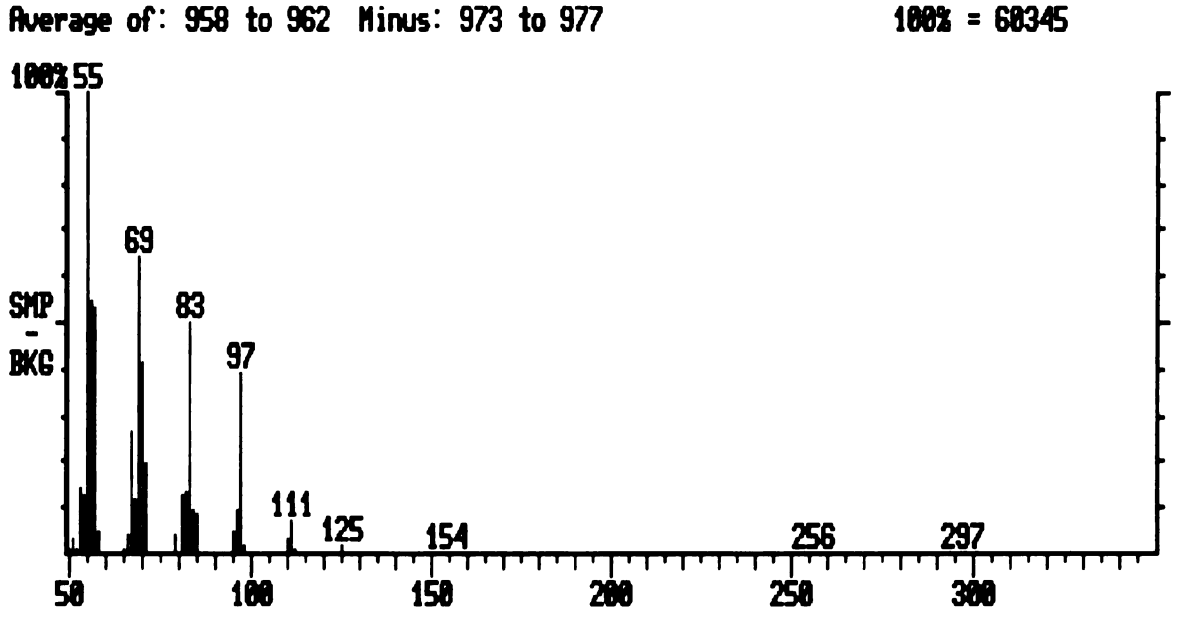

Figure A.11. GC/MS spectrum for peak #10 of strain D2 incubated with glucose and H-PFOSA.

Mass	Intensity	% Base	Mass	Intensity	% Base
51	6,670	5.48	81	20,012	16.43
52	4,213	3.46	82	16,501	13.54
53	20,012	16.43	83	84,262	69.16
54	16,501	13.54	84	14,394	11.82
55	121,829	100.00	85	16,852	13.83
56	73,378	60.23	95	12,288	10.09
57	87,071	71.47	96	11,937	9.80
58	9,830	8.07	97	70,569	57.93
65	4,213	3.46	98	6,319	5.19
66	3,862	3.17	110	4,564	3.75
67	37,215	30.55	111	12,990	10.66
68	15,799	12.97	125	4,915	4.03
69	93,390	76.66	154	1,755	1.44
70	62,143	51.01			
71	36,162	29.68			
79	4,915	4.03			

Table A.10. GC/MS listing for peak #10 of strain D2 incubated with glucose and H-PFOSA.

GC/MS DATA USING ELECTRON IMPACT WITH QUADRAPOLE DETECTOR

GC/MS (Quadrapole) analysis:

GC:	Hewlett-Packard 5995 Series II GC/MS
Column:	DB624 capillary column (30 m x 0.25 mm x1.4 μm) from J & W Scientific (Folsom, CA)
Operation:	40°C for 4 minutes, 10°C/minute ramp to 200°C
Injector:	250°C
Transfer Line:	225°C
Sample:	Headspace for 15-30 minutes with a solid phase microextraction (SPME) fiber assembly with a 100 μM polydimethylsiloxane coating (Supelco, Inc., Bellefonte, PA)

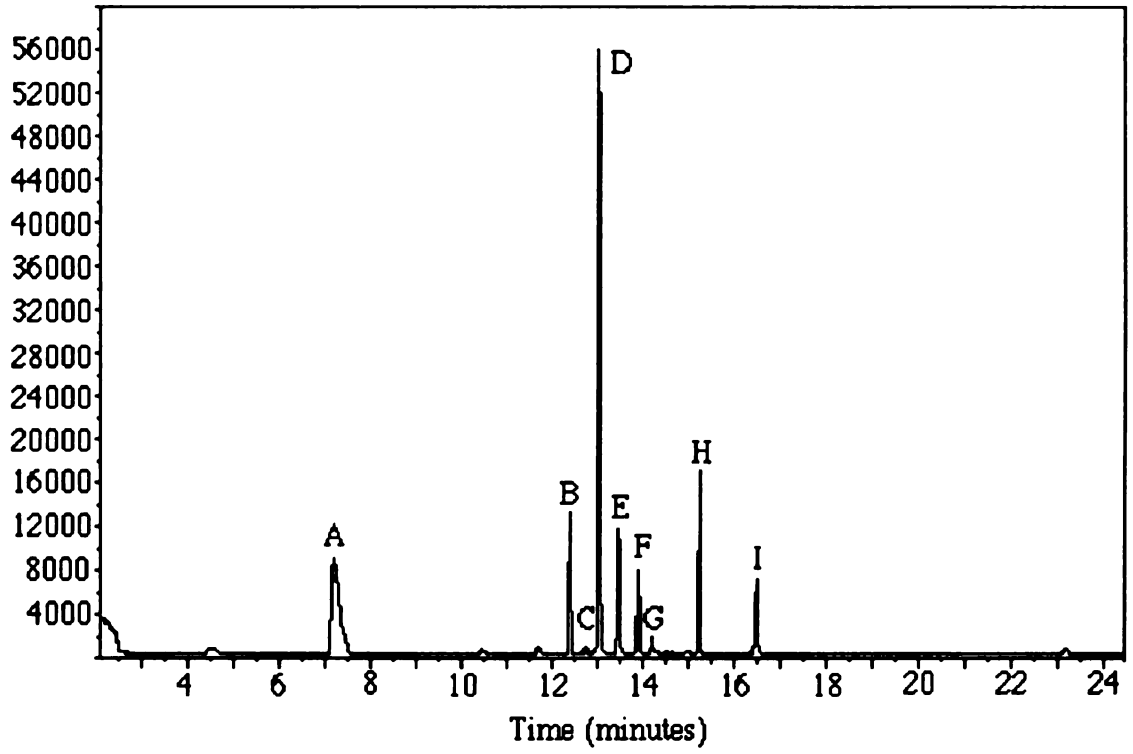

Figure A.12. GC/MS chromatogram for strain D2 incubated with glucose and H-PFOSA.

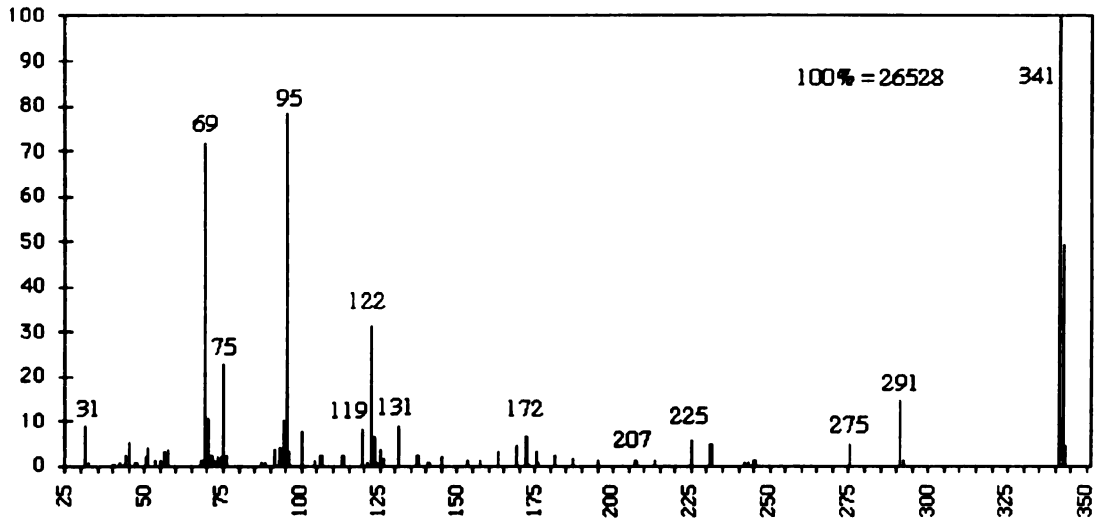

Figure A.13. GC/MS spectrum for peak A of strain D2 incubated with glucose and H-PFOSA.

