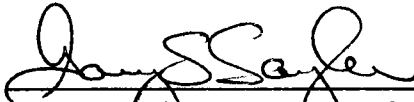
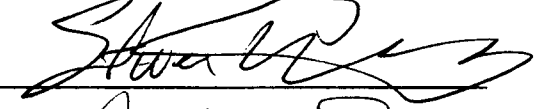
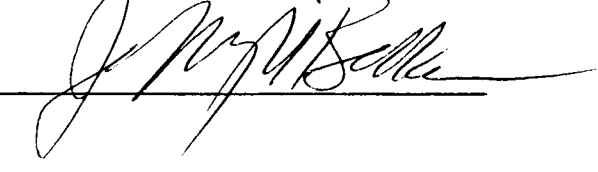


To the Graduate Council:

I am submitting herewith a dissertation written by James Franklin Rice, Jr. entitled "Effects of 2,4-D and 2,4,5-T Metabolites on Degradation of Chlorinated Phenoxyacetic Acids, Dibenzofuran, and Dibenzo-*p*-Dioxin by Environmental Bacteria." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.

  
Dr. Gary S. Sayler, Major Professor

We have read this dissertation  
and recommend its acceptance:

  
  
  
\_\_\_\_\_

Accepted for the Council:

  
Associate Vice Chancellor  
and Dean of the Graduate School

Effects of 2,4-D and 2,4,5-T Metabolites on Degradation of  
Chlorinated Phenoxyacetic Acids, Dibenzofuran, and Dibenzo-*p*-  
Dioxin by Environmental Bacteria.

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

James Franklin Rice, Jr.

December 1999

## ACKNOWLEDGMENTS

I would like to thank my committee members: Dr. Jeffrey M. Becker, Dr. Terry W. Schultz, Dr. Steven W. Wilhelm, and Dr. Gary S. Sayler, my major professor, for their guidance and support during this research project. I would also like to extend my gratitude to Dr. James T. Fleming for his invaluable consultation and friendship during my Ph.D. studies. I would like to thank Drs. Anthony Hay, Alice Layton, Bruce Applegate, John Sanseverino, and Fu-Menn Menn for their guidance and discussions on various topics concerning this research project. Additionally, I would like to acknowledge Dr. Gary Stacey whose influences have positively impacted my scientific development. Generally, I would like to thank the other people with whom I have had stimulating intellectual discussions regarding this research project, and I appreciate the openness of the research environment in our laboratory, in which the focus is on a consolidated team effort. For his expert practical guidance, I would like to give my most sincere thanks to my major professor, Dr. Gary Sayler, who helped keep this research project focused and relevant. Finally, I would like to thank my wife, Amy E. Rice, for her persistent support and encouragement through these difficult times.

## ABSTRACT

Agent Orange herbicide contaminated soils were utilized in enrichment culture studies to isolate 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) degrading bacteria. HPLC analyses of these soils demonstrated the presence of 22.4 mg/kg 2,4-D and 73.8 mg/kg 2,4,5-T. Two bacteria, *Burkholderia* species strain JRB1 and *Burkholderia* species strain JR7B3, were isolated from these soils. These strains were able to mineralize 2,4-D and 2,4,5-T, respectively. Similar enrichment culture studies were unsuccessful in identifying dibenzofuran (DBF) and dibenzo-*p*-dioxin (DD) degrading bacteria. PCR experiments utilizing known genetic sequences from other 2,4-D and 2,4,5-T degrading bacteria have shown that these organisms contain gene sequences corresponding to the *tfdA*, *B*, *C*, *E*, and *R* genes (2,4-D degrading strain) and the *tftA*, *C*, and *E* genes (2,4,5-T degrading strain). These results are the first to indicate that both 2,4-D and 2,4,5-T metabolic pathways can exist in Agent Orange contaminated soils. *Burkholderia* species strain JR7B3 represents the first bacterium isolated directly from 2,4,5-T contaminated soil, which contains genes specific for 2,4,5-T degradation. Future studies comparing this environmental isolate with *Burkholderia cepacia* AC1100 and other 2,4,5-T degrading bacteria will provide valuable information regarding the evolution, abundance, and activity of these *tft* genes in association with contaminated sites.

Growing cell assays coupled with messenger RNA analyses were utilized to examine the ability of these bacteria to degrade 2,4-D and 2,4,5-T in the presence of metabolic intermediates, which may affect catabolism of these compounds in Agent

Orange contaminated soils. Degradation of 2,4-D by *Burkholderia* species strain JRB1 was enhanced in the presence of glucose and RNA hybridization studies demonstrated a two-fold increase of *tfdB* mRNA transcripts. Addition of succinate resulted in a buildup of 2,4-dichlorophenol (2,4-DCP), a cell death phase, delayed degradation of 2,4-D, and a two-fold decrease of *tfdB* mRNA transcripts. Growth on 2,4,5-T by a bacterial consortium containing *Burkholderia* species strain JR7B3 and *Burkholderia* species strain JR7B2 in the presence of glucose and succinate resulted in increased 2,4,5-trichlorophenol (2,4,5-TCP) accumulation. *Burkholderia* species strain JR7B3 grown auxotrophically on 2,4,5-T in the presence of glucose resulted in 2,4,5-TCP concentrations of 22.4 mg/L, and no subsequent degradation or growth occurred. When succinate was provided as a co-carbon source 2,4,5-TCP concentrations remained below 300 µg/L and 2,4,5-T was slowly metabolized. 10 mg/L and 25 mg/L 2,4-DCP amendments induced degradation of 2,4,5-T relative to cultures containing only 2,4,5-T whereas 500 mg/L 2,4-D inhibited 2,4,5-T degradation for this strain.

*Sphingomonas* species strain RW1 was chosen as a model bacterium to examine DBF degradation in the presence of metabolic intermediates of 2,4-D and 2,4,5-T. Random arbitrarily primed reverse transcriptase-PCR (RAP-PCR) was used to detect a mRNA transcript, putatively designated *OB1*, which was upregulated in the presence of DBF versus acetate. This previously unidentified gene sequence, *OB1*, was found to be located downstream of the *dxnB* gene and is clustered with other genes involved in DBF/DD metabolism. Bacterial growth experiments demonstrated that 2,4-DCP and 2,4,5-TCP inhibited growth when present at concentrations of 10 mg/L, and that 500

mg/L 2,4-D and 500 mg/L 2,4,5-T delayed growth of *Sphingomonas* species strain RW1 on acetate. Bacterial cultures containing DBF and 2,4,5-T had a lower overall biomass relative to cultures grown with only DBF. Cultures containing acetate and 2,4-D or DBF and 2,4-D had increased overall biomass and metabolized all of the 2,4-D present. During growth on DBF/2,4-D and acetate/2,4-D 2,4-dichlorophenol concentrations were maintained below 1.0 mg/L. However, during growth on acetate/2,4,5-T, 2,4,5-trichlorophenol concentrations reached a maximum of 11.9 mg/L. RNA hybridization studies showed that addition of 2,4-D, 2,4-DCP, and 2,4,5-TCP to acetate and DBF growing cells resulted in decreased *dxnA1*, *dbfB* and *OBI* transcript abundance. These results illustrate that strain RW1 has the capacity to completely metabolize 2,4-D and 2,4-DCP in the presence of either DBF or acetate. Strain RW1 can also cometabolize 2,4,5-T to a minor degree, resulting in production of 2,4,5-TCP, which was inhibitory to utilization of DBF as a growth substrate.

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## LIST OF ABBREVIATIONS

|           |                                                 |
|-----------|-------------------------------------------------|
| 2,4,5-T   | 2,4,5-trichlorophenoxyacetic acid               |
| 2,4,5-TCP | 2,4,5-trichlorophenoxyacetic acid               |
| 2,4-D     | 2,4-dichlorophenoxyacetic acid                  |
| 2,4-DCP   | 2,4-dichlorophenoxyacetic acid                  |
| 3,5-DCC   | 2,5-dichlorocatechol                            |
| 4C2MP     | 4-chloro-2-methylphenol                         |
| AOE       | Agent Orange enrichment                         |
| CFU       | colony forming unit                             |
| CRS       | core soil                                       |
| CS        | control soil                                    |
| DBF       | dibenzofuran                                    |
| DD        | dibenzo- <i>p</i> -dioxin                       |
| DMF       | dimethylformamide                               |
| dNTP      | deoxynucleosidetriphosphate                     |
| DTT       | dithiothreitol                                  |
| MMLV      | moloney murine leukemia virus                   |
| PCDD      | polychlorinated dibenzo- <i>p</i> -dioxin       |
| PCDF      | polychlorinated dibenzofuran                    |
| PCR       | polymerase chain reaction                       |
| RT-PCR    | reverse transcription polymerase chain reaction |
| SS        | surface soil                                    |

## **PART I**

### **INTRODUCTION**

Agent Orange consists of a mixture of 50% 2,4,5-trichlorophenoxy acetic acid (2,4,5-T) and 50% 2,4-dichlorophenoxyacetic acid (2,4-D). This herbicide was used frequently in the United States, and although the use of Agent Orange was discontinued in the 1980s environmental sinks (such as compost, sewage sludges, and sediments) may contribute to current deposition. During the manufacture of Agent Orange many chlorinated dibenzofurans (DBF) and dibenzo-*p*-dioxins (DD) are unintentionally produced (EPA 1995). These contaminating "dioxins" have also been shown to arise from other industrial processes such as incineration of industrial and domestic wastes, and the bleaching of paper pulp (EPA 1995 and Fortnagel 1989). As a result of these processes, polychlorinated DBFs and DDs (PCDD/F) are now widespread contaminants in the environment. The abundance of these PCDDs and PCDFs poses a significant contamination problem, due to their associated toxicity to both humans and animals and their recalcitrance under environmental conditions.

Biodegradation of Agent Orange potentially involves a multitude of different microorganisms having diverse potentials for metabolic transformations of 2,4-D, 2,4,5-T, and associated polychlorinated dibenzo-*p*-dioxins/dibenzofurans (PCDD/DBF). Previous research addressing the biodegradability of Agent Orange in the environment has mainly focused on furthering the understanding of specific metabolic pathways involved in degradation of 2,4-D, 2,4,5-T, DD, and DBF. Consequently, fundamental knowledge concerning the interactions of mixtures of these compounds in association with metabolism by environmental bacteria is lacking. Previous research studies have indicated that in the environment biodegradation processes are not occurring in isolation,

and that experiments examining metabolic processes in isolation from secondary metabolites may not be indicative of what is actually occurring in the environment.

One of the primary components in Agent Orange, 2,4-D, has been found to be rapidly degraded in the environment (Fulthorpe et al. 1995), and the microorganisms capable of biodegrading 2,4-D are common in all geographical locations examined (Kamagata et al. 1997). Specific metabolic pathways involved in 2,4-D degradation have predominately been found in the  $\alpha$ ,  $\beta$ , and  $\gamma$ -*proteobacteria* (Tiedje et al. 1997), and are located on large catabolic plasmids and/or within the chromosome (Top et al. 1996). One of the best-studied bacterial systems for 2,4-D degradation is found in the  $\beta$ -*Proteobacteria Ralstonia eutrophus* JMP134. *R. eutrophus* JMP134 is considered a model bacterium for 2,4-D degradation by many scientists (Harker et al. 1989), and contains a large 80 kb catabolic plasmid designated, pJP4, containing the genes necessary for conversion of 2,4-D to the TCA intermediates succinate and acetal-CoA (Don and Pemberton 1985). Many laboratories around the world have extensively studied this degradative pathway, and researchers are now beginning to understand the molecular mechanisms involved in degradation of 2,4-D in this organism.

Degradation of the other primary constituent of Agent Orange, 2,4,5-T, has been well studied in *Burkholderia cepacia* AC1100 (Zaborina et al. 1998). This bacterium was generated in the laboratory using plasmid assisted molecular breeding techniques (Kellogg et al. 1981), and the metabolic pathways necessary for utilization 2,4,5-T are almost entirely characterized for this strain. Metabolism of 2,4,5-T by *B. cepacia* AC1100 has been shown to be distinct from 2,4-D metabolism by *R. eutrophus* JMP134

(Zaborina et al. 1998). Interestingly, the catabolic genes necessary for 2,4,5-T mineralization in *B. cepacia* AC1100 are localized in three separate operons, and have been found to reside on replicons III (0.53 Mbp) and IV (0.34 Mbp). It has been observed that insertion elements are disproportionately concentrated on these two smaller replicons, which may facilitate the plasticity of the genome in this organism (Hubner et al. 1998). The three major gene clusters involved in 2,4,5-T degradation are the *tftAB*, *tftCD*, and the *tftEFGH* operons (Hubner et al. 1998). The *tftCD* operon is present in duplicate (Hubner et al. 1998), and has been proposed to provide a selective advantage by protecting against buildup of the toxic intermediate 2,4,5-trichlorophenol (2,4,5-TCP) during 2,4,5-T metabolism (Hubner et al. 1998).

In the absence of complete mineralization, 2,4-D and 2,4,5-T can be incompletely transformed by abiotic and biotic processes (Amy et al. 1985). Abiotic processes can only contribute to partial degradation and subsequent accumulation of intermediates and never to the complete removal of these compound such as occurs during mineralization processes (Somerville et al. 1978). In the absence of a lower pathway for degradation of these compounds, growth may still occur because the organism can gain carbon and energy from the acetate, which is generated from cleavage from the phenoxy group (Top et al. 1996). Also, incomplete metabolism of 2,4,5-T can lead to accumulation of the metabolic intermediates 2,4,5-TCP and 5-chloro-2-hydroquinone (Haugland et al. 1990). Likewise, partial metabolism of 2,4-D can lead to the formation of 2,4-dichlorophenol (2,4-DCP) and 3,5-dichlorocatechol (3,5-DCC) (Haugland et al. 1990). Co-cultures containing the 2,4,5-T degrading *Burkholderia cepacia* AC1100 and *Alcaligenes*

*eutrophus* JMP134 have been used to identify potential inhibition problems between these two pathways (Haugland et al. 1990). In these studies, it was demonstrated that although either organism mineralized their respective single contaminant easily, 2,4-D co-metabolism by *B. cepacia* AC1100 produced chlorohydroquinone, which inhibited both its own 2,4,5-T metabolism and 2,4-D degradation, by *R. eutrophus* JMP134.

Degradation pathways for other constituents of Agent Orange such as "Dioxins" have been poorly defined. Recently, a degradation pathway in *Sphingomonas species* strain RW1, which allows this organism to co-metabolize several mono- and di-chlorinated DBFs and DDs, during the mineralization of unchlorinated DBFs and DDs has been identified (Wilkes et al. 1996). Similar activities, by *Burkholderia sp.* strain LB400, have been observed for polychlorinated biphenyl (PCB) metabolism utilizing biphenyl as the growth substrate where PCB congeners containing up to six chlorine substituents are degraded (Layton et al. 1992). Previous studies addressing the biodegradability of Agent Orange in the environment have focused on the 2,4-D and 2,4,5-T. Consequently, fundamental knowledge concerning the ability of environmental bacteria from Agent Orange contaminated soils to mediate degradation of DBFs and DDs is lacking. Since many of the toxicological effects of Agent Orange are now believed to actually be the result of contaminating PCDD/Fs within this herbicide mixture, it is necessary to develop a more thorough understanding of how environmental bacteria from these soils affect the degradation of the "dioxin" class of compounds.

Because of the complexity of microbially mediated degradation of Agent Orange constituents, a scheme was devised consisting of three primary objectives. The first

objective was to characterize bacterial species present in contaminated soils through direct isolation and subsequent phenotypic characterization. The second objective was to use these organisms in experiments designed to examine the effects of 2,4-D, 2,4-DCP, 2,4,5-T and 2,4,5-TCP on the metabolic pathways responsible for 2,4-D, 2,4,5-T, DBF, and DD conversion associated with polluted soils. The third object was to utilize gene probes, which have been developed using both DDRT-PCR and PCR based techniques applied to pollutant degrading bacteria, in order to determine mRNA expression levels associated with contaminant degradation processes. The goal of this research project is to determine the extent to which intermediates of 2,4-D and 2,4,5-T degradation modulate 2,4-D, 2,4,5-T, DBF, and DD metabolism in bacteria associated with Agent Orange contaminated soil. In order to accomplish this goal, traditional enrichment culture methods coupled with growing cell assays were used to study the degradative potential of bacteria endemic to soils containing Agent Orange. In addition to these experiments, modern differential display reverse transcriptase-PCR (DDRT-PCR, Yao et al. 1996) technologies were utilized to examine the repression and/or activation of gene expression, during growth of *Sphingomonas species* strain RW1 on DBF/DD, as a consequence of potential byproducts of 2,4-D and 2,4,5-T degradation processes. This research project furthers understanding of the microbial processes surrounding microbially mediated degradation of Agent Orange constituents, and will facilitate the advancement of future cleanup strategies by providing valuable information regarding the degradation of 2,4,5-T, 2,4-D, DBF, and DD within contaminant mixtures.

## **PART II**

### **LITERATURE REVIEW**

## 2.1. Agent Orange as a priority pollutant

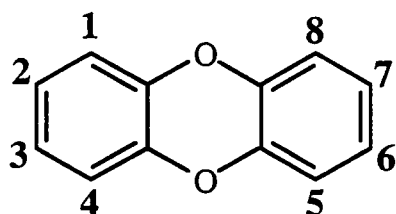
The Environmental Protection Agency has postulated that in 1970 up to one half of the environmental emissions of dioxins was due to the spraying of chlorophenolic herbicides and pesticides. Although the use of these herbicides and pesticides was discontinued in the 1980s, it is believed that environmental sinks, such as compost, sewage sludges, and sediments, may be a significant contributor to current dioxin deposition (EPA 1995). Herbicide Orange, which is more commonly known as Agent Orange, was frequently used as a defoliant in the United States and was sprayed in large quantities in Southeast Asia during the Vietnam war. Agent Orange is composed of a mixture of 50% 2,4,5-trichlorophenoxy acetic acid (2,4,5-T) and 50% 2,4-dichlorophenoxyacetic acid (2,4-D). During the synthesis of these phenoxyacetic acids, many chlorinated congeners of dibenzo-*p*-dioxin (DD) and dibenzofuran (DBF) are generated as contaminants due to the chemical processes used (Thomas et al. 1996). These dioxins are found in most, if not all, Agent Orange samples from the environment and are responsible for many of the potential health risks associated with this herbicide.

## 2.2 Dibenzo-*p*-dioxins and dibenzofurans in the environment

Polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs) are a group of tricyclic compounds, containing two benzene rings interconnected through either a di- or mono- ether bridge, respectively (Figure 1; EPA 1995). Commonly referred to as dioxins, these compounds are generated as byproducts of incineration processes of

domestic and industrial waste, synthesis of chloroaromatics, metal smelting, chlorine bleaching of pulp and paper, and other industrial processes (EPA 1995, Fortnagel et al.

A)



B)

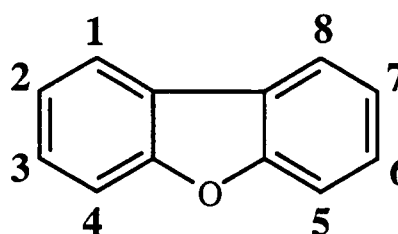


**Figure 2.1.** Structure and positions of halogen substitution for A) dibenzo-*p*-dioxin and B) dibenzofuran.

1990, Fortnagel et al. 1989). Dioxins usually occur as complex mixtures, which may theoretically contain 75 and 135 different chlorinated congeners of PCDD and PCDF, respectively (Tysklind et al. 1993). Deposition of PCDD/Fs into the environment can occur via atmospheric loading from combustion processes, waste effluent from manufacturing processes, and from the use of chlorinated herbicides and pesticides, such as Agent Orange. A recent study has postulated that these chloroaromatic compounds may not be strictly anthropogenic (Jong et al. 1994). In this study, it was shown that common wood- and forest litter- degrading fungi are responsible for the production of anisyl metabolites, which can be converted into chlorinated phenols. *In vitro* experiments using these chlorinated phenols have demonstrated that peroxidase-mediated oxidation can produce PCDD/Fs. In another study it has been postulated that these chlorinated

biaryl ethers can be generated in wastewater treatment plants (Oberg et al. 1993). The extent to which these biological processes contribute to the deposition of chlorinated biaryl ethers in the environment remains to be seen.

Dioxins are of great public concern because of their associated health risks.

2,3,7,8 tetrachlorodibenzo-*p*-dioxin (TCDD) is the most toxic of this class of compounds and is suspected to be both a human and animal carcinogen (Berg et al. 1994, Parrott et al. 1995). Dioxins have been linked with immunological dysfunction (EPA 1995), liver and nervous system disorders (Servos et al. 1994), and both embryotoxic and teratogenic effects (EPA 1995). These compounds are very lipophilic, due to their co-planar structure, and there is a tendency for their bioaccumulation in the environment (Friesen et al. 1995). Dioxins have been termed environmental endocrine disrupters, specifically binding to and activating the Ah receptor (Zacharewski et al. 1995). Activation of the Ah receptor releases HSP90, which allows the receptor to heterodimerize with ARNT, the Ah receptor nuclear translocator protein. The ARNT then induces gene expression in target genes containing dioxin response elements (Zacharewski et al. 1995).

### **2.3 Microbial degradation of dibenzofuran and dibenzo-*p*-dioxin**

Although the degradation pathways of PCDD/F are becoming better understood, there is limited knowledge concerning the fate of PCDD/F in contaminated soils. This is in part a result of the difficulties in studying bacterially mediated degradation of these biaryl ethers. These compounds have very low solubilities and have been shown to adhere to colloidal materials, such as soils (Greene, R. IT Corporation, Personal

Communication). Chlorinated congeners of DBF and DD are largely commercially unavailable in quantities necessary for experiments where bacterial growth is necessary and represent significant toxicological hazards. Because of these problems, research efforts have focused on understanding the degradation of the parent compounds DBF and DD. Similar experimental strategies have been utilized for polychlorinated biphenyl (PCB) degradation using biphenyl (Mohn et al. 1997). Transformation of the more highly chlorinated PCDD/Fs by anaerobic dechlorination often results in an increase in the concentrations of the less chlorinated compounds in sediments (Beurskens et al. 1995). Akin to PCB metabolism, as related to biphenyl, PCDD/Fs containing fewer chlorine substituents are degradable by bacteria through the co-metabolism of DBF and DD (Arensdorf and Focht 1994, Wilkes et al. 1996). Because of the significant toxicity and the importance of DBF and DD in PCDD/F metabolism, this investigation will focus primarily on the unchlorinated surrogate compounds, DBF and DD. The study of the non-chlorinated forms of these pollutants will further the knowledge base concerning the environmental fate of these recalcitrant compounds.

Previous attempts to study DD and DBF degradation by bacterial systems that degrade many other polyaromatic hydrocarbon (PAH) compounds have yielded limited information, and when metabolic transformations of PCDD/Fs do occur the results are generally dead end metabolites that the organism is unable to mineralize further (Klecka and Gibson 1979, 1980). It has been observed that *Alcaligenes eutrophus* A5 and *Sphingomonas* sp. strain A8AN3 are able to co-metabolize DBF during growth and degradation of biphenyl and naphthalene, respectively (Leblond 1997 and Menn et al.

1993). These soil isolates have been utilized in several studies in our laboratory (Layton, et al. 1992, Nadeau et al. 1994, Ahn, 1996), but were unable to mediate complete degradation of DBF or DD. Recently, an extradiol dioxygenation pathway has been identified which mediates the complete mineralization of DBF and DD (Figure 2.2 and Figure 2.3, Happe et al. 1993). This pathway represents a slightly different mechanism by which phenolic rings are cleaved. Initial dioxygenation is achieved through the activity of a three component angular extradiol dioxygenase, which attacks the 4,4a position between the two aromatic rings (Armengaud and Timmis 1997 and Schmid et al. 1997). The ferredoxin subunit of this dioxygenase has been purified, sequenced and found to be similar to the putidaredoxin of *Pseudomonas putida*, while the two isofunctional flavoreductases show no similarities to any of the flavoproteins characterized to date (Armengaud and Timmis 1997). Therefore the necessity of this class IIA ring-hydroxylating dioxygenase for DBF and DD mineralization may have been a limiting factor in increasing the understanding of bacterially mediated DBF and DD removal from contaminated soils and sediments.

The class IIA dioxygenase, dibenzofuran-4,4a-dioxygenase, catalyzes the initial dioxygenation of DBF/DD. Dihydroxylation of the biaryl either requires the interaction of a short electron transport chain, consisting of a 12 kDa ferredoxin and a monomeric flavoreductase, with the three component dioxin dioxygenase (Butler and Mason 1997). This reaction requires both  $\text{NADH}_2$  and  $\text{O}_2$  and results in the formation of a diene-diol-hemiacetal, which is believed to spontaneously rearomatize to form 2,2',3-trihydroxybiphenyl (Bunz and Cook 1993). 2,2',3-Trihydroxybiphenyl is then *meta*

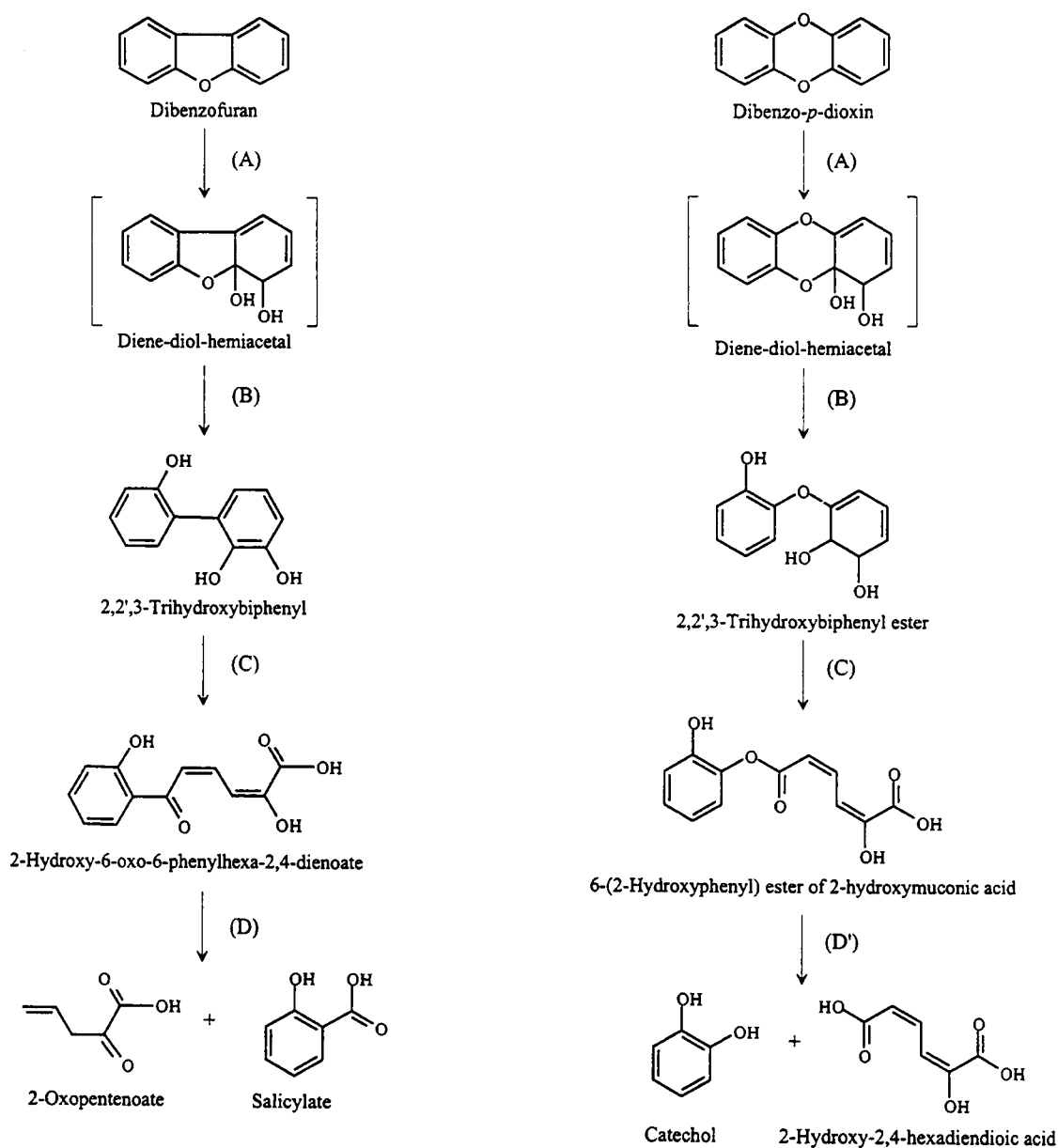


**Figure 2.2.** Proposed metabolic pathway for biodegradation of dibenzofuran and dibenzo-*p*-dioxin to salicylate and catechol, respectively, by *Sphingomonas* sp. strain RW1 (Figure adapted from Bunz et al. 1993, and Wittich et al. 1992). Enzymes for the pathways are: A) dibenzofuran 4,4a-dioxygenase (DxnA1A2, Bunz and Cook 1993), B) spontaneous rearomatization (Bunz and Cook 1993), C) 2,2',3-trihydroxybiphenyl dioxygenase (DbfB, Happe et al. 1993), D) hydrolase H1 (DxnB) and/or H2 (Bunz et al. 1993), D') No information concerning the mechanism of this reaction has been published.

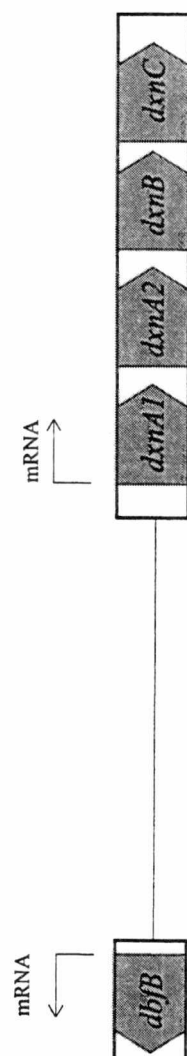
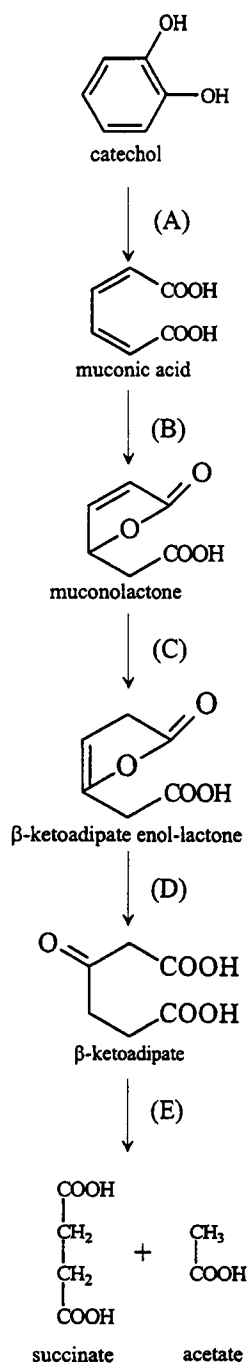


**Figure 2.3** Organization of two major operons encoding some of the dibenzofuran/dibenzo-*p*-dioxin metabolic genes in *Sphingomonas* species strain RW1.

cleaved by the product of the *dbfB* gene, 2,2',3-trihydroxybiphenyl dioxygenase, to form either 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate or the 6-(2-hydroxyphenyl) ester of 2-hydroxymuconic acid, depending on whether DBF or DD was the initial substrate (Happe et al. 1993). This extradiol dioxygenase is active as a monomer, requires another molecule of dioxygen, but does not require an additional external reductant. 3-chlorocatechol inhibits this dioxygenase, as has been observed for other dioxygenases of this type (Happe et al. 1993).

Currently, it is thought that both DBF and DD metabolism proceeds using a single pathway for degradation rather than two separate pathways. 2-Hydroxy-6-oxo-6-phenylhexa-2,4-dienoate has been shown to be hydrolyzed by the H1 (DxnB) and/or H2 dibenzofuran hydrolase to generate 2-oxopentanoate and salicylic acid. These hydrolases were identified in Strain RW1 using protein purification and subsequent enzyme assays (Bunz et al. 1993). However, the corresponding substituted dienoate from DD has not been demonstrated to be a substrate for these hydrolases, and no data has been presented speculating on whether or not these hydrolases are involved in DD mineralization. By whatever mechanism, the 6-(2-hydroxyphenyl) ester of 2-hydroxymuconic acid is postulated to be converted to 2,6-hydroxy-2,4-hexadiendioic acid and catechol (Wittich et al. 1992). The product of the H1/H2 hydrolases, salicylate, is believed to be converted to catechol via an unidentified mechanism (Happe et al. 1993). Catechols derived from DBF and DD are believed to be further metabolized through the activity of an unknown catechol-2,3-dioxygenase, which is distinct from the *DbfB* enzyme (Figure 2.4, Bunz et al. 1993). There has been no catechol-1,2-dioxygenase activity found in Strain RW1 to

## Ortho Pathway



## Meta Pathway

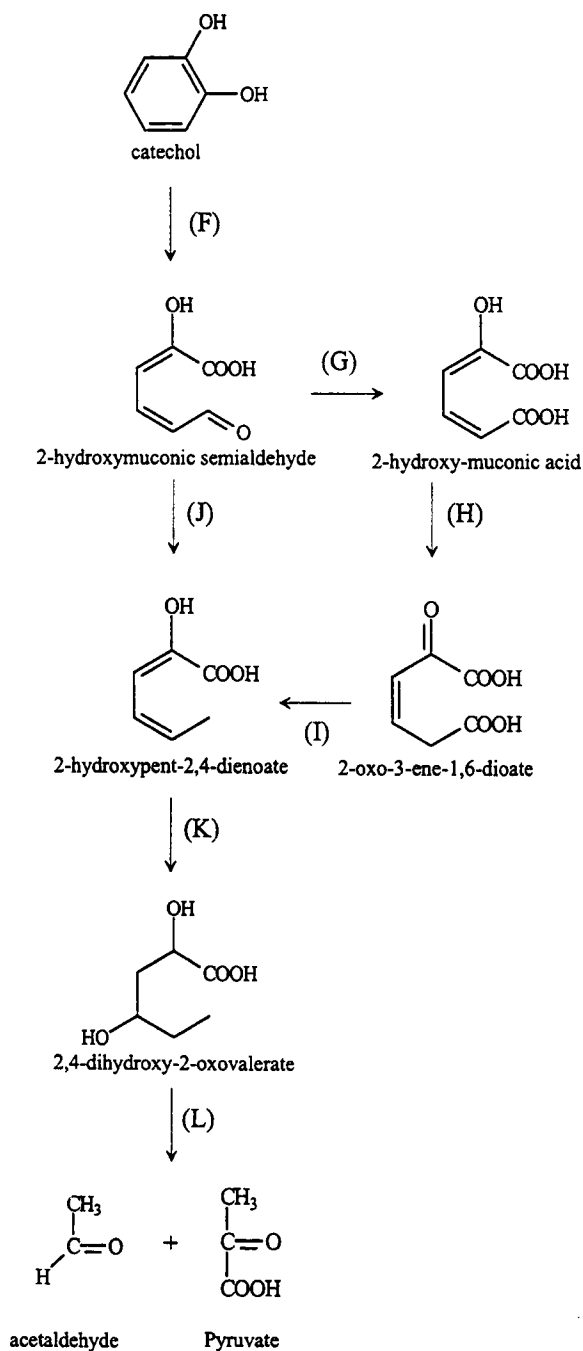


**Figure 2.4** *Ortho* and *meta* cleavage pathways found in many *Pseudomonas* species (Harayama and Timmis 1995 and Harwood and Parales 1996). Enzymes for these pathways are: A) catechol-1,2-dioxygenase, CatA; B) *cis,cis*-muconate lactonizing enzyme, CatB; C) muconolactone isomerase, CatC; D) enol-lactone hydrolase, CatD; E)  $\beta$ -ketoadipate succinyl-CoA transferase, CatIJ AND  $\beta$ -ketoadipyl-CoA thiolase, Cat F (Note: This conversion is the result of two reactions); F) Catechol-2,3-dioxygenase, XylE; G) 2-hydroxymuconic semialdehyde, XylG; H) 2-hydroxy-muconic acid tautomerase, XylH; I) 2-oxo-3-ene-1,6-dioate decarboxylase, XylI; K) 2-hydroxypent-2,4-dienoate hydratase, Xyl J; L) 2,4-dihydroxy-2-oxovalerate aldolase, XylK.

date (Bunz et al. 1993), and no data concerning the further metabolism of this compound has been published. Clearly, there is much more to be learned about the mechanisms and genetic organization of this interesting angular dioxygenation pathway. Additionally, the importance of this angular dioxygenation pathway in DBF and DD metabolism in Strain RW1 raises the questions of whether this pathway is commonly found at Agent Orange contaminated sites and what factors at these sites influence bacterial metabolism of these compounds.

## **2.4 Microbial degradation of 2,4-Dichlorophenoxyacetic acid**

### ***2.4.1 Microbial Ecology of 2,4-D degrading bacteria***

One of the first xenobiotic compounds demonstrated to be biodegradable was 2,4-D (Duxbury et al. 1970). Since that time, it has been shown that 2,4-D degrading bacteria are ubiquitously distributed in environmental soils (Kamagata et al. 1997). Bacteria able to mediate degradation of 2,4-D have been found to reside in diverse geographic regions (Tiedje et al. 1997). Several studies have indicated that pristine soils, which have not been previously exposed to 2,4-D, demonstrate 2,4-D degrading activity (Fulthorpe et al. 1996 and Tiedje et al. 1997). This degrading activity is primarily associated with primary and secondary enrichment cultures and axenic cultures were obtained from less than 1% of these soils (Tiedje et al. 1997). This is in contrast to 3-chlorobenzoate degrading activity, which was observed to occur with approximately twice the frequency, and axenic cultures were common. Based on these data, Tiedje et al. (1997) believe that culturable bacteria able to completely degrade 2,4-D are not as widespread as has

previously been thought and that 2,4-D metabolism by microbial consortia is more common.

#### **2.4.2 Metabolic pathways involved in 2,4-D degradation**

The broadleaf herbicide 2,4-D has been found to be rapidly degraded in the environment (Ka et al. 1994), and the microorganisms capable of biodegrading 2,4-D are common in all geographical locations examined (Fulthorpe et al. 1996). The Metabolic pathways involved in 2,4-D degradation have predominately been found in the  $\alpha$ ,  $\beta$ , and  $\gamma$  proteobacteria (Fulthorpe et al. 1995), and are located on large catabolic plasmids and/or within the chromosome. One of the best-studied bacterial systems for 2,4-D degradation is found in the  $\beta$  proteobacter *Ralstonia eutrophus* JMP134. *Ralstonia eutrophus* JMP134 is considered a model bacterium for 2,4-D degradation by many scientists (Harker et al. 1989), and contains a large 80 kb catabolic plasmid, designated pJP4, that contains the genes necessary for conversion of 2,4-D to the TCA intermediates succinate and acetal-CoA (Figure 2.5). This degradative pathway has been extensively studied in many laboratories around the world, and researchers are now beginning to understand the molecular mechanisms involved in degradation of 2,4-D in this organism.

The metabolic pathway necessary for 2,4-D catabolism by *R. eutrophus* JMP134, is localized on the pJP4 plasmid (Figure 2.5 and Figure 2.6). Initially 2,4-D enters the bacterial cell via passive diffusion through the bacterial membranes and via TfdK (a proposed 2,4-D transport protein and member of the major facilitator superfamily of transport proteins, Leveau et al. 1998) which is able to increase the intracellular

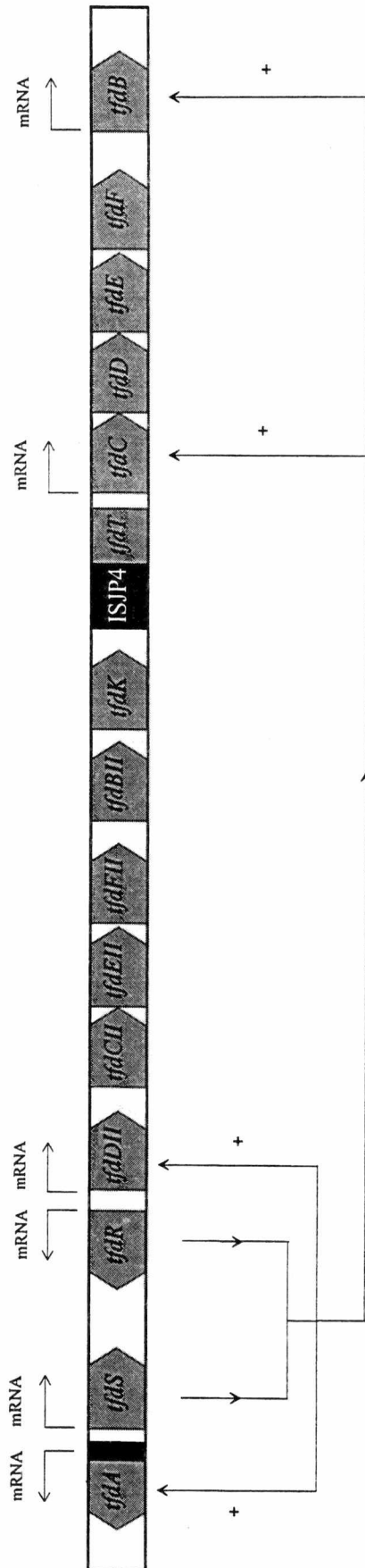


**Figure 2.5** Organization of two major operons involved in the regulation and synthesis of 2,4-D metabolic genes on the catabolic plasmid pJP4 in *Ralstonia eutrophus* JMP134. Location of the ISJP4 elements (■) and the structural genes (▤).

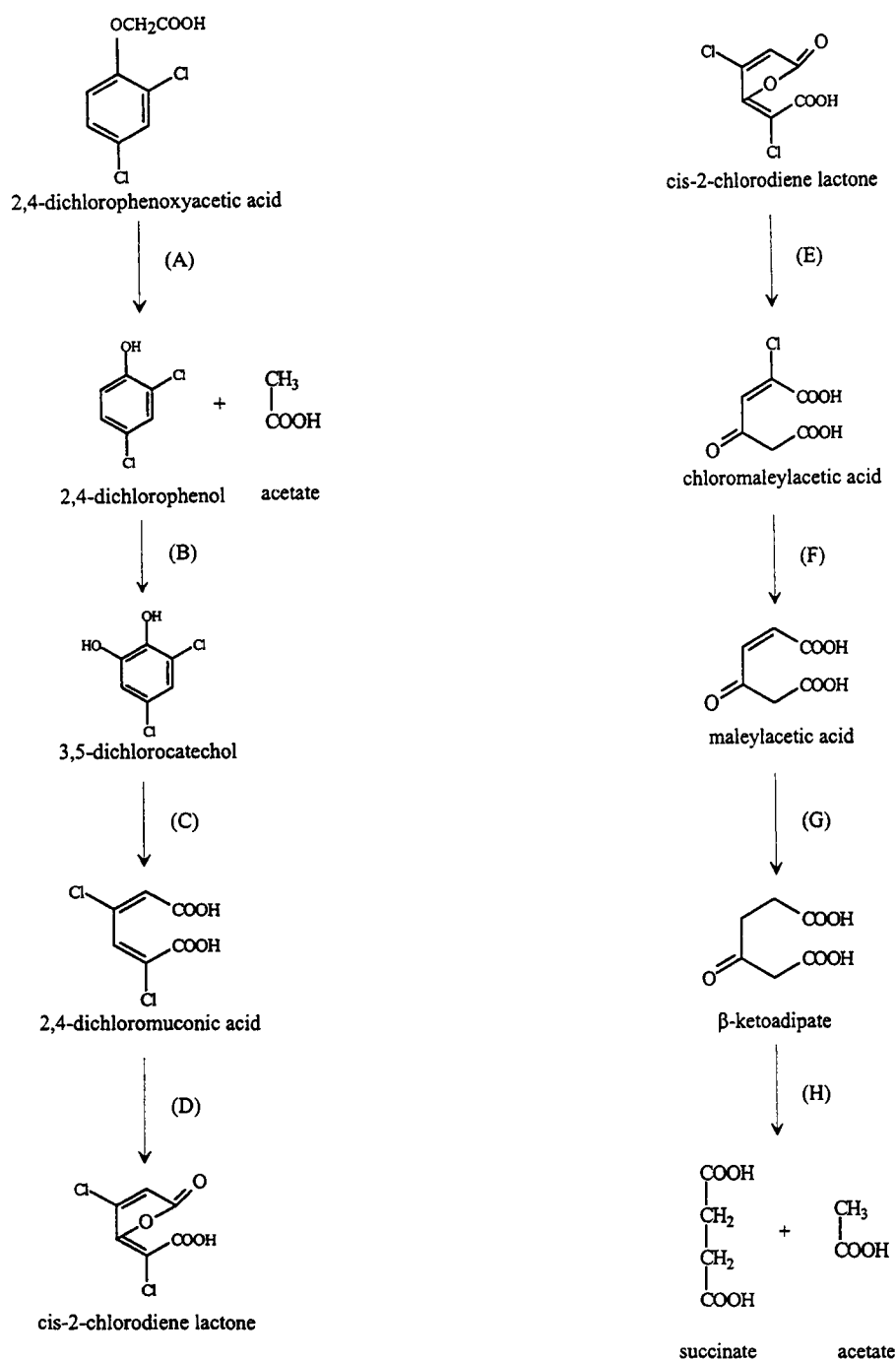


**Figure 2.6** Proposed metabolic pathway for biodegradation of 2,4-dichlorophenoxyacetic acid to succinate and acetate by *Ralstonia eutrophus* JMP134 (Kaphammer et al. 1990). A) 2,4-dichlorophenoxyacetic acid monooxygenase, TfdA; B) 2,4-dichlorophenol hydrolase, TfdB; C) 3,5-dichlorocatechol-1,2-dioxygenase, TfdC; D) 2,4-dichloromuconic acid cycloisomerase, TfdD; E) cis-2-chlorodiene lactone hydrolase, TfdE; F) chloromaleylacetic acid reductase; G) maleylacetic acid reductase, TfdF; H)  $\beta$ -ketoadipate succinyl-CoA transferase, CatIJ AND  $\beta$ -ketoadipyl-CoA thiolase, CatF (Note: This conversion is the result of two reactions).

concentration of 2,4-D. As 2,4-D becomes available the 2,4-diochlorophenoxyacetic acid/ $\alpha$ -ketoglutarate dioxygenase, TfdA, catalyzes the monohydroxylation of 2,4-D resulting in the formation of 2,4-dichlorophenol (2,4-DCP) and glyoxylate (Harker et al. 1989, Top et al. 1996). 2,4-DCP is then monooxygenated by TfdB to form 3,5 dichlorocatechol (3,5-DCC). Subsequent metabolism is mediated by the TfdC (2,4-dichlorocatechol dioxygenase), TfdD, (chloromuconate cycloisomerase), TfdE (dienelactone hydrolase), and TfdF(chloromaleylacetate reductase), generating succinate and acetal-CoA, which then feeds into the Citric Acid Cycle.

Recently, McGowen et al. (1998) have proposed three classes of *tfdA* genes. This categorization scheme is based on: 1) whether the genes necessary for 2,4-D degradation are localized on a plasmid or the chromosome, and 2) Sequence relatedness of the 2,4-D dioxygenase gene *tfdA* (McGowen et al. 1998). Class I 2,4-D degrading bacteria were defined as containing the pJP4 type *tfdA* gene which is highly conserved among 2,4-D metabolizing bacteria. Sequence variation associated with this *tfdA* gene is only approximately 2 base pairs. The class I degradation system is proposed to be plasmid based. The Class II 2,4-D degrading system encompasses *tfdA* genes with significantly lower sequence conservation. The *tfdA* genes in this class are approximately 76% similar to the class I type *tfdA* genes and are also believed to be localized on large catabolic plasmids. In contrast to the Class I and Class II systems, the Class III system is found predominantly on the chromosome (McGowen et al. 1998). The *tfdA* gene within this class of 2,4-D degrading bacteria demonstrated only 77% similarity to the class I *tfdA* gene and 80% similarity to the Class II gene. In addition to these three classes of 2,4-D

dioxygenase genes a fourth class can be postulated based on McGowen's findings that several  *$\alpha$ -Proteobacteria* demonstrated no homology to any of the known *tfdA* classes.

TfdA catalyzes the incorporation of single hydroxyl group onto the C1 carbon of the acetate moiety generating 2,4-dichlorophenoxy-1-hydroxy-acetic acid. TfdA is active as a homodimer with a  $M_r$  32,000 and absolutely requires Ferrous iron for its activity (Hausinger et al. 1997). This reaction also requires as a cofactor  $\alpha$ -ketoglutarate and oxygen and generates 2,4-dichlorophenoxy-1-hydroxy-acetic acid, succinate, and carbon dioxide as products (Hausinger et al. 1997). The 2,4-dichlorophenoxy-1-hydroxy-acetic acid spontaneously decomposes to yield glyoxylate and 2,4-DCP. The *tfdA* gene product can also use 2,4,5-trichlorophenoxy acetic acid or 4-nitrophenoxyacetic acid as a substrate yielding 2,4,5-trichlorophenol or 4-nitrophenol, respectively, and the associated byproducts (Hausinger et al. 1997). 4-nitrophenoxyacetic acid is often used experimentally to detect *tfdA* activity since 4-nitrophenol is an easily detectable chromophore (Hausinger et al. 1997).

The *tfdA* gene is found in single copy on the pJP4 plasmid with a divergently transcribed *tfdS* element encoding the TfdS regulatory protein. Experimental evidence suggests that the *tfdA* is negatively regulated by the TfdR (TfdR is a homologue of TfdS see below) and positively regulated by TfdS (Wright and Snyder 1997). It has been shown experimentally that the *tfdS* and the *tfdR* are identical to each other (100% sequence identity, (Matrubutham and Harker 1994), and that the predicted protein products share sequence homology with the LysR family of activator proteins. The

inducer molecule for the TfdR/S has been identified and is the product of the TfdC reaction, 2,4-dichloromuconate (Filer and Harker 1997).

## **2.5 Microbial degradation of 2,4,5-Trichlorophenoxyacetic acid**

### ***2.5.1 Microbial biology of 2,4,5-T degrading bacteria***

Like its dichlorinated counterpart, 2,4-D, the herbicide 2,4,5-T is a major component of Agent Orange (Danganan et al. 1995). However, less is known about the metabolism of 2,4,5-T compared to 2,4-D, and only a few researchers have addressed the degradation of 2,4,5-T by bacteria. Predominantly, most of the research efforts thus far have been associated with Dr. A.M. Chakrabarty's laboratory at the University of Illinois College of Medicine, beginning with isolation of the only bacterium characterized to date able to mediate complete mineralization of 2,4,5-T (Kilbane et al. 1982) in liquid culture. The lack of information gleaned from 2,4,5-T degrading organisms is puzzling considering the enormous amount of information available concerning 2,4-D degradation processes. To date, after 17 years of research, only two bacteria have been demonstrated to grow on 2,4,5-T as a sole carbon and energy source. The first and best studied is the bacterial strain *Burkholderia cepacia* AC1100 which is capable of growing on 2,4,5-T as a sole carbon and energy source (Daubaras et al. 1995), and the second is *Nocardiodes simplex* strain 3E which was isolated in 1990 by Golovleva et al. Both of these bacteria

were isolated from enrichment cultures after many months of incubation under artificial laboratory selection conditions.

### ***2.5.2 Metabolic pathways involved in 2,4,5-T degradation***

2,4,5-T metabolism by *B. cepacia* AC1100 has been well studied, and the metabolic pathway necessary for utilization of this chlorinated phenoxyacetic acid are almost completely characterized in this strain (Figure 2.7). However, several questions remain unanswered. *B. cepacia* AC1100 has recently been shown to contain 5 chromosomal elements, which have been designated replicons I through V (Hubner et al., 1998). The catabolic genes necessary for 2,4,5-T mineralization in *B. cepacia* AC1100 are localized in three separate operons, and have been found to reside on replicons III (0.53 Mbp) and IV (0.34 Mbp) (Hubner et al. 1998). Interestingly it has been observed that insertion elements are disproportionately concentrated on these two smaller replicons, which may facilitate the genome plasticity in this organism (Hubner et al. 1998). The three major gene clusters involved in 2,4,5-T degradation are the *tftAB*, *tftCD*, and the *tftEFGH* operons, and the *tftCD* operon is present in duplicate (Figure 2.8, Hubner et al. 1998). The duplication of *tftCD* has been proposed to provide a selective advantage by protecting against buildup of the toxic intermediate 2,4,5-trichlorophenol (2,4,5-TCP) during 2,4,5-T metabolism (Hubner et al. 1998).

The first two genes in the 2,4,5-T degradation pathway, *tftAB*, have been proposed to be constitutively expressed (Haugland et al. 1991). The products of the *tftAB* genes mediate the bioconversion of 2,4,5-T to 2,4,5-TCP

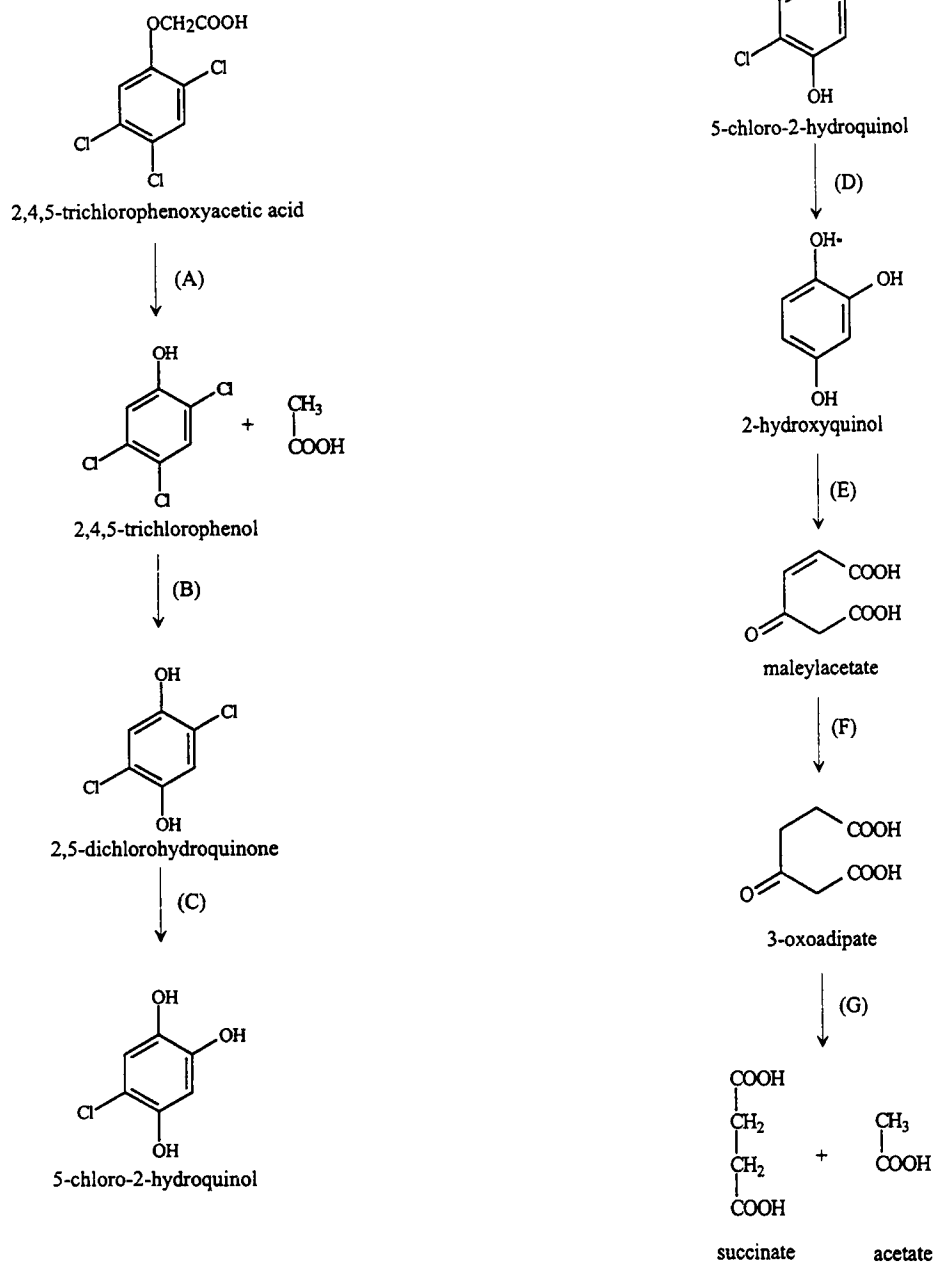


**Figure 2.7** Proposed metabolic pathway for biodegradation of 2,4,5-trichlorophenoxyacetic acid to succinate and acetate by *Burkholderia cepacia* AC1100 (Daubaras et al. 1996). Enzymes for the pathways are: A) 2,4,5-trichlorophenoxyacetic acid dioxygenase, TftAB; B,C) 2,4,5-trichlorophenol hydrolase, TftCD D) 2,5-dichloroquinone hydrolase, TftG E) 5-chloro-2-hydroxyquinol-1,2-dioxygenase, TftH; F) maleylacetate reductase, TftE; H) 3-oxoadipate is oxidized to succinate and acetate but this reaction is still not characterized, although TftF,G, and/or H are involved. Reactions B-F are not completely characterized at this time.

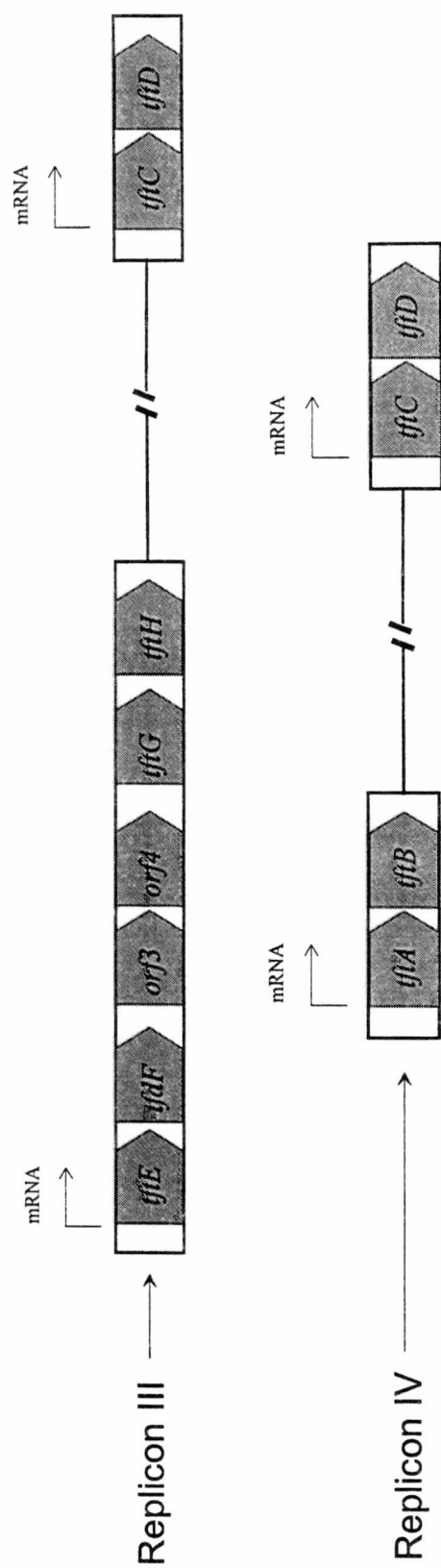


**Figure 2.8** Organization of four major operons encoding 2,4,5-T metabolic genes on separate chromosomal replicons in *Burkholderia cepacia* AC1100.

(Danganan et al. 1995). The *tftA* and *tftB* are found on replicon IV within a single operon under transcriptional activation by a fusion promoter apparently created by the Insertion Sequence (IS) IS1490 (Hubner and Hendrickson 1997). The substrate range of TftAB includes both 2,4,5-T and 2,4-D oxidation to 2,4,5-TCP and 2,4-DCP, respectively, and phenoxyacetate oxidation to phenol (Danganan et al. 1995). The TftA and TftB share sequence similarities with many other 1,2-dioxygenases from other bacterial degradation pathways (Danganan et al. 1995). Many of the related dioxygenases, such as XylXYZ, are modulated by regulatory genes such as the XylS and the BenR (Harayama and Timmis 1995). Therefore, it is in contrast to these systems that the *tftAB* genes are constitutive in *B. cepacia* AC1100. Also it has been observed that the activity of the 2,4,5-T oxygenase is decreased in response to increasing concentrations of 2,4,5-T possibly because of the toxicity of accumulated 2,4,5-TCP (Danganan et al. 1995). The precise enzymatic mechanism for the TftAB has been postulated to be similar to the TfdA dioxygenase but no conclusive mechanistic studies have been published to date.

Recently Hubner et al. (1998) have cloned, sequenced, and characterized a locus encoding the *tftC* and *tftD* genes. The TftCD monooxygenase catalyzes a two stage reaction involving the conversion of 2,4,5-TCP to 2,5-dichlorohydroquinone in stage I followed by dechlorination of 2,5-dichlorohydroquinone in stage II to yield 5-chlorohydroxy-quinol (Xun 1996). The TftD shares strong similarities with other aromatic monooxygenases including the HadA which is associated with 2,4,6-trichlorophenol-4-dechlorinase Hubner et al. (1998), but the TftC shares similarity not with the Had system

but rather with the HpaB monooxygenase like enzymes. The TftC is capable of NADH-dependent reduction of cytochrome c, in the presence of flavin adenine dinucleotide, and has been postulated to be involved in electron transport processes (Hubner et. al. 1998). Unlike the *tftAB* operon the *tftCD* operon is not constitutive and despite a high level of background transcription the *tftCD* genes are upregulated in the presence of both 2,4,5-T and 2,4,5-TCP (Hubner et. al. 1998). The precise mode of regulation of this operon is unknown at present, although glucose, succinate, or lactate grown cells lose the ability to dechlorinate 2,4,5-TCP.

Daubaras et al. (1996) have identified a gene cluster, containing the *tftEFGH* genes, which is responsible for the further metabolism of 2,4,5-TCP to 3-oxoadipate. This operon is located on replicon III, and although it is known that this gene cluster is positively regulated during 2,4,5-T metabolism, the precise inducer has not been identified (Danganan et al. 1995). Zaborina et al. (1998) have identified a novel dechlorinase enzyme, TftG, which is involved in the first reactions of this lower pathway. The TftG in concert with a novel reductase component is able to dechlorinate 5-chloro-hydroxyquinol to yield hydroquinol. Currently the reductase component is unidentified, although, a putative hydroxy-1,4-benzoquinone is being investigated (Zaborina et al. 1998). TftG catalyzes the removal of a single chlorine from 5-chloro-hydroxyquinol to yield the nonchlorinated 2-hydroxy-1,4-benzoquinone (HBQ). HBQ is then subsequently reduced via a proposed hydroxy-1,4-benzoquinone reductase which requires an NADH + H<sup>+</sup>. During the next stage of reactions the hydroquinol dioxygenase, TftH, catalyzes ring fission to yield maleylacetate. Maleylacetate reductase, TftE, facilitates reduction of

maleylacetate to yield 3-oxoadipate, which is then converted by an uncharacterized enzyme(s) to form tricarboxylic acid cycle intermediates.

## **2.6 Inhibition of microbial degradative pathways via 2,4-D and 2,4,5-T metabolites**

In the absence of complete mineralization, 2,4-D and 2,4,5-T can be incompletely transformed by abiotic and biotic processes (Amy et al. 1985). Abiotic processes can only contribute to partial degradation and subsequent accumulation of intermediates and never to the complete removal of these compound such as occurs in mineralization processes (Somerville et al. 1978). In the absence of a lower pathway for degradation of these compounds, growth may still occur because the organism can gain carbon and energy from the acetate, which is generated from cleavage of the phenoxy group (Top et al. 1996). Also, incomplete metabolism of 2,4,5-T can lead to accumulation of the metabolic intermediates 2,4,5-TCP, 5-chloro-2-hydroquinone and 3,4,6-trichlorocatechol (3,4,6-TCC; Haugland et al. 1990). Likewise, partial metabolism of 2,4-D can lead to the formation of 2,4-DCP and 3,5-DCC (Haugland et al. 1990). Co-cultures containing the 2,4,5-T degrading *B. cepacia* AC1100 and *R. eutrophus* JMP134 have been used to identify potential inhibition problems between these two pathways (Haugland et al. 1990). In these studies, it was demonstrated that although either organism mineralized their respective single contaminant easily, 2,4-D co-metabolism by *B. cepacia* AC1100 produced chlorohydroquinone, which inhibited both its own 2,4,5-T metabolism and 2,4-D degradation, by *R. eutrophus* JMP134.

The extent to which 2,4-D and 2,4,5-T degradation affects the metabolism of DBF and DD needs to be examined. The presence of 2,4-DCP, 2,4,5-TCP, 3,4,6-TCC, and 3,5-DCC in association with bacteria degrading DBF and DD may cause repression of genes necessary for the cell's continued metabolic fitness. Compounds, such as 3-chlorocatechol, are known inhibitors of *meta*-ring cleavage enzymes (Klecka and Gibson 1981, Bartels et al. 1984). These compounds form acyl halides, which bind to and inactivate *meta*-pyrocatechase type enzymes. The presence of chlorocatechols inhibits the enzymes responsible for one type of biphenyl metabolism (Arensdorf and Focht 1994). Many of the studies dealing with the effects of these chlorinated phenolic compounds involve monitoring changes in the rate of oxygen uptake and/or substrate conversion (Bartels et al. 1984), and consequently, little has been elucidated concerning the changes in gene expression in response to these compounds. Discerning specific changes in gene expression will provide information not only on the activity of particular enzymes, but on potential secondary genetic elements which are involved with DBF and DD metabolism.

## **2.7 Catabolite repression of biodegradative pathways**

Carbon catabolite repression in *Pseudomonads* appears to be regulated differently than the model cAMP mediated mechanism previously characterized for *Escherichia coli* associated with the phosphotransferase system (Staijen et al. 1999). This is based on studies by several independent research laboratories (Staijen et al. 1999, Mcfall et al. 1997, and Holtel et al. 1994). One of the most recent research papers addressing this

particular issue demonstrated that a plasmid encoded biodegradative pathway, involving utilization of n-alkanes, which demonstrated catabolite repression in *Pseudomonas oleovorans* was no longer subject to carbon catabolite repression when transferred to *E. coli alk<sup>+</sup>* strains (Staijen et al. 1999). In *Pseudomonas aeruginosa* and *Pseudomonas putida*, the levels of cAMP are not modulated in response to varying carbon sources to the degree observed in *E. coli* (Yuste et al. 1998), and the only cAMP receptor protein analog known is not involved in catabolite repression but rather is involved in quorum sensing (Yuste et al. 1998).

### **2.7.1 Repression of *n*-alkane degradation**

The metabolic pathway necessary for *n*-alkane degradation is under the influence of catabolite repression (Staijen et al. 1999 and Yuste et al. 1998). This negative regulation occurs, specifically, in association with AlkB monooxygenase when glucose or lactate is available as a secondary carbon source, and was confirmed by *alkB* mRNA analysis in *P. oleovorans* (Staijen et al. 1999). In an earlier paper, Yuste et al. (1998), a *lacZ*-reporter containing the *alkB* promoter and the *alkS* regulatory protein was utilized to examine expression of the *PalkB* promoter in response to organic acids. These experiments demonstrated that this promoter was repressed in the presence of lactate, succinate, and that catabolite repression associated with Lauria-Bertani broth appeared to occur via a different mechanism. Furthermore, in the Yuste et al. (1998) studies, transcription of the *alkS* gene was unaffected by succinate and lactate. Based on these observations two possible models have been proposed. The first model is that there is a

putative negative regulatory factor which may reduce *alkS* (*alkS* is positive regulatory element necessary for transcriptional activation of the *alkB* promoter) expression under conditions of catabolite repression. The second model is that a putative negative regulatory factor may compromise the ability of the AlkS to initiate transcription at the *alkB* promoter by physically interacting with the *alkS* binding site. These data also suggest that several mechanisms for catabolic repression may exist in *Pseudomonas* species.

### ***2.7.2 Repression of phenol degradation***

Phenol degradation by *P. putida* strain H is subject to organic acid mediated catabolite repression (Muller et al. 1996). These experiments utilized a *PphlA* promoter *lacZ* fusion to demonstrate that transcription from the *phlA* promoter is reduced in the presence of the rapidly metabolizable organic acids succinate, lactate, and acetate, as well as the carbohydrates glucose and gluconate. Similar to observations of repression in *P. oleovorans* associated with *n*-alkane degradation, evidence supported the action of a negative controlling factor that inhibited the *phl* gene specific activator PhlR. Muller et al. (1996) proposed that the phosphotransferase system is not important in catabolite repression associated with phenol degradation because all of the carbon sources found to repress activity in these studies are taken into the cell by special transport systems. Additionally, they proposed that a general requirement of catabolite repression by inhibition of the specific activator, such as PhlR, requires that a positive control mechanism be involved in the gene or operon's transcription and that a general

mechanism of inhibition may be through masking of the nucleic acid binding region by the negative effector.

### **2.7.3 Repression of chlorocatechol degradation**

Chlorocatechol is an important metabolic intermediate in many catabolic pathways, which degrade chlorinated aromatic compounds. Catabolite repression of the evolutionarily related *catBCA* and *clcABD* operons in *P. putida* has recently been examined, by McFall et al. 1997. The *catBCA* and *clcABD* operons are regulated by the LysR-type regulators CatR and ClcR respectively, and are 32.5% identical and 43% similar in their amino acid sequences. Previous experiments have shown that the CatR can functional replace the ClcR for growth on 3-chlorobenzoate, but that the ClcR cannot substitute for the CatR. In vitro transcription analyses, using *clcR-AB-lacZ* and *catB-lacZ* transcriptional fusions, demonstrated that the *clcABD*, but not the *catBCA* was catabolite repressed when cells were grown in the presence of succinate, citrate, or fumarate (McFall et al. 1997). LacZ reporter assays showed that this repression was mediated specifically by fumarate and that fumarate most probably was acting as an anti-inducer by competing for the 2-chloro-cis-cis-muconate binding site on the ClcR. Inhibition of the ClcR was reversible. Gel retardation assays demonstrated that fumarate did not interfere with the binding of ClcR to the *clc* operator. McFall et al. (1997) suggested that the utilization of fumarate as a key signaling molecule would provide the cell with an elegant sensing mechanism to facilitate catabolism of a myraid of chloroaromatic in the absence of preferred carbon and energy sources.

## **2.8 Methods for monitoring gene expression in environmental bacteria**

An alternative approach to biochemical or enzymatic analysis of biodegradative gene activity is an analysis of gene expression directly using mRNA based techniques. This approach has the advantage of providing information regarding gene induction while isolating mRNAs coding for proteins necessary for the conversion of DBF and DD. At the forefront of this area of research are three techniques: differential display, subtractive hybridization, and electronic subtraction. Each of these techniques has advantages and disadvantages, as recently reviewed by Wan et al. (1996). Electronic subtraction has the advantage of providing digital and reusable data. This method entails sequencing randomly selected cDNAs from the cDNA library of interest. Generally a single study requires the sequencing of between 1000 – 3000 different cDNAs. Unfortunately, this method cannot detect rare mRNAs, which are speculated to comprise 99% of the mRNA transcripts (Wan et al. 1996). Also, electronic subtraction has a high cost of operation and results obtained from electronic subtraction are dependent on mRNA abundance.

Subtractive hybridization eliminates the need for randomly processing the large number of cDNA samples required for electronic subtraction. Briefly, subtractive hybridization involves the hybridization of cDNAs from one uninduced RNA pool and a second induced RNA pool. Hybridization is followed by separation of double stranded species from single stranded species, and construction/characterization of a subtracted cDNA library. This method has several advantages over electronic subtraction, such as the ability to detect both abundant and rare mRNAs, and is technically more efficient and

cost effective. However, subtractive hybridization has several disadvantages, including: the potential to subtract out mRNA molecules belonging to similar families and sharing regions of DNA homology, expression of some mRNAs may not be an all or none event resulting in the subtracting out of up regulated mRNA molecules, abundant mRNAs will be preferentially identified, and results from this method are dependent on mRNA abundance.

Differential display reverse transcriptase PCR (DDRT-PCR) is evolving as the superior method for identifying induced genes using mRNA populations (Liang and Pardee 1993, Wang et al. 1996, and Wong et al. 1994). This method involves initial cDNA synthesis using a reverse transcriptase reaction followed by a PCR reaction, which amplifies the cDNA products. These cDNA products are then electrophoresed using a denaturing polyacrylamide gel to produce a cDNA fingerprint. Bands are then excised from the sequencing gel, purified, and cloned for subsequent analysis and characterization. DDRT-PCR shares with subtractive hybridization the advantage of identifying both abundant and rare mRNAs, and requires only a small quantity of RNA. Another advantage of using DDRT-PCR is that the technique is not as sensitive to mRNA abundance and is dependent on the sequence of the primers used. This allows the DDRT-PCR technique to be fine tuned for the experimental system. DDRT-PCR also has the advantages of more rapid output of data, is highly reproducible, allows simultaneous evaluation of multiple experimental conditions, and requires no prior sequence information.

Studies on DBF and DD metabolism in *Sphingomonas* sp. strain RW1 have mainly focused on enzyme purification and subsequent analyses based on these protein products (Bunz and Cook 1993, Harms et al. 1995, Harms et al. 1995b). While these experiments have provided valuable information concerning the identity and activity of many necessary proteins, they have not provided much information concerning the expression of genes or the regulatory potential involved in the metabolism of these compounds. The examination of the gene repression effects, due to byproducts of 2,4-D and 2,4,5-T transformations, is ideally suited to further the understanding of how this metabolic pathway could be regulated in a mixed pollutant scenario. Data from these experiments can be applied to help elucidate degradation potential of dibenzofuran and dibenzo-*p*-dioxin in Agent Orange contaminated soils.

## **PART III**

### **DEGRADATION OF 2,4-D AND 2,4,5-T BY BACTERIA ISOLATED FROM AGENT ORANGE CONTAMINATED SOIL**

### 3.1 ABSTRACT

Agent Orange contaminated soils were utilized in enrichment culture studies to isolate 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) degrading bacteria. Two bacterial cultures able to grow at the expense of 2,4-D and/or 2,4,5-T were isolated from these soils. Subsequent characterization of the 2,4-D degrading culture showed that one bacterium, *Burkholderia* species strain JRB1, was able to utilize 2,4-D as a sole carbon and energy source in pure culture. The 2,4,5-T degrading culture was identified as a consortium containing two bacteria, *Burkholderia* species strain JR7B2 and *Burkholderia* species strain JR7B3. This consortium was able to metabolize 2,4,5-T as the sole source of carbon and energy, and demonstrated the ability to affect metabolism of 2,4-D to a lesser degree. Strain JR7B3 was able to mineralize 2,4,5-T in pure culture and utilized 2,4,5-T as a carbon and energy source in the presence of 0.01% yeast extract. Polymerase chain reaction (PCR) experiments utilizing known genetic sequences from other 2,4-D and 2,4,5-T degrading bacteria have shown that these organisms contain gene sequences similar to *tfdA*, *B*, *C*, *E*, and *R* genes (*Burkholderia* sp. strain JRB1) and the *tftA*, *C*, and *E* genes (*Burkholderia* sp. Strain JR7B3). Additionally, a genetic element induced in Strain JR7B2 during consortial growth on 2,4,5-T was identified using Differential Display Reverse Transcriptase -PCR (DDRT-PCR). Particularly interesting, is the ability of *Burkholderia* species strain JR7B3 to mineralize 2,4,5-T-ring-UL-<sup>14</sup>C in pure culture. Future research using this strain will provide valuable information about the environmental fate of 2,4,5-T in Agent Orange contaminated sites.

### 3.2 INTRODUCTION

Herbicide Orange, which is more commonly known as Agent Orange, was frequently used as a defoliant in the United States and was sprayed in large quantities in Southeast Asia during the Vietnam war. Agent Orange is composed of a mixture of 50% 2,4,5-trichlorophenoxy acetic acid (2,4,5-T) and 50% 2,4-dichlorophenoxyacetic acid (2,4-D). It has been shown that 2,4-D degrading bacteria are ubiquitously distributed in environmental soils (Kamagata et al. 1997). The broadleaf herbicide 2,4-D has been found to be rapidly degraded in the environment, and the microorganisms capable of biodegrading 2,4-D are common in all geographical locations examined (Tiedje et al. 1997). The metabolic pathways involved in 2,4-D degradation have predominately been found in the  $\alpha$ ,  $\beta$ , and  $\gamma$ -*Proteobacteria*, and are located on large catabolic plasmids and/or within the chromosome. One of the best-studied bacterial systems for 2,4-D degradation is found in the  $\beta$ -*Proteobacteria*, *Ralstonia eutrophus* JMP134. *R. eutrophus* JMP134 is considered a model bacterium for 2,4-D degradation by many scientists (Harker et al. 1989), and contains a large ~80 kb catabolic plasmid designated, pJP4, containing the genes necessary for conversion of 2,4-D to  $\beta$ -ketoadipate which is subsequently metabolized to succinate and acetyl-CoA by chromosomally encoded enzymes (Fulthorpe et al. 1995). This degradative pathway has been extensively studied by many laboratories around the world, and researchers are now beginning to understand the molecular mechanisms involved in degradation of 2,4-D in this organism.

Like its dichlorinated counterpart, 2,4-D, the herbicide 2,4,5-T is a primary component of Agent Orange (Danganan et al. 1995). However, less is known about the

metabolism of 2,4,5-T compared to 2,4-D, and only a few researchers have addressed the degradation of 2,4,5-T by bacteria. Predominantly, most of the research efforts thus far have been associated with Dr. A. M. Chakrabarty's laboratory at the University of Illinois College of Medicine, beginning with the isolation of the only bacterium characterized to date able to mediate complete mineralization of 2,4,5-T (Kilbane et al. 1982) in liquid culture. The lack of information gleaned from 2,4,5-T degrading organisms is puzzling considering the enormous amount of information available concerning 2,4-D degradation processes. To date, after 17 years of research, only two bacteria have been demonstrated to grow on 2,4,5-T as a sole carbon and energy source. The first and best studied is the bacterial strain *Burkholderia cepacia* AC1100 which is capable of growing on 2,4,5-T as a sole carbon and energy source (Daubaras et al. 1995), and the second is *Nocardioides simplex* strain 3E which was isolated in 1990 by Golovleva et al. Both of these bacteria were isolated from enrichment cultures after many months of incubation under artificial laboratory selection conditions.

The genes for 2,4,5-T degradation by *B. cepacia* AC1100 are chromosomally encoded, specifically localized on replicons III and IV. These genes have been previously characterized and found to reside within 3 distinct operons (Hubner et al., 1998); *tftAB* (replicon IV), *tftCD* (replicon III and a second copy found on replicon IV), and the *tftEFGH* (replicon III). Together these genes facilitate conversion of 2,4,5-T to 3-oxoadipate, which is further broken down to generate succinate and acetate that is then channeled into the tricarboxylic acid cycle. Biodegradation of the herbicide Agent

Orange potentially involves a multitude of different microorganisms having diverse potentials for metabolic transformations of 2,4-D and 2,4,5-T.

Because of the complexity of microbially mediated biodegradation of Agent Orange constituents, a scheme was devised consisting of two primary goals: 1) to isolate and identify 2,4-D and 2,4,5-T degrading bacteria present in Agent Orange contaminated soils through direct isolation and subsequent phenotypic and genotypic analyses, and 2) to characterize 2,4-D and 2,4,5-T degradation by environmental bacteria isolated from Agent Orange contaminated soils. The fundamental goal of this research is to determine the potential for bacterial catabolism of 2,4-D and 2,4,5-T associated with Agent Orange, contaminated soils. An understanding of the metabolic potential present in these contaminated soils will facilitate risk assessment and potential cleanup strategies at polluted sites containing mixtures of 2,4-D and 2,4,5-T.