m/z	intensity	m/z	intensity
31	8.919	122	31.416
32	0.516	123	6.472
40	0.345	124	0.833
42	0.456	125	3.675
43	0.422	126	1.421
44	2.337	131	8.617
45	5.259	137	2.119
47	0.75	140	0.456
50	1.76	141	0.818
51	3.966	145	1.764
53	1.172	153	1.218
55	0.95	157	1.12
56	3.012	163	3.061
57	3.679	169	4.471
68	0.912	172	6.416
69	71.592	173	0.426
70	10.63	175	3.023
71	2.198	176	0.539
72	1.022	181	2.247
73	2.077	187	1.598
74	1.685	195	1.116
75	22.542	207	1.138
76	2.284	213	1.239
87	0.841	225	5.602
88	0.494	231	4.821
91	3.613	242	0.66
93	3.837	243	0.622
94	9.993	245	0.988
95	78.04	275	4.667
96	2.94	291	14.438
100	7.641	292	1.225
104	1.037	341	100
106	2.258	342	49.389
113	2.36	343	4.412
119	8.041		
121	0.471		

Table A.11. GC/MS listing for peak A of strain D2 incubated with glucose and H-PFOSA.

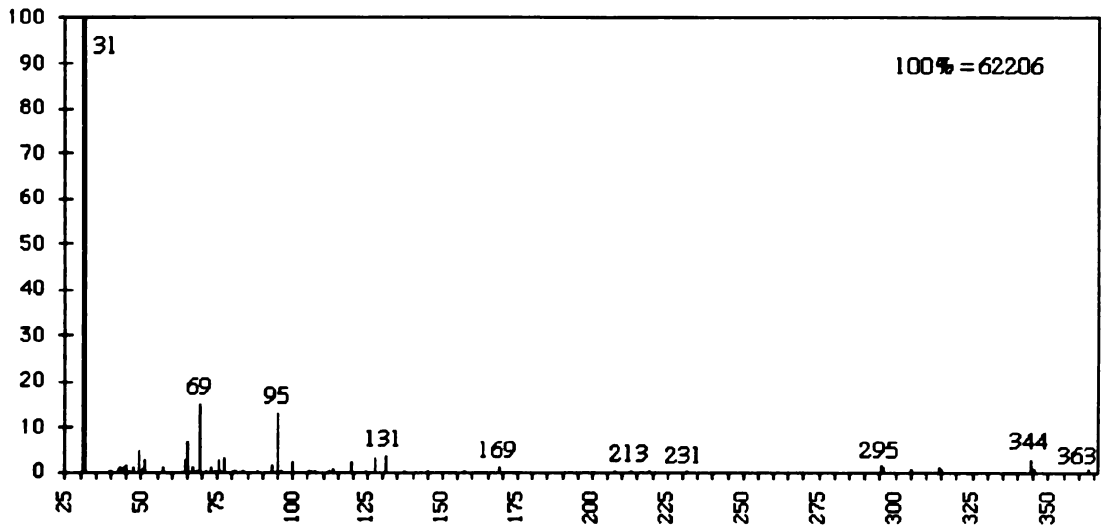

Figure A.14. GC/MS spectrum for peak B of strain D2 incubated with glucose and H-PFOSA.

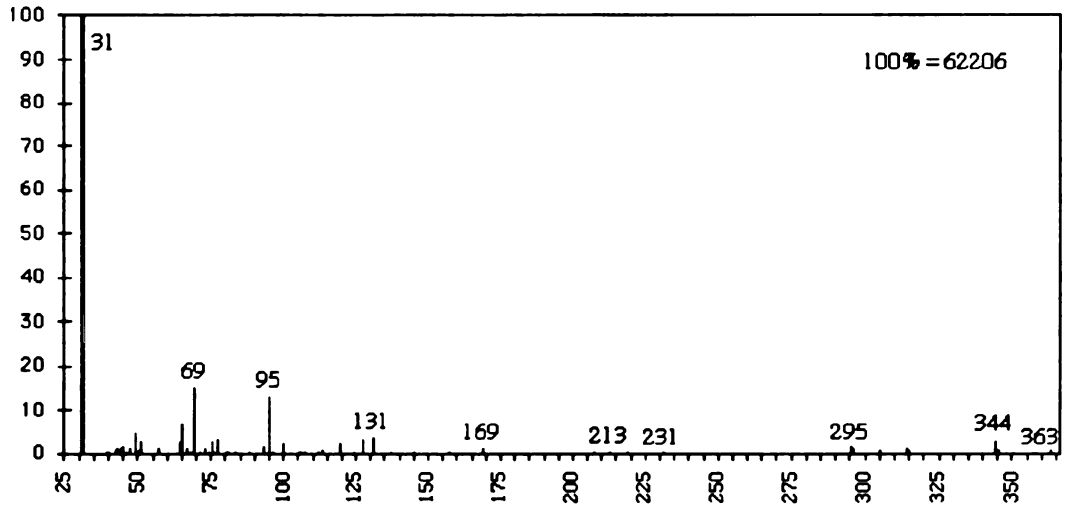

Figure A.14. GC/MS spectrum for peak B of strain D2 incubated with glucose and H-PFOSA.

m/z	intensity	m/z	intensity
31	100	106	0.254
39	0.186	107	0.305
40	0.164	112	0.233
42	0.524	113	0.725
43	1.163	119	2.249
44	1.088	124	0.296
45	1.629	127	3.207
46	0.412	131	3.559
47	1.008	137	0.268
49	4.6004	145	0.416
50	0.593	157	0.296
51	2.677	169	0.897
57	0.995	207	0.252
64	2.514	213	0.362
65	6.884	219	0.35
67	0.858	231	0.436
68	0.262	295	1.27
69	14.741	296	1.174
71	0.217	305	0.453
73	0.95	314	1.101
74	0.354	315	0.497
75	2.762	344	2.516
76	0.379	345	0.701
77	2.99	363	0.701
80	0.24		
81	0.256		
83	0.212		
88	0.166		
93	1.534		
95	12.926		
96	0.423		
100	2.262		
105	0.198		

Table A.12. GC/MS listing for peak B of strain D2 incubated with glucose and H-PFOSA.

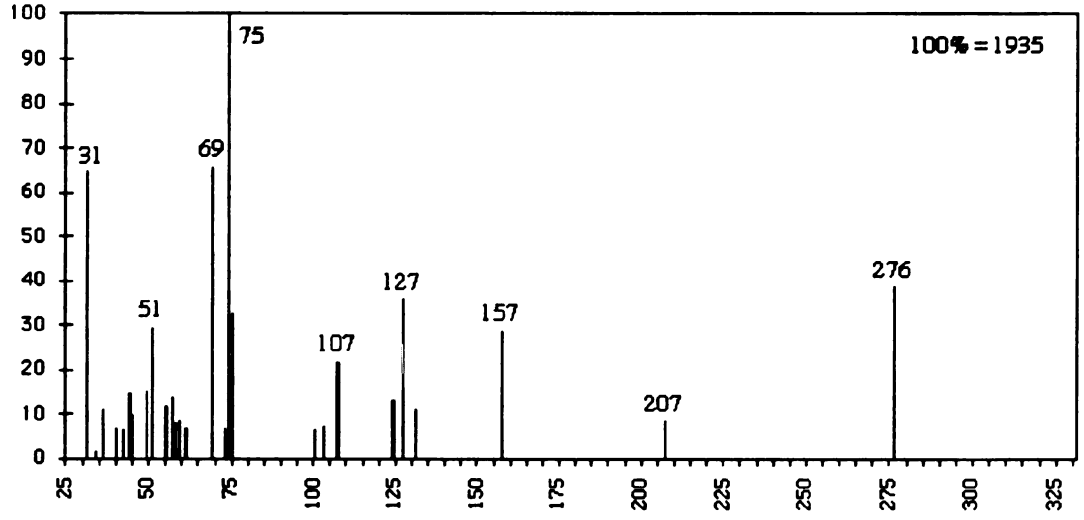

Figure A.15. GC/MS spectrum for peak C of strain D2 incubated with glucose and H-PFOSA.

m/z	intensity
31	64.91
34	1.525
36	10.956
40	6.718
42	6.305
44	14.522
45	9.612
49	14.884
51	29.147
55	11.473
57	13.54
58	7.907
59	8.217
61	6.615
69	65.426
73	6.822
74	100
75	32.558
100	6.357
103	7.183
107	21.499
124	12.765
127	35.711
131	10.853
157	28.217
207	8.269
276	38.76

Table A.13. GC/MS listing for peak C of strain D2 incubated with glucose and H-PFOSA.