In this paper we report the isolation, identification, and characterization of a 2,4-D degrading bacterium, *Burkholderia* species strain JRB1 and a 2,4,5-T degrading bacterium, *Burkholderia* species strain JR7B3, from Agent Orange contaminated soils. Specific primers for amplification of the *tfdA*, B, C, E and *tftA*, C, E genes were designed and synthesized. These primers were then used in conjunction with the 2,4-D and 2,4,5-T-degrading bacteria isolated from Agent Orange contaminated soils. It was postulated that these organisms might have genes similar to those previously characterized for *R. eutrophus* JMP 134 and *B. cepacia* AC1100. These bacteria were shown to contain genes similar to those previously characterized for 2,4-D degradation by *R. eutrophus* JMP 134 and 2,4,5-T degradation in *B. cepacia* AC1100, respectively.

### 3.3 MATERIALS AND METHODS

#### 3.3.1 *Bacteria, plasmids, media, and chemicals.*

*Escherichia coli* strain DH5 $\alpha$  (Invitrogen, San Diego, California) was grown in Luria Bertani broth (10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract) and used in combination with the pCR 2.1 (Invitrogen, San Diego, California) plasmid vector for all cloning experiments as described by the manufacturer. *R. eutrophus* JMP 134 (Amy et al. 1985) and *B. cepacia* AC1100 (Kilbane et al. 1982) were grown in phosphate-buffered minimal salts medium (PAS), containing in grams per liter; 4.43 g K<sub>2</sub>HPO<sub>4</sub>, 1.72 g KH<sub>2</sub>PO<sub>4</sub>, 2.16 g NH<sub>4</sub>Cl, 0.195 g MgSO<sub>4</sub>·H<sub>2</sub>O, 0.01 g FeSO<sub>4</sub>·2H<sub>2</sub>O, and 0.003 g CaCl<sub>2</sub>·2H<sub>2</sub>O. Solidified PAS medium was prepared by addition of 15 g/L of Agarose (Difco) added prior to autoclaving. The large catabolic plasmid, pJP4 (Don and Pemberton 1985) was isolated from *R. eutrophus* JMP 134 using the alkali lysis method as described by Sambrook et al. (1989). 2,4-dichlorophenol (2,4-DCP, 99%) and 2,4-D (99+%) were purchased from Acros Organics (Pittsburgh, PA). 2,4,5-trichlorophenol (2,4,5-TCP, 99%) was purchased from Fluka Chemicals (Milwaukee, WI). 2,4,5-T (97%) was obtained from Aldrich chemical Company, Inc. (Milwaukee, WI). N,N-dimethylformamide (DMF, 99.9%), 2,4,5-T-ring-UL-<sup>14</sup>C, and 2,4-D-ring-UL-<sup>14</sup>C were purchased from Sigma Chemical Company (St. Louis, MO). Acetonitrile (HPLC grade) and Water (HPLC grade) were obtained from Fisher Scientific. All other chemicals were of analytical grade.

### ***3.3.2 Enrichment cultures.***

Traditional enrichment cultures were used to isolate 2,4-D and 2,4,5-T-degrading bacteria from Agent Orange contaminated soils. Briefly, 50 ml of PAS medium was combined with 5 grams of soil from the surface contaminated soil for one set of cultures and contaminated soil from the 6 inch core sample for a second set of samples. To these soil slurries, 100  $\mu$ l of a 500 mg/ml stock solution of 2,4-D or 2,4,5-T dissolved in DMF was added. These soil slurries were incubated at 28°C and 150 rpm. After one week of incubation, aliquots of the soil slurries were serially diluted and plated on PAS media amended with the appropriate carbon source. These plates were incubated at 28°C and monitored for colony formation. Additionally, all enrichment culture samples were plated on PAS media without 2,4-D or 2,4,5-T to control for false positives. Soil slurries were also sub-cultured into fresh PAS media containing the appropriate chemical and incubated for another week. Bacterial colonies that formed during the enrichment experiments, were re-inoculated into PAS liquid medium (without yeast extract) amended with the respective carbon source and grown to an approximate optical density (600 nm) of 0.25. Culture aliquots (1.0 ml) were routinely amended with 1.0 ml 30% glycerol and frozen at -80°C for long-term storage and further analyses.

### ***3.3.3 Mineralization assays.***

0.5 ml aliquots from freezer stocks were washed 2 times with 1X PAS medium and used to inoculate starter cultures containing 50 ml of PAS medium amended with 1 mg/ml of 2,4-D or 2,4,5-T. These starter cultures were incubated at 28°C until mid-log

growth phase was achieved (an optical density of 0.2 at 600 nm). From these starter cultures 0.5 ml was transferred to 250 ml Erlenmeyer flasks containing 50 ml of fresh PAS medium containing 1 mg/ml 2,4-D or 2,4,5-T. These cultures were allowed to incubate at 28°C until mid-log phase was attained and then 5.0 ml was transferred to 40 ml EPA mineralization vials. An 8 ml glass vial, containing 0.5 ml of freshly prepared 0.5 N NaOH, was then inserted into the EPA mineralization vial, and these vials were sealed using open top screw caps fitted with Teflon-lined rubber septa. To each reaction vial ~100,000 dpm of 2,4,5-T-ring-UL- $^{14}\text{C}$  or 2,4-D-ring-UL- $^{14}\text{C}$  was then added. For each experiment, an abiotic control and a biotic control was prepared. Abiotic controls consisted of PAS media amended with 1 mg/ml 2,4-D or 2,4,5-T without any added cells. Biotic controls were prepared in the same manner as the test samples except that prior to addition of the 2,4,5-T-ring-UL- $^{14}\text{C}$  or 2,4-D-ring-UL- $^{14}\text{C}$  200  $\mu\text{l}$  of 2 N  $\text{H}_2\text{SO}_4$  was added. All reaction vials were then incubated in the dark at 28°C for 7 days. Test cultures were then amended with 200  $\mu\text{l}$  2 N  $\text{H}_2\text{SO}_4$  and incubated for an additional 2 hours. In order to determine  $^{14}\text{C}$  concentrations associated with biomass, liquid media and evolved  $\text{CO}_2$  the following procedure was used. The 0.5 ml of NaOH from the  $\text{CO}_2$  trap and 0.5 ml of culture from the 40 ml EPA mineralization vial was transferred to 20 ml scintillation vials containing 10 ml of Ready Safe Scintillation cocktail (Beckman, Fullerton, CA). Additionally, 1.0 ml of culture from the 40 ml EPA mineralization vial was transferred to 1.5 ml microcentrifuge tubes and centrifuged at 14,000 rpm for 10 min. Subsequently, 0.5 ml of supernatant was then transferred to 20 ml scintillation vials containing Ready Safe Scintillation cocktail. Scintillation vials were then vortexed and

allowed to incubate 2 days at room temperature prior to analysis on a Beckman LS 5000 TD scintillation counter (Beckman, Fullerton, CA).

### **3.3.4 PCR analyses.**

PCR primers were developed using nucleic acid sequences obtained from the Genbank sequence database (National Center for Biotechnological Information, NCBI, <http://www.ncbi.nlm.nih.gov>). Specific primer sequences were chosen with the help of the Primer3 software algorithm (Whitehead Institute for Biomedical Research, <http://www.genome.wi.mit.edu>) and Blast 2.0 algorithm (NCBI). Primer sequences are listed in Table 3.1. All primers were synthesized using a Beckman oligo 100 DNA synthesizer (Beckman Instruments, Fullerton, CA) according to the manufacturer's directions.

A modified touchdown PCR protocol (Hecker et al. 1996) was used for amplification of 400 - 2300 bp nucleic acid sequences. PCR amplification was achieved using PCR beads (Amersham Pharmacia, Piscataway, New Jersey) according to manufacturer's recommendations. Initial PCR analyses were conducted using whole bacterial cells from solid media. One small bacterial colony was transferred to a sterile 200 µl thin wall PCR tube (Perkin Elmer, Foster City, CA) and combined with the following components: 1.5 µl Primer A (2.0 µM stock), 1.5 µl Primer B (2.0 µM stock), 23.5 µl ultrapure H<sub>2</sub>O, and 1 PCR bead. Subsequent amplification of cloned PCR products was achieved using 1.5 µl Primer A (2.0 µM stock), 1.5 µl Primer B (2.0 µM

**Table 3.1.** PCR primers used in this study

| Target Gene        | Primers                                            | Primer Target Region | Target Accession Number | Target Size |
|--------------------|----------------------------------------------------|----------------------|-------------------------|-------------|
| <i>tfdR - tfdA</i> | L1 5'-cgt tcg tag tgc agg tgc-3'                   | 52-69                | M16730                  | 1610        |
|                    | R1 5'-ggg ggc tac atc ttg tgg-3'                   | 1662-1647            |                         |             |
| <i>tfdA</i>        | <i>tfdA</i> L2 5'-cga aga cat cga cct tcg ag-3'    | 795 - 814            | M16730                  | 589         |
|                    | <i>tfdA</i> R2 5'-gcc gat gaa gag aaa ctt gc-3'    | 1383 - 1364          |                         |             |
| <i>tfdB</i>        | <i>tfdB</i> L1 5'-tgt tag tca atg cgt ttc gtg-3'   | 4010-4030            | M35097                  | 2218        |
|                    | <i>tfdB</i> R1 5'-cgt ctc acc tcg aat aga tcg-3'   | 6227-6207            |                         |             |
| <i>tfdC</i>        | <i>tfdC</i> L1 5'-caa gga tgt tgc cga tgc g-3'     | 351-369              | M35097                  | 591         |
|                    | <i>tfdC</i> R1 5'-gcc ggg ata tga aac gtt gac c-3' | 921-942              |                         |             |
| <i>tfdE</i>        | <i>tfdE</i> L1 5'-gtc gct ttg gtc cct acc t-3'     | 2328-2346            | M35097                  | 655         |
|                    | <i>tfdE</i> R1 5'-tcc ttc aac att gca agc ag-3'    | 2982-2963            |                         |             |
| <i>fftA</i>        | <i>fftA</i> L1 5'-agc tcg att ctg gtg tac gg-3'    | 7-26                 | U80795                  | 798         |
|                    | <i>fftA</i> R1 5'-cgc att cag atc aag caa ga-3'    | 804-785              |                         |             |
| <i>fftC</i>        | <i>fftC</i> L1 5'-ggg cca gca gct caa gaa ag-3'    | 909-928              | U83405                  | 522         |
|                    | <i>fftC</i> R1 5'-tat tcc ggc agc gat cta gt-3'    | 1430-1411            |                         |             |
| <i>fftE</i>        | <i>fftE</i> L1 5'-gtg acg tcg tgc ttt act cg-3'    | 71-90                | U19883                  | 1299        |
|                    | <i>fftE</i> R1 5'-cta caa acg cga acc atc at-3'    | 1369-1350            |                         |             |
| <i>fftE</i>        | <i>fftE</i> L2 5'-gac aac cta tgc ggg tag tga-3'   | 642-662              | U19883                  | 617         |
|                    | <i>fftE</i> R2 5'-cat gtc tgc agc gag ctc ta-3'    | 1257-1238            |                         |             |
| <i>D20</i>         | L1 5'-aag ggg tgg gac cta taa cc-3'                | -NA-                 | -NA-                    | 415         |
|                    | R1 5'-gac gaa ggg gga aaa tat gc-3'                |                      |                         |             |

stock), 22.5 µl ultrapure H<sub>2</sub>O, 1.0 µl Template DNA (100 ng), and 1 PCR bead. All PCR products were stored at -20°C prior to cloning, sequencing, and future analyses.

PCR products were cloned using Invitrogen's TA cloning kit (Invitrogen, San Diego, California) and analyzed further. Freshly amplified PCR bands were ligated into the pCR 2.1 vector, overnight at 14°C, and transformed into *E. coli* DH5α cells (Invitrogen, San Diego, California). Plasmid DNA was extracted using the alkali lysis method as described by Sambrook et al. (1989) or the BIO-RAD Quantum Prep<sup>®</sup> plasmid miniprep kit (BIO-RAD Laboratories, Hercules, CA) and characterized using *Eco*R1 endonuclease digestion followed by agarose gel electrophoresis. Plasmid inserts matching the molecular weight of the expected cDNA band were sequenced by the Department of Microbiology Molecular Biology Resource Facility (The University of Tennessee, Knoxville) using either T7 or M17 based sequencing primers. Sequences obtained were analyzed using the Blast 2.0 algorithm (NCBI) in order to determine similarities to published gene sequences.

### **3.3.5 RT-PCR analyses.**

To determine if the identified genetic sequences were expressed RT-PCR was utilized. Starter cultures containing 50 ml of PAS medium amended with 1 mg/ml of 2,4-D or 2,4,5-T were incubated at 28°C until mid-log growth phase was achieved (an optical density of 0.2 at 600 nm). These starter cultures were the source of an inoculum for test cultures. Briefly, 0.5 ml was transferred to 250 ml Erlenmeyer flasks containing 50 ml of PAS medium containing 1 mg/ml 2,4-D or 2,4,5-T. These cultures were allowed to

incubate at 28°C until mid-log phase was attained. Reverse transcription reaction components were as follows: 200 µM dNTP, 5.0 mM DTT, 50 units of MMLV reverse transcriptase (Gibco BRL, Grand Island, New York), 1X MMLV reaction buffer, 0.4 µM random primer and 1.0 µg of total RNA was used as the template. Reaction conditions for the reverse transcription were: an initial ramping down from 55°C to 40°C for primer annealing, reverse transcription at 37°C for 60 min, and a final 5.0 min incubation at 75°C for enzyme inactivation. Reverse transcription reactions were stored at -20°C prior to PCR. PCR beads (Amersham Pharmacia, Piscataway, New Jersey) were used for subsequent PCR amplification. 1 PCR bead was combined with 21 µl HPLC grade H<sub>2</sub>O, 1.5 µl 2.0 µM primer A, 1.5 µl 2.0 µM primer B, and 1.0 µl reverse transcription reaction. A modified touchdown PCR protocol (Hecker et al. 1996) consisted of an initial denaturation at 92°C for 4.0 min, 20 cycles of PCR including a denaturation at 92°C for 15 sec, annealing at (65 - 45 °C, 1°C degrees/cycle) for 30 sec, and primer extension at 72°C for 30 sec. Finally, a 7.0 min extension was followed by storage at 4°C until gel electrophoresis.

### ***3.3.6 Strain identification.***

Bacterial strains obtained from enrichment cultures were identified using 16S rRNA analysis. Bacterial strains were grown to mid-log phase in their respective media and chromosomal DNA was extracted using the alkali lysis method (Sambrook et al. 1989). PCR was used to amplify 16S rDNA from these total chromosomal DNA preparations using 1492r and 27f primers (Weisburg et al. 1991). These primers are

highly homologous to the conserved regions of the rRNA, as previously described (Weisburg et al. 1991). In some cases, PCR amplification was accomplished using whole cells obtained directly from plates with similar results. Briefly, 100 ng of genomic DNA was used as the template for PCR amplification using these primers (stock concentrations of 2  $\mu$ M). PCR conditions consisted of 40 cycles of 95°C (5 min), 50°C (30 sec), and 72°C (2 min), followed by a final extension time of 7 min at 72°C and storage at 4°C. Subsequently, 10  $\mu$ l of the PCR reaction was electrophoresed through a 1.5% agarose gel to verify the specificity of the PCR amplification. Samples with the correct band sized were cloned into the TA cloning vector and sequenced, as described above.

### ***3.3.7 RISA-RFLP analysis.***

Ribosomal intergenic spacer analysis (RISA) coupled with Restriction fragment length polymorphism (RFLP) was utilized to examine relatedness among members of the 2,4,5-T degrading bacterial consortia. For these studies a modified touchdown PCR protocol (Hecker et al. 1996) was used for amplification of the intergenic region between the 23S and 16S rDNA. Specific primers for PCR amplification included 1055f (5'-ATG GCT GTC GTC AGC T-3') and 23Sr (5'-CAA GGC ATC CAC CGT-3'). PCR was achieved using PCR beads (Amersham Pharmacia, Piscataway, New Jersey) according to manufacturer's recommendations. PCR analyses were conducted using whole bacterial cells from solid media. One small bacterial colony was transferred to a sterile 200  $\mu$ l thin wall PCR tube (Perkin Elmer, Foster City, CA) and combined with the following components: 1.5  $\mu$ l Primer A (2.0  $\mu$ M stock), 1.5  $\mu$ l Primer B (2.0  $\mu$ M stock), 23.5  $\mu$ l

ultrapure H<sub>2</sub>O, and 1 PCR bead. The PCR conditions consisted of an initial denaturation at 92°C for 4.0 min, 20 cycles of PCR including a denaturation at 92°C for 15 sec, annealing at (65 - 45 °C, 1°C degrees/cycle) for 30 sec, and primer extension at 72°C for 30 sec, followed by a final extension time of 7 min at 72°C and storage at 4°C. To the 25 µl PCR reaction was added 3 µl of an appropriate 10X-reaction buffer and 1.0 ul each of either the HhaI and Rsa or MspI and AluI endonucleases. These digestions were incubated at 37°C for 3 hours, supplemented with 3 µl of DNA loading dye, and then electrophoresed using 1.5% agarose mini-gels. Gels were quantitated using the AlphaImager<sup>TM</sup> 2200 Gel Documentation System (Alpha Innotech Corporation, San Leandro, CA).

### **3.3.8 HPLC analyses.**

Analytical determination of 2,4-D, 2,4,5-T, 2,4-DCP, and 2,4,5-TCP concentrations in culture supernatants was determined using HPLC. Samples for analysis were obtained directly from culture supernatants. Aliquots, 100 µl were taken from growing bacterial cultures and transferred to 1.5 ml microcentrifuge tubes. These were then microcentrifuged at 14,000 rpm to pellet bacterial cells, and the supernatant was frozen at -80°C for HPLC studies. Immediately prior to analysis, samples were warmed at 37°C for 5 min and 70 µl was transferred to an 1.8 ml amber autosampler vial containing 730 µl HPLC grade H<sub>2</sub>O and 50 µg 4-bromobenzoic acid (4-BBA, internal standard) dissolved in 200 µl acetonitrile. Sample injection was facilitated by a Perkin Elmer LC 600 autoinjector (Perkin Elmer, Foster City, CA) fitted with a 50 µl injection

loop. A Supelcosil LC-18-T reverse phase  $C_{18}$  column (Supelco, Bellefonte, PA) was fitted onto a Perkin Elmer Binary LC 250 series pump (Perkin Elmer, Foster City, CA) and a Perkin Elmer model LC-235 diode array detector (Perkin Elmer, Foster City, CA). Data analysis was achieved using the Turbochrome version 4.1 software package (Perkin Elmer, PE Nelson Division, San Jose, CA). Conditions for HPLC analysis were optimized for detection of the above compounds in 20% acetonitrile:80%  $H_2O$ . Samples were eluted at a flow rate of 1.0 ml/min with 0.025%  $H_3PO_4$ -acetonitrile gradient, as follows: 0% acetonitrile (isocratic, 5.0 min), 0 to 50% acetonitrile (linear, 5.0 min), 50% acetonitrile (isocratic, 20 min), 50 to 0% acetonitrile (linear, 5.0 min). Absorbance of eluted compounds was monitored at 210 nm. Sample retention times were compared to known standards prepared and analyzed using identical protocols. Retention times for compounds of interest were 4-BBA (16.2), 2,4-D (17.1), 2,4-DCP (18.0), 2,4,5-T (19.2), and 2,4,5-TCP (21.5).

### 3.3.9 Differential display reverse transcriptase - PCR.

DDRT-PCR was utilized to identify potentially novel genetic elements, which are induced in the presence of 2,4,5-T. For these studies, a bacterial consortium harboring two previously characterized *Burkholderia* species was used. One control culture inoculated with the consortium was grown in PAS + succinate and a second culture inoculated with the consortia was grown in PAS + 2,4,5-T. For these cultures, a starter culture was grown in PAS medium supplemented with 2,4,5-T to an approximate optical density (600 nm) of 0.2, and 0.5 ml was used to inoculate both bacterial cultures. Mid-

log phase cultures, approximately at optical density of 0.2, were then the source of a series of samples for RNA extractions. First, 6.0 ml of each culture was aliquoted into four Eppendorf tubes and immediately placed in an ice bath. Rifampicin was then added to each culture to a final concentration of 0.2 mg/ml. Rifampicin was added as a transcriptional inhibitor, and allows mRNA transcripts to be differentiated from rRNA during DDRT-PCR (Nagel et al. 1999). The cultures were then allowed to incubate for 60 min after adding rifampicin, and then 6.0 ml of culture from each flask was transferred to four 1.5 ml Eppendorf tubes and placed in the ice bath.. After samples were collected, they were centrifuged at 14,000 rpm in a 4°C cold room. Cell pellets were washed with ice cold 1X PAS media, re-centrifuged and stored at -80°C.

Total RNA was extracted using the Hot Phenol method, as previously described (Fleming et al. 1993), and stored at -80°C prior to reverse transcription. Reverse transcription reaction components were as follows: 200  $\mu$ M dNTP, 5.0 mM DTT, 50 units of MMLV reverse transcriptase (Gibco BRL, Grand Island, New York), 1X MMLV reaction buffer, 0.4  $\mu$ M random primer and 1.0  $\mu$ g of total RNA was used as the template. Reaction conditions for the reverse transcription were: an initial ramping down from 55°C to 40°C for primer annealing, reverse transcription at 37°C for 60 min, and a final 5.0 min incubation at 75°C for enzyme inactivation. Reverse transcription reactions were stored at -20°C prior to PCR. Components for the PCR were as follows: 20.0  $\mu$ M dNTP, 1X PCR Buffer (Gibco BRL, Grand Island, New York), 0.3  $\mu$ l Taq Polymerase (Gibco BRL, Grand Island, New York), 1.8  $\mu$ l DMSO, 0.2  $\mu$ M anti-sense primer (Same as used in reverse transcription), 0.2  $\mu$ M Shine Dalgarno primer (Fleming et al. 1998), 0.3  $\mu$ l

10% TritonX-100, and 0.25  $\mu\text{l}$   $\alpha\text{-}^{32}\text{P}$  CTP (800  $\mu\text{C}/\text{mM}$ ), 3.0  $\mu\text{l}$  reverse transcription reaction. PCR reactions volumes were adjusted to 30.0  $\mu\text{l}$  using ultrapure  $\text{H}_2\text{O}$ . Following an initial denaturation at 92°C for 2.0 min, 40 cycles of PCR consisted of: denaturation at 92°C for 15 sec, annealing at 40°C for 30 sec, and primer extension at 72°C for 30 sec.

PCR reactions were visualized on sequencing gels using a Genomix DNA sequencing apparatus (Genomix, Foster City, CA). Four  $\mu\text{l}$  of each PCR reaction were combined with 4.0  $\mu\text{l}$  of loading dye and incubated for 2.0 min at 100°C. Four  $\mu\text{l}$  of this mixture was electrophoresed on a 4.5% denaturing polyacrylamide gel for 2.0 hours at 2700 V. The polyacrylamide gel was dried directly on the glass plate, and urea residues were removed by washing with distilled  $\text{H}_2\text{O}$  and subsequent drying according to manufacturer's directions. This washing procedure was repeated until no traces of urea were visible. After washing, the dried gel was exposed to autoradiography film (Genomix, Foster City, CA) for 12 – 96 hours.

Differential cDNA bands were eluted from the gel for further analysis. Using the autoradiogram as a template, cDNA bands were cut out using a razorblade and softened in 5.0  $\mu\text{l}$  of HPLC grade water. These bands were then diluted in 100  $\mu\text{l}$   $\text{H}_2\text{O}$ , incubated at 68°C for 2.0 hours, and re-amplified using PCR (same conditions as above but omitting the radionucleotide). Reamplified PCR products were then separated using a 1.0 % low melting point agarose gel. Reamplified bands were cut from the agarose gel and used in subsequent studies.

Because of the presence of rRNAs in the RNA extractions, it was necessary to verify that the cDNA obtained through DDRT-PCR is actually the product messenger RNA transcripts, prior to cloning. Total RNA from control and 2,4,5-T cultures was vacuum blotted onto nylon membranes (ICN Biochemical, Cleveland, OH). The reamplified cDNAs were then radiolabeled using T4 Kinase (Gibco BRL, Grand Island, New York) according to the manufacture's protocol. Membranes were pretreated in a solution (pH 7.3) containing, 0.5 M  $\text{Na}_2\text{HPO}_4$ , 1.0 mM EDTA, and 7.0 % SDS and incubated at 65°C in a shaking water bath. After 4.0 hours, the denatured cDNA probe was added to the solution and allowed to incubate with the membrane for up to 24 hours. Membranes were then washed four times with a high stringency wash buffer containing, in g/L: 0.59 g NaCl, 3.15 g Tris-HCl, 0.37 g EDTA, and 5.0 g SDS. Hybridized blots were then exposed to Kodak Biomax<sup>TM</sup> MR film (Eastman Kodak, Rochester, NY) and allowed to incubate for 24 – 96 hours at -80°C or analyzed using the Storm<sup>TM</sup> 840 Phosphoimager (Molecular Dynamics, Sunnyvale, CA). In addition to the RNA hybridization verification, standard RT-PCR (as described above) was utilized to re-detect the band of interest and exclude the possibility of chimeric artifacts.

### **3.4 RESULTS**

#### **3.4.1 Soil characterization.**

Three soil samples contaminated with Agent Orange were received from Dr. James Cornette (Tyndall Air Force Base, Florida). These soil samples are from a

loading/unloading area at Hardstand 7, Eglin Air Force Base (Niceville, Florida). The approximate depths of these samples were 0 inches (surface soil, SS) and 6 inches (core soil, CRS). A control soil (CS) was also obtained which was not contaminated with this herbicide. Visually, contaminated soils were dark brownish with a red-orange to red-violet tint. HPLC analyses of hexane:acetone (1:1) soil wash extractions approximated the 2,4-D and 2,4,5-T concentrations of these soils to be 22.4 mg/kg and 73.8 mg/kg, respectively (Figure 3.1), and very low concentrations of 2,4-DCP and 2,4,5-TCP were detected (<1.0 ppm). Total heterotrophic colony counting techniques demonstrated that these soils contained  $5.2 \times 10^7$  cfu/g for the control soil,  $5.2 \times 10^7$  cfu/g for the surface soil, and  $2.8 \times 10^6$  cfu/g for the core soil.

#### ***3.4.2 Isolation of 2,4-D and 2,4,5-T degrading bacteria.***

Traditional enrichment culture experiments, using these Agent Orange contaminated soils, readily identified two bacterial cultures capable of using either 2,4-D or 2,4,5-T as a sole source of carbon and energy. These cultures were designated Agent Orange enrichment culture JRB1 and JR7B, respectively. Transmission electron micrographs of JRB1 cultures revealed an organism of approximately  $1.2 \mu\text{m} \times 0.6 \mu\text{m}$  (Figure 3.2). Similar electron micrographs of JR7B revealed several different sized bacterial cells (Figure 3.3). Culturing of JR7B onto  $\frac{1}{4}$  strength YEPG plates containing 1000 mg/L 2,4,5-T identified two distinct colony morphotypes (Figure 3.4). Further, subculturing of these colony types identified one bacterial strain, designated Strain

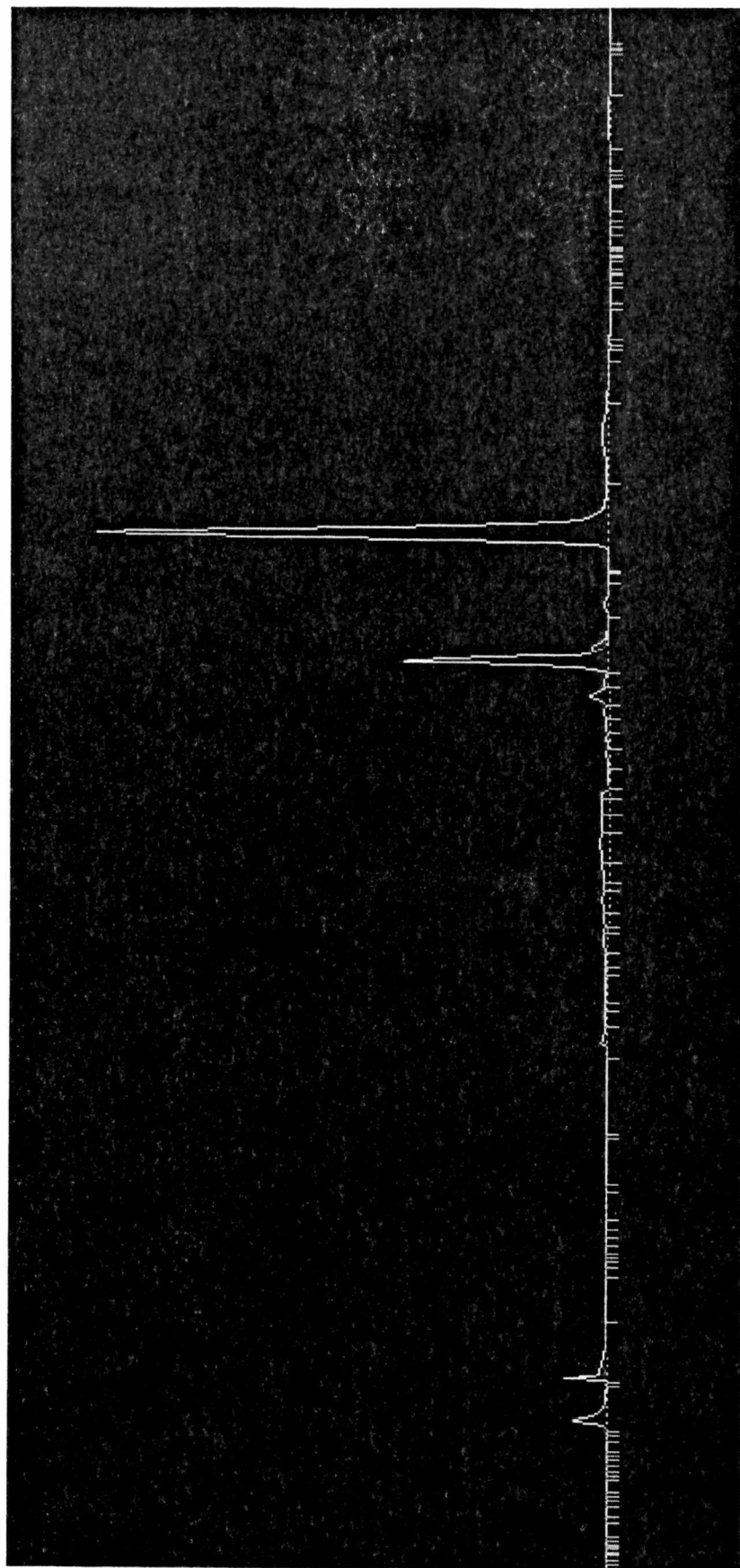


**Figure 3.1** HPLC chromatograph of hexane:acetone (1:1) extractions from Agent Orange contaminated soil samples. 2,4-dichlorophenoxyacetic acid (17.5) and 2,4,5-trichlorophenoxyacetic acid (19.8) were found at concentrations of 22.4 mg/L and 73.8 mg/L, respectively.

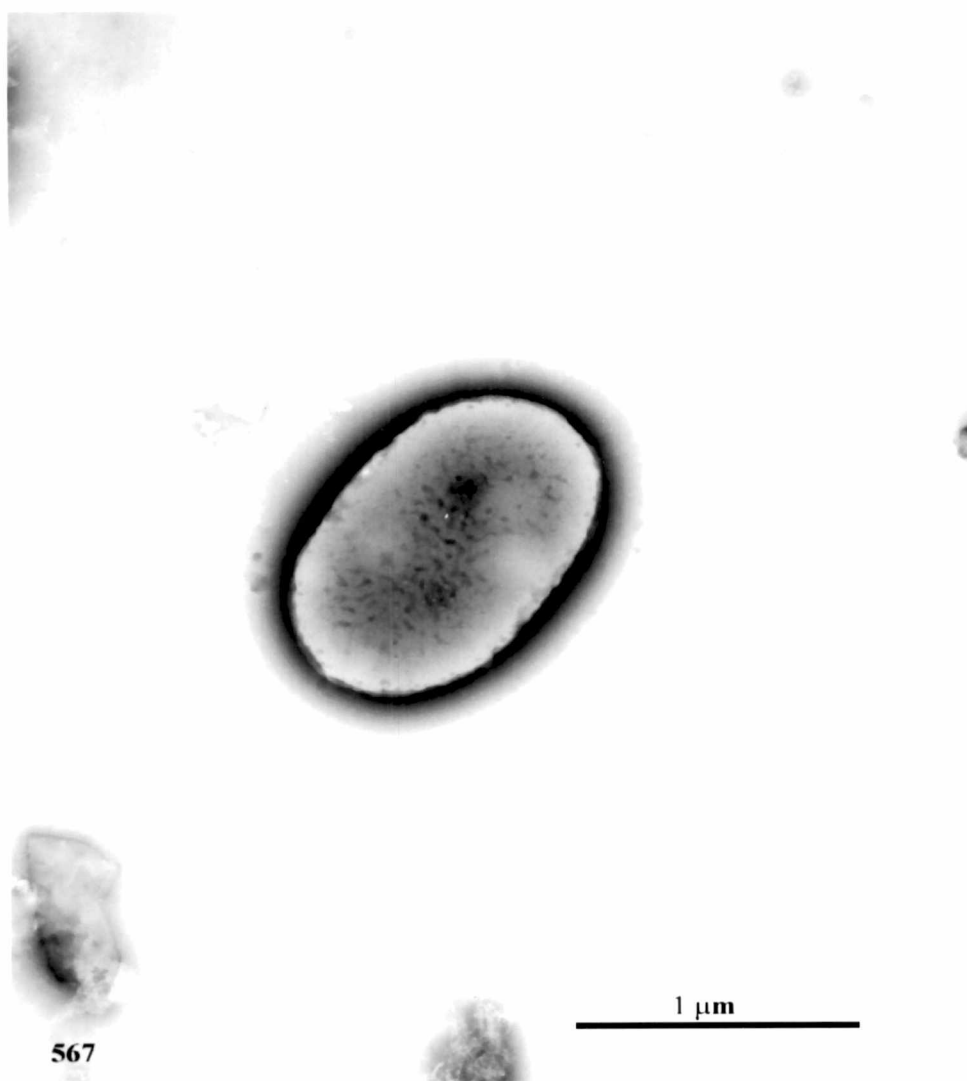
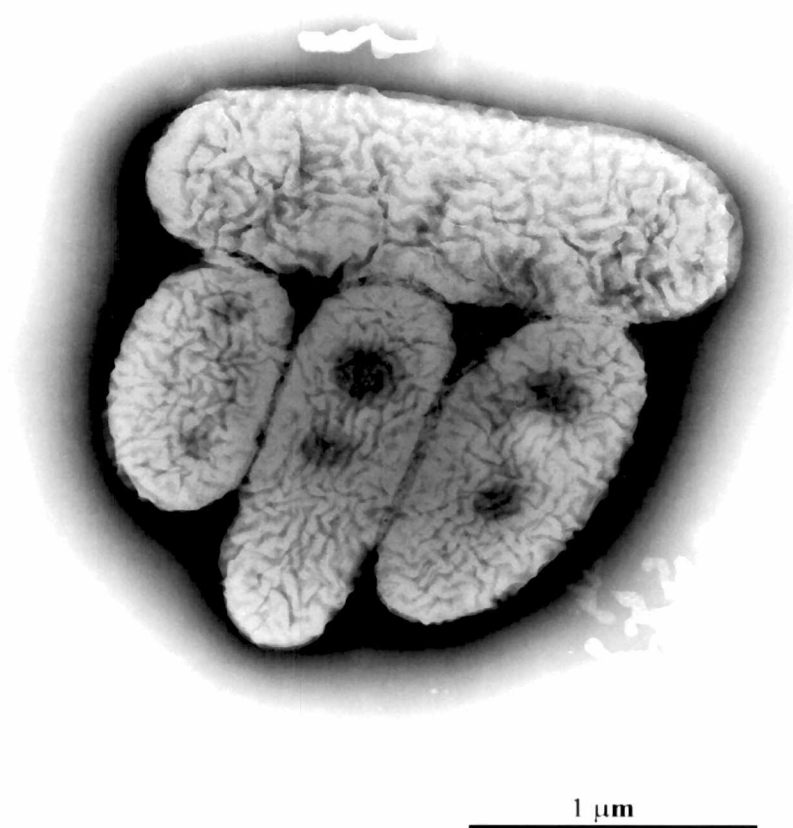


**Figure 3.2** Transmission electron micrograph of *Burkholderia* species strain JRB1.



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**Figure 3.3** Transmission electron micrograph of a 2,4,5-T degrading microbial consortium containing *Burkholderia* species strain JR7B2 and *Burkholderia* species strain JR7B3

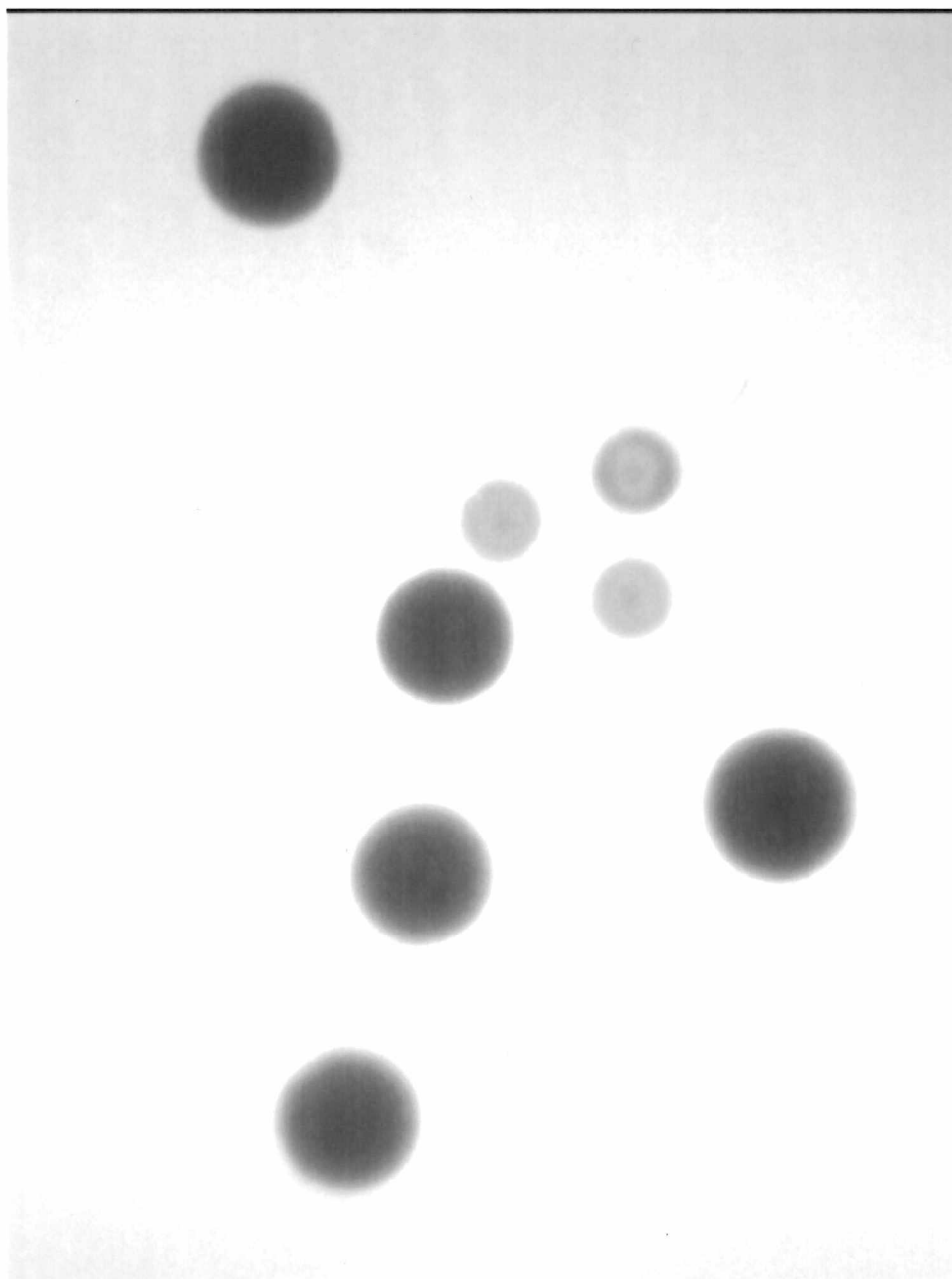


**Figure 3.4** Small and large colony types associated with the 2,4,5-T degrading Agent Orange enrichment culture JR7B

JR7B3 which was able to mediate degradation of 2,4,5-T in the presence of additional nutrients (0.01% yeast extract). The other bacterial strain was designated JR7B2 and demonstrated no 2,4,5-T or 2,4,5-TCP transformation capacity. Transmission electron micrographs of Strain JR7B3 revealed a bacterial cell approximately 1.5  $\mu\text{m}$  X 0.7  $\mu\text{m}$  (Figure 3.5). Ribosomal intergenic spacer analysis (RISA) coupled with restriction fragment length polymorphism (RFLP) analysis of JRB1, JR7B3, and JR7B2 demonstrated that each one of these organisms had a unique banding pattern and therefore were genotypically distinct from each other (Figure 3.6). RISA-RFLP analysis of Strain JR7B3 and *B. cepacia* AC1100 revealed that these organisms might be closely related as evidenced by identical banding patterns (Figure 3.6).