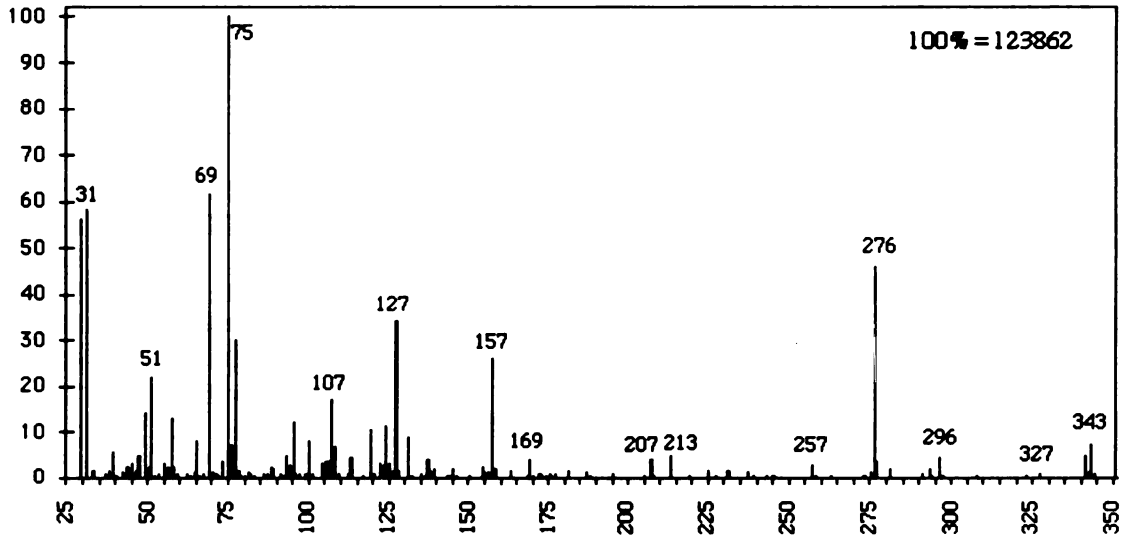

Figure A.16. GC/MS spectrum for peak D of strain D2 incubated with glucose and H-PFOSA.

m/z	intensity	m/z	intensity	m/z	intensity	m/z	intensity
29	56.226	83	0.314	145	2.055	291	0.782
31	58.158	86	0.655	146	0.109	293	1.998
33	1.515	87	0.806	150	0.352	294	0.146
37	0.678	88	2.274	154	2.189	296	4.308
38	1.62	89	1.891	155	1.035	297	0.389
39	5.529	91	0.808	156	1.02	308	0.235
40	0.318	92	0.177	157	26.085	323	0.188
42	1.106	93	4.683	158	1.931	327	0.775
43	2.238	94	2.895	163	1.648	341	4.622
44	2.235	95	12.162	168	0.304	342	1.248
45	3.008	96	0.77	169	3.924	343	7.364
46	1.577	97	0.75	170	0.154	344	0.61
47	4.797	99	0.802	172	0.477		
49	14.25	100	7.814	173	0.412		
50	2.388	101	0.528	174	0.134		
51	21.958	104	3.132	175	0.683		
52	0.357	105	3.323	176	0.201		
53	0.758	106	3.354	177	0.566		
55	3.067	107	17.056	181	1.476		
56	2.467	108	6.964	187	0.877		
57	12.894	109	0.554	188	0.325		
58	2.149	112	0.559	195	0.677		
59	0.58	113	4.28	205	0.142		
62	0.558	117	0.359	207	4.068		
63	0.309	119	10.463	208	0.363		
64	1.117	120	0.501	213	4.802		
65	7.982	122	2.962	219	0.433		
66	0.258	123	2.674	225	1.382		
67	0.703	124	11.42	227	0.412		
69	61.566	125	3.099	231	1.389		
70	0.936	126	1.547	237	0.899		
71	0.603	127	34.241	239	0.121		
72	0.22	128	1.547	243	0.315		
73	3.354	131	8.779	245	0.323		
75	100	132	0.287	257	2.793		
76	7.297	135	0.828	258	0.16		
77	30.108	136	0.323	263	0.3		
78	1.611	137	3.776	273	0.416		
79	0.15	138	1.333	275	0.948		
80	0.273	139	1.867	276	46.02		
81	0.902	143	0.437	277	3.567		
82	0.665	144	0.277	281	1.786		

Table A.14. GC/MS listing for peak D of strain D2 incubated with glucose and H-PFOSA.

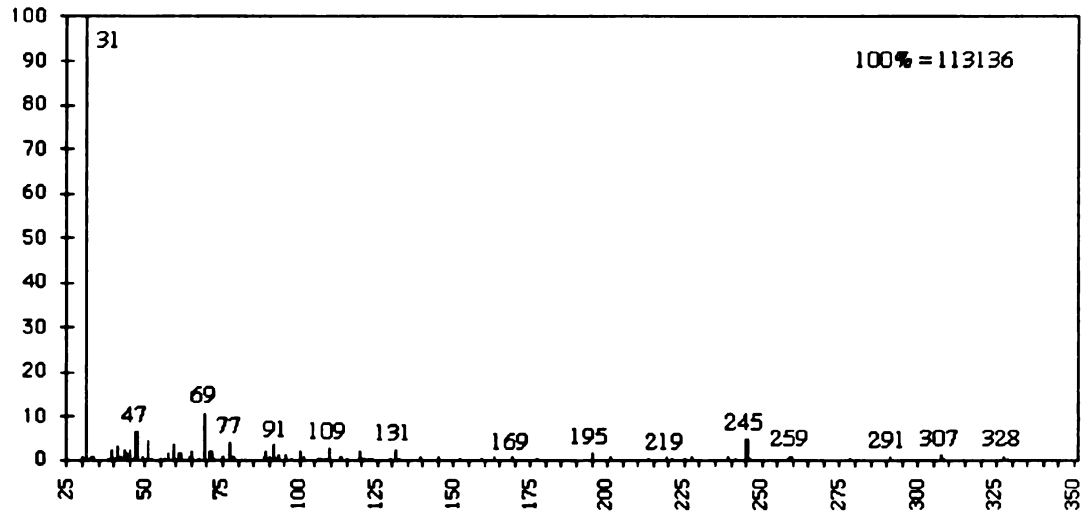

Figure A.17. GC/MS spectrum for peak E of strain D2 incubated with glucose and H-PFOSA.

m/z	intensity	m/z	intensity	m/z	intensity
30	0.59	78	0.725	201	0.479
31	100	88	0.486	213	0.411
32	0.402	89	1.849	219	0.681
33	0.814	90	0.698	221	0.271
37	0.025	91	3.643	225	0.218
38	0.369	92	0.285	227	0.51
39	2.139	93	1.003	239	0.688
40	0.246	95	1.115	241	0.253
41	3.042	97	0.13	245	4.596
42	0.821	100	1.863	246	0.301
43	2.097	101	0.468	258	0.32
44	1.652	106	0.182	259	0.462
45	2.181	107	0.21	278	0.209
46	0.27	108	0.371	291	0.789
47	6.299	109	2.586	307	0.944
49	0.633	113	0.742	308	0.105
50	0.416	115	0.171	327	0.737
51	4.173	119	1.813	328	0.41
52	0.262	120	0.209		
55	0.366	121	0.381		
56	0.133	122	0.364		
57	1.536	123	0.122		
59	3.707	129	0.313		
60	0.354	131	2.3		
61	1.424	132	0.226		
64	0.811	139	0.468		
65	1.707	145	0.619		
67	0.392	152	0.195		
69	10.311	159	0.108		
71	1.732	163	0.51		
72	0.077	169	0.655		
75	0.701	177	0.252		
77	4.02	195	1.39		

Table A.15. GC/MS listing for peak E of strain D2 incubated with glucose and H-PFOSA.

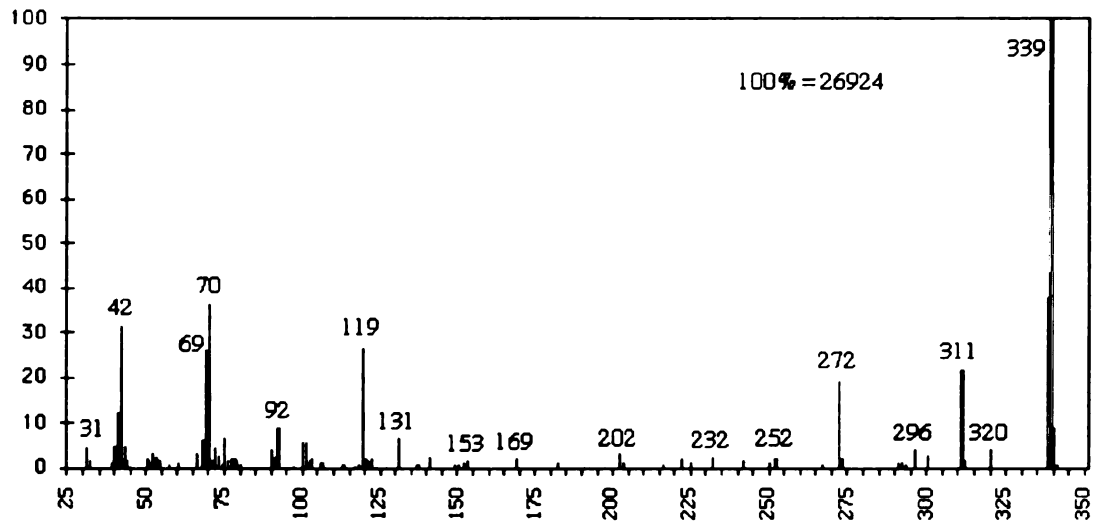

Figure A.18. GC/MS spectrum for peak F of strain D2 incubated with glucose and H-PFOSA.

m/z	intensity	m/z	intensity	m/z	intensity
31	4.513	97	0.416	273	1.72
32	1.549	100	5.59	291	0.917
39	1.192	101	5.649	292	1.252
40	4.598	102	1.601	293	0.813
41	12.073	103	2.002	296	3.87
42	31.411	106	0.94	300	2.722
43	4.895	113	0.579	311	21.579
44	1.445	117	0.379	312	1.612
45	0.42	118	0.602	320	3.759
50	1.753	119	26.326	338	37.695
51	1.319	120	1.909	339	100
52	3.283	121	1.645	340	8.799
53	2.429	122	1.801	341	0.602
54	1.419	131	6.47		
57	0.758	137	0.773		
60	0.977	141	2.254		
66	3.124	149	0.825		
68	5.868	150	0.813		
69	26.122	152	0.984		
70	36.272	153	1.553		
71	1.606	169	1.772		
72	4.3347	182	1.062		
73	2.563	202	2.897		
74	0.739	203	1.255		
75	6.47	216	0.728		
76	1.616	222	1.701		
77	1.679	225	1.189		
78	2.046	232	2.403		
79	1.437	242	1.4		
80	0.49	250	0.895		
90	4.085	252	1.994		
91	2.414	267	0.661		
92	8.877	272	19.009		

Table A.16. GC/MS listing for peak F of strain D2 incubated with glucose and H-PFOSA.

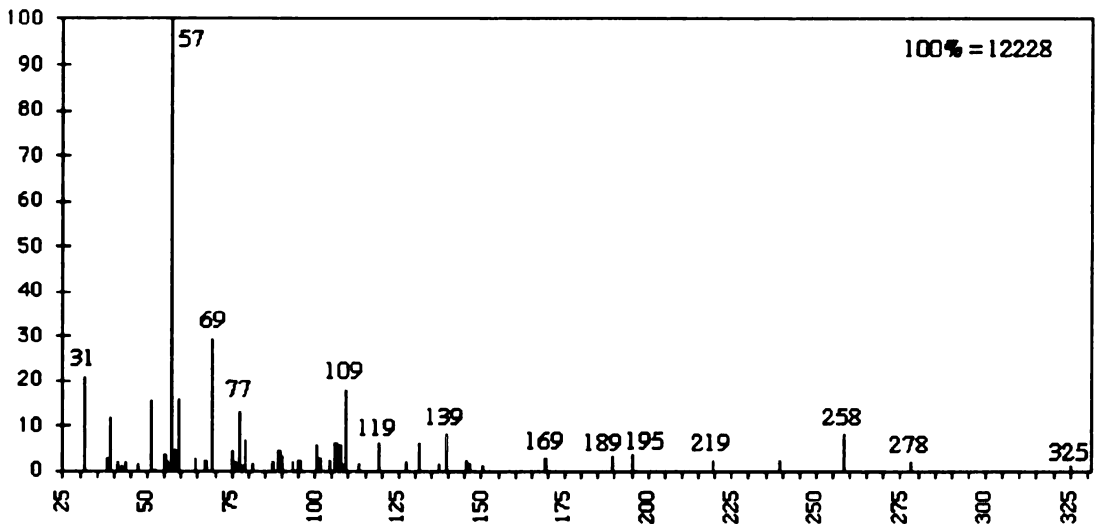

Figure A.19. GC/MS spectrum for peak G of strain D2 incubated with glucose and H-PFOSA.

m/z	intensity	m/z	intensity
31	20.527	108	1.382
38	2.535	109	17.722
39	11.833	113	1.497
41	2.077	119	5.757
42	1.047	127	1.914
43	1.922	131	5.904
47	1.652	137	1.398
51	15.289	139	8.096
52	0.131	145	2.412
55	3.439	146	1.333
56	1.865	150	1.079
57	100	169	2.543
58	4.899	189	3.304
59	15.718	195	3.688
64	2.584	219	2.355
67	2.486	239	2.241
69	29.31	258	7.924
75	4.342	278	1.717
76	1.832	325	0.99
77	13.036		
78	1.088		
79	6.608		
81	1.317		
87	1.946		
89	4.236		
90	3.042		
93	1.807		
95	2.453		
100	5.684		
101	2.633		
104	2.085		
106	5.806		
107	5.397		

Table A.17. GC/MS listing for peak G of strain D2 incubated with glucose and H-PFOSA.

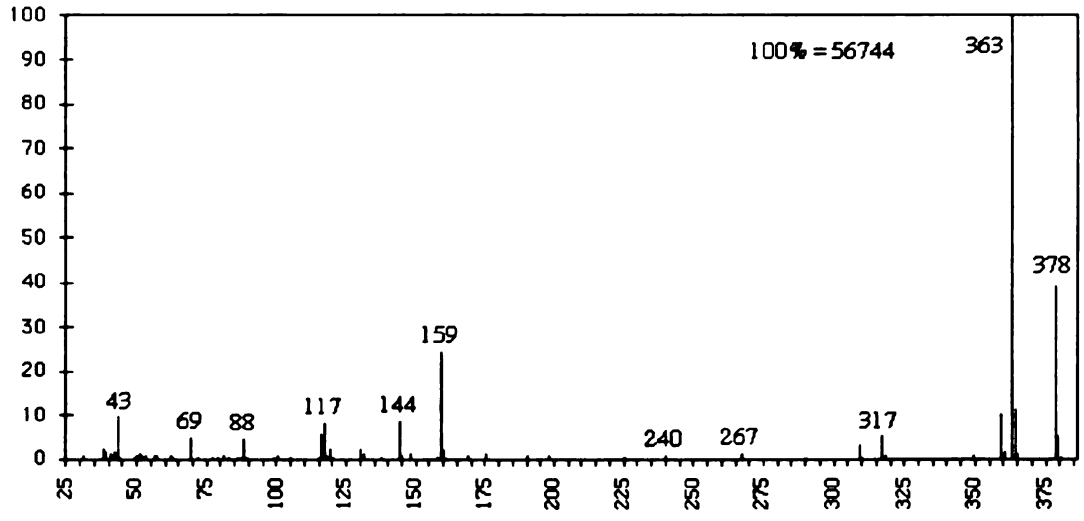

Figure A.20. GC/MS spectrum for peak H of strain D2 incubated with glucose and H-PFOSA.

m/z	intensity	m/z	intensity
31	0.511	119	2.192
38	2.226	120	0.289
39	1.353	130	2.132
41	0.906	131	1.165
42	1.269	138	0.296
43	9.821	144	8.319
44	0.236	145	0.737
49	0.303	148	1.163
50	0.684	158	0.34
51	0.927	159	24.129
52	0.633	160	1.914
53	0.687	169	0.624
56	0.465	175	0.888
57	0.732	190	0.497
62	0.46	198	0.62
63	0.405	225	0.293
69	4.786	240	0.793
72	0.375	267	0.936
77	0.363	309	3.221
79	0.43	310	0.226
81	0.513	317	5.072
83	0.189	318	0.522
86	0.384	349	0.809
87	0.33	359	10.177
88	4.504	360	1.348
89	0.441	363	100
99	0.342	364	11.192
100	0.691	365	0.895
105	0.264	378	39.053
115	0.208	379	5.139
116	5.694	380	0.4
117	8.154		
118	0.541		

Table A.18. GC/MS listing for peak H of strain D2 incubated with glucose and H-PFOSA.