16S phylogenetic analysis revealed that the 16S rRNA of the 2,4-D degrading bacterium (Strain JRB1, Figure 3.7, Table 3.2) was 97% similar to *Burkholderia glathei* accession number U96935. Phylogenetic analyses of *Burkholderia* species JR7B2 and *Burkholderia* species strain JR7B3 revealed that they were 99% similar to *Burkholderia caribiensis* (accession number Y17009, Table 3.2) and 97% similar to *Burkholderia graminis* (accession number U96941), respectively (Figure 3.8, Table 3.2).

### **3.4.3 Analysis of 2,4-D and 2,4,5-T degradation by environmental bacteria from Agent Orange contaminated soil.**

Degradation of 2,4-D and 2,4,5-T by *Burkholderia* species strain JRB1 and *Burkholderia* species strain JR7B3, respectively, was examined using mineralization and growing cell assays. Degradation studies utilizing *Burkholderia* species strain JRB1

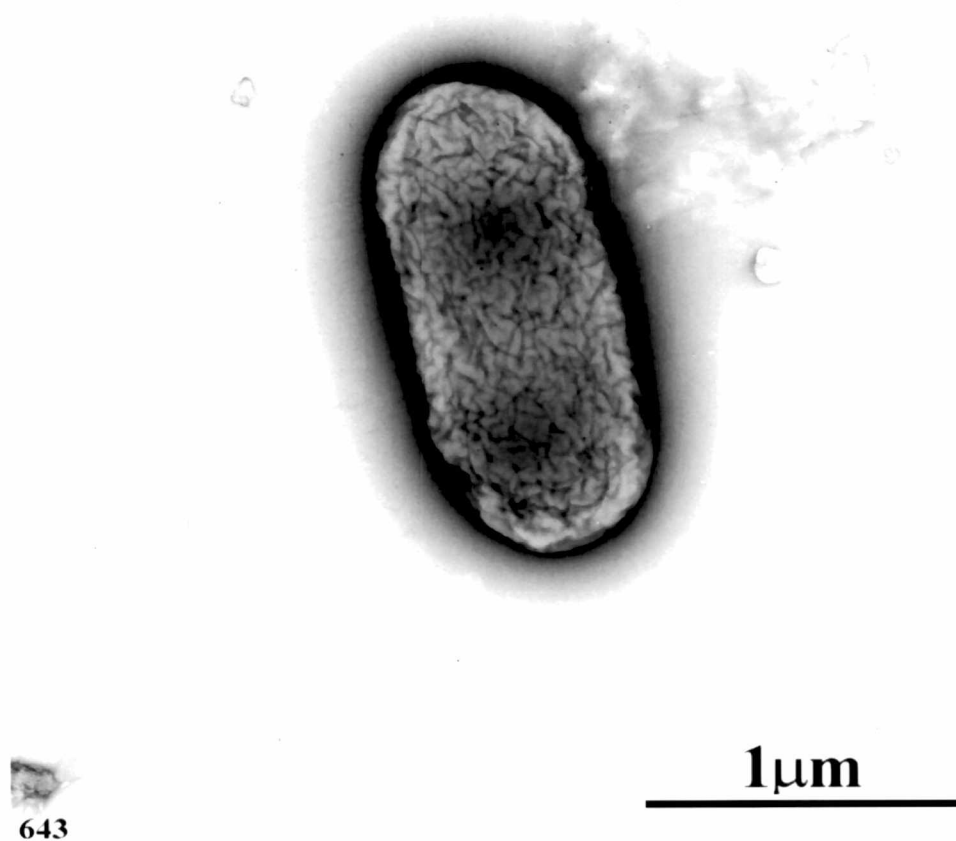


**Figure 3.5** Transmission electron micrograph of *Burkholderia* species strain JR7B3

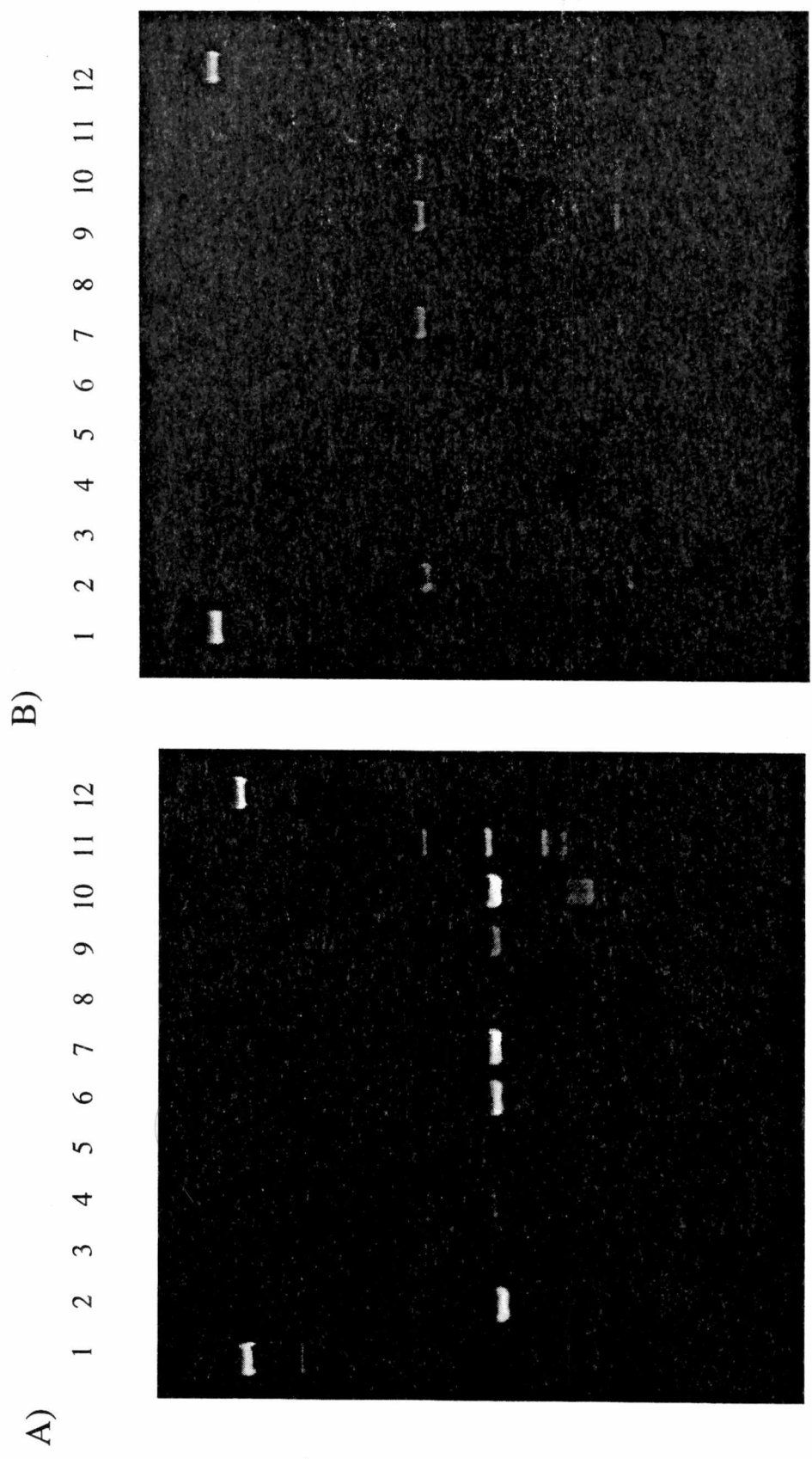


**Figure 3.6** RISA-RFLP analysis of a 2,4,5-T degrading Agent Orange enrichment culture containing *Burkholderia* species strain JR7B2 and *Burkholderia* species strain JR7B3. PCR was used to amplify the 23S-16S intergenic region using primers 1055f and 23Sr. PCR reactions were then digested using HhaI + RsaI (A) or MspI + AluI (B) endonucleases. Incubation was 3 hours at 37°C. Lane designations are as follows: 1 kb ladder (1, 12), culture JR7B (2), strain JR7B3 (3), large white colony (4), strain JR7B2 (5), large yellow colony (6), colony G7 (7), colony N7 (8), colony 2 (9), colony 5 (10), *B. cepacia* AC1100 (11).