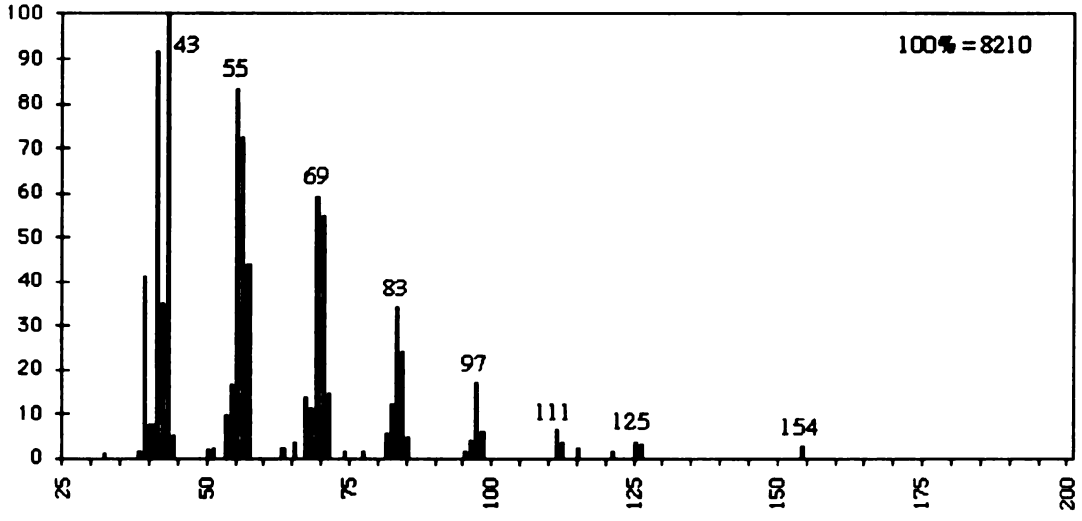

Figure A.21. GC/MS spectrum for peak I of strain D2 incubated with glucose and H-PFOSA.

m/z	intensity	m/z	intensity
32	1.23	111	6.164
38	1.309	112	3.63
39	40.977	115	2.473
40	7.759	121	1.34
41	91.711	125	3.435
42	34.728	126	3.131
43	100	154	2.631
44	5.019		
50	1.961		
51	2.351		
53	9.733		
54	16.725		
55	83.044		
56	72.587		
57	44.095		
63	2.217		
65	3.386		
67	13.557		
68	11.304		
69	59.005		
70	54.875		
71	14.483		
74	1.3852		
77	1.376		
81	5.652		
82	11.974		
83	33.973		
84	23.881		
85	4.726		
95	1.571		
96	4.032		
97	16.773		
98	6.042		

Table A.19. GC/MS listing for peak I of strain D2 incubated with glucose and H-PFOSA.

APPENDIX B

APPENDIX B

GC/MS DATA USING CHEMICAL IONIZATION WITH QUADRAPOLE DETECTOR

GC/MS (Chemical Ionization) analysis:

GC:	Varian 3400
MS:	Axtrel ELQ 400 (Methane as ionization agent)
Column:	Hewlett-Packard HP-FFAP (25 m x 0.32 mm x 0.52 μm)
Operation:	-20°C for 4 minutes, 25°C/minute ramp to 225°C, hold 10minutes
Injector:	250°C
Transfer Line:	225°C
Sample:	Headspace for 15-30 minutes with a solid phase microextraction (SPME) fiber assembly with a 100 μM polydimethylsiloxane coating (Supelco, Inc., Bellefonte, PA)

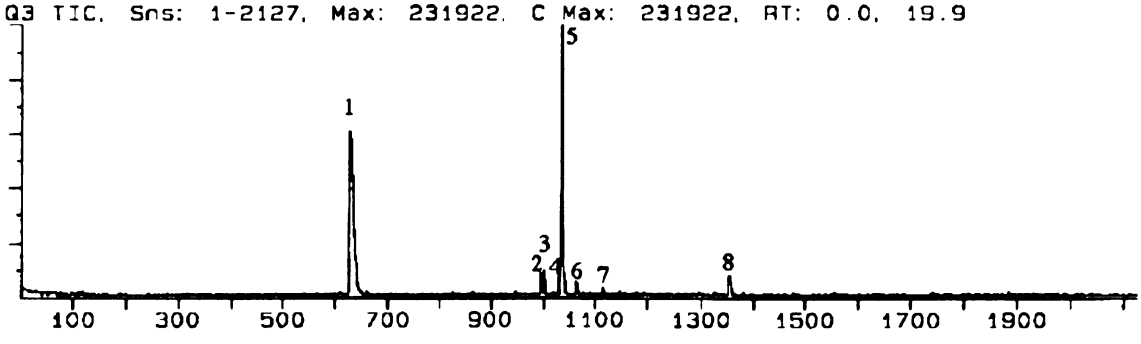

Figure B.1. GC/MS chromatogram for strain D2 incubated with glucose and H-PFOSA.

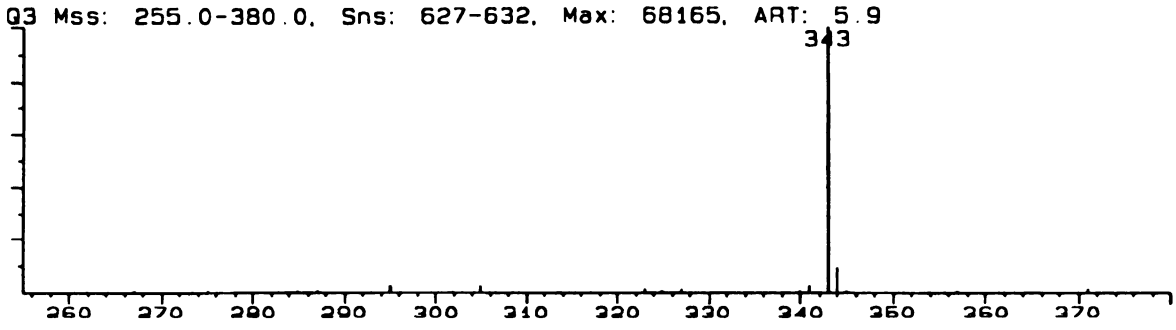

Figure B.2. GC/MS spectrum for peak #1 strain D2 incubated with glucose and H-PFOSA.

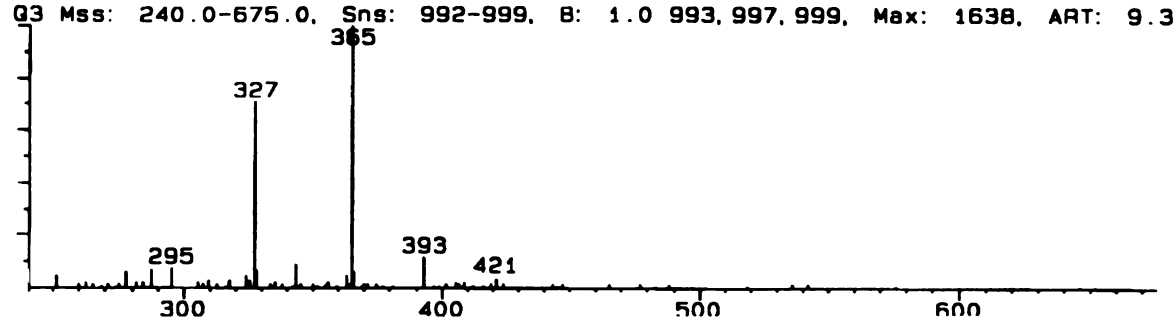

Figure B.3. GC/MS spectrum for peak #2 strain D2 incubated with glucose and H-PFOSA.