***Burkholderia* species strain JRB1**

16S sequence Length: 1449

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TGAACGCTGG CGGCATGCCT TACACATGCA AGTCGAACGG CAGCACGGGG
GCAACCCTGG TGGCGAGTGG CGAACGGGTG AGTAATACAT CGGAACGTGT
CCTGTAGTGG GGGATAGCCC GGCGAAAGCC GGATTAATAC CGCATACGAC
CTAAGGGAGA AAGCGGGGGA TCTTCGGACC TCGCGCTATA GGGGCGGCCG
ATGGCAGATT AGCTAGTTGG TGGGGTAAAG GCCTACCAAG GCGACGATCT
GTAGCTGGTC TGAGAGGACG ACCAGCCACA CTGGGACTGA GACACGGCCC
AGACTCCTAC GGGAGGCAGC AGTGGGGAAT TTTGGACAAT GGGGGCAACC
CTGATCCAGC AATGCCGCGT GTGTGAAGAA AGCCTTCGGG TTGTAAAGCA
CTTTTGTCCG GAAAGAAAAC TTCGTCCCTA ATATGGATGG AGGATGACGG
TACCGGAAGA ATAAGCACCG GCTAACTACG TGCCAGCAGC CGCGGTAATA
CGTAGGGTGC GAGCGTTAAT CGGAATTACT GGGCGTAAAG CGTGCGCAGG
CGGTCTGTTA AGACCGATGT GAAATCCCCG GGCTTAACCT GGGAAGTGCA
TTGGTGACTG GCAGGCTTTG AGTGTGGCAG AGGGAGGTAG AATTCCACGT
GTAGCAGTGA AATGCGTAGA GATGTGGAGG AATACCGATG GCGAAGGCAG
CCTCCTGGGC CAACACTGAC GCTCATGCAC GAAAGCGTGG GGAGCAAACA
GGATTAGATA CCCTGGTAGT CCACGCCCTA AACGATGTCA ACTAGTTGTT
GGGGATT CAT TCCCTTAGTA ACGTAGCTAA CGCGTGAAGT TGGCCGCCTG
GGGAGTACGG TCGCAAGATT AAAACTCAAA GGAATTGACG GGGACCCGCA
CAAGCGGTGG ATGATGTGGA TTAATTCGAT GCAACGCGAA AAATCTTACC
TACCCTTGAC ATGGTCGGAA CCCTGCTGAA AGGTGGGGGT GCTCGAAAGA
GAACCGGCGC ACAGGTGCTG CATGGCTGTC GTCAGCTCGT GTCGTGAGAT
GTTGGGTTAA GTCCCGCAAC GAGCGCAACC CTTGTCCTTA GTTGCTACGC
AAGAGCACTC TAAGGAGACT GCCGGTGACA AACCGGAGGA AGGTGGGGAT
GACGTCAAGT CCTCATGGCC CTTATGGGTA GGGCTTCACA CGTCATACAA
TGGTCGGAAC AGAGGGTTGT CAAGCCGCGA GGTGGAGCCA ATCCCAGAAA
ACCGATCGTA GTCCGGATCG CAGTCTGCAA CTCGACTGCG TGAAGCTGGA
ATCGCTAGTA ATCGCGGATC AGCATGCCGC GGTGAATACG TTCCCGGGTC
TTGTACACAC CGCCCGTCAC ACCATGGGAG TGGGTTTCAC CAGAAGTAGG
TAGCCTAACC GCAAGGAGGC GCTTACCACG GTGGGTTCAT GACTGGGTG

```

**Figure 3.7** *Burkholderia* species strain JRB1 16S sequence

***Burkholderia* species strain JR7B3**

16S sequence Length: 1449

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GAACGCTGGC GGCATGCCTT ACACATGCAA GTCGGACGGC AGCGCGGGCT
TCGGCCTGGC GCGAGTGGC GAACGGGTGA GTAATACATC GGAACGTGTC
CTGTAGTGGG GGATAGCCCG GCGAAAGCCG GATTAATACC GCATACGCTC
TGCGGAGGAA AGCGGGGGAT CCTTCGGGAC CTCGCGCTAC AGGGGCGGCC
GATGGCAGAT TAGCTAGTTG GTGGGGTAAA GGCCTACCAA GGCGACGATC
TG TAGCTGGT CTGAGAGGAC GACCAGCCAC ACTGGGACTG AGACACGGCC
CAGACTCCTA CGGGAGGCAG CAGTGGGGAA TTTTGGACAA TGGGGGCAAC
CCTGATCCAG CAATGCCGCG TGTGTTGAAG AAGCCTTTTCG GGTGTAAG
CAATTTTGTC CGAAAGAAA CGTTGTGCTA ATACCCGTGG CGGATGACGG
TACCGGAAGA ATAAGCACCG GCTAACTAAC GTGCCAGCAG CCGCGGTAAT
ACGTAGGGTG CAAGCGTTAA TCGGAATTAC TGGGCGTAAA GCGTGCGCAG
GCGGTTTCGCT AAGACAGATG TGAAATCCCC GGGCTTAACC TGGGAAGTGC
ATTTGTGACT GCGGGGCTAG AGTATGGCAG AGGGGGGTAG AATCCACGT
GTAGCAGTGA AATGCGTAGA GATGTGGAGG AATACCGATG GCGAAGGCAG
CCCCCTGGGC CAATACTGAC GCTCATGCAC GAAAGCGTGG GGAGCAAACA
GGATTAGATA CCCTGGTAGT CCACGCCCTA AACGATGTCA ACTGGTTGTC
GGGCCTTCAT TGGCTTGTA GCGTAGCTAA CCGGTGAAGT TGACCGCCTG
GGGAGTACGG TCGCAAGATT AAAACTCAA GGAATTGACG GGGACCCGCA
CAAGCGGTGG ATGATGTGGA TTAATTCGAT GCAACGCGAA AAACCTTACC
TACCCTTGAC ATGGATGGAA TCCCGCTGAG AGGTGGGAGT GCCCGAAAGG
GAGCCATCAC ACAGGTGCTG CATGGCTGTC GTCAGCTCGT GTCGTGAGAT
GTTGGGTTAA GTCCCGCAAC GAGCGCAACC CTTGTCCCTA GTTGCTACGC
AAGAGCACTC CAGGGAGACT GCCGGTGACA AACCGGAGGA AGGTGGGGAT
GACGTCAAGT CCTCATGGCC CTTATGGGTA GGGCTTCACA CGTCATACAA
TGGTCGGAAC AGAGGGTCGC CAAGCCGCGA GGTGGAGCCA ATCCCAGAAA
ACCGATCGTA GTCCGGATCG CACTCTGCAA CTCGAGTGCG TGAAGCTGGA
ATCGCTAGTA ATCGCGGATC AGCATGCCGC GGTGAATACG TTCCCGGGTC
TTGTACACAC CGCCCGTCAC ACCATGGGAG TGGGTTTTAC CAGAAGTGGC
TAGTCTAACC GCAAGGAGGA CGGTCACCAC GGTAGGATCA TGAAGTGGG

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**Figure 3.8** *Burkholderia* species strain JR7B3 16S sequence

**Table 3.2.** Results from 16S sequence analysis of bacterial isolates obtained from Agent Orange soil enrichments (AOE). PCR primers 27f, 1492r, 907r, 926f, and 530f were used to amplify 16S segments from strains *Burkholderia* species strain JRB1, *Burkholderia* species strain JR7B2, and *Burkholderia* species strain JR7B3.

| Organism  | Region of Similarity | Similarity Score | Name/Accession Number                  |
|-----------|----------------------|------------------|----------------------------------------|
| AOE JRB1  | 30 - 1447            | 1379/1420 (97%)  | <i>Burkholderia glathei</i> U96935     |
| AOE JR7B2 | 525 - 999            | 475/476 (99%)    | <i>Burkholderia caribiensis</i> Y17009 |
| AOE JR7B3 | 35 - 1430            | 1370/1398 (97%)  | <i>Burkholderia graminis</i> U96941    |

demonstrated that 2,4-D was metabolized as a sole carbon and energy source (Figure 3.9). In addition mineralization experiments demonstrated 63.0% conversion of 2,4-D-ring-UL-<sup>14</sup>C (Table 3.3, Figure 3.10). A similar experiment using culture 7B demonstrated that 2,4,5-T was completely metabolized by the co-culture (Figure 3.11). Mineralization experiments using the Agent Orange enrichment culture 7B demonstrated 75.7% conversion of 2,4,5-T-ring-UL-<sup>14</sup>C to CO<sub>2</sub> (Table 3.4, Figure 3.12). Surprisingly, the JR7B enrichment culture also mineralized 2,4-D-ring-UL-<sup>14</sup>C (32.1%, Table 3.3, Figure 3.13). Strain JR7B3 was able to utilize 2,4,5-T as a carbon and energy source when grown in PAS containing 0.01% yeast extract (Figure 3.14), but strain JR7B2 was unable to degrade 2,4,5-T or 2,4,5-TCP (data not shown).

#### 3.4.4 Characterization of 2,4-D and 2,4,5-T degradative genes.

Previous studies have demonstrated that the *tfdA* gene is highly conserved among  $\gamma$  and  $\beta$ -*Proteobacteria* (McGowen et al. 1998). Based on this conservation it was

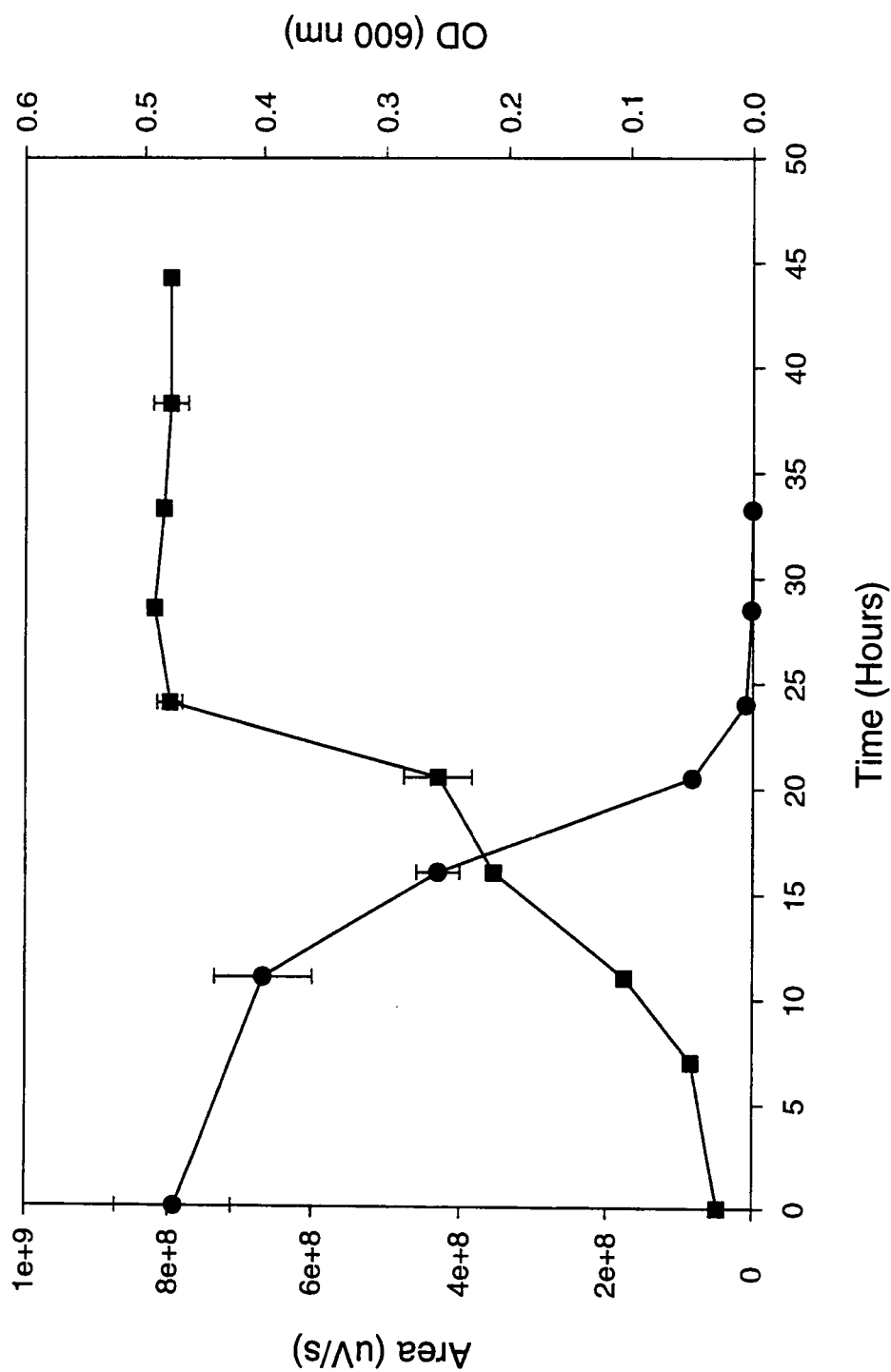


**Figure 3.9** *Burkholderia* species strain JRB1 2,4-D degradation and growth curves. Bacterial cells were grown in PAS media supplemented with 1000 mg/L 2,4-D. 2,4-D concentrations (●) were determined using HPLC analysis. Bacterial biomass (■) was determined by estimating the optical density of the culture (600 nm) using a DU-70 Spectrophotometer. All data points represent the average of three separate experiments. Error bars represent the standard deviation between these data.

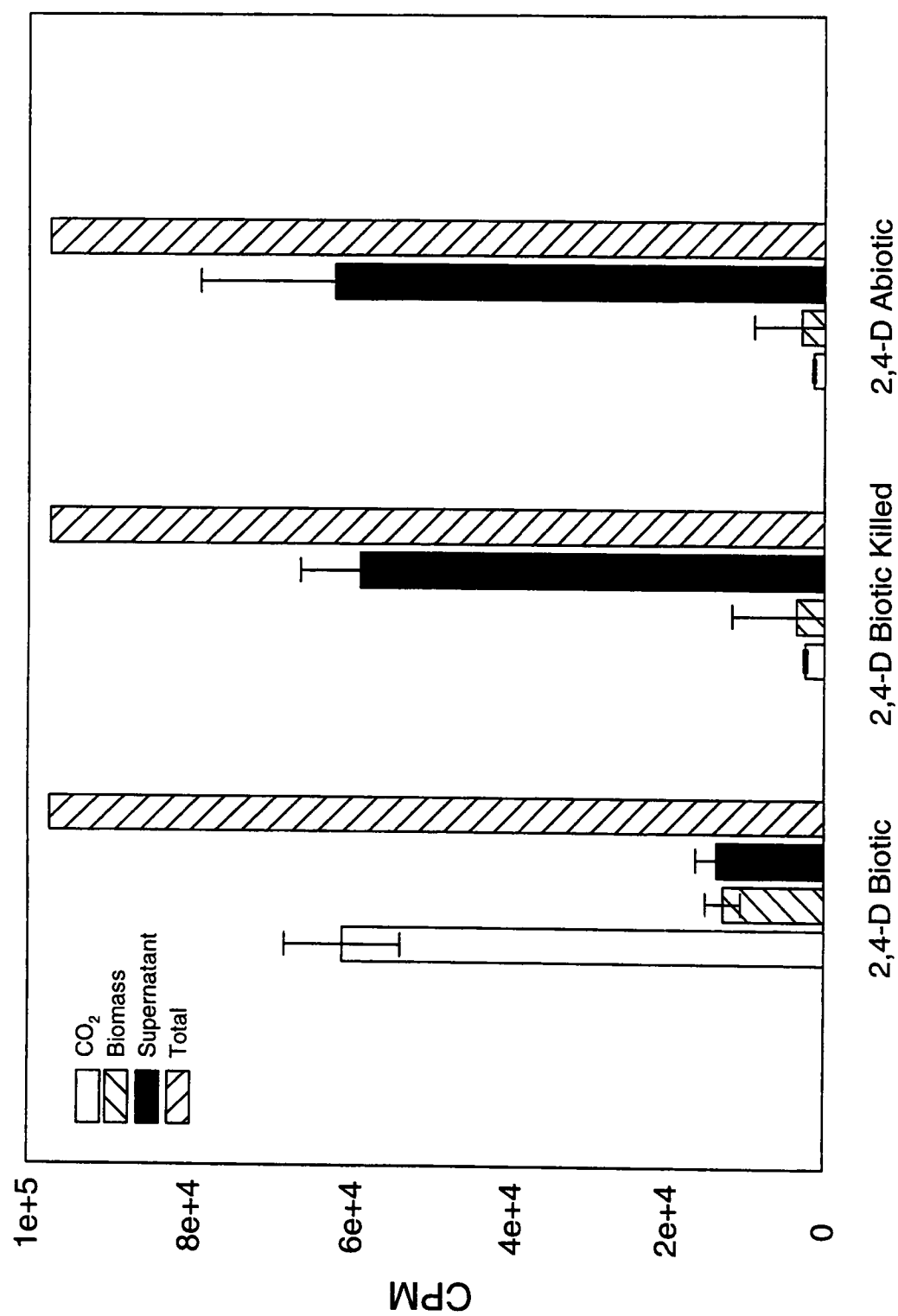


**Figure 3.10** 2,4-D mineralization assays for *Burkholderia* species strain JRB1. All data points represent the average of three separate experiments. Error bars represent the standard deviation between these data.

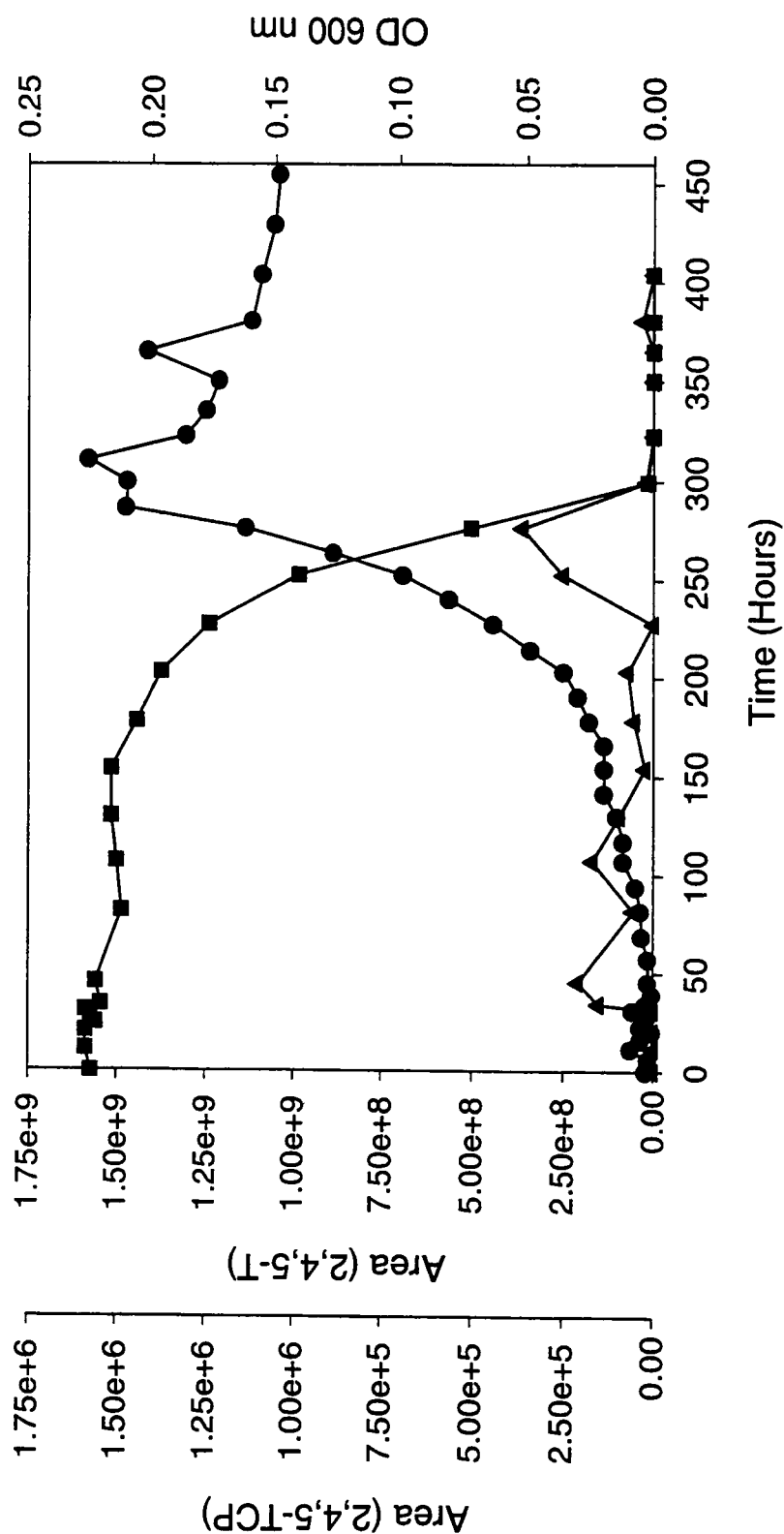


**Figure 3.11** 2,4,5-T degradation and growth curves for the Agent Orange enrichment culture JR7B. The enrichment culture was grown in PAS media supplemented with 1000 ppm 2,4,5-T. 2,4,5-T concentrations (■) and 2,4,5-TCP (▲) were determined by HPLC analysis, and the culture's optical density (●) was determined spectrophotometrically (600 nm).

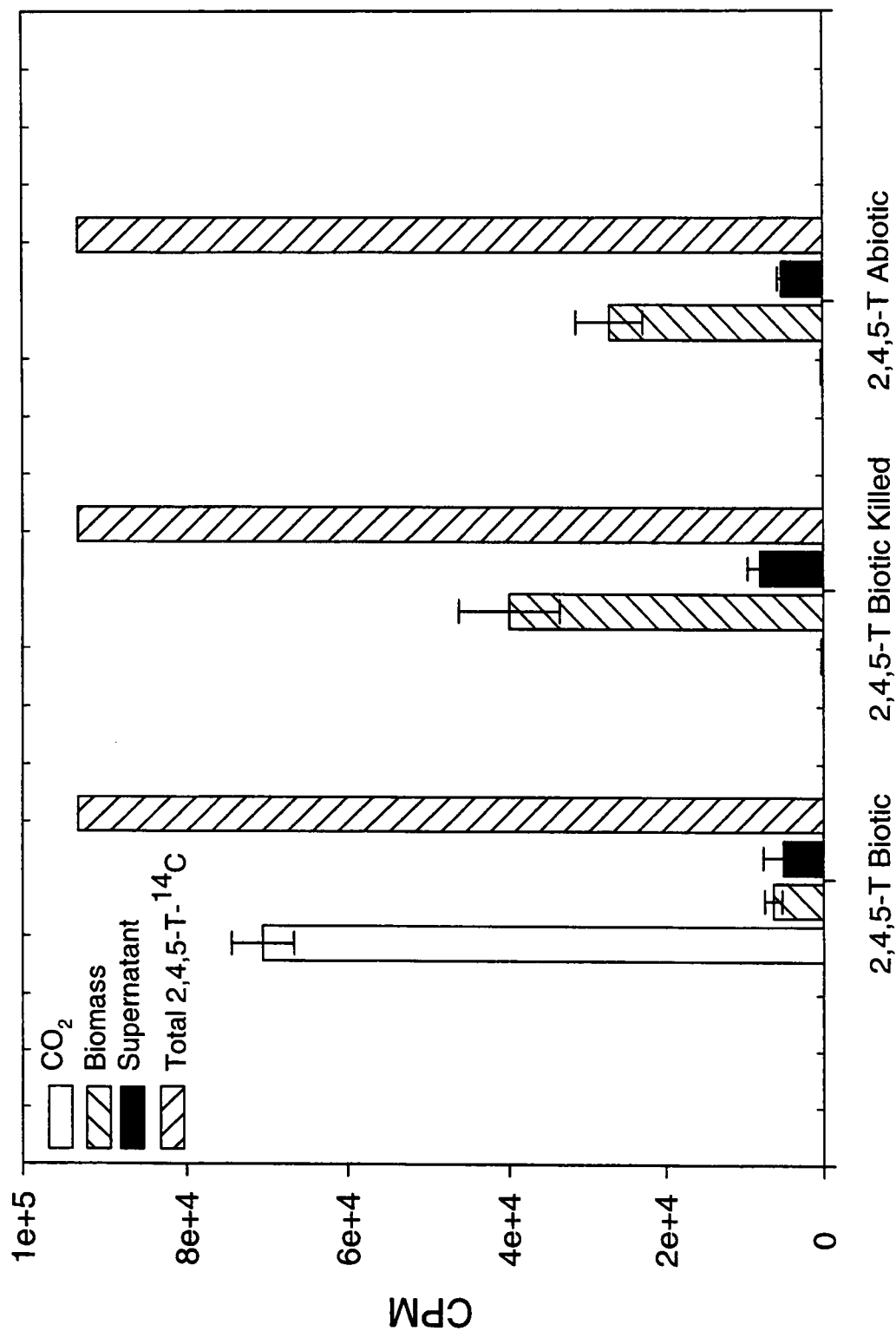


**Figure 3.12** 2,4,5-T mineralization assays for *Burkholderia* species strain JR7B3. All data points represent the average of three separate experiments. Error bars represent the standard deviation between these data.

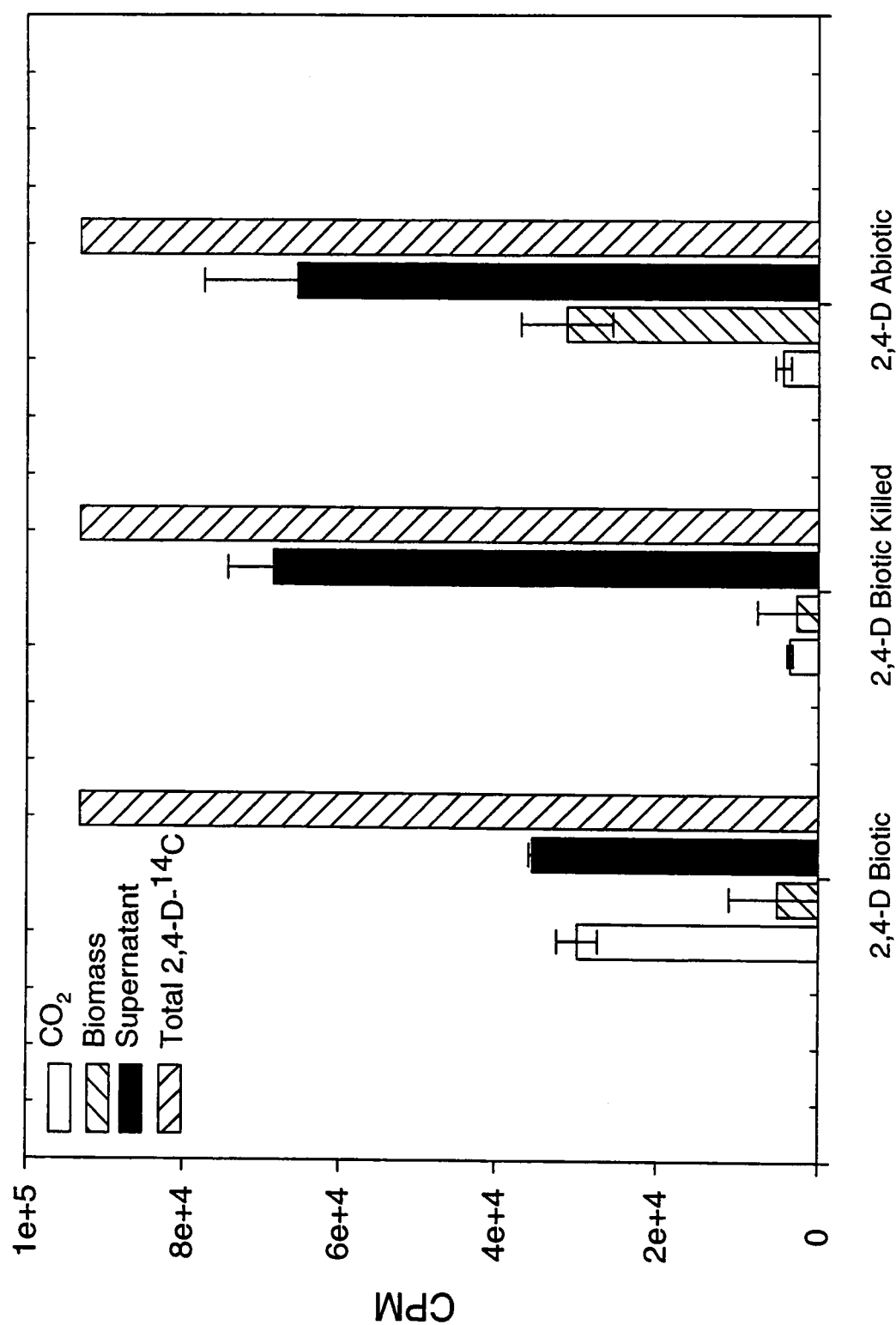


**Figure 3.13** 2,4-D mineralization assays for *Burkholderia* species strain JR7B3. All data points represent the average of three separate experiments. Error bars represent the standard deviation between these data.

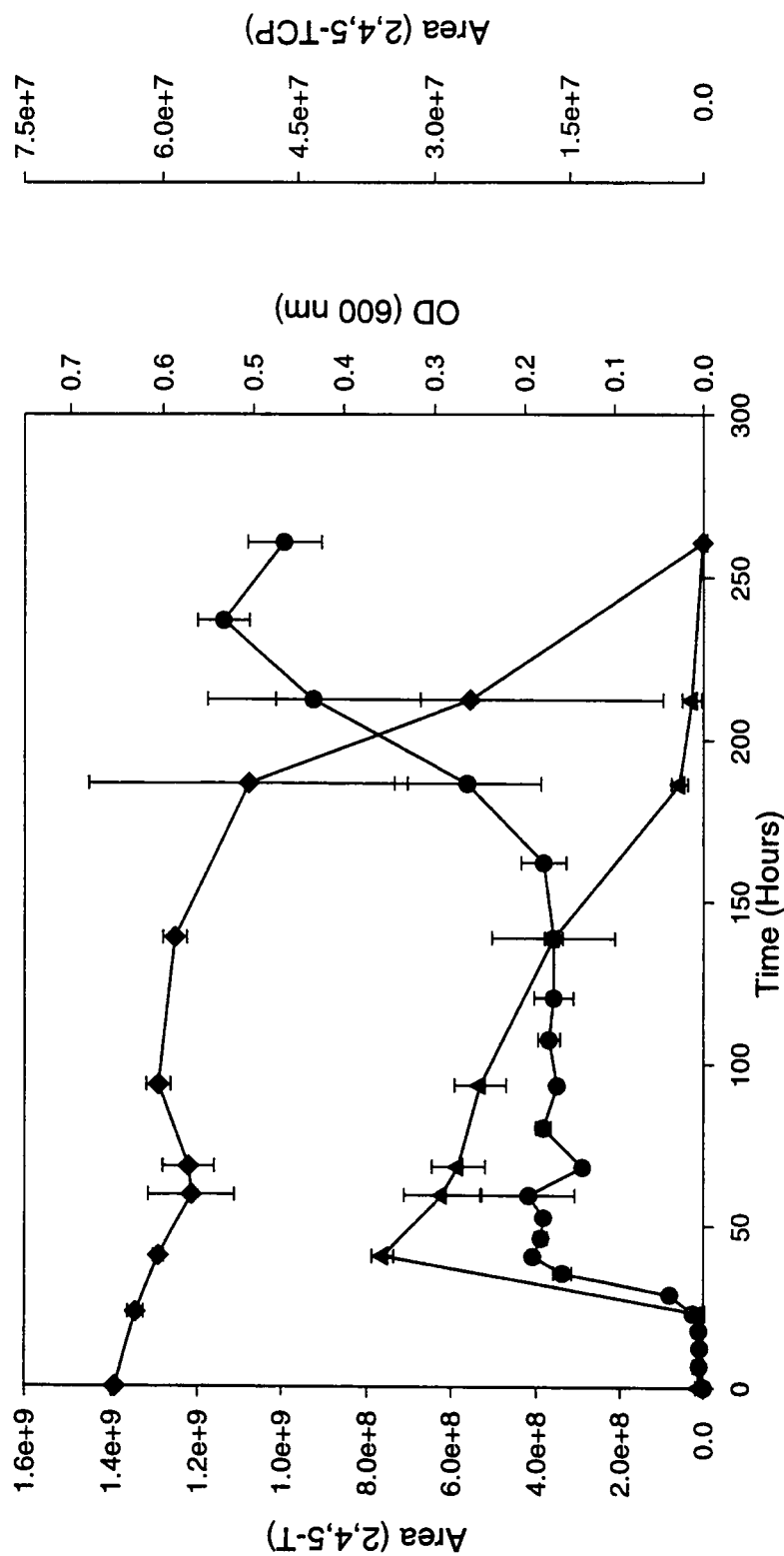


**Figure 3.14** 2,4,5-T degradation and growth curves for *Burkholderia* species strain JR7B3. Cultures were grown in PAS media containing 0.01% yeast extract and 1000 ppm 2,4,5-T. 2,4,5-T concentrations (◆) and 2,4,5-TCP concentrations (▼) were determined by HPLC, and culture optical density (●) was determined spectrophotometrically. Error bars represent the standard deviation between three individual cultures. All data points represent the average from these three cultures.

**Table 3.3.** 2,4-Dichlorophenoxyacetic acid mineralization assays for bacteria from Agent Orange contaminated soil. Values represent percent  $^{14}\text{C}$  detected as evolved  $\text{CO}_2$  or present in cell biomass or culture supernatants. Each data point represents the average of three individual experiments. Numbers in parentheses are the standard deviations of this mean.

|                                                          | $\text{CO}_2$    | Biomass/solids   | Supernatant       |
|----------------------------------------------------------|------------------|------------------|-------------------|
| <b>JRB1 Culture</b>                                      | 63.0 (+/- 7.4) % | 13.3 (+/- 2.3) % | 14.1 +/- (2.8) %  |
| <b>JRB1 Culture + <math>\text{H}_2\text{SO}_4</math></b> | 2.4 (+/- 0.3) %  | 3.5 (+/- 8.6) %  | 60.7 +/- (7.6) %  |
| <b>Abiotic Control</b>                                   | 1.4 (+/- 0.2) %  | 2.9 (+/- 6.3) %  | 64.0 +/- (17.0) % |
|                                                          |                  |                  |                   |
| <b>JR7B Culture</b>                                      | 32.1 (+/- 2.6) % | 5.4 (+/- 6.5) %  | 37.9 (+/- 0.5) %  |
| <b>JR7B Culture + <math>\text{H}_2\text{SO}_4</math></b> | 3.8 (+/- 0.4) %  | 2.8 (+/- 5.3) %  | 73.5 (+/- 6.3) %  |
| <b>Abiotic Control</b>                                   | 4.7 (+/- 1.1) %  | 33.5 (+/- 6.0) % | 70.3 (+/- 12.8) % |

**Table 3.4** 2,4,5-Trichlorophenoxyacetic acid mineralization assays for bacteria from Agent Orange contaminated soil. Values represent percent  $^{14}\text{C}$  detected as evolved  $\text{CO}_2$  or present in cell biomass or culture supernatants. Each data point represents the average of three individual experiments. Numbers in parentheses are the standard deviations of this mean.

|                                                          | $\text{CO}_2$    |  | Biomass/solids   |  | Supernatant     |
|----------------------------------------------------------|------------------|--|------------------|--|-----------------|
| <b>JR7B Culture</b>                                      | 75.7 (+/- 4.1) % |  | 6.7 (+/- 1.2) %  |  | 5.4 (+/- 2.7) % |
| <b>JR7B Culture + <math>\text{H}_2\text{SO}_4</math></b> | 0.3 (+/- 0.01) % |  | 42.9 (+/- 6.9) % |  | 8.5 (+/- 1.7) % |
| <b>Abiotic Control</b>                                   | 0.3 (+/- 0.01) % |  | 29.1 (+/- 4.5) % |  | 5.6 (+/- 0.6) % |
|                                                          |                  |  |                  |  |                 |

hypothesized that the mercury resistant 2,4-D degrading bacteria isolated from Agent Orange contaminated soil samples contained the *tfdA* and *tfdB* genes and that a modified lower pathway corresponding to the *tfdCDEF* was present. To test this hypothesis a PCR based strategy was adopted in which computer-based analysis was used to design specific primers for amplification of the *tfdA* (2,4-dichlorophenoxyacetic acid dioxygenase), *B* (2,4-dichlorophenol hydrolase), *C* (3,5-dichlorocatachol 1,2-dioxygenase, and *E* (*cis*-2-chlorodiene lactone hydrolase) genes. All primers designed for this study are listed in Table 3.1.

PCR analyses of Strain JRB1 revealed that the *tfdA*, *B*, *C*, and *E* genes were present in this organism (Figure 3.15). Sequence analysis and subsequent bioinformatic studies of these PCR products revealed that these gene sequences were highly similar to 2,4-D degradative genes previously identified for strain *R. eutrophus* strain JMP 134 (Table 3.5). The *tfdA* fragment sequence was 96% similar to the previous published *R. eutrophus* sequence (accession number M16730). The PCR amplification of this gene was designed to target the intergenic region between the *tfdR* and the *tfdA*, and the size of the expected sequence was identical to that predicted from JMP 134. From this fragment, a 129 bp sequence of the *tfdR* was found to be 100% homologous to the JMP 134 sequence. PCR products were identified which were 99%, 98%, and 98% similar to previously published *tfdB*, *tfdC*, and *tfdE* gene sequences (Accession numbers M35097, M36280, and M35097, respectively).

Examinations of *Burkholderia* species strain JR7B3 by PCR demonstrated that gene sequences similar to the *tfdA*, *C*, and *E* genes were present in this organism (Figure

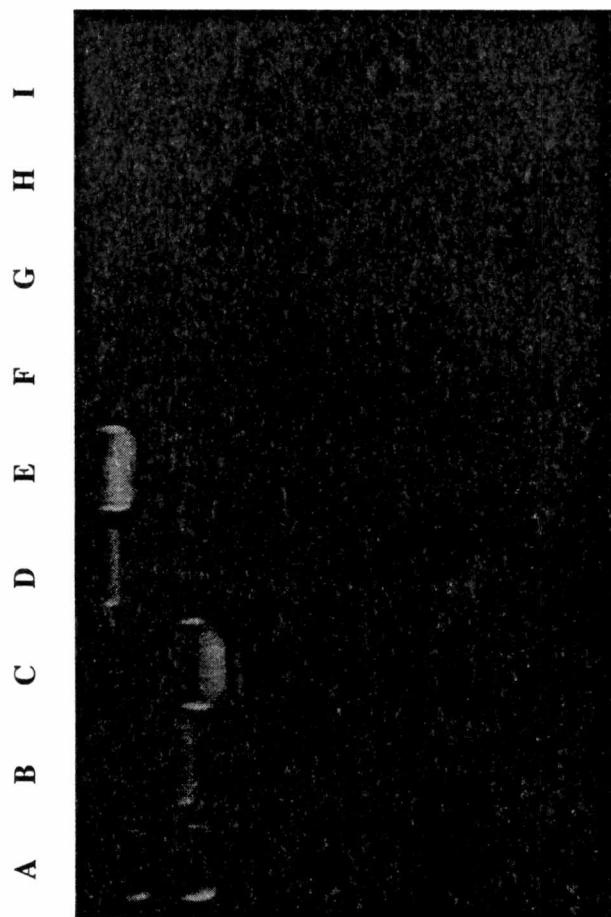


**Figure 3.15** PCR analysis of important 2,4-D catabolic genes in *Burkholderia* species strain JRB1. Figure illustrates gel electrophoresis of PCR reactions using specific primers for 2,4-D catabolic genes. Sample designations are as follows: 1 kb DNA ladder (A), strain JRB1 (B) and pJP4 (C) using *tfdA* specific primers, strain JRB1 (D) and pJP4 (E) using *tfdB* specific primers, strain JRB1 (F) and pJP4 (G) using *tfdC* specific primers, and strain JRB1 (H) and pJP4 (I) using *tfdE* specific primers.

**Table 3.5.** Summary of important bioinformatic information obtained from analysis of sequenced PCR products.

| Clone designation | Size of fragment analyzed | Similarity Score | Gene                      | Accession Number for known genes |
|-------------------|---------------------------|------------------|---------------------------|----------------------------------|
| <i>B1A(2)</i>     | 129 bp                    | 129/129 (100%)   | <i>tfdR</i> (477 - 605)   | S80112                           |
| <i>B1A(2)</i>     | 373 bp                    | 359/373 (96%)    | <i>tfdA</i> (1662 - 1293) | M16730                           |
| <i>B1B(5)</i>     | 310 bp                    | 307/310 (99%)    | <i>tfdB</i> (6360 - 6052) | M35097                           |
| <i>B1C(6)</i>     | 590 bp                    | 583/592 (98%)    | <i>tfdC</i> (35 - 626)    | M36280                           |
| <i>B1E(8)</i>     | 655 bp                    | 648/655 (98%)    | <i>tfdE</i> (2328 - 2982) | M35097                           |
| <i>WA(13)</i>     | 324 bp                    | 321/325 (98%)    | <i>tftA</i> (19 - 342)    | U11420                           |
| <i>7C</i>         | 300 bp                    | 298/300 (99%)    | <i>tftC</i> (909 - 1208)  | U83405                           |
| <i>7E</i>         | 350 bp                    | 338/343 (98%)    | <i>tftE</i> (1010 - 1349) | U83405                           |

3.16). None of the *tft* genes identified in strain JR7B3 were present in strain JR7B2 (Figure 3.16). Identified genes were found to be similar to those previously described for *B. cepacia* AC1100 (Table 3.5). PCR amplification products from these studies corresponded with expected band sizes associated with control amplifications using *B. cepacia* AC1100 (Figure 3.16). Subsequent cloning, sequencing, and computer analysis revealed gene sequences which were 98% similar to the 19 -342 bp region of the *tftA* gene (Accession number U11420), 99% similar to the 909-1208 region of the *tftC* gene (Accession number U83405), and 98% similar to the 1010 - 1349 region of the *tftE* gene (Accession number U83405).

#### **3.4.5 Messenger RNA analysis for 2,4-D and 2,4,5-T-degrading bacteria.**

PCR primers designed to examine degradative genes in *Burkholderia* species strain JRB1 and *Burkholderia* species strain JR7B3 were used to examine mRNA expression levels for the *tfdA*, *B*, *C* and the *tftA*, *C*, *E* genes to determine if these gene sequences were expressed during growth on 2,4,5-T. Expression analysis of the *tfdA* gene illustrated that the *tfdA* gene was transcribed during growth on 2,4-D (Figure 3.17A) and that the size of the observed PCR product corresponded with amplification of control DNA (pJP4). Similar results were obtained using primers specific for the *tfdB* and the *tfdC* genes (Figure 3.17C and 3.17B). Analogous experiments using the 2,4,5-T degrading consortia JR7B demonstrated that the *tftA*, *C*, and *E* genes were expressed during growth on 2,4,5-T as a sole carbon and energy source (Figure 3.18A, C, and B). These experiments also demonstrated that the *tftA*, *C*, and *E* genes were not expressed

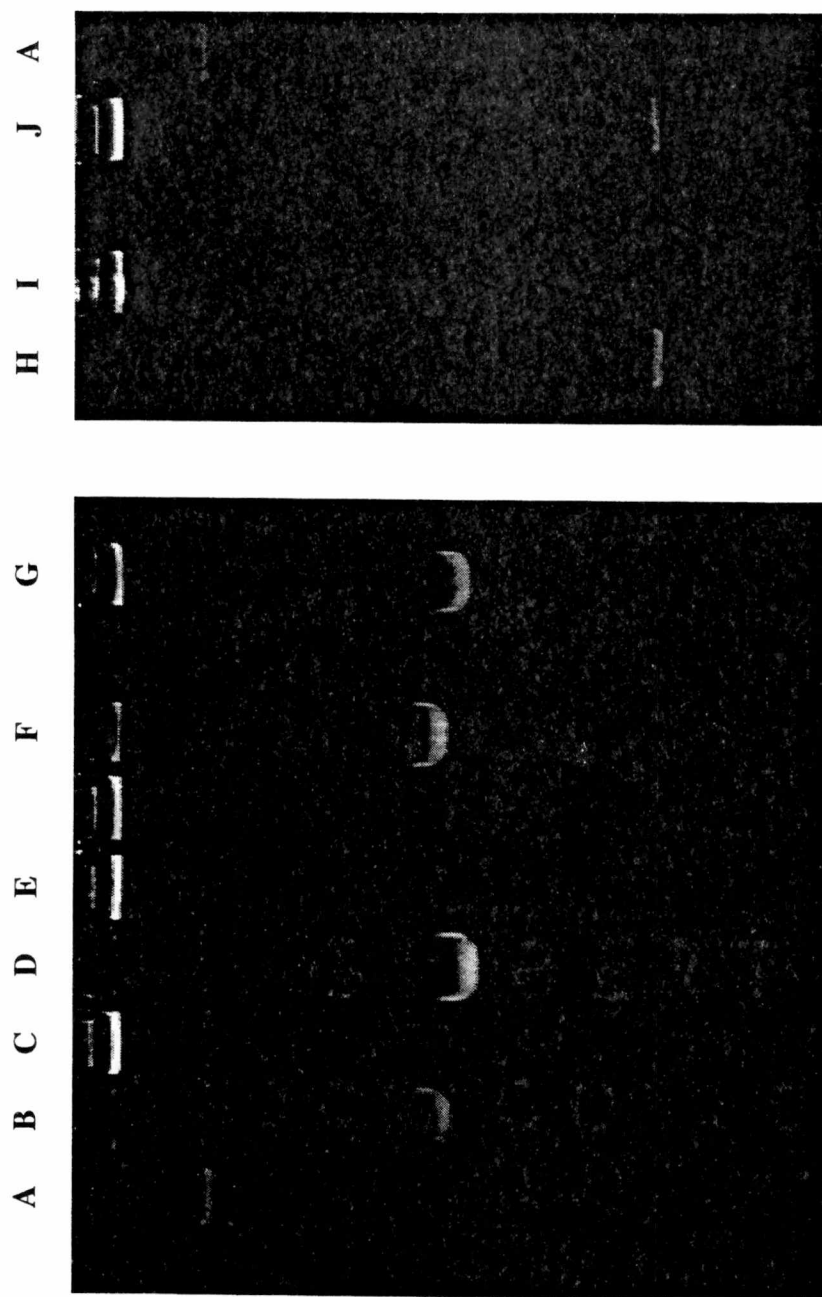


**Figure 3.16** PCR analysis of important 2,4,5-T catabolic genes in *Burkholderia* species strain JR7B2 and JR7B3. Figure illustrates gel electrophoresis of PCR reactions using specific primers for 2,4,5-T catabolic genes. Sample designations are as follows: 1 kb DNA ladder (A), strain JR7B3 (B), strain JR7B2 (C), and *B. cepacia* AC1100 (F) using *tftA* specific primers; strain JR7B3 (D), strain JR7B2 (E), and *B. cepacia* AC1100 (G) using *tftC* specific primers; strain JR7B3 (B), strain JR7B2 (I), and *B. cepacia* AC1100 (J) using *tftE* specific primers.

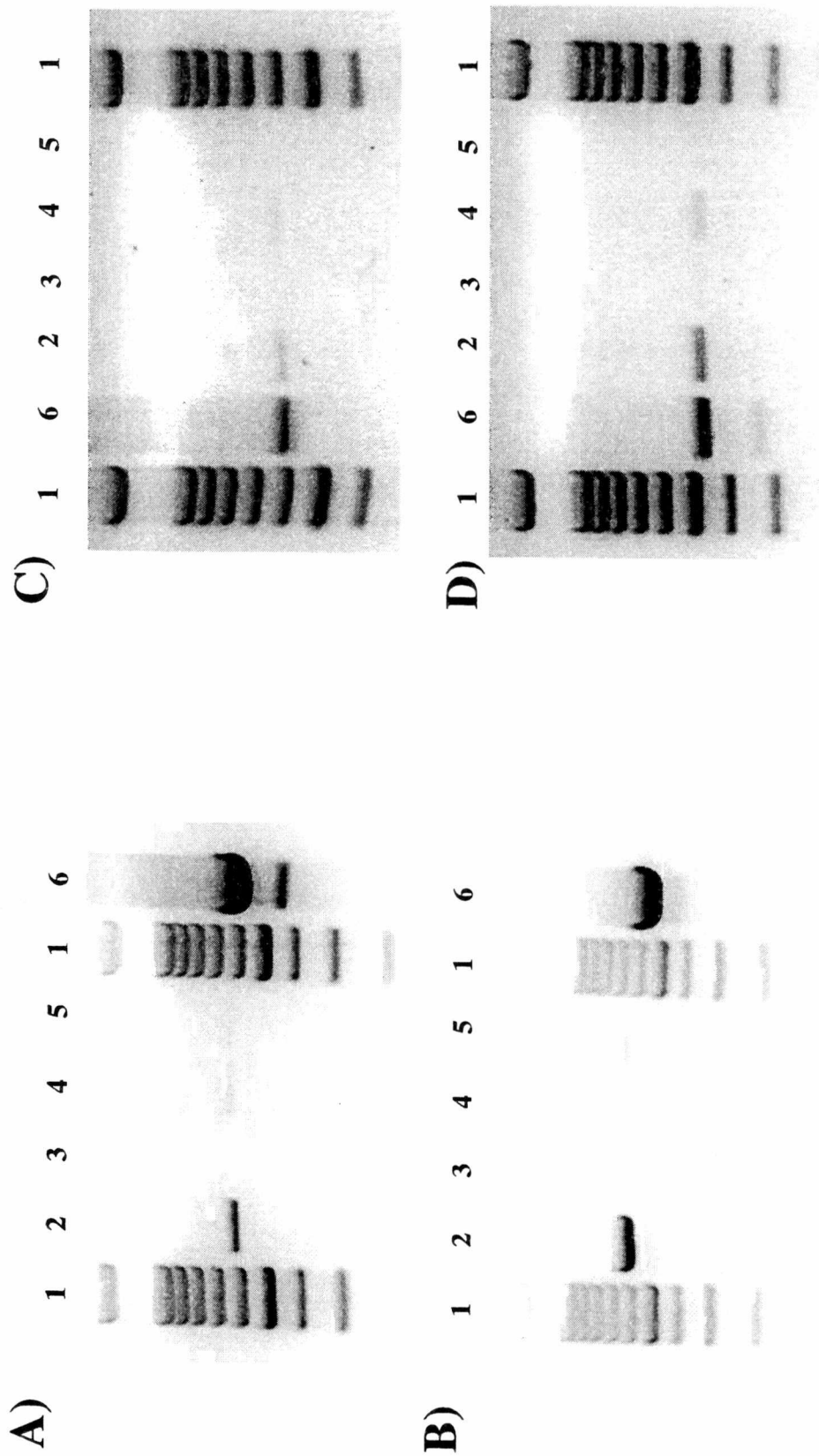


**Figure 3.17** RT-PCR analysis of *tfdA*, *tfdB*, and *tfdC* in *Burkholderia* species strain JRB1. Figure illustrates gel electrophoresis of RT-PCR reactions using specific primers for A) *tfdA*, B) *tfdB*, C) *tfdC*, D) 16S rRNA. For these studies total RNA was isolated from cultures containing: 2) PAS + 2,4-D, 3) PAS + 2,4-D (Rif), 4) PAS + succinate, and 5) PAS + succinate (Rif). Control DNA for each primer was included (6), and 100 bp DNA ladders (1) were used for size references. The abbreviation "Rif" denotes cultures that were treated with rifampicin for 64 minutes prior to total RNA extraction.

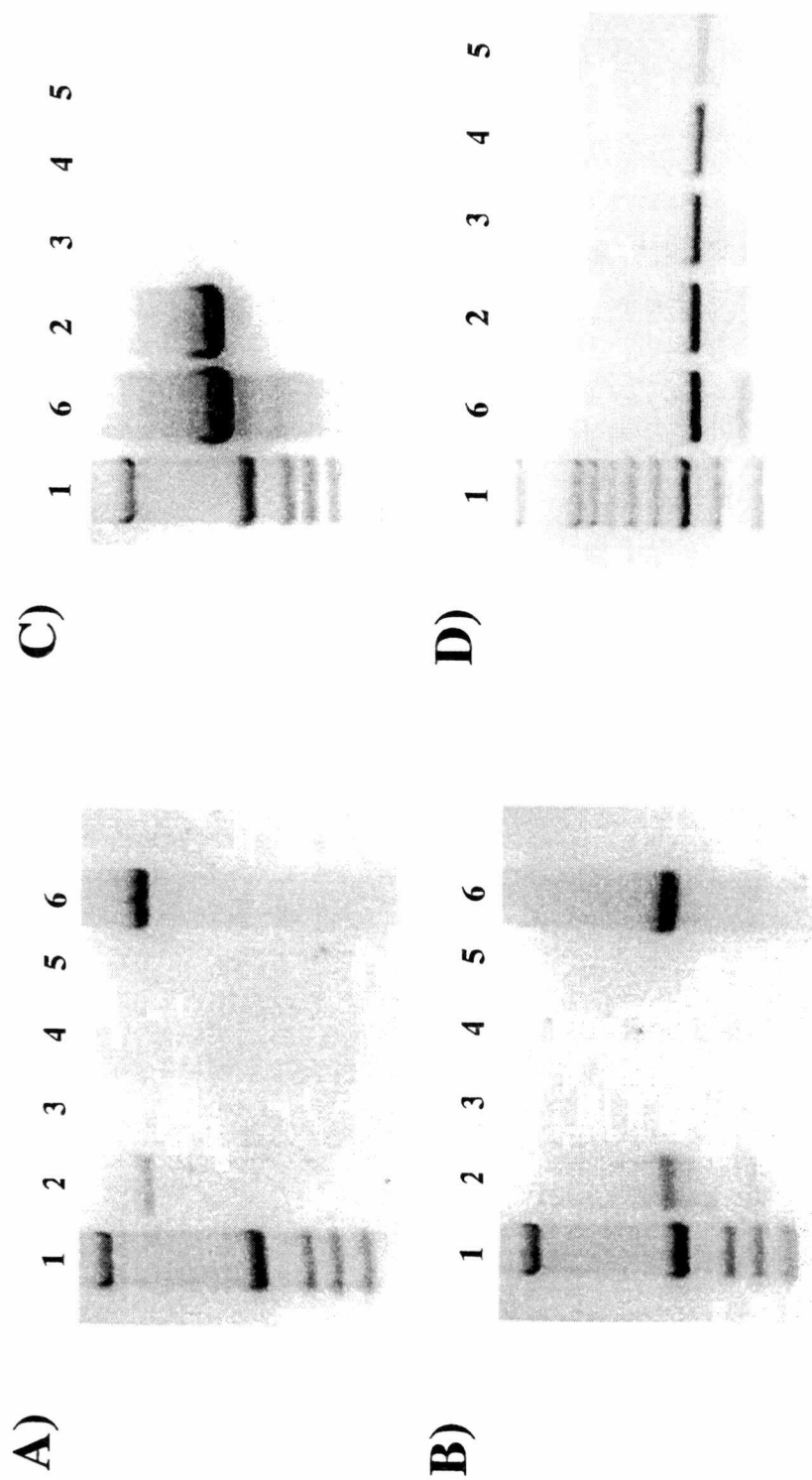