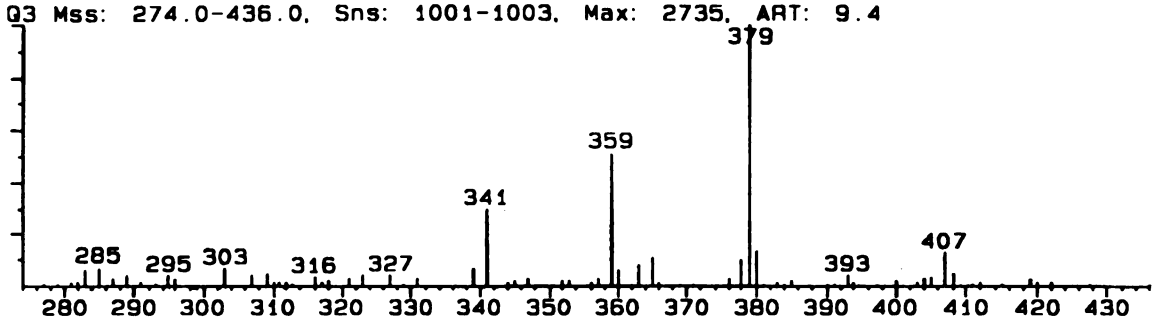

Figure B.4. GC/MS spectrum for peak #3 strain D2 incubated with glucose and H-PFOSA.

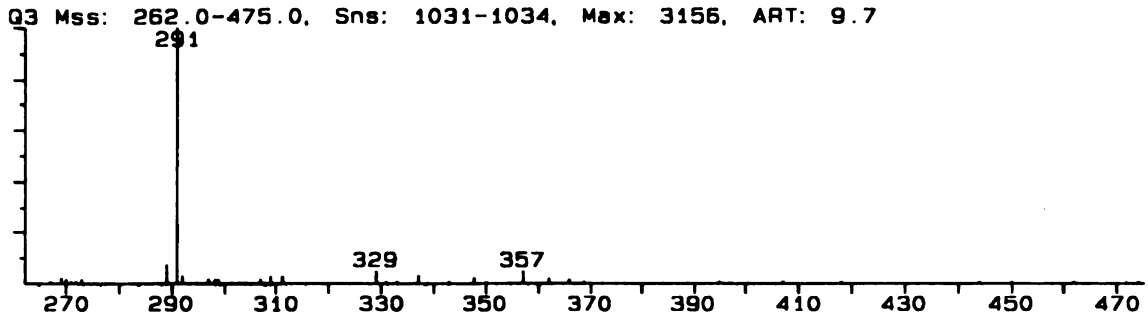

Figure B.5. GC/MS spectrum for peak #4 strain D2 incubated with glucose and H-PFOSA.

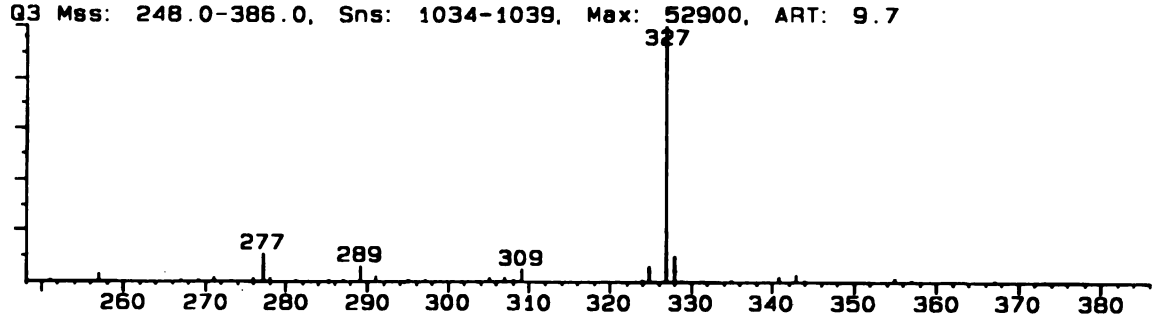

Figure B.6. GC/MS spectrum for peak #5 strain D2 incubated with glucose and H-PFOSA.

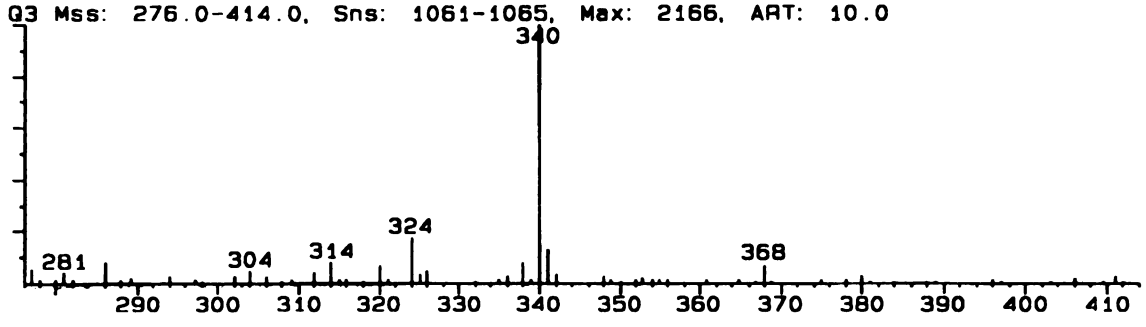

Figure B.7. GC/MS spectrum for peak #6 strain D2 incubated with glucose and H-PFOSA.

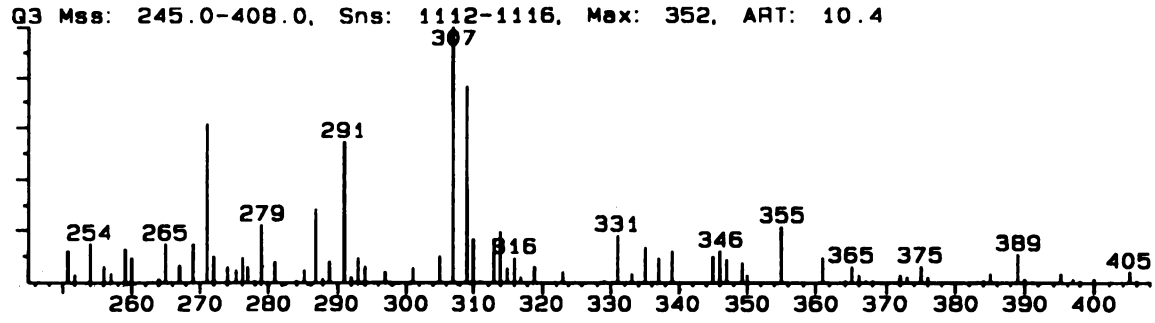

Figure B.8. GC/MS spectrum for peak #7 strain D2 incubated with glucose and H-PFOSA.

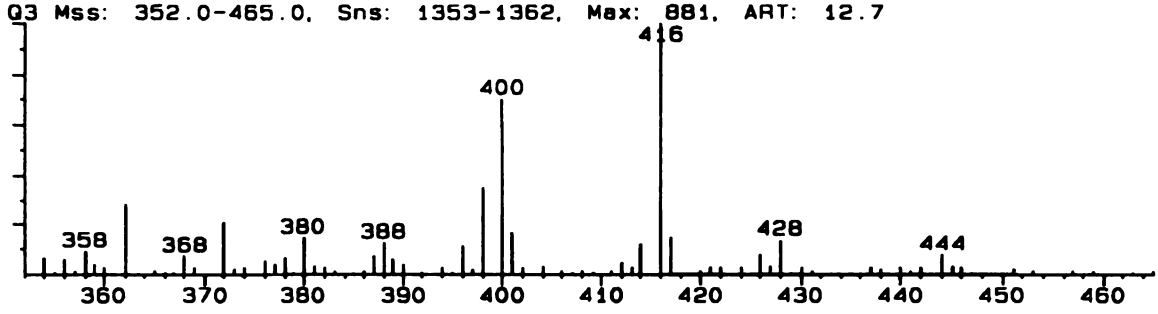

Figure B.9. GC/MS spectrum for peak #8 strain D2 incubated with glucose and H-PFOSA.

APPENDIX C

APPENDIX C

GC/ATOMIC EMISSION DETECTION

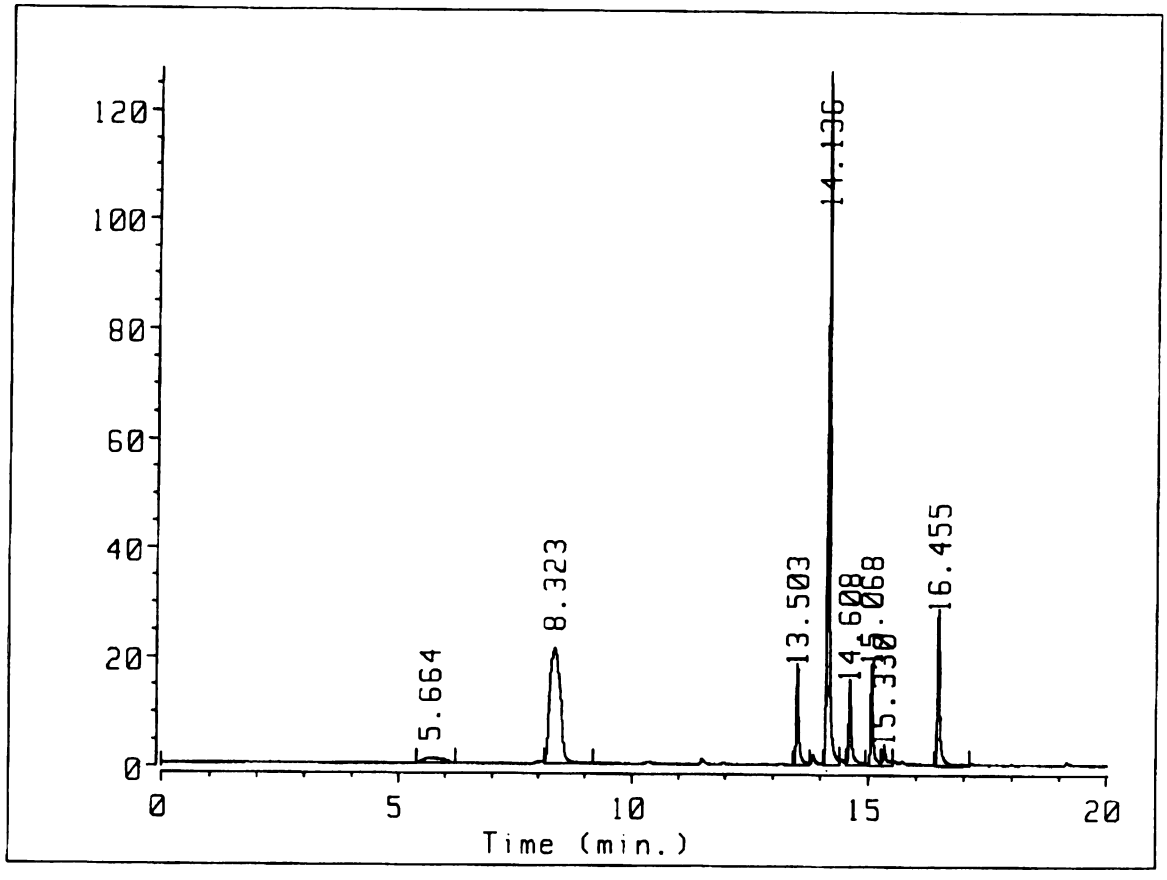

Figure C.1. AED chromatogram for fluorine (at 690 nm) strain D2 incubated with glucose and H-PFOSA.

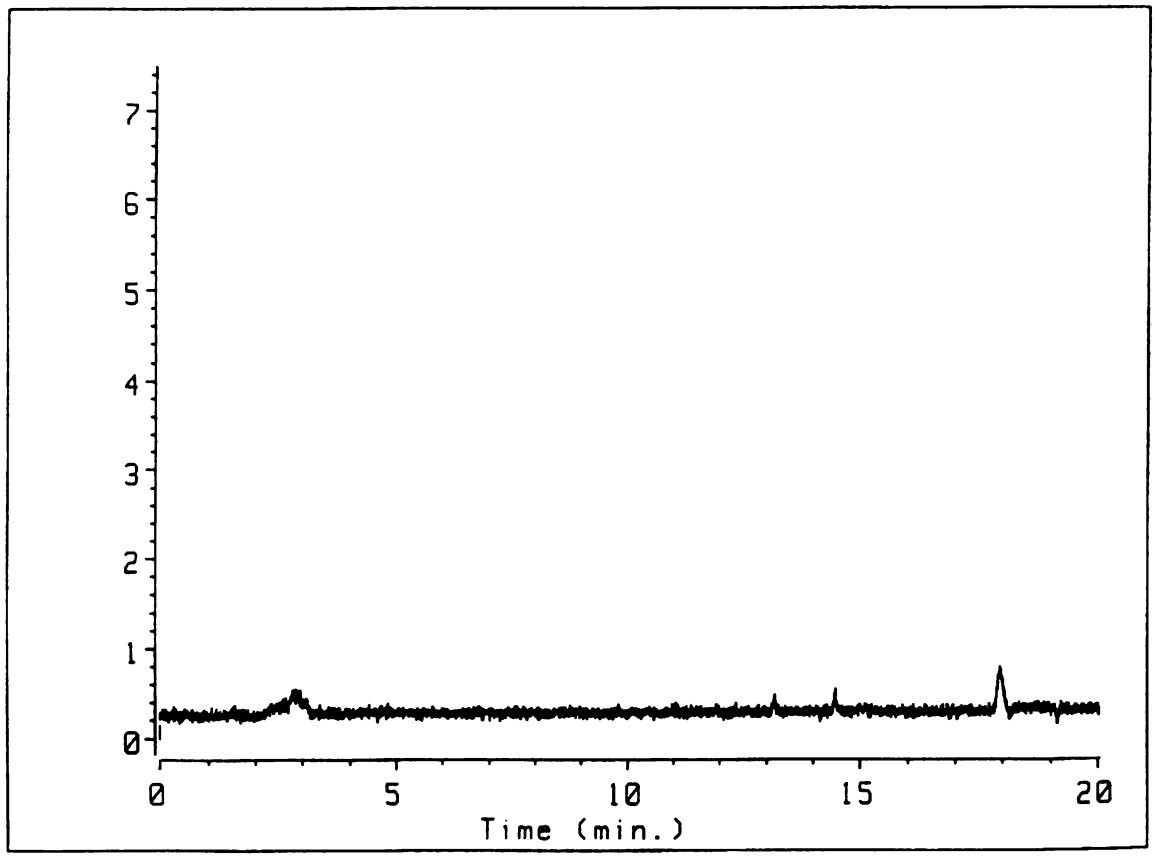

Figure C.2. AED chromatogram for sulfur (at 181 nm) strain D2 incubated with glucose and H-PFOSA.