**Figure 3.18** RT-PCR analysis of *tftA*, *tftC*, and *tftE* in Agent Orange enrichment culture JR7B. Figure illustrates gel electrophoresis of RT-PCR reactions using specific primers for A) *tftA*, B) *tftC*, C) *tftE*, D) 16S rRNA. For these studies total RNA was isolated from cultures containing: 2) PAS + 2,4,5-T, 3) PAS + 2,4,5-T (Rif), 4) PAS + succinate, and 5) PAS + succinate (Rif). Control DNA for each primer was included (6), and 100 bp DNA ladders (1) were used for size references. The abbreviation "Rif" denotes cultures that were treated with rifampicin for 64 minutes prior to total RNA extraction.

during growth on succinate as the sole carbon and energy source (Figure 3.18A, C, and B). Rifampicin controls for JR7B experiments were consistent and provided a control for DNA contamination during the experiment. Note that in Figure 3.18D 16S control amplifications are similar for 2,4,5-T, 2,4,5-T + rifampicin, and succinate cultures. Decreases in 16S amplification product for succinate + rifampicin cultures probably indicates RNA degradation associated with this sample.

#### **3.4.6 Differential Display Reverse Transcriptase-PCR (DDRT-PCR) Analysis.**

DDRT-PCR experiments using the 2,4,5-T degrading consortium JR7B identified a genetic element, designated D20, which was differentially expressed in *Burkholderia* species strain JR7B2 (Figure 3.19). Transcription of this genetic element was verified by RT-PCR analysis (Figure 3.20), and confirmed that this band was not ribosomally derived nor a chimeric artifact. PCR analyses using strains JR7B2 and JR7B3 demonstrated that this putative gene was specifically localized in Strain JR7B2 and not present in Strain JR7B3. Currently, no significantly similar genes could be found in the online databases. This genetic element represents a putative gene that is activated in a non-degrading member of a 2,4,5-T-degrading consortium in association with utilization of 2,4,5-T as a sole carbon and energy source.

### **3.5 DISCUSSION**

This study describes the isolation and characterization of two *Burkholderia* species capable of mediating mineralization of 2,4-D and 2,4,5-T. *Burkholderia* species

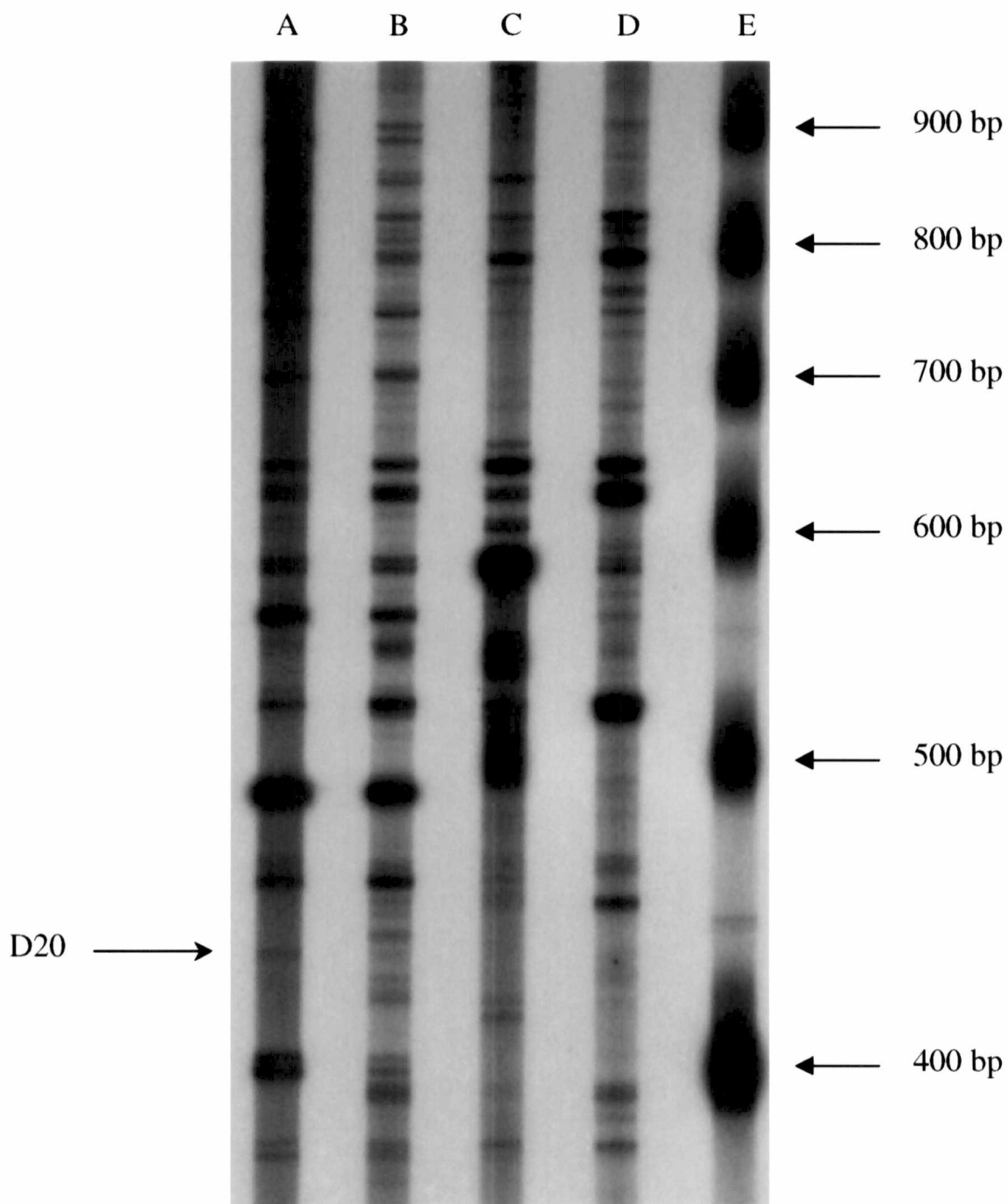


**Figure 3.19** DDRT-PCR experiment using Agent Orange enrichment culture JR7B grown in the presence of either 2,4,5-T or succinate. Lanes are A) 2,4,5-T, B) 2,4,5-T + rifampicin, C) succinate, D) succinate + rifampicin, E) 100 bp DNA ladder. Arrow denotes position of Band D20

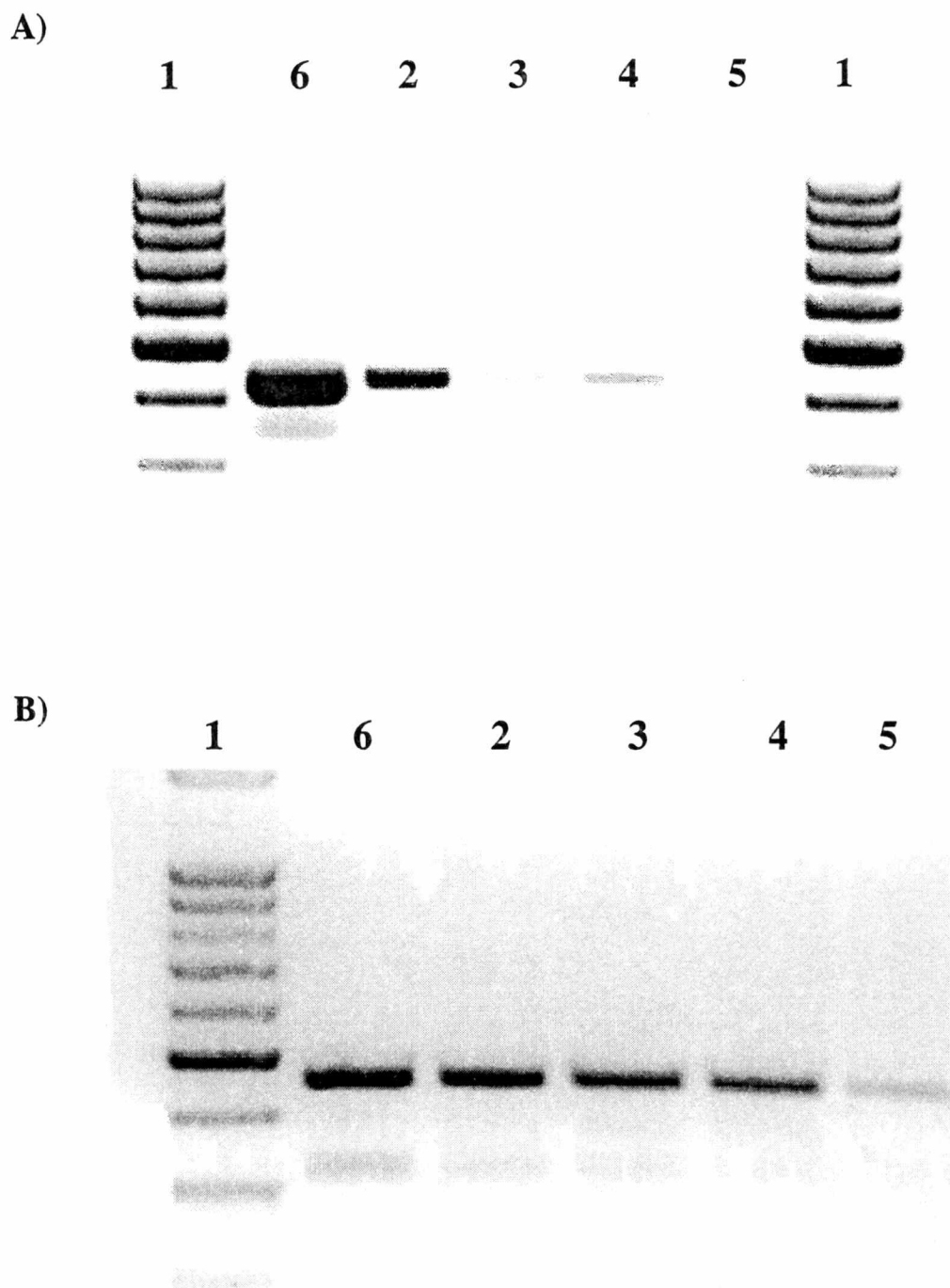


**Figure 3.20.** RT-PCR analysis of a differentially expressed band from Agent Orange enrichment culture JR7B. For these studies total RNA was isolated from cultures containing: 2) PAS + 2,4,5-T, 3) PAS + 2,4,5-T (Rif), 4) PAS + succinate, and 5) PAS + succinate (Rif). Control DNA for PCR was included (6), and 100 bp DNA ladders (1) were used for size references. The abbreviation "Rif" denotes cultures that were treated with rifampicin for 64 minutes prior to total RNA extraction.

strain JRB1 was capable of mineralizing 2,4-D, and *Burkholderia* species strain JR7B3 was capable of mineralizing 2,4-D (32.1%) and 2,4,5-T (75.7%). The 16S sequence of the 2,4-D degrading bacterium, *Burkholderia* species strain JRB1, is 97% similar to *B. glathei*. The 16S sequence of *Burkholderia* species strain JR7B3 was found to be 97% similar to *B. graminis* and contained genes similar to those involved in 2,4,5-T metabolism by *B. cepacia* AC1100. *Burkholderia* species strain JRB1 was shown to completely degrade 2,4-D during growth on this compound as a sole carbon and energy source. *Burkholderia* species strain JR7B3 was able to utilize 2,4,5-T as a carbon and energy source in the presence of additional growth nutrients or in association with a consortium containing *Burkholderia* species strain JR7B2. Differences in the growth characteristics for strain JRB1 and strain JR7B3 were very apparent with each having doubling times of hours versus days. Transmission electron micrographs of these bacterial strains demonstrated similar cell morphologies as those observed for *B. cepacia* AC1100. One notable difference was the presence of exopolysaccharide in association with *B. Cepacia* AC1100 cells during growth on PAS containing 1000 mg/L 2,4,5-T.

Agent Orange contaminated soil samples were the source of inoculation for these bacterial strains. These soils originated from a loading/unloading area and were historically heavily contaminated with Herbicide Orange. Current 2,4-D and 2,4,5-T concentrations were only approximately 22.4 and 73.8 mg/kg of soil, and it is therefore possible that much of the chlorophenoxy- herbicide remaining in these soils is recalcitrant to bacterial degradation due to sorption to the soil organic matter. Isolation of 2,4-D and 2,4,5-T degrading bacterial cultures from these soils required very little enrichment

culturing, and growth was apparent after the 1<sup>st</sup> week for 2,4-D degrading cultures and after only 3 weeks for 2,4,5-T degrading cultures. Growth of 2,4-D degrading bacteria was fairly rapid on PAS minimal media plates containing 1000 mg/L 2,4-D with visible colonies appearing with 2 - 3 days. Growth of 2,4,5-T degrading bacteria was much slower with visible colonies appearing only after 1 - 1 ½ weeks. Additionally, the Agent Orange enrichment culture JR7B was able to form minute colonies on 2,4-D plates after 2 - 2 ½ weeks.

Genetic analysis of these bacterial strains using PCR identified 2,4-D and 2,4,5-T degradative genes. *Burkholderia* species strain JRB1 was shown to contain genes that were similar to previously published 2,4-D degradative genes. Gene sequences identified in this strain had regions of high similarity to the *tfdA* (96%), *tfdB* (99%), *tfdC* (98%), *tfdE* (98%), and *tfdR* (100%). Genetic elements similar to published sequences were also found in the 2,4,5-T degrading *Burkholderia* species strain JR7B3. Specifically, these included genic regions similar to the *tftA* (98%), *tftC* (99%), and *tftE* (98%) genes. In addition to these previously reported genetic sequences, an apparently novel gene sequence was identified in *Burkholderia* species strain JR7B2. This genetic element was identified using DDRT-PCR applied to 2,4,5-T versus succinate grown minimal media cultures inoculated with the Agent Orange enrichment culture JR7B. No significant match for this gene was found in the current databases available for web based sequence analyses. It is interesting that this gene is induced in the presence of 2,4,5-T in the non-degrading *Burkholderia* species strain JR7B2. One implication of this finding is that this organism is responding to changing growth conditions as would be expected during

acclimation of the microbial consortium for growth on different carbon sources. Another possibility is that this gene is involved in adaptation of this organism to cellular stresses that may result during metabolism of 2,4,5-T by strain JR7B3. Future experiments would be able to examine the role of this genetic element in 2,4,5-T degradation by this microbial consortium, and determine the implications associated with its differential expression and probable role in growth of this organism during metabolism of 2,4,5-T by other members of the consortium.

This study is the first to demonstrate that 2,4-D and 2,4,5-T degrading bacteria can co-exist in Agent Orange contaminated soils. Previous studies have relied on prolonged selective conditions within the laboratory, such as plasmid assisted molecular breeding (Kilbane et al. 1982), to allow bacterial organisms to adapt to growth on 2,4,5-T as a sole carbon and energy source. It has been postulated that microbial consortia may be more widely spread in association with 2,4-D breakdown in the environments than has previously been thought (Tiedje et al. 1997). Based on these experiments, this certainly seems to be likely for 2,4,5-T degradation as well. It is interesting, however, that most of the important metabolic potential necessary for 2,4,5-T transformation exists in a single microorganism. Further analysis of these bacterial strains will allow the potential dynamic interaction of 2,4-D and 2,4,5-T degradation pathways to be studied. Results from such studies have the potential for facilitating bioremediation and bioaugmentation efforts at sites contaminated 2,4-D and 2,4,5-T mixtures.

## **ACKNOWLEDGMENTS**

The authors would like to thank Dr. James Cornette for generously supplying soil samples for this research. This research project was supported by The United States Air Force Office of Scientific Research (AASERT Award) and the Dow Foundation (SPHERE Award).

## **PART IV**

### **EFFECTS OF METABOLIC INTERMEDIATES ON DEGRADATION OF 2,4,5-T AND 2,4-D BY ENVIRONMENTAL BACTERIA ISOLATED FROM AGENT ORANGE CONTAMINATED SOIL**

## 4.1 ABSTRACT

*Burkholderia* species strain JRB1 and *Burkholderia* species JR7B3 contain 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) degradative genes which are similar to those found in *Ralstonia eutrophus* JMP 134 and *Burkholderia cepacia* AC1100, respectively (Rice, Part III). Growing cell assays coupled with messenger RNA analyses were utilized in this study to examine the ability of these bacteria to degrade 2,4-D and 2,4,5-T in the presence of metabolic intermediates, which may affect catabolism of these compounds in Agent Orange contaminated soils. Degradation of 2,4-D by *Burkholderia* species strain JRB1 was enhanced in the presence of glucose and corresponding RNA hybridization studies demonstrated a 2X-fold increase of *tfdB* mRNA transcripts. Addition of succinate, however, resulted in a buildup of 2,4-dichlorophenol (2,4-DCP), a cell death phase, delayed degradation of 2,4-D, and a 2X-fold decrease of *tfdB* mRNA transcripts. Addition of 10 mg/L of 2,4,5-trichlorophenol (2,4,5-TCP) delayed degradation of 2,4-D, but did not significantly affect *tfdA*, *tfdB*, or *tfdC* mRNA abundance. Similar studies using 100 - 500 mg/L of 2,4,5-T demonstrated only slight delays in 2,4-D metabolism. Growth on 2,4,5-T by a bacterial consortium containing *Burkholderia* species strain JR7B3 and *Burkholderia* species strain JR7B2 in the presence of glucose and succinate resulted in increased 2,4,5-TCP accumulation, 11.3 mg/L and 14.8 mg/L respectively, relative to control cultures containing only 2,4,5-T (28.0 µg/L 2,4,5-TCP). For *Burkholderia* species strain JR7B3 grown auxotrophically on 2,4,5-T, it was found that growth on glucose resulted in 2,4,5-TCP concentrations of 22.4 mg/L, and no subsequent degradation or growth occurred.

When succinate was provided as a co-carbon source 2,4,5-TCP concentrations remained below 300 µg/L and 2,4,5-T was slowly metabolized. Similar studies using 10 mg/L and 25 mg/L 2,4-DCP induced degradation of 2,4,5-T relative to cultures containing only 2,4,5-T. These findings indicate that the dynamics of 2,4-D and 2,4,5-T metabolism are subject to the influences of metabolic intermediates such as 2,4-DCP and 2,4,5-TCP. Furthermore, alternative carbon sources can dramatically affect how these organisms degrade 2,4-D and 2,4,5-T through catabolite modulation of the *tfdB* gene.

## 4.2 INTRODUCTION

Agent Orange consists of a mixture of 50% 2,4,5-trichlorophenoxy acetic acid (2,4,5-T) and 50% 2,4-dichlorophenoxyacetic acid (2,4-D) and was frequently used as a defoliant in the United States. Although the use of this herbicide was discontinued in the 1980s, environmental sinks, such as compost, sewage sludges, and sediments, may contribute to current deposition. Bacterial mediated degradation of each of these compounds have been demonstrated (Duxbury et al. 1970 and Kilbane et al. 1982), but the extent to which this herbicide mixture influences bacterial degradation of 2,4-D and 2,4,5-T has not been adequately assessed. Preliminary research by Haugland et al. (1990) has identified potential problems associated with degradation by 2,4-D and 2,4,5-T degrading bacteria, through misrouting of the respective metabolic pathways.

The broadleaf herbicide 2,4-D has been found to be rapidly degraded in the environment (Fulthorpe et al. 1995), and the microorganisms capable of biodegrading 2,4-D are common in all geographical locations examined (Kamagata et al. 1997).

Specific metabolic pathways involved in 2,4-D degradation have predominately been found in the  $\alpha$ ,  $\beta$ , and  $\gamma$  *proteobacteria* (Tiedje et al. 1997), and are located on large catabolic plasmids and/or within the chromosome (Top et al. 1996). One of the best-studied bacterial systems for 2,4-D degradation is found in the  $\beta$ -*Proteobacter*, *Ralstonia eutrophus* JMP134. *R. eutrophus* JMP134 is considered a model bacterium for 2,4-D degradation by many scientists (Harker et al. 1989), and contains a large 80 kb catabolic plasmid designated, pJP4, containing the genes necessary for conversion of 2,4-D to the TCA intermediates succinate and acetal-CoA (Don and Pemberton 1985). Many laboratories around the world have extensively studied this degradative pathway, and researchers are now beginning to understand the molecular mechanisms involved in degradation of 2,4-D in this organism.

Initiation of 2,4-D catabolism by *R. eutrophus* JMP134, begins as 2,4-D enters the bacterial cell via passive diffusion through the bacterial membranes and via TfdK (a proposed 2,4-D transport protein and member of the major facilitator superfamily of transport proteins, Leveau et al. 1998) which is able to increase the intracellular concentration of 2,4-D. As 2,4-D becomes available the 2,4-diochlorophenoxyacetic acid/ $\alpha$ -ketoglutarate dioxygenase, TfdA, catalyzes the monohydroxylation of 2,4-D resulting in the formation of 2,4-dichlorophenol (2,4-DCP) and glyoxylate (Harker et al. 1989, Koh et al. 1997). 2,4-DCP is then monooxygenated by TfdB to form 3,5-dichlorocatechol (3,5-DCC). Subsequent metabolism is mediated by the TfdC (2,4-dichlorocatechol dioxygenase), TfdD, (chloromuconate cycloisomerase), TfdE (dienelactone hydrolase), and TfdF(chloromaleylacetate reductase), generating succinate

and acetal-CoA, which then feeds into the Citric Acid Cycle. The regulation of these genes is mediated by 2,4-dichloromuconic acid. This metabolic intermediate has been shown to activate transcription of the *tfdA*, *tfdCDEF* and potentially the *tfdB* and *tfdD<sub>II</sub>C<sub>III</sub>E<sub>III</sub>B<sub>III</sub>K* operons. The TfdR, belonging to the *lysR* family of transcriptional regulators (Matrubutham et al. 1994) facilitates this transcriptional activation.

Metabolism of 2,4,5-T by *Burkholderia cepacia* AC1100 has been well studied (Zaborina et al. 1998), and the metabolic pathways necessary for utilization of this chlorinated phenoxyacetic acid are almost completely characterized in this strain. *B. cepacia* AC1100 has recently been shown to contain 5 chromosomal elements designated replicons I through V (Hubner et al. 1998). The catabolic genes necessary for 2,4,5-T mineralization in *B. cepacia* AC1100 are localized in three separate operons, and have been found to reside on replicons III (0.53 Mbp) and IV (0.34 Mbp). Interestingly it has been observed that insertion elements are disproportionally concentrated on these two smaller replicons, which may facilitate the plasticity genome in this organism (Hubner et al. 1998). The three major gene clusters involved in 2,4,5-T degradation are the *tftAB*, *tftCD*, and the *tftEFGH* operons. The *tftCD* operon is present in duplicate (Hubner et al. 1998), and has been proposed to provide a selective advantage by protecting against buildup of the toxic intermediate 2,4,5-trichlorophenol (2,4,5-TCP) during 2,4,5-T metabolism (Hubner et al. 1998).

The degradation pathway responsible for degradation of 2,4,5-T in *B. cepacia* AC1100 is distinct from 2,4-D metabolism by *R. eutrophus* JMP134. Degradation of 2,4,5-T proceeds via production of 2,4,5-TCP through the action of the 2,4,5-T

oxygenase. The 2,4,5-TCP produced is then monohydroxylated by the TftCD yielding 2,5-dichlorohydroxyquinone. The TftCD catalyzes a second hydroxylation of 2,5-dichlorohydroxyquinone producing 5-chlorohydroxyquinol. 5-Chlorohydroxyquinol is then dechlorinated through the activity of TftG producing 2-hydroxy-1,4-benzoquinone which is then oxidized by an unidentified reductase to yield 2-hydroxyquinol. 2-Hydroxyquinol is further degraded by TftH to yield maleylacetate, which is then metabolized to  $\beta$ -ketoadipate by TftE.  $\beta$ -Ketoadipate is then converted to succinate and glyoxylate and channeled into the tricarboxylic acid cycle.

In the absence of complete mineralization, 2,4-D and 2,4,5-T can be incompletely transformed by abiotic and biotic processes (Amy et al. 1985). Abiotic processes can only contribute to partial degradation and subsequent accumulation of intermediates and never to the complete removal of these compounds such as occurs during mineralization processes (Somerville et al. 1978). In the absence of a lower pathway for degradation of these compounds, growth may still occur because the organism can gain carbon and energy from the acetate, which is generated from cleavage of the phenoxy group (Top et al. 1996). Also, incomplete metabolism of 2,4,5-T can lead to accumulation of the metabolic intermediates 2,4,5-TCP and 5-chloro-2-hydroquinone (Haugland et al. 1990). Likewise, partial metabolism of 2,4-D can lead to the formation of 2,4-DCP and 3,5-DCC (Haugland et al. 1990). Co-cultures containing the 2,4,5-T degrading *B. cepacia* AC1100 and *R. eutrophus* JMP134 have been used to identify potential inhibition problems between these two pathways (Haugland et al. 1990). In these studies, it was demonstrated that although either organism mineralized their respective single

contaminant easily, 2,4-D co-metabolism by *B. cepacia* AC1100 produced chlorohydroquinone, which inhibited both its own 2,4,5-T metabolism and 2,4-D degradation, by *R. eutrophus* JMP134.

Recently, a 2,4,5-T degrading bacteria, *Burkholderia* species strain JR7B3, from Agent Orange contaminated soil has been characterized by Rice (Part III), which utilized 2,4,5-T as a source of carbon and energy. PCR based experiments confirmed the presence of the *tftA*, *tftC*, and *tftE* within strain JR7B3 and, mRNA expression studies demonstrated that these genes were actively transcribed during growth on 2,4,5-T. In addition to this 2,4,5-T degrading *Burkholderia* species, a 2,4-D degrading bacteria, *Burkholderia* species strain JRB1, was isolated from Agent Orange contaminated soils and shown to contain degradative genes similar to those associated with pJP4 (Rice, Part III). Both of these organisms were isolated from the same Agent Orange contaminated soil sample, which has been shown to contain both 2,4-D and 2,4,5-T (Rice, Part III). Also, low levels of 2,4-DCP and 2,4,5-TCP were detected during HPLC analysis of these soil samples.

To understand how bacteria associated with Agent Orange contaminated soils metabolized 2,4-D and 2,4,5-T, the influences of pollutant mixtures on bacterial degradation of 2,4-D and 2,4,5-T needs to be examined. The presence of 2,4-DCP, 2,4,5-TCP, and 3,5-DCC in association with 2,4-D and 2,4,5-T degrading bacteria may cause repression of genes necessary for the cell's continued metabolic fitness. The goal of this research project was to study the interactions of 2,4-D and 2,4,5-T metabolic intermediates on bacterial catabolism of 2,4-D and 2,4,5-T by *Burkholderia* species strain

JRB1 and *Burkholderia* species strain JR7B3. The results reported here will facilitate the advancement of future cleanup strategies by providing valuable information regarding the degradation of 2,4,5-T and 2,4-D within contaminant mixtures.

## 4.3 MATERIALS AND METHODS

### 4.3.1 Materials.

Most chemicals used in this study were of the highest grade commercially available. 2,4-dichlorophenol (2,4-DCP, 99%) and 2,4-dichlorophenoxyacetic acid (2,4-D, 99+%) were obtained from Acros Organics (Pittsburgh, PA). 2,4,5-trichlorophenol (2,4,5-TCP, 99%) was purchased from Fluka Chemicals (Milwaukee, WI). 2,4,5-trichlorophenoxyacetic acid (2,4,5-T, 97%) was obtained from Aldrich chemical Company, Inc. (Milwaukee, WI). Acetonitrile (HPLC grade) and Water (HPLC grade) were obtained from Fischer Scientific (Fairlawn, NJ). All other chemicals were of analytical grade.

### 4.3.2 Media.

Bacterial cultures were grown on phosphate-buffered minimal salts media, PAS, containing in grams per Liter: 4.43 g  $K_2HPO_4$ , 1.72 g  $KH_2PO_4$ , 2.16 g  $NH_4Cl$ , 0.195 g  $MgSO_4 \cdot H_2O$ , 0.01 g  $FeSO_4 \cdot 2H_2O$ , and 0.003 g  $CaCl_2 \cdot 2H_2O$ . For auxotrophic culture studies PAS media was amended with 0.01% yeast extract to provide additional nutrients

for growth. For all cultures, the pH was adjusted to 7.2. 2,4-D and 2,4,5-T were added to the growth media prior to autoclaving at concentrations up to but not exceeding 1000 mg/L. HPLC analysis of sterile PAS media containing 2,4-D or 2,4,5-T was used to confirm the stability of these compounds during heating. These examinations did not detect significant concentrations of 2,4-DCP or 2,4,5-TCP in autoclaved media. Once prepared all media containing 2,4-D and 2,4,5-T were stored at 28°C in the dark. Addition of succinate and glucose to PAS media was accomplished using filter sterilized (0.2  $\mu$ m) stock solutions and media volumes were adjusted to maintain equivalent concentrations of media constituents. 2,4-DCP and 2,4,5-TCP were solublized in HPLC grade H<sub>2</sub>O and added at appropriate concentrations.

#### **4.3.3 Growing cell assays.**

Growing cell assays were used to examine degradation and growth of *Burkholderia* species strain JRB1, *Burkholderia* species strain JR7B3, and a bacterial consortium containing *Burkholderia* species JR7B2 and *Burkholderia* species JR7B3. For these experiments, starter cultures containing 50 ml of PAS medium amended with 1 mg/ml of 2,4-D or 2,4,5-T were inoculated with single colonies from PAS plates containing 1000 mg/L 2,4-D or 2,4,5-T. These cultures were then incubated at 28°C until mid-log growth phase was achieved. A 0.1 ml aliquot from these starter cultures was then transferred to 50 ml sterile mineralization vials containing 10 ml of PAS medium amended with the appropriate concentration of 2,4-D and/or 2,4,5-T and supplemented with the appropriate test compound. In cases where 2,4-D and 2,4,5-T were both present,

a maximum concentration of 500 mg/L for the primary growth compound was used rather than the standard 1000 mg/L. During the course of the experiment, 200  $\mu$ l samples were taken for optical density measurements and analytical determination of 2,4-D, 2,4,5-T, 2,4-DCP, and 2,4,5-TCP concentrations. Spectrophotometric monitoring was routinely done using a Beckman DU-70 spectrophotometer (Beckman, Fullerton, CA) at 600 nm. Analytical analysis was achieved using High Pressure Liquid Chromatography (HPLC) as described below.

#### **4.3.4 HPLC analyses.**

Analytical determination of 2,4-D, 2,4,5-T, 2,4-DCP, and 2,4,5-TCP concentrations in culture supernatants was determined using HPLC. Samples for analysis were obtained directly from culture supernatants. Aliquots, 100  $\mu$ l were taken from growing bacterial cultures and transferred to 1.5 ml microcentrifuge tubes. These were then microcentrifuged at 14,000 rpm to pellet bacterial cells, and the supernatant was frozen at -80°C for HPLC studies. Immediately prior to analysis, samples were warmed at 37°C for 5 min and 70  $\mu$ l was transferred to an 1.8 ml amber autosampler vial containing 730  $\mu$ l HPLC grade H<sub>2</sub>O and 50  $\mu$ g 4-bromobenzoic acid (4-BBA, internal standard) dissolved in 200  $\mu$ l acetonitrile. Sample injection was facilitated by a Perkin Elmer LC 600 autoinjector (Perkin Elmer, Foster City, CA) fitted with a 50  $\mu$ l injection loop. A Supelcosil LC-18-T reverse phase C<sub>18</sub> column (Supelco, Bellefonte, PA) was fitted onto a Perkin Elmer Binary LC 250 series pump (Perkin Elmer, Foster City, CA) and a Perkin Elmer model LC-235 diode array detector (Perkin Elmer, Foster City, CA).

Data analysis was achieved using the Turbochrome version 4.1 software package (Perkin Elmer, PE Nelson Division, San Jose, CA). Conditions for HPLC analysis were optimized for detection of the above compounds in 20% acetonitrile:80% H<sub>2</sub>O. Samples were eluted at a flow rate of 1.0 ml/min with 0.025% H<sub>3</sub>PO<sub>4</sub>-acetonitrile gradient, as follows: 0% acetonitrile (isocratic, 5.0 min), 0 to 50% acetonitrile (linear, 5.0 min), 50% acetonitrile (isocratic, 20 min), 50 to 0% acetonitrile (linear, 5.0 min). Absorbance of eluted compounds was monitored at 220 nm. Sample retention times were compared to known standards prepared and analyzed using identical protocols. Retention times for compounds of interest were 4-BBA (16.2), 2,4-D (17.1), 2,4-DCP (18.0), 2,4,5-T (19.2), and 2,4,5-TCP (21.5).

#### **4.3.5 RNA hybridization.**

RNA hybridization experiments were used to help elucidate the effects of glucose, succinate, 2,4-D, 2,4,5-T, 2,4-DCP, and 2,4,5-TCP on expression of key genes involved in 2,4-D and 2,4,5-T metabolism. For these experiments, *Burkholderia* species strain JRB1 and *Burkholderia* species strain JR7B3 were grown on PAS containing 2,4-D and PAS (amended with 0.1% Casamino Acids, which have been shown to be a effective non-inducing carbon source) containing 2,4,5-T, respectively. These cultures were incubated at 28°C until mid-log phase was achieved. Aliquots from these cultures were then transferred into 20 ml sterile scintillation vials containing PAS media amended with the test compounds. For *Burkholderia* species strain JRB1, the following treatments were tested: 0, 100, 250, and 500 mg/L 2,4,5-T; 0, 10, and 25 mg/L 2,4,5-TCP; and 5 and 10

mM each of succinate and glucose. For *Burkholderia* species strain JR7B3 the following treatments were tested: 0, 100, 250, and 500 mg/L 2,4-D; 0, 10, and 25 mg/L 2,4-DCP; and 5 and 10 mM each of succinate and glucose. Following amendments with these compounds, cultures were allowed to incubate for 1 generation time. Total RNA was extracted from these test cultures using the hot phenol method (Fleming et al. 1993), and vacuum blotted onto nylon membranes (ICN Biochemical, Cleveland, OH) as previously described (Sambrook et al. 1989). RNA hybridization analysis was conducted as previously described (Sambrook et al. 1989).

PCR coupled with Stratagene's Prime-It<sup>®</sup> Random primer labeling kit was used to generate *tfdA,B, C* and *tftA,B,C* probes. For the PCR amplifications, primers previously designed for detection of these genes in these organisms (Rice, Part III; Table 3.1) were used in combination with PCR beads (Amersham Pharmacia, Piscataway, New Jersey) according to manufacture's recommendations. The following components were used for these PCR reactions: 100 ng of plasmid DNA as template DNA (Specific clones for these amplifications are detailed in Part III), 1.5  $\mu$ l Primer A (2.0  $\mu$ M stock), 1.5  $\mu$ l Primer B (2.0  $\mu$ M stock), 23.5  $\mu$ l ultrapure H<sub>2</sub>O, and 1 PCR bead. PCR products were electrophoresed using 1.0 % low melting point agarose (Sea Kemp GTG<sup>®</sup>). Bands were then cut from the gel and incubated at 60°C to soften the agarose. A 10  $\mu$ l aliquot from the softened band was then combined with 10  $\mu$ l of random primer and 14  $\mu$ l H<sub>2</sub>O and incubated at 100°C for 5 min. To this mixture 10  $\mu$ l of 5X primer buffer, 5  $\mu$ l  $\alpha$ -<sup>32</sup>P-dCTP, and 1  $\mu$ l Klenow enzyme was added. The reaction was incubated at 37°C for 15 min. A 2  $\mu$ l aliquot of stop mix was then added and the mixture was push column

purified according to manufacturer's instructions (Stratagene). Probes were then boiled for 10 min, cooled quickly to 4°C and added directly to the hybridization solutions. For these hybridizations, Ambion's Ultrasensitive hybridization buffer was used as described by the manufacturer. The hybridization solution was preheated at 60°C, and 10 ml was added to hybridization bags and allowed to prehybridize in a 42°C water bath for 2 hours. Radiolabeled probes were then added directly to the hybridization bags and allowed to incubate 16 - 18 hours. These blots were then washed 3 X using a high stringency wash buffer containing 0.1X SSC and 0.5% SDS. RNA blots were then analyzed using the Storm<sup>TM</sup> 840 Phosphoimager (Molecular Dynamics, Sunnyvale, CA).

#### **4.3.6 Data analysis**

Experiments for this study were done in triplicate. For Growing cell assays, one starter culture was used to inoculate three independent cultures. Each of these replicate cultures was then sampled throughout the time course of the experiment. These samples were analyzed to determine optical density and compound concentrations. For messenger RNA analyses, three bacterial cultures were independently treated with the test compound and allowed to incubate for one generation time. Total RNA from these cultures was then extracted, applied to nylon membranes, and hybridized to a radiolabeled probe. Numerical data was obtained after densitometry using a phosphoimager (described above). These data were then entered into Microsoft Excel 97 (Microsoft Corporation, Redmond, WA) and an average value was calculated using the AVERAGE function. A standard deviation was also calculated for these values using the

STDDEVP function. This standard deviation value is a measure of how widely values are dispersed from the mean value. No further statistical analysis was utilized. Conclusions from these experiments were based on separation of the average values by at least one standard deviation.

## 4.4 RESULTS

### *4.4.1 Growth of Burkholderia species strain JRB1 in the presence of alternative carbon sources resulted in modulation of 2,4-D degrading activity.*

Degradation of 2,4-D for *Burkholderia* species strain JRB1 on PAS media containing 1000 mg/L 2,4-D and 5.6 mM Glucose was slightly enhanced compared to PAS cultures without glucose (Figure 4.1). During metabolism of 2,4-D by PAS cultures with and without glucose, 2,4-DCP was maintained below HPLC detection limits. Compared to PAS cultures containing only glucose, initiation of growth for PAS culture supplemented with glucose was only slightly inhibited (Figure 4.1). Examination of TfdA messenger RNA transcripts revealed that no significant change in response to glucose addition was observed (Figure 4.2). 2,4-Dichlorophenol hydrolase (TfdB) expression was enhanced by glucose addition by 2.1 and 2.32 fold for 5 mM and 10 mM glucose, respectively (Figure 4.3). No significant increase in 3,5-dichlorocatechol dioxygenase (*tfdC*) mRNA transcripts was observed (Figure 4.4).

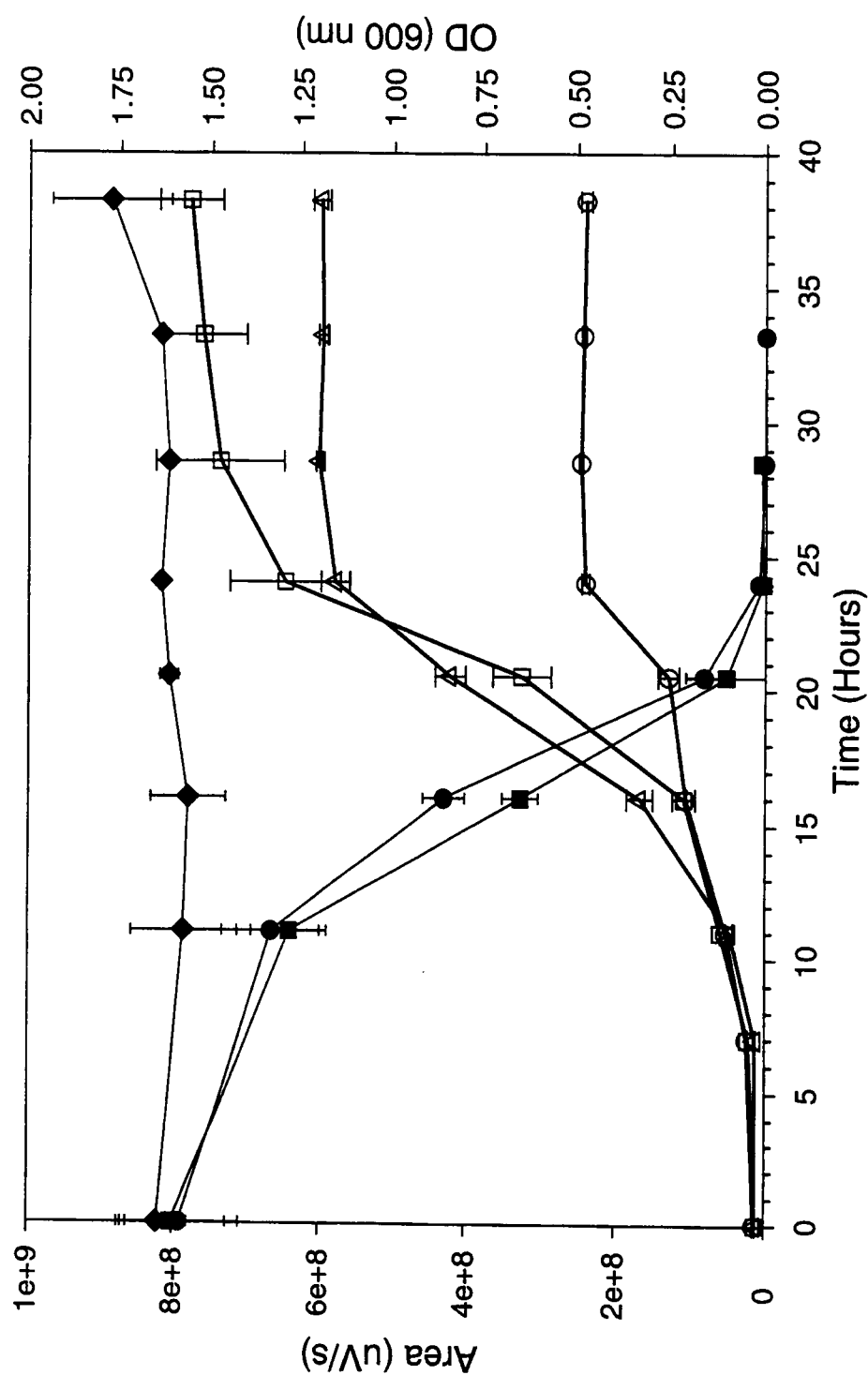
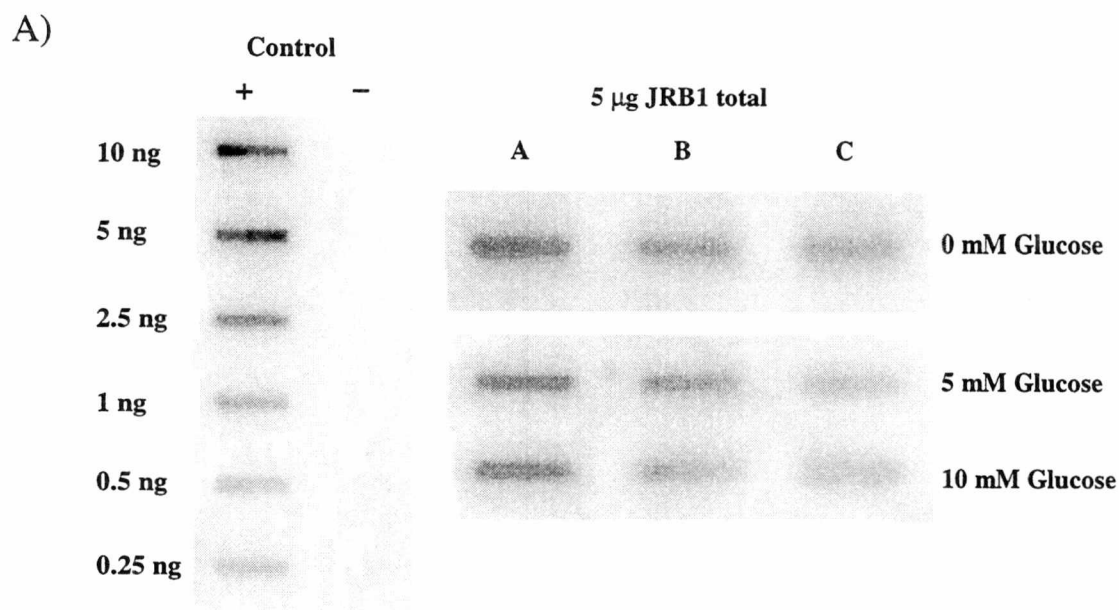


**Figure 4.1** *Burkholderia* species strain JRB1 growing cell assay. Symbols are as follows: (●) 2,4-D concentration for PAS + 2,4-D cultures, (■) 2,4-D concentration for PAS + 2,4-D + glucose cultures, (○) optical density for PAS + 2,4-D cultures, and (□) optical density for PAS + 2,4-D + glucose cultures, (◆) 2,4-D concentration for abiotic control, and (▼) optical density for PAS + glucose cultures. 2,4-DCP was maintained below detection limits (50  $\mu\text{g/ml}$ ) in these cultures. Data points represent the average of triplicate cultures. Error bars represent the standard deviation of among these cultures.



B)

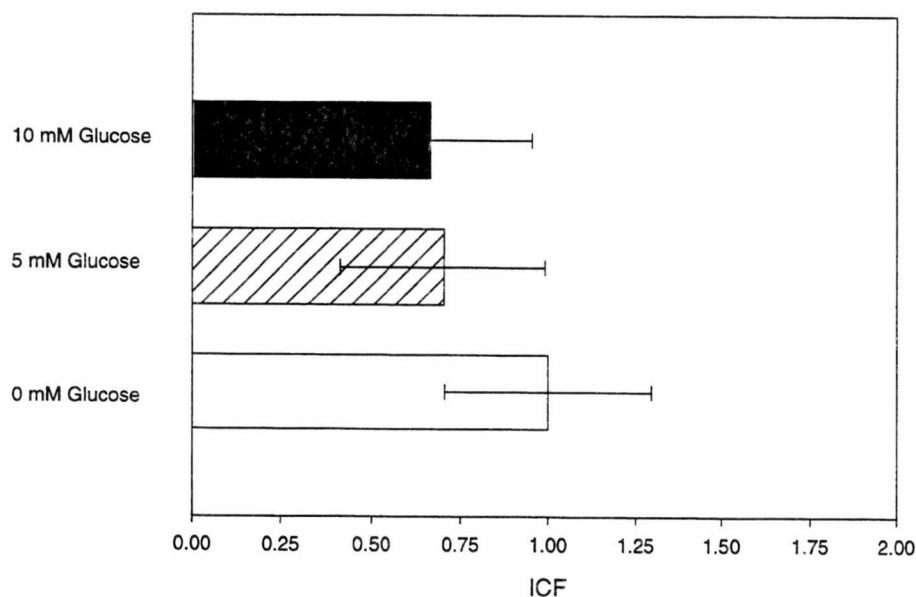
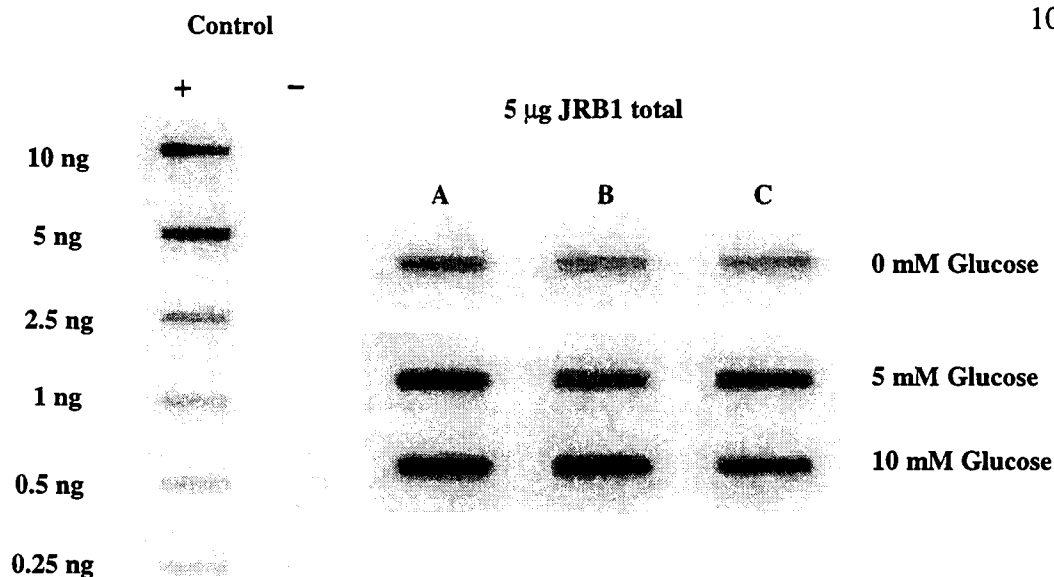


**Figure 4.2.** Analysis of *tfdA* mRNA expression in response to glucose addition for *Burkholderia* species strain JRB1. A) RNA hybridization analysis. Positive controls are shown on the left side of the figure and cover a concentration range from 0.25 ng to 10 ng. A 10 ng negative control was blotted adjacent to the 10 ng positive control. Five  $\mu$ g of total RNA isolated from cultures amended with glucose was applied to the nylon membranes, and probed using a *tfdA* specific,  $\alpha$ - $^{32}$ P-CTP, probe. B) Graphical representation of Induction Factors (IF); IF values were determined by dividing results for each treatment condition by the control value and represent the ratio of condition mRNA/control mRNA. Error bars represent the standard deviations among three separate experiments.

A)

103



B)

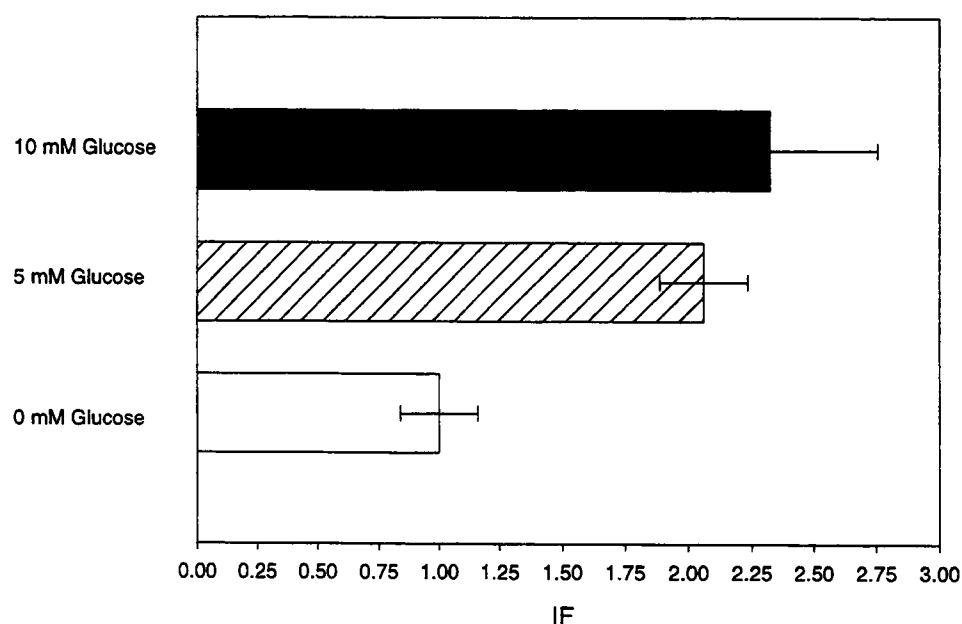
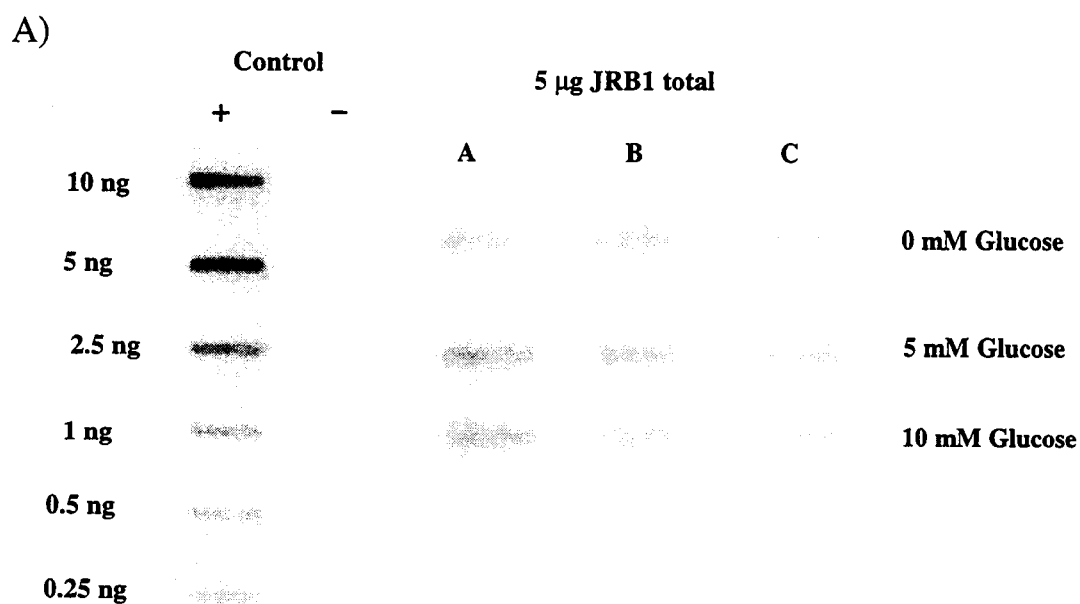


**Figure 4.3** Analysis of *tfdB* mRNA expression in response to glucose addition for *Burkholderia* species strain JRB1. A) RNA hybridization analysis. Positive controls are shown on the left side of the figure and cover a concentration range from 0.25 ng to 10 ng. A 10 ng negative control was blotted adjacent to the 10 ng positive control. Five µg of total RNA isolated from cultures amended with glucose was applied to the nylon membranes, and probed using a *tfdB* specific,  $\alpha$ - $^{32}$ P-CTP, probe. B) Graphical representation of Induction Factors (IF); IF values were determined by dividing results for each treatment condition by the control value and represent the ratio of condition mRNA/control mRNA. Error bars represent the standard deviations among three separate experiments.



B)

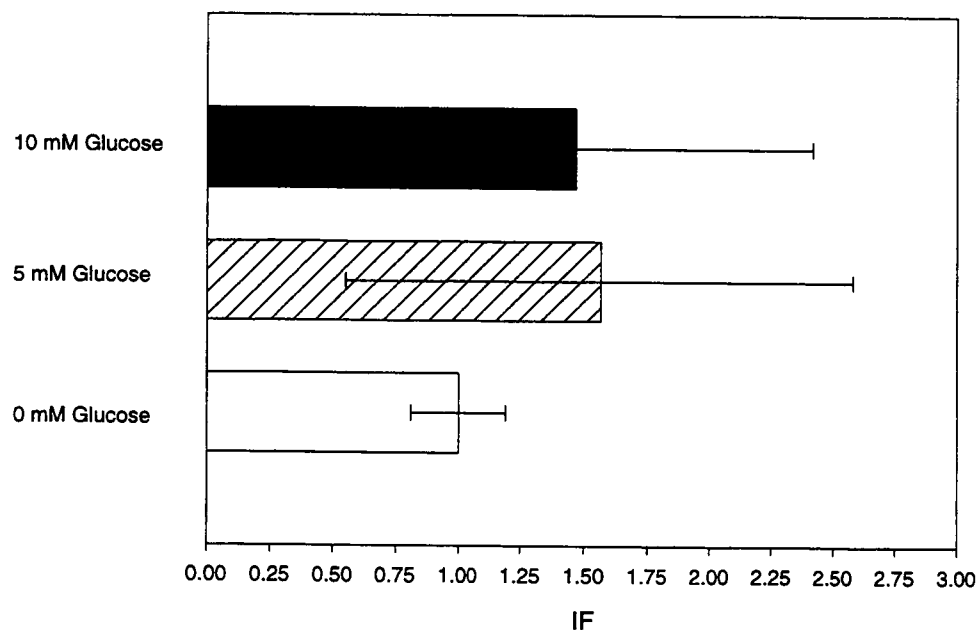