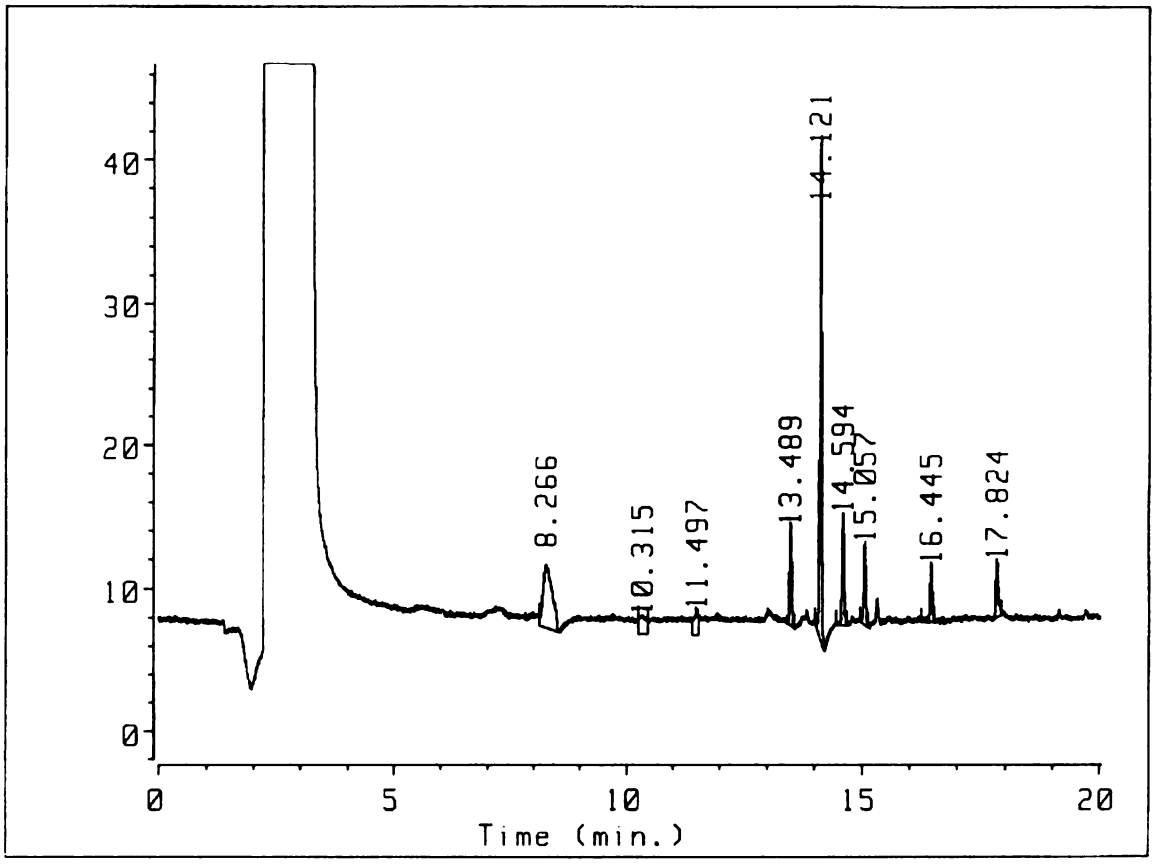

Figure C.3. AED chromatogram for hydrogen (at 486 nm) strain D2 incubated with glucose and H-PFOSA.

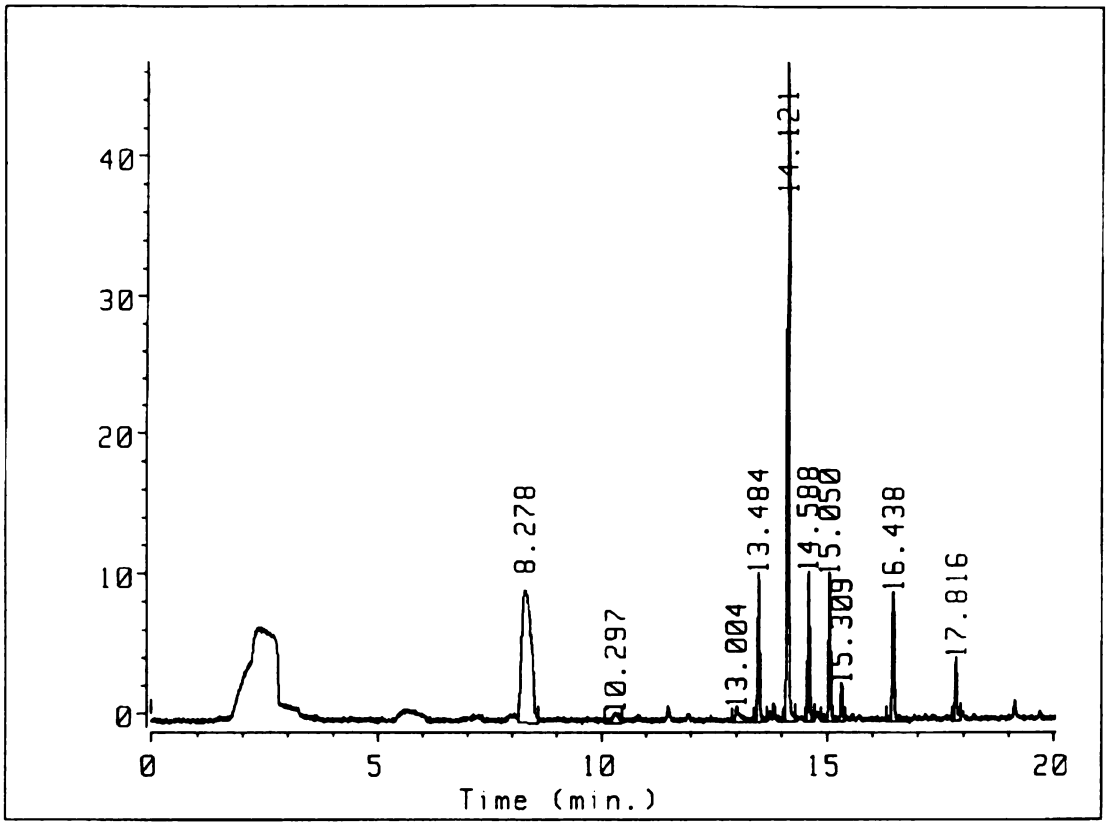

Figure C.4. AED chromatogram for carbon (at 496 nm) strain D2 incubated with glucose and H-PFOSA.

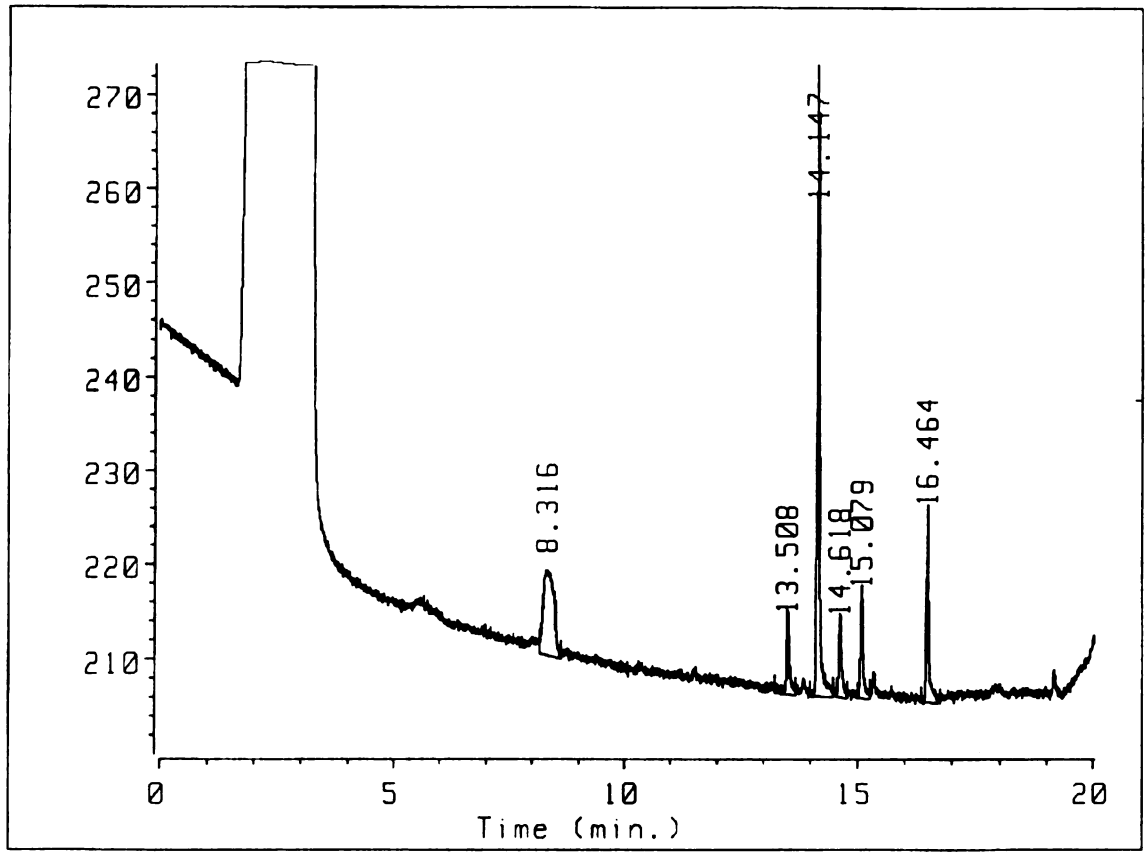

Figure C.5. AED chromatogram for oxygen (at 777 nm) strain D2 incubated with glucose and H-PFOSA.