**Figure 4.4** Analysis of *tfdC* mRNA expression in response to Glucose addition for *Burkholderia* species strain JRB1. A) RNA hybridization analysis. Positive controls are shown on the left side of the figure and cover a concentration range from 0.25 ng to 10 ng. A 10 ng negative control was blotted adjacent to the 10 ng positive control. 5  $\mu$ g of total RNA isolated from cultures amended with glucose was applied to the nylon membranes, and probed using a *tfdC* specific,  $\alpha$ - $^{32}$ P-CTP labeled, probe. B) Graphical representation of Induction Factors (IF). IF values were determined by dividing results for each treatment condition by the control value and represent the ratio of Condition mRNA/Control mRNA. Error bars represent the standard deviations among three separate experiments.

During the first 15 hours of growth, *Burkholderia* species strain JRB1 cultures containing 2,4-D + 3.7 mM succinate had similar 2,4-D degradation profiles relative to cultures containing only 2,4-D (Figure 4.5). Similar to 2,4-D cultures containing glucose there was a slight growth delay compared to cultures containing succinate only (Figure 4.5). During 2,4-D degradation in the presence of succinate, 2,4-DCP accumulated to approximately 24.2 mg/L and resulted in a cell death phase and delayed 2,4-D degradation (Figure 4.5). Accumulation of 2,4-DCP for cultures containing only 2,4-D or 2,4-D + glucose was not observed. An analysis of *tfdA* expression revealed a decrease in mRNA abundance of approximately 0.62 and 0.49 fold for 5 mM and 10 mM succinate, respectively (Figure 4.6). Similarly, *tfdB* expression was decreased by 0.48 and 0.55 fold (5 mM and 10 mM, respectively) compared to controls without succinate (Figure 4.7). There was also a 0.56 and 0.65 fold decrease in *tfdC* mRNA under these treatment conditions (Figure 4.8).

#### **4.4.2 *Burkholderia* species strain JRB1 completely metabolized 2,4-D in the presence of 2,4,5-T.**

Initiation of 2,4-D metabolism was not affected by the presence of 2,4,5-T at concentrations up to 500 mg/L (Figure 4.9). During metabolism of 2,4-D, transformation of 2,4,5-T was negligible (data not shown). The rate of transformation of 2,4-D was not significantly effected by 100 mg/L and 250 mg/L 2,4,5-T, and 500 mg/L 2,4,5-T only

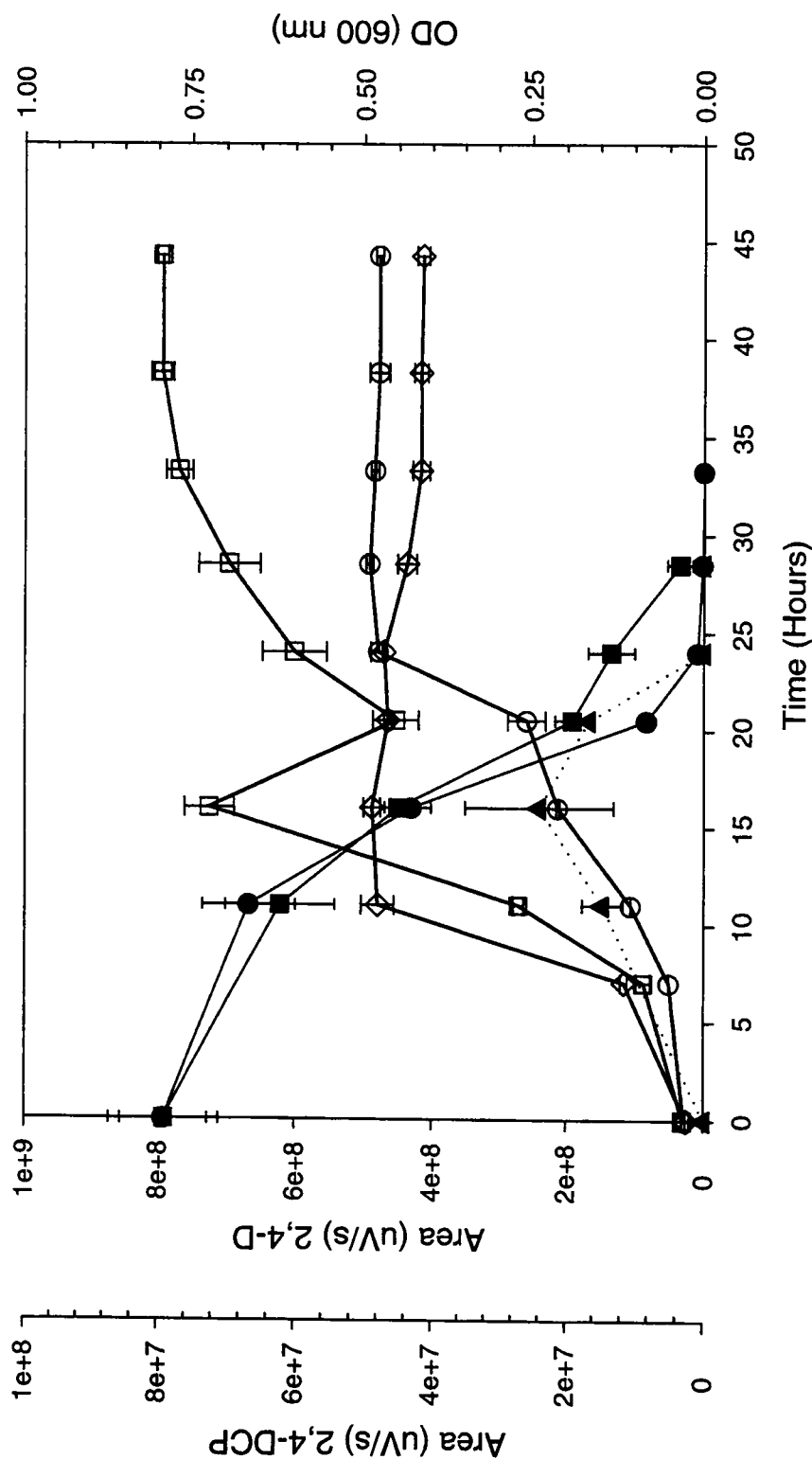


**Figure 4.5** *Burkholderia* species strain JRB1 growing cell assay. Symbols are as follows: (●) 2,4-D concentration for PAS + 2,4-D cultures, (■) 2,4-D concentration for PAS + 2,4-D + succinate cultures, (○) optical density for PAS + 2,4-D cultures, and (□) optical density for PAS + 2,4-D + succinate cultures, (◊) PAS + succinate cultures, and (▲) 2,4-DCP concentrations for PAS + 2,4-D + succinate. 2,4-DCP was below detection limits in cultures without succinate. The abiotic control is displayed in Figure 1. Data points represent the average of triplicate cultures. Error bars represent the standard deviation of among these cultures.

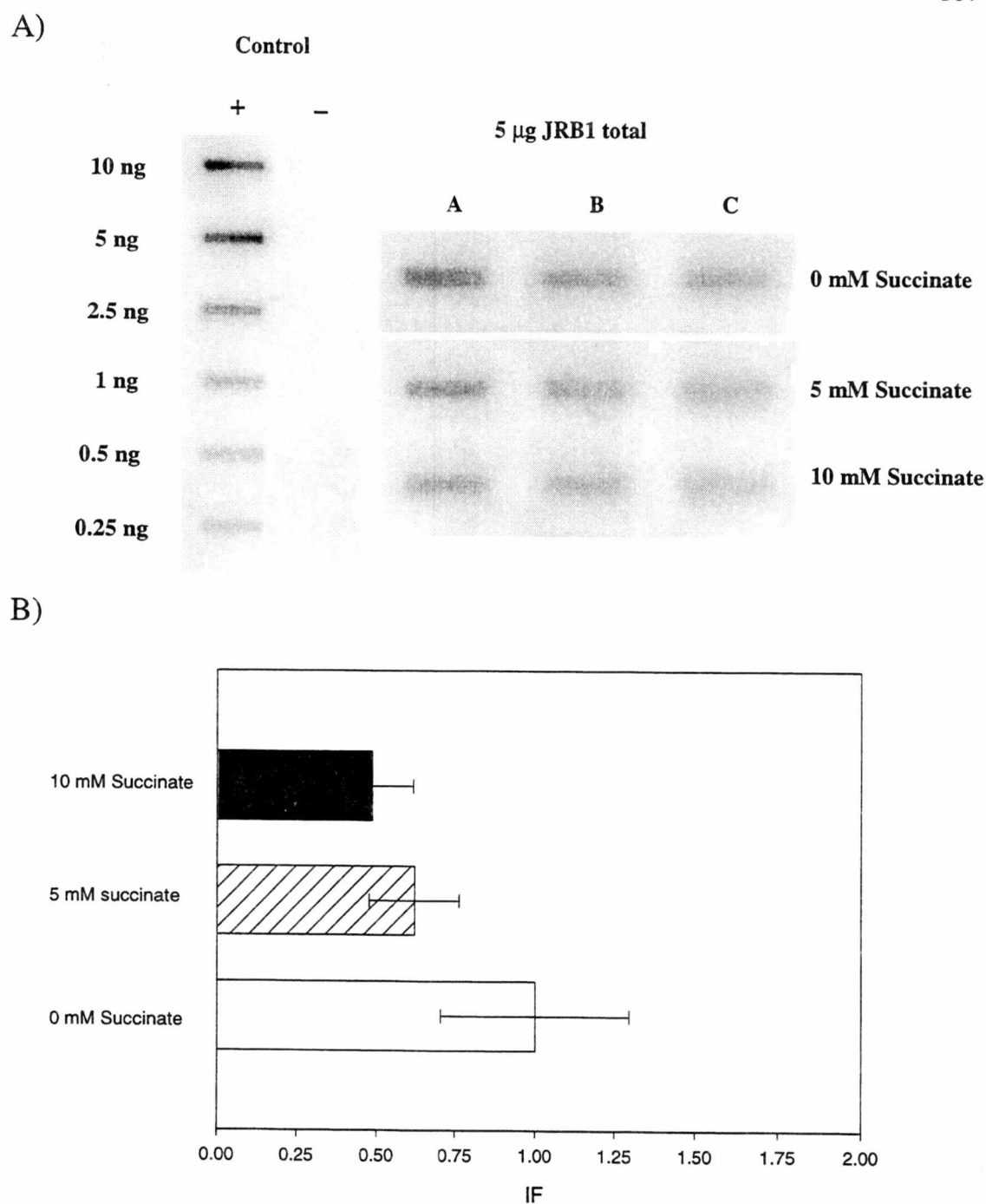


**Figure 4.6** Analysis of *tfdA* mRNA expression in response to succinate addition for *Burkholderia* species strain JRB1. A) RNA hybridization analysis. Positive controls are shown on the left side of the figure and cover a concentration range from 0.25 ng to 10 ng. A 10 ng negative control was blotted adjacent to the 10 ng positive control. Five  $\mu$ g of total RNA isolated from cultures amended with succinate was applied to the nylon membranes, and probed using a *tfdA* specific,  $\alpha$ - $^{32}$ P-CTP labeled, probe. B) Graphical representation Induction Factor (IF); IF values were determined by dividing results for each treatment condition by the control value and represent the ratio of condition mRNA/control mRNA. Error bars represent the standard deviations among three separate experiments.

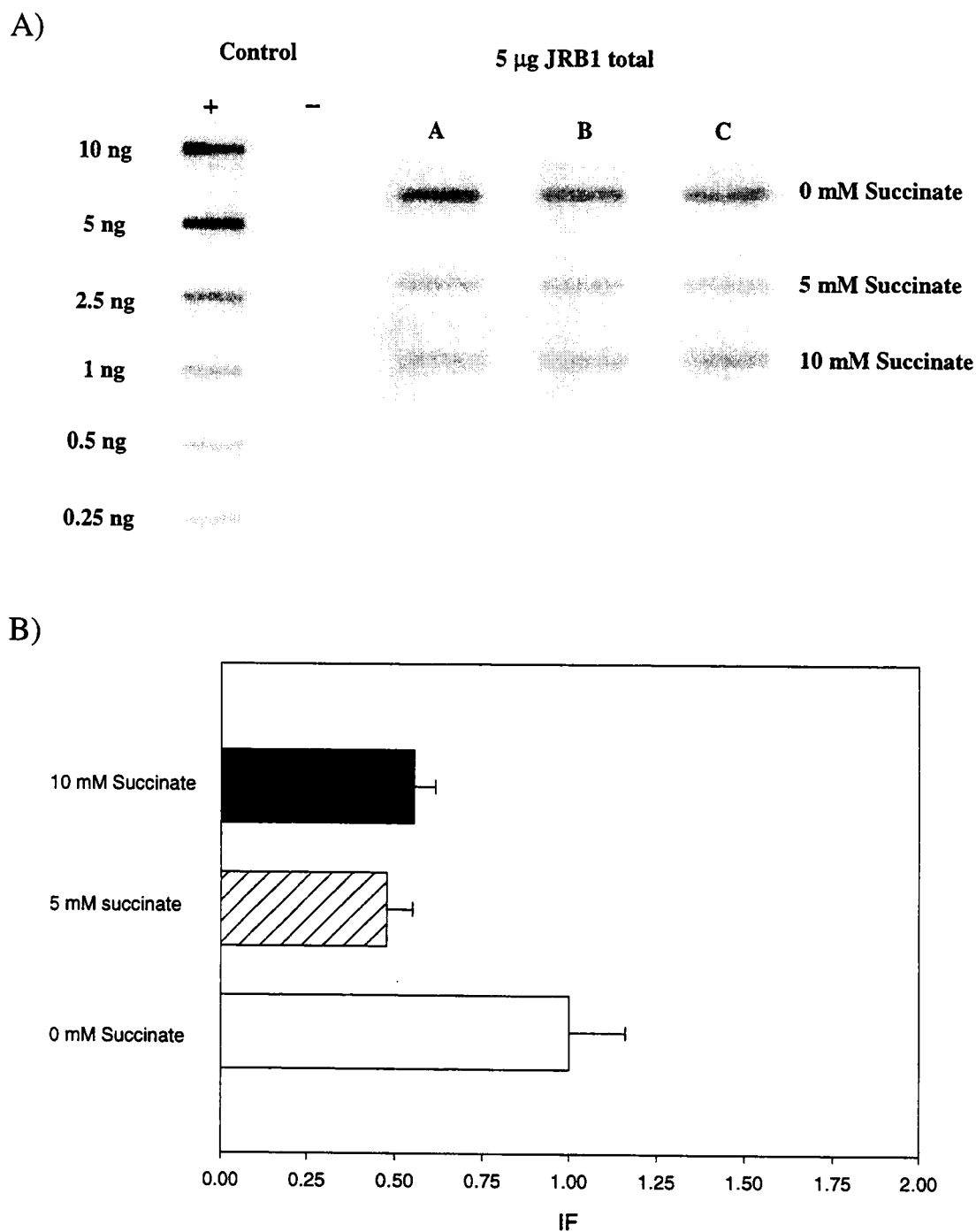
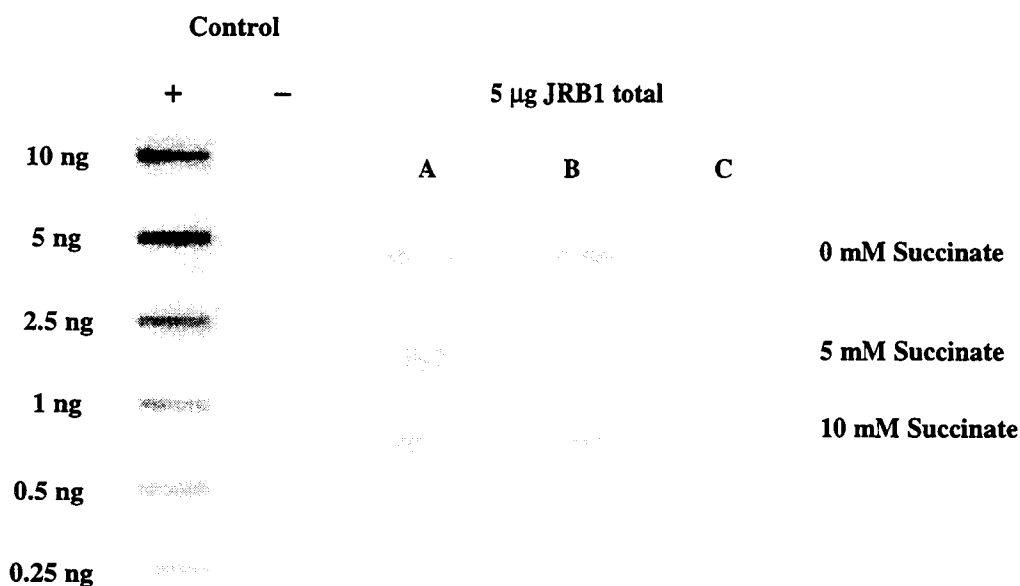


**Figure 4.7** Analysis of *tfdB* mRNA expression in response to succinate addition for *Burkholderia* species strain JRB1. A) RNA hybridization analysis. Positive controls are shown on the left side of the figure and cover a concentration range from 0.25 ng to 10 ng. A 10 ng negative control was blotted adjacent to the 10 ng positive control. Five  $\mu$ g of total RNA isolated from cultures amended with succinate was applied to the nylon membranes, and probed using a *tfdB* specific,  $\alpha$ - $^{32}$ P-CTP labeled, probe. B) Graphical representation Induction Factor (IF); IF values were determined by dividing results for each treatment condition by the control value and represent the ratio of condition mRNA/control mRNA. Error bars represent the standard deviations among three separate experiments.

A)



B)

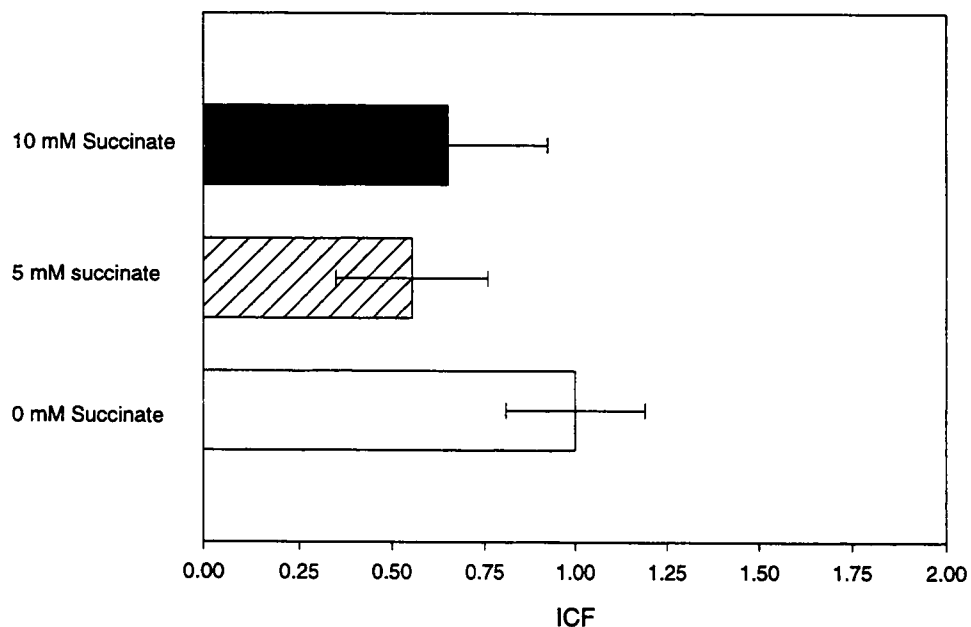


**Figure 4.8** Analysis of *tfdC* mRNA expression in response to succinate addition for *Burkholderia* species strain JRB1. A) RNA hybridization analysis. Positive controls are shown on the left side of the figure and cover a concentration range from 0.25 ng to 10 ng. A 10 ng negative control was blotted adjacent to the 10 ng positive control. Five  $\mu$ g of total RNA isolated from cultures amended with succinate was applied to the nylon membranes, and probed using a *tfdC* specific,  $\alpha$ - $^{32}$ P-CTP labeled, probe. B) Graphical representation Induction Factor (IF); IF values were determined by dividing results for each treatment condition by the control value and represent the ratio of condition mRNA/control mRNA. Error bars represent the standard deviations among three separate experiments.

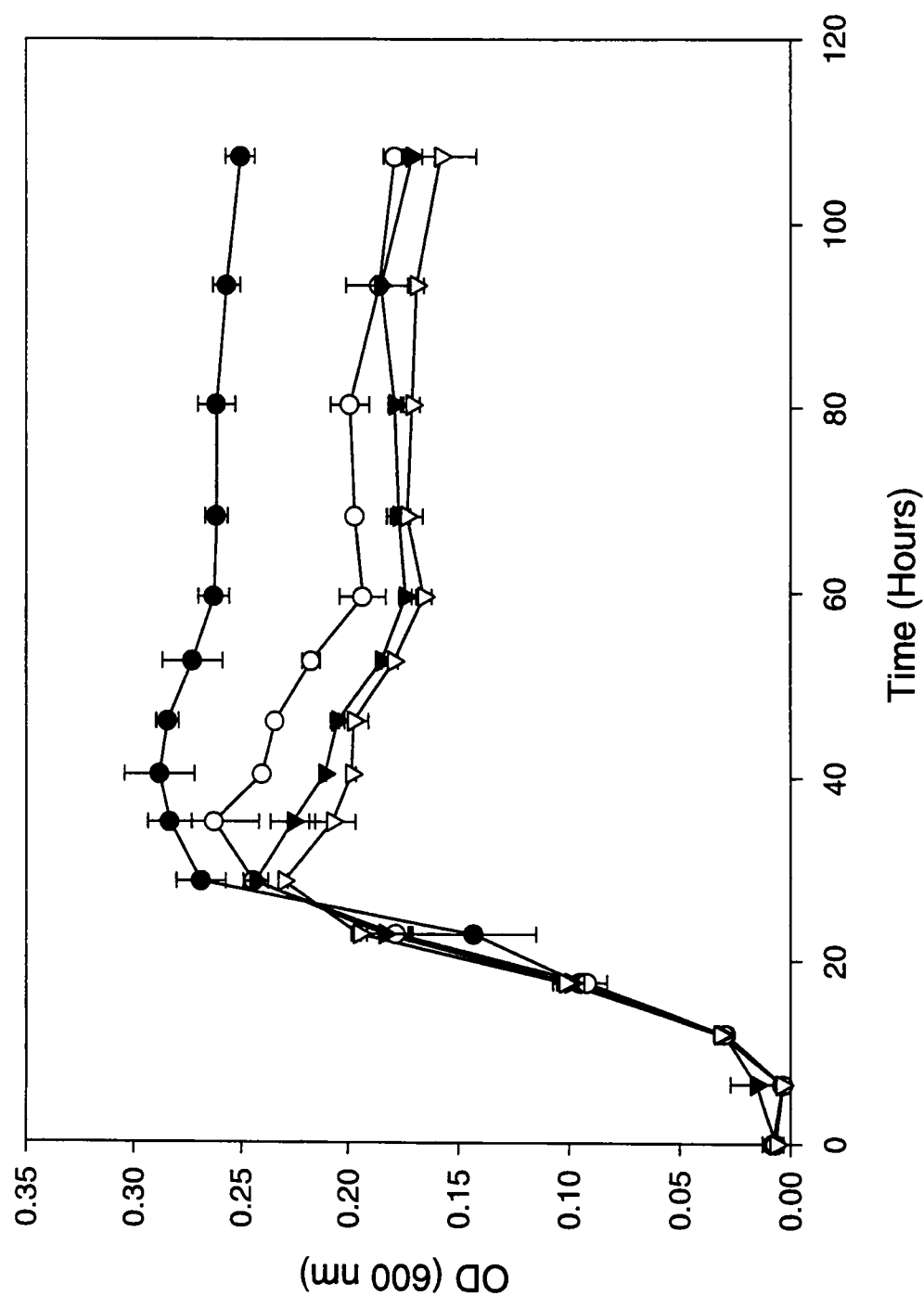


**Figure 4.9** Growth of *Burkholderia* species strain JRB1 in the presence of 0 mg/L (●), 100 mg/L (○), 250 mg/L (▼), or 500 mg/L (▽) 2,4,5-T. Each culture was grown in PAS containing 500 mg/L 2, 4-D and amended with the appropriate amount of 2,4,5-T. Data points represent the average of triplicate cultures. Error bars represent the standard deviation of among these cultures.

slightly delayed 2,4-D degradation (Figure 4.10). During log phase growth of *Burkholderia* species strain JRB1 on 2,4-D, 1.4 mg/L, 1.9 mg/L, 1.6 mg/L, and 1.0 mg/L 2,4,5-DCP was generated during peak growth periods in the presence of 500 mg/L, 250 mg/L, 100 mg/L, and 0 mg/L 2,4,5-T, respectively (Figure 4.11A). Also 2,4,5-TCP was generated during metabolism of 2,4-D to maximum concentrations of 1.4 mg/L, 0.4 mg/L, and 0.1 mg/L for cultures amended with 500 mg/L, 250 mg/L, and 100 mg/L 2,4,5-T (Figure 4.11B). Interestingly, 2,4,5-TCP was removed from all cultures tested by 40 hours (Figure 4.11B), and 2,4,5-TCP buildup and subsequent removal corresponded with a decrease in cellular biomass and 2,4-D degradation rate. An analysis of *tfdA* and *tfdC* mRNA demonstrated that there was no significant alteration in expression of these genes upon addition of 100 mg/L and 250 mg/L 2,4,5-T (Figure 4.12 and 4.13). There was however a slight increase in the abundance of *tfdB* mRNA for 100 mg/L (2.3 fold) and 250 mg/L (1.9 fold) 2,4,5-T additions (Figure 4.14).

#### 4.4.3 2,4,5-TCP delayed degradation of 2,4-D by *Burkholderia* species strain JRB1.

Growth of *Burkholderia* species strain JRB1 was inhibited in the presence of 10 mg/L and 25 mg/L 2,4,5-TCP (Figure 4.15). In the case of 25 mg/L, growth was completely inhibited for PAS cultures containing 2,4-D or succinate amended with 2,4,5-TCP, and no degradation of 2,4-D was observed (Figure 4.16). Whereas, the addition of 10 mg/L 2,4,5-TCP resulted in delayed initiation of 2,4-D degradation and subsequent

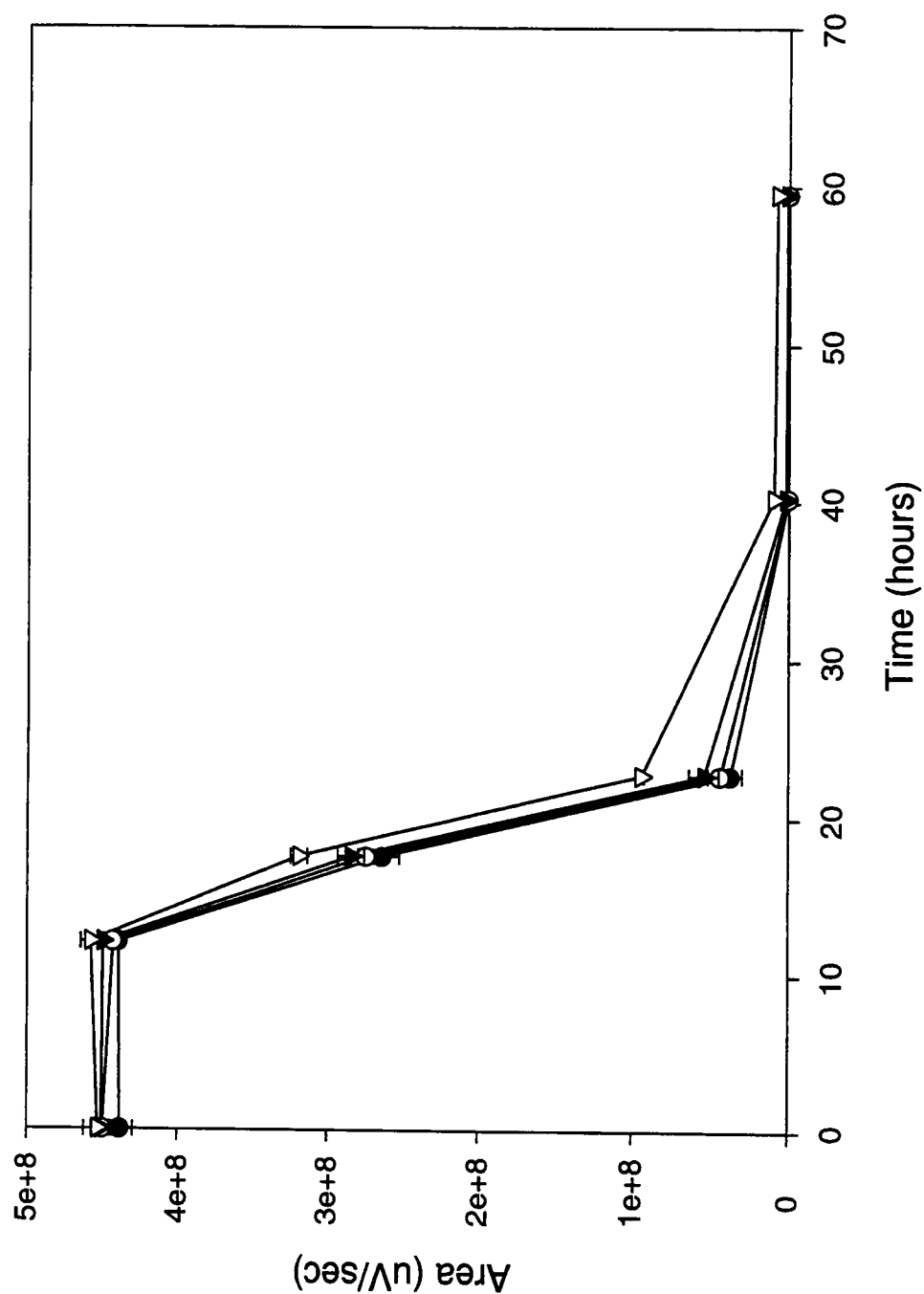
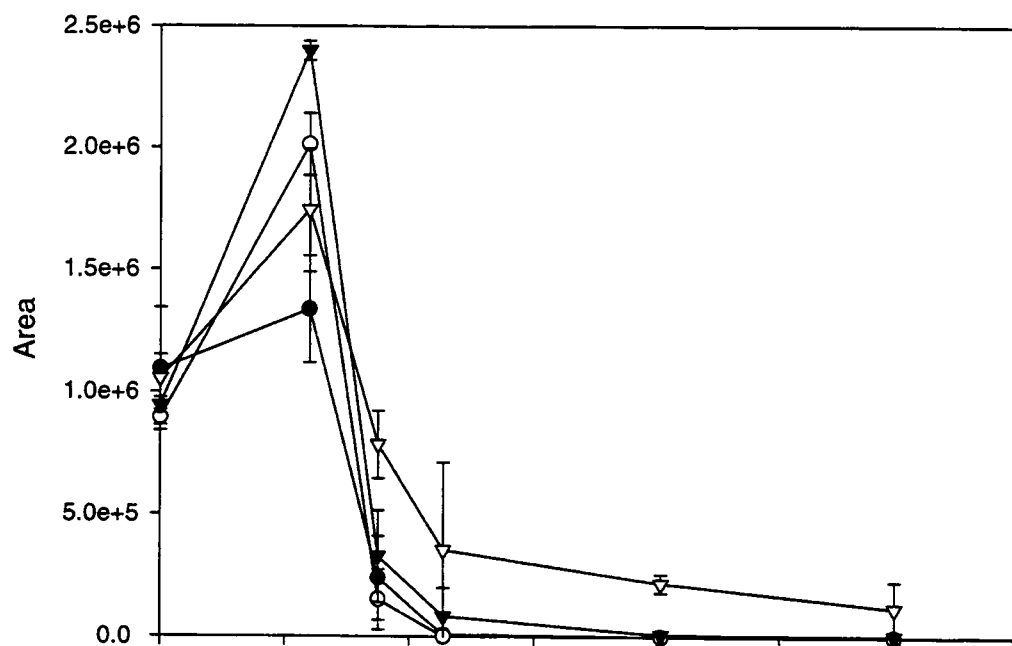


**Figure 4.10** 2,4-D degradation curves for *Burkholderia* species strain JRB1 in the presence of 0 mg/L (●), 100 mg/L (○), 250 mg/L (▼), or 500 mg/L (▽) 2,4,5-T. Each culture was grown in PAS containing 500 mg/L 2, 4-D and amended with the appropriate amount of 2,4,5-T. Data points represent the average of triplicate cultures. Error bars represent the standard deviation of among these cultures.

A)



B)

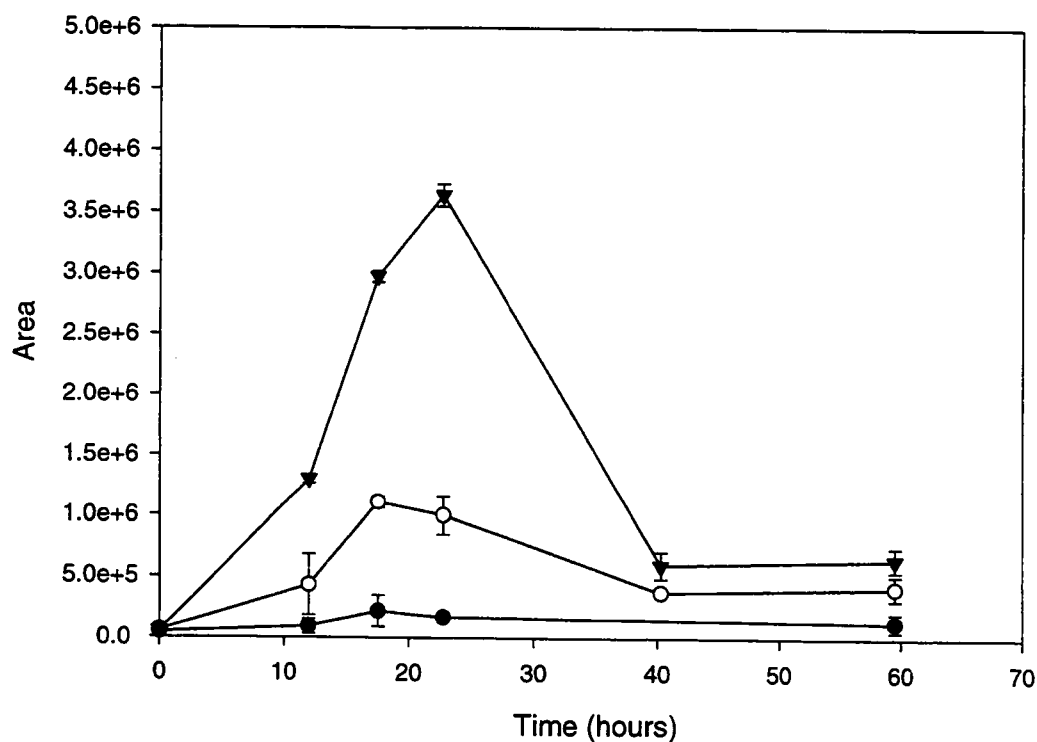


**Figure 4.11** A) 2,4-DCP and B) 2,4,5-TCP accumulation and degradation curves for *Burkholderia* species strain JRB1 in the presence of 0 mg/L (●), 100 mg/L (○), 250 mg/L (▼), or 500 mg/L (▽) 2,4,5-T. Each culture was grown in PAS containing 500 mg/L 2, 4-D and amended with the appropriate amount of 2,4,5-T. Data points represent the average of triplicate cultures. Error bars represent the standard deviation of among these cultures.

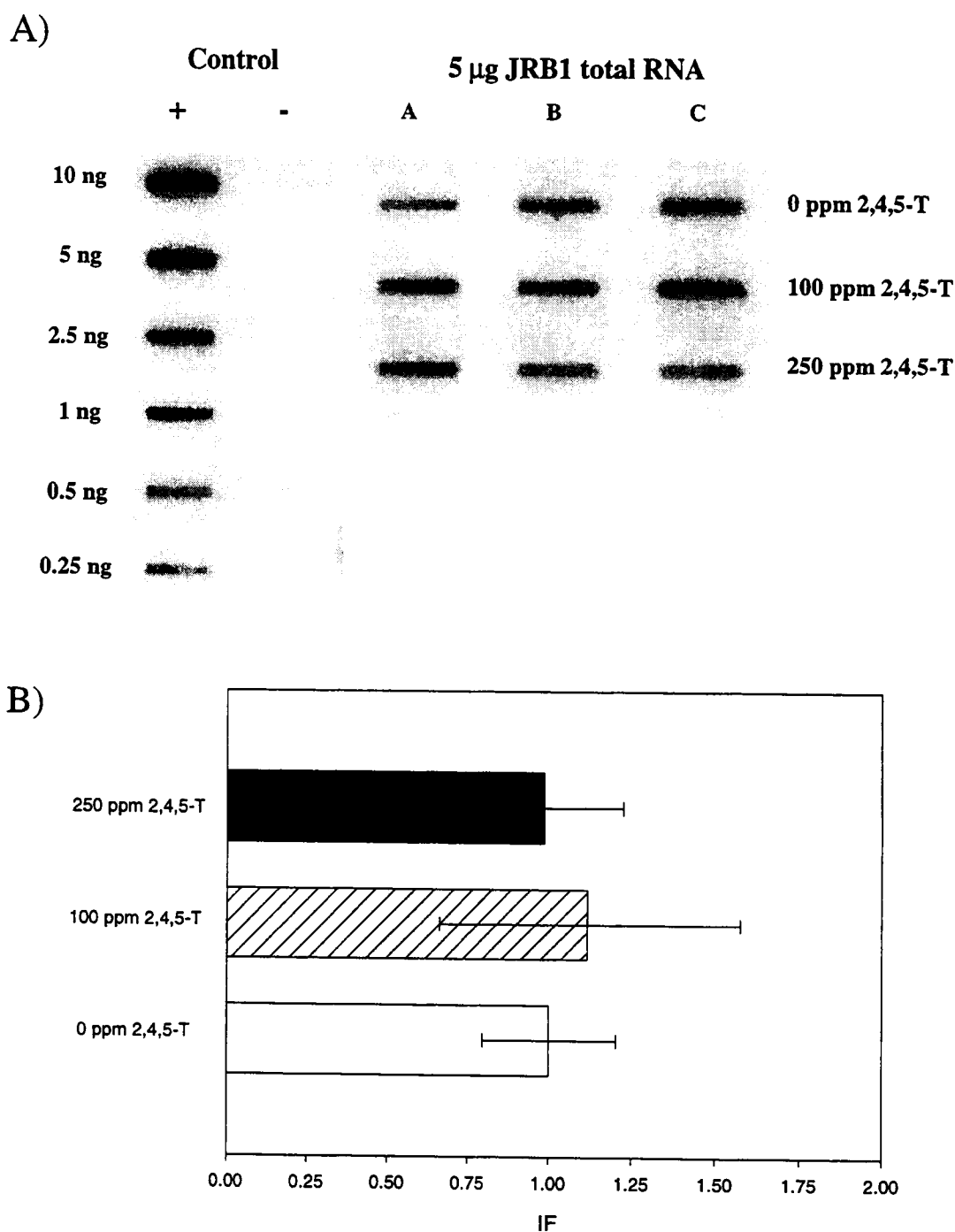


**Figure 4.12** Analysis of *tfdA* mRNA expression in response to 2,4,5-T addition for *Burkholderia* species strain JRB1. A) RNA hybridization analysis. Positive controls are shown on the left side of the figure and cover a concentration range from 0.25 ng to 10 ng. A 10 ng negative control was blotted adjacent to the 10 ng positive control. Five  $\mu$ g of total RNA isolated from cultures amended with 2,4,5-T was applied to the nylon membranes, and probed using a *tfdA* specific,  $\alpha$ - $^{32}$ P-CTP labeled, probe. B) Graphical representation of Induction Factors (IF); IF values were determined by dividing results for each treatment condition by the control value and represent the ratio of condition mRNA/control mRNA. Error bars represent the standard deviations among three separate experiments.

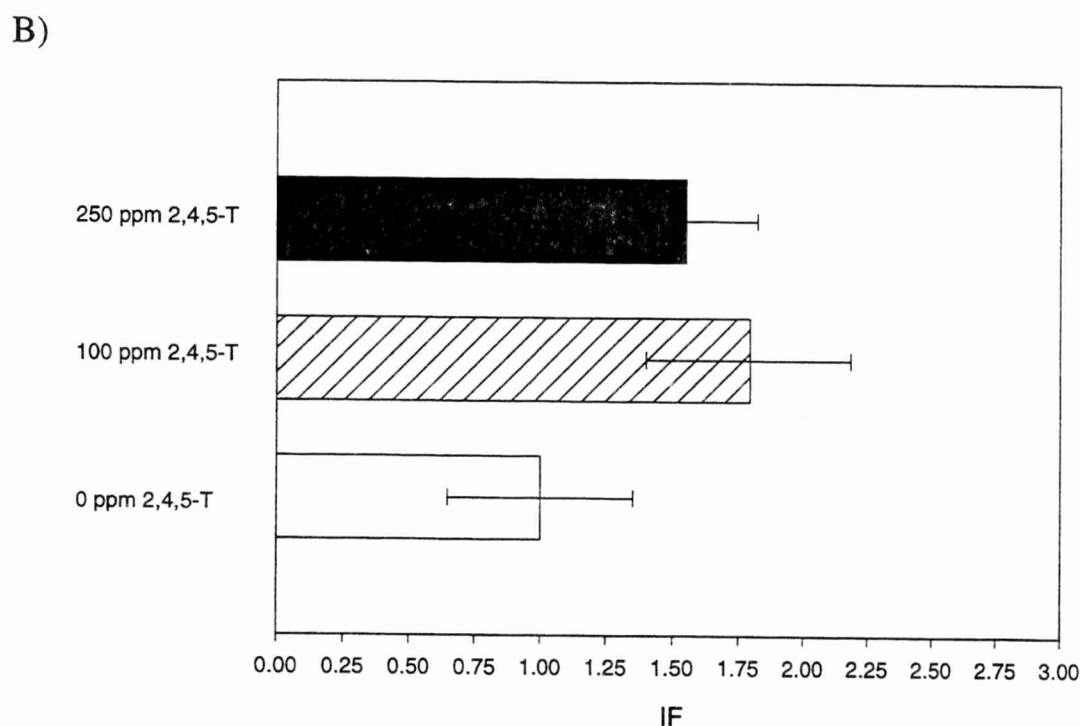
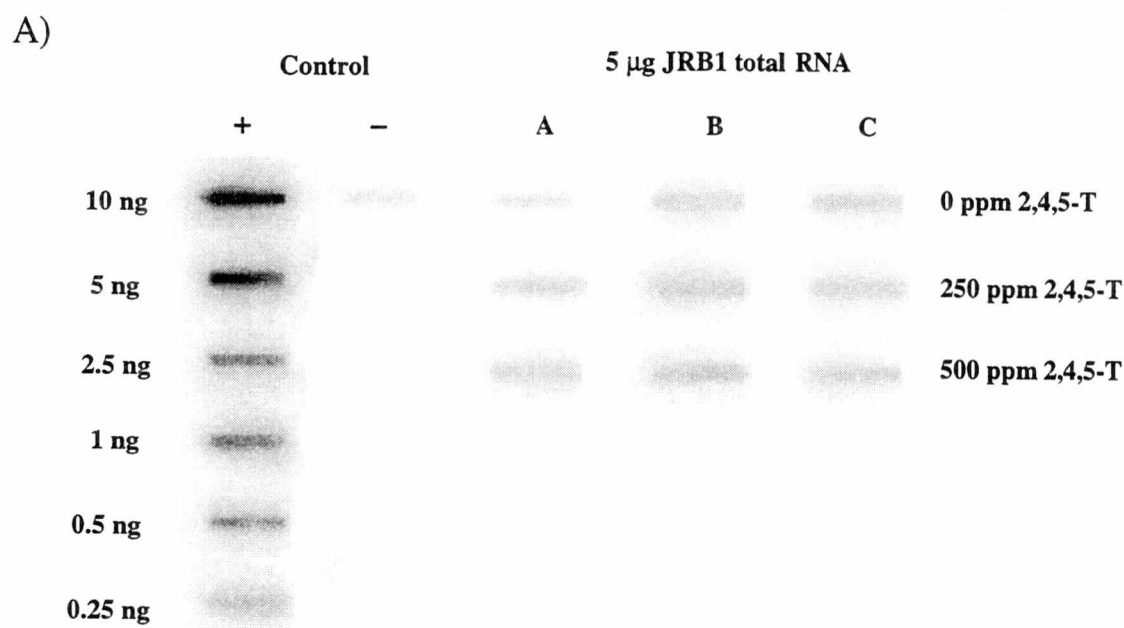


**Figure 4.13** Analysis of *tfdC* mRNA expression in response to 2,4,5-T addition for *Burkholderia* species strain JRB1. A) RNA hybridization analysis. Positive controls are shown on the left side of the figure and cover a concentration range from 0.25 ng to 10 ng. A 10 ng negative control was blotted adjacent to the 10 ng positive control. Five µg of total RNA isolated from cultures amended with 2,4,5-T was applied to the nylon membranes, and probed using a *tfdC* specific,  $\alpha$ - $^{32}$ P-CTP labeled, probe. B) Graphical representation of Induction Factors (IF); IF values were determined by dividing results for each treatment condition by the control value and represent the ratio of condition mRNA/control mRNA. Error bars represent the standard deviations among three separate experiments.

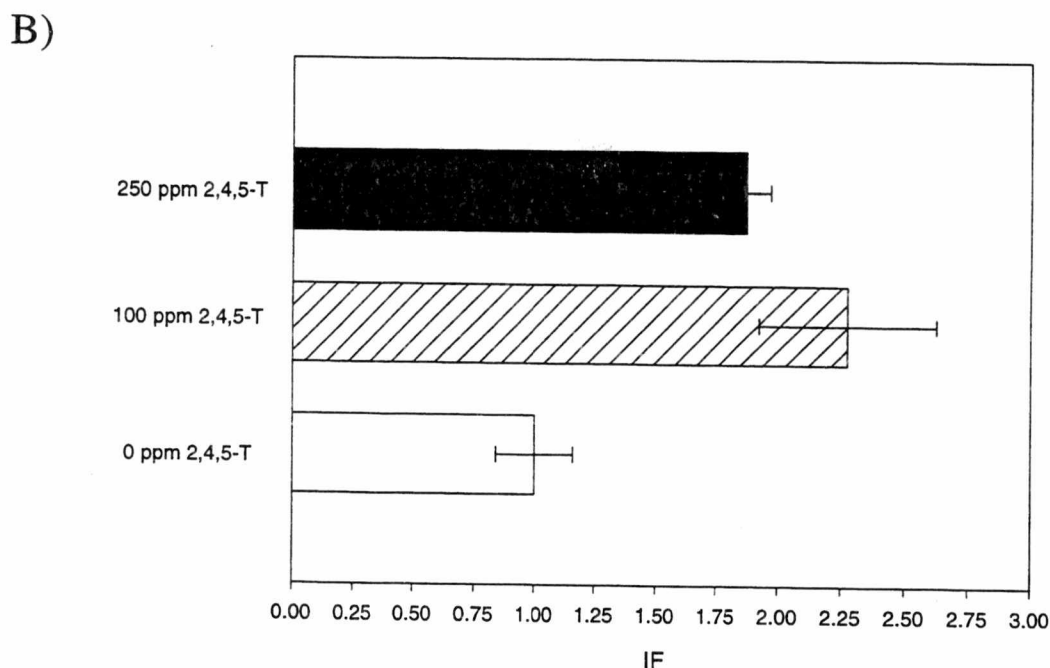
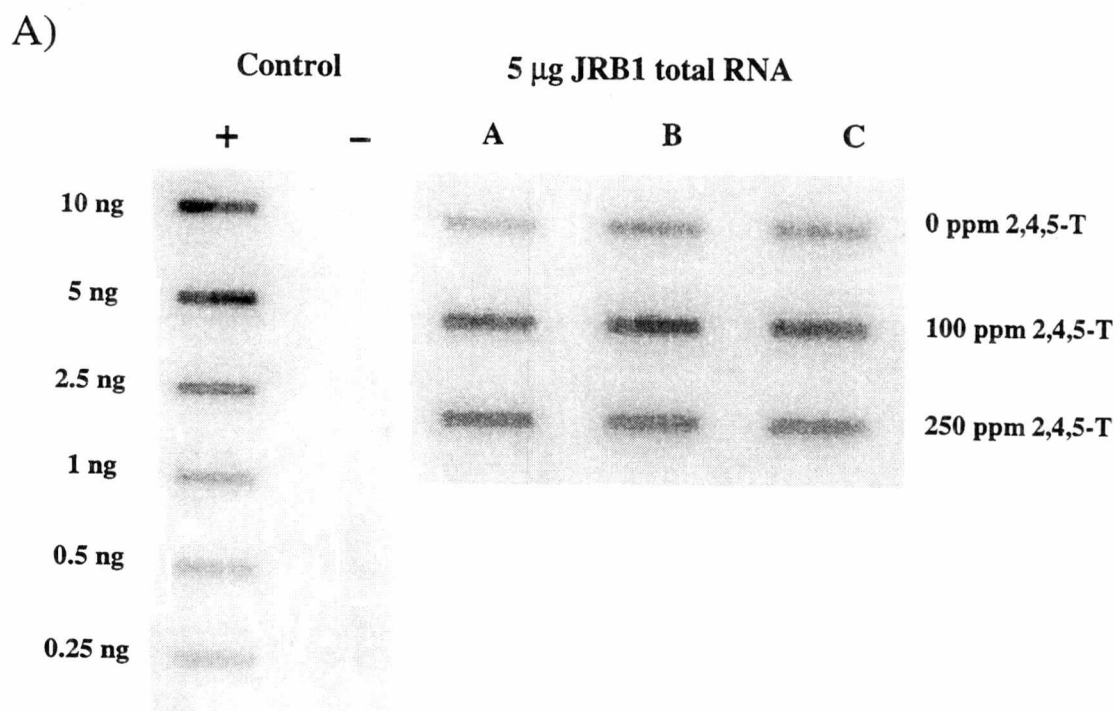


**Figure 4.14** Analysis of *tfdB* mRNA expression in response to 2,4,5-T addition for *Burkholderia* species strain JRB1. A) RNA hybridization analysis. Positive controls are shown on the left side of the figure and cover a concentration range from 0.25 ng to 10 ng. A 10 ng negative control was blotted adjacent to the 10 ng positive control. Five  $\mu$ g of total RNA isolated from cultures amended with 2,4,5-T was applied to the nylon membranes, and probed using a *tfdB* specific,  $\alpha$ - $^{32}$ P-CTP labeled, probe. B) Graphical representation of Induction Factors (IF); IF values were determined by dividing results for each treatment condition by the control value and represent the ratio of condition mRNA/control mRNA. Error bars represent the standard deviations among three separate experiments.

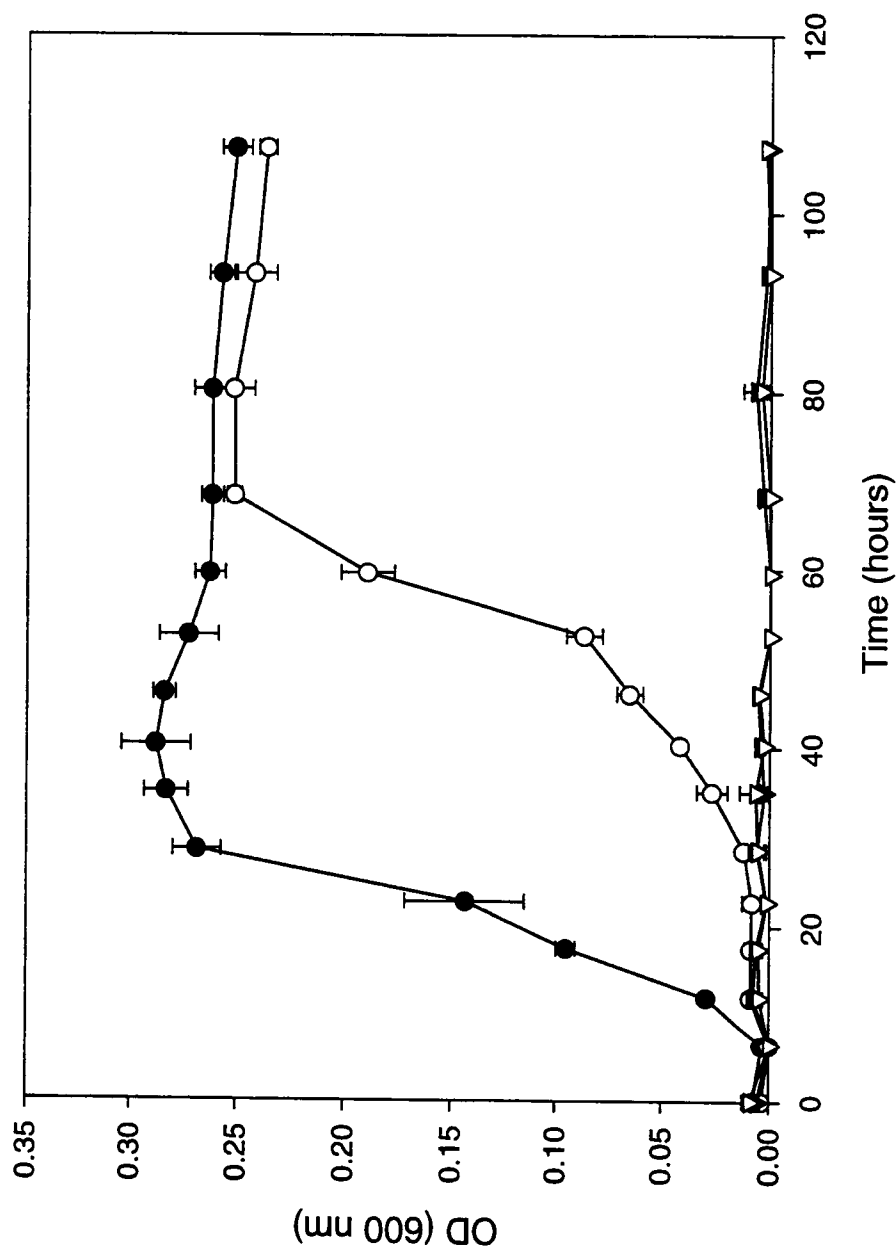


**Figure 4.15** Growth of *Burkholderia* species strain JRB1 during growth on 2,4-D in the presence of 2,4,5-TCP. Strain JRB1 was grown in PAS media containing 500 ppm 2,4-D and 0 mg/L (●), 10 mg/L (○), or 25 mg/L (▼) 2,4,5-TCP. One control culture containing 3.7 mM succinate and 25 mg/L 2,4,5-TCP (▽) was also included in this study. Data points represent the average of triplicate cultures. Error bars represent the standard deviation of among these cultures.

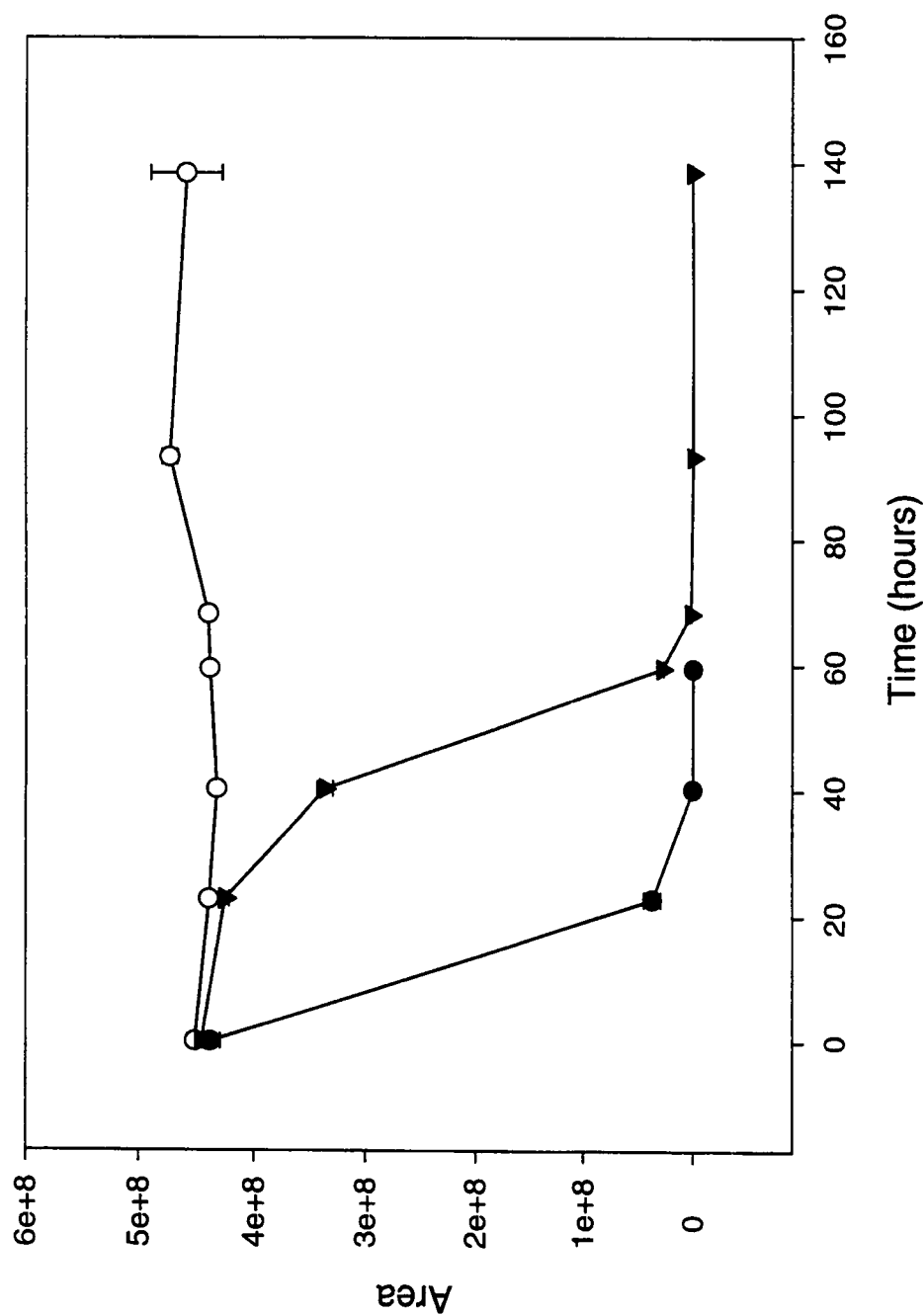


**Figure 4.16** 2,4-D degradation curves for *Burkholderia* species strain JRB1 during growth on 2,4-D in the presence of 2,4,5-TCP. Strain JRB1 was grown in PAS media containing 500 ppm 2,4-D and 0 mg/L (●), 10 mg/L (O), or 25 mg/L (▼) 2,4,5-T. Data points represent the average of triplicate cultures. Error bars represent the standard deviation of among these cultures.

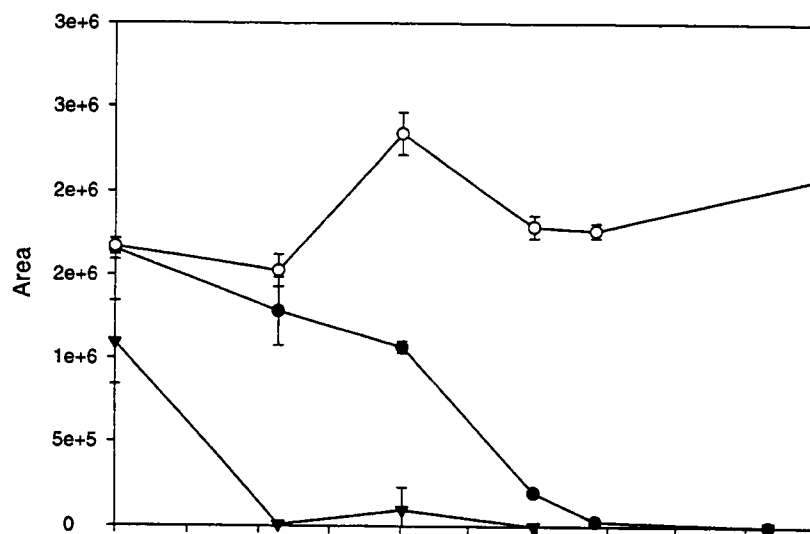
growth (Figure 4.15 and 4.16). Degradation of 2,4-D corresponded with decreasing 2,4,5-TCP concentrations in culture supernatants (Figure 4.17B), and an increased growth rate was observed once 2,4,5-TCP was removed. Removal of 2,4-DCP from these cultures mirrored 2,4,5-TCP disappearance (Figure 4.17A). Initial production of 2,4-DCP up to 0.8 mg/L and 1.3 mg/L for 0 mg/L 2,4,5-TCP and both 10 mg/L and 25 mg/L 2,4,5-TCP, respectively (Figure 4.17A) was followed by a complete removal of 2,4-DCP over after 20 hours for control cultures and 60 hours for culture containing 10 mg/L 2,4,5-TCP. Cultures containing 25 mg/L demonstrated a maximum 2,4-DCP concentration of 1.88 mg/L but exhibited no further production or degradation of 2,4-DCP. An examination of 10 mg/L and 25 mg/L treatments on expression of the *tfdA* and *tfdC* genes demonstrated that neither of treatments apparently affected the abundance of these transcripts (Figure 4.18 and 4.19). A similar experiment examining *tfdB* expression showed that although 10mg/L 2,4,5-TCP had no affect, 25 mg/L 2,4,5-TCP resulted in an apparent upregulation of this catabolic gene (Figure 4.20).

***4.4.4 Increases in 2,4,5-trichlorophenol concentrations were observed during growth of a microbial consortium containing Burkholderia species strain JR7B2 and Burkholderia species strain JR7B3 on 2,4,5-T in the presence of either glucose or succinate.***

Growth on glucose was delayed for Agent Orange enrichment cultures inoculated into PAS media supplemented with 1000 mg/ml 2,4,5-T and 5.6 mM glucose compared to control cultures containing only glucose (Figure 4.21). For these cultures, 1.9 ppm

A)

120



B)

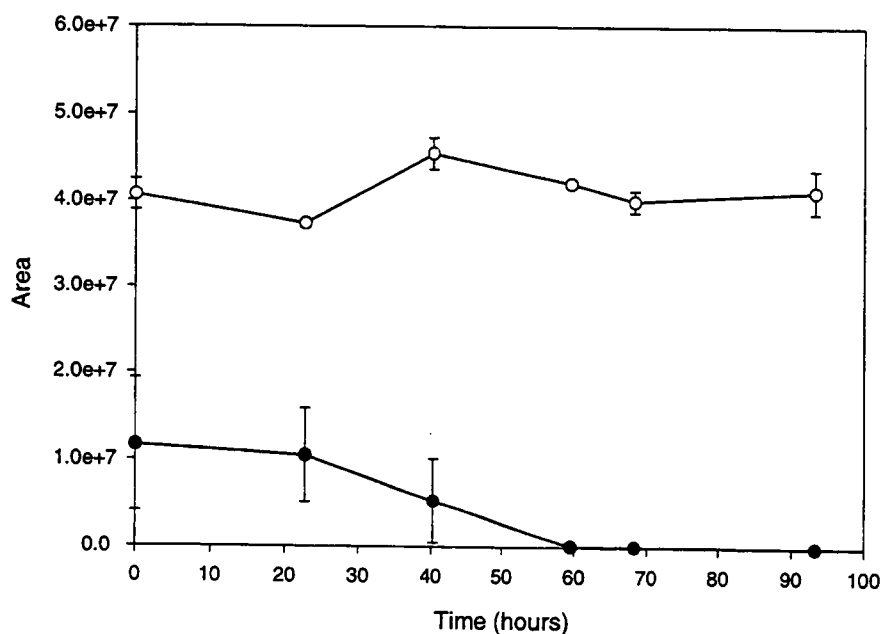


**Figure 4.17** 2,4-DCP (A) and 2,4,5-TCP (B) accumulation and degradation curves for *Burkholderia* species strain JRB1 in the presence of 0 mg/L (▼), 10 mg/L (●), 25 mg/L (○) 2,4,5-TCP. Each culture was grown in PAS containing 500 mg/L 2, 4-D and amended with the appropriate amount of 2,4,5-TCP. Data points represent the average of triplicate cultures. Error bars represent the standard deviation of among these cultures.

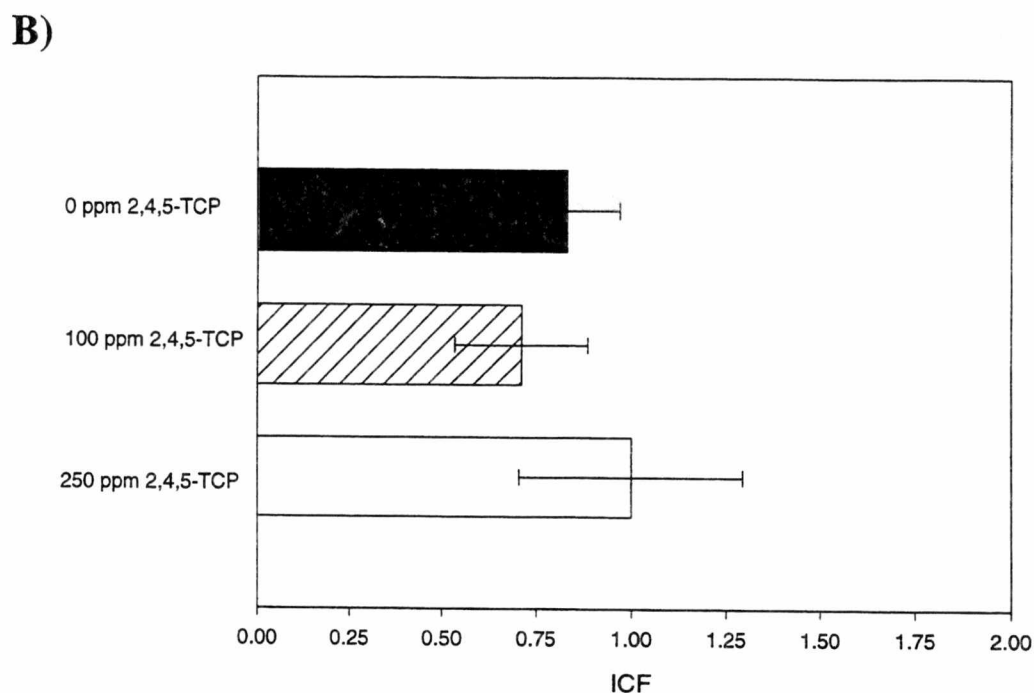
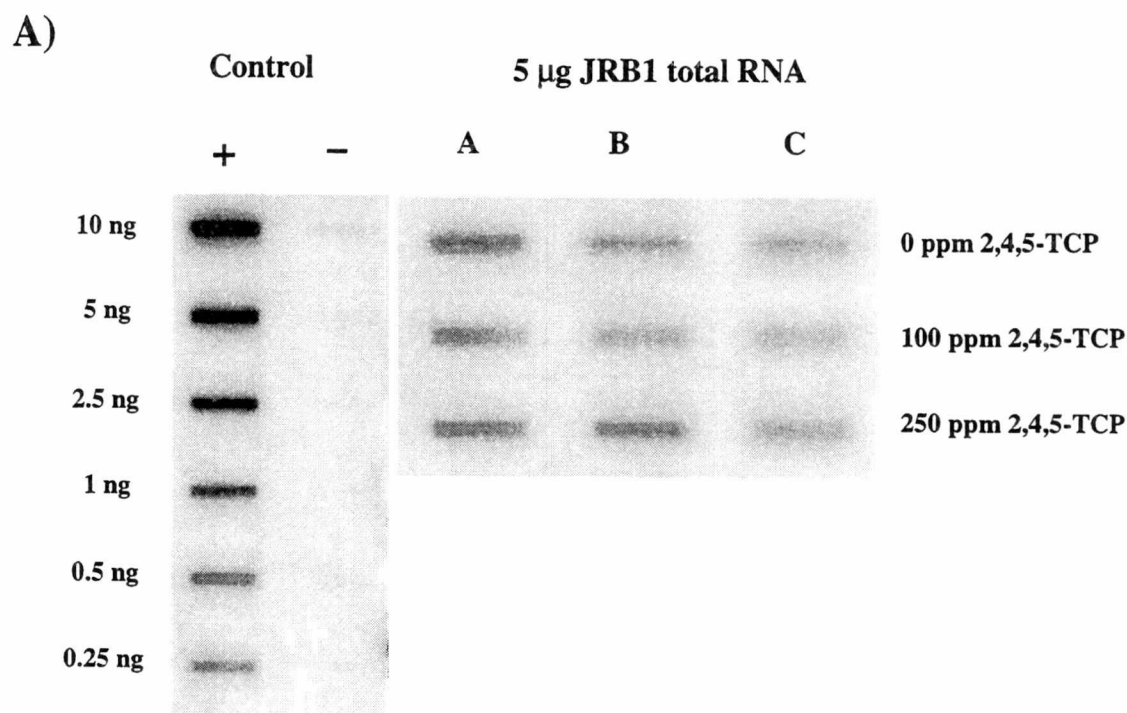
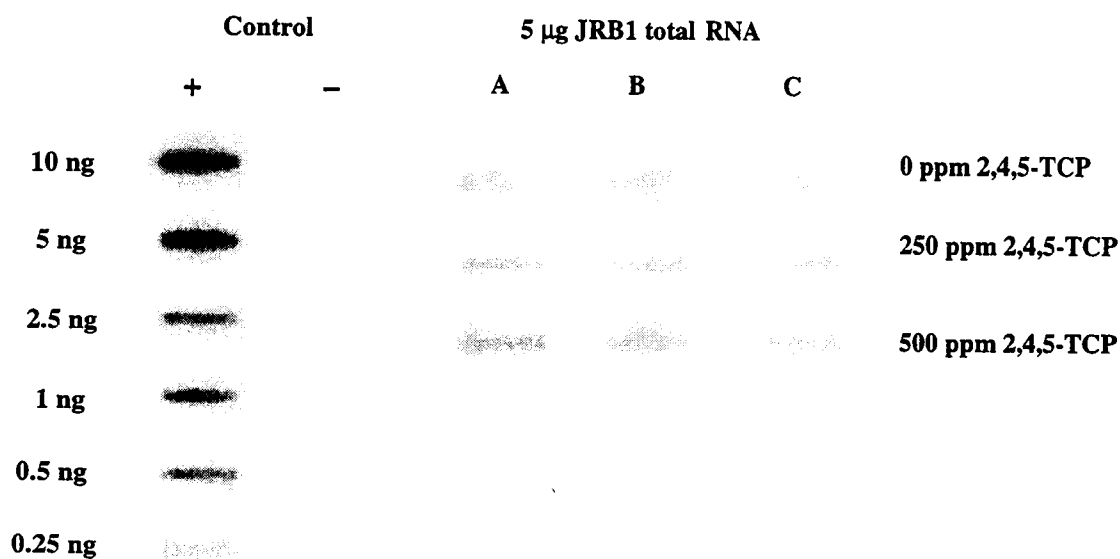


**Figure 4.18** Analysis of *tfdA* mRNA expression in response to 2,4,5-TCP addition for *Burkholderia* species strain JRB1. A) RNA hybridization analysis. Positive controls are shown on the left side of the figure and cover a concentration range from 0.25 ng to 10 ng. A 10 ng negative control was blotted adjacent to the 10 ng positive control. Five  $\mu$ g of total RNA isolated from cultures amended with 2,4,5-TCP was applied to the nylon membranes, and probed using a *tfdA* specific,  $\alpha$ - $^{32}$ P-CTP labeled, probe. B) Graphical representation of Induction Factors (IF); IF values were determined by dividing results for each treatment condition by the control value and represent the ratio of condition mRNA/control mRNA. Error bars represent the standard deviations among three separate experiments.

A)



B)

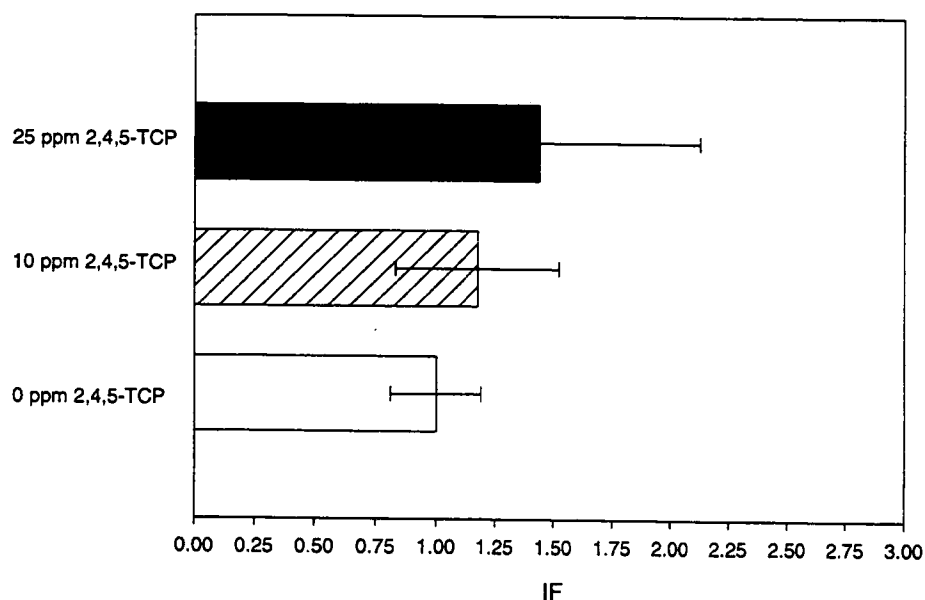


**Figure 4.19** Analysis of *tfdC* mRNA expression in response to 2,4,5-TCP addition for *Burkholderia* species strain JRB1. A) RNA hybridization analysis. Positive controls are shown on the left side of the figure and cover a concentration range from 0.25 ng to 10 ng. A 10 ng negative control was blotted adjacent to the 10 ng positive control. Five µg of total RNA isolated from cultures amended with 2,4,5-TCP was applied to the nylon membranes, and probed using a *tfdC* specific,  $\alpha$ - $^{32}$ P-CTP labeled, probe. B) Graphical representation of Induction Factors (IF); IF values were determined by dividing results for each treatment condition by the control value and represent the ratio of condition mRNA/control mRNA. Error bars represent the standard deviations among three separate experiments.

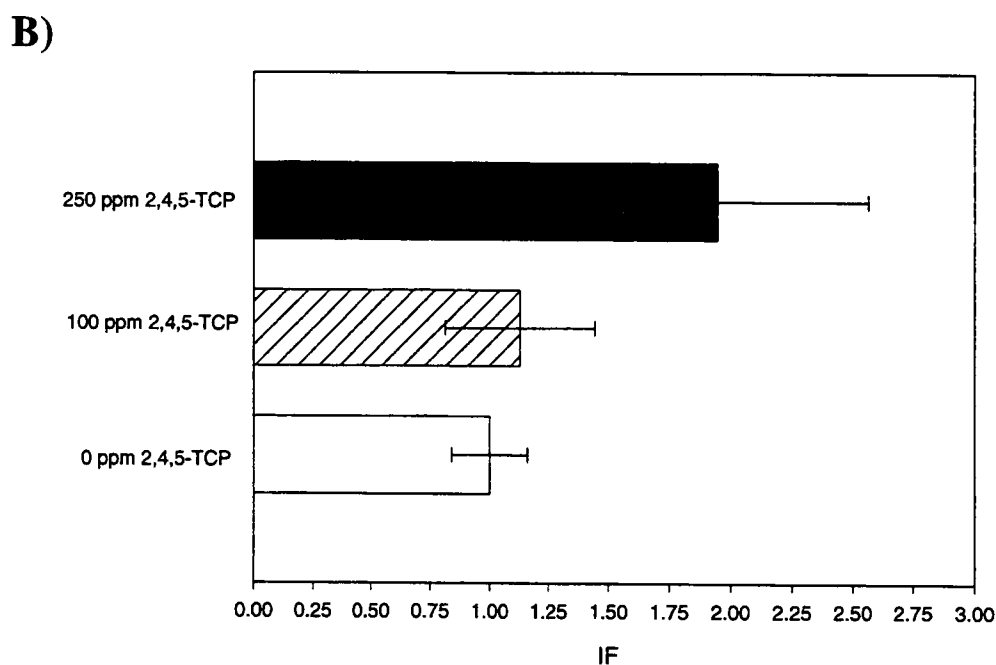
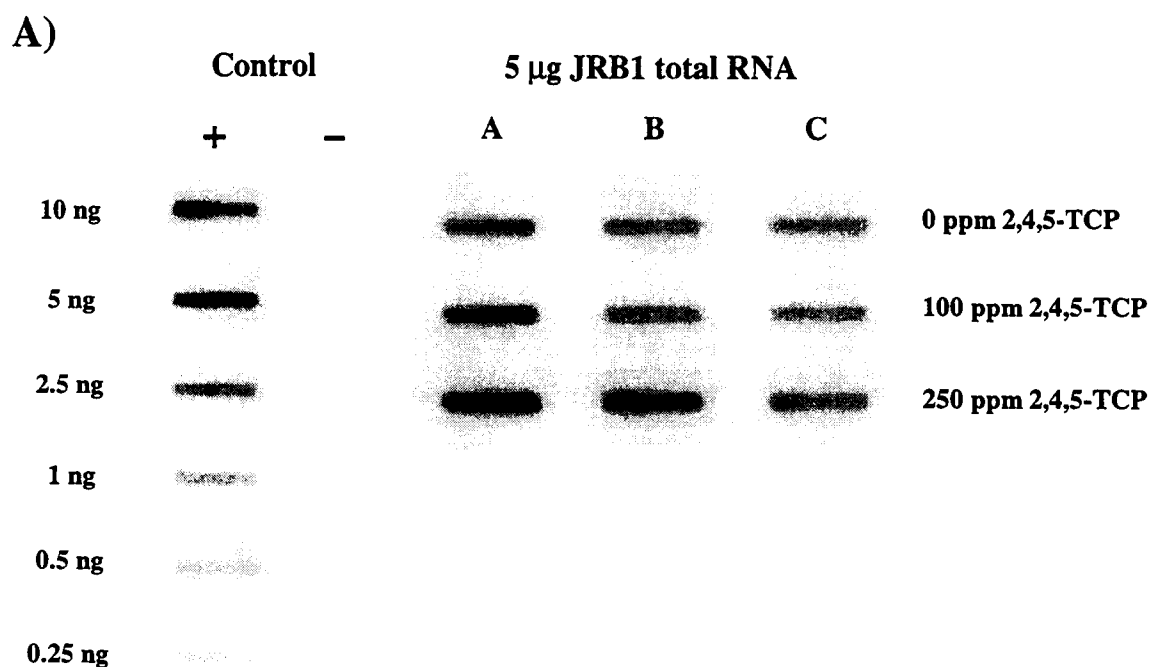


**Figure 4.20** Analysis of *tfdB* mRNA expression in response to 2,4,5-TCP addition for *Burkholderia* species strain JRB1. A) RNA hybridization analysis. Positive controls are shown on the left side of the figure and cover a concentration range from 0.25 ng to 10 ng. A 10 ng negative control was blotted adjacent to the 10 ng positive control. Five µg of total RNA isolated from cultures amended with 2,4,5-TCP was applied to the nylon membranes, and probed using a *tfdB* specific,  $\alpha$ - $^{32}$ P-CTP labeled, probe. B) Graphical representation of Induction Factors (IF); IF values were determined by dividing results for each treatment condition by the control value and represent the ratio of condition mRNA/control mRNA. Error bars represent the standard deviations among three separate experiments.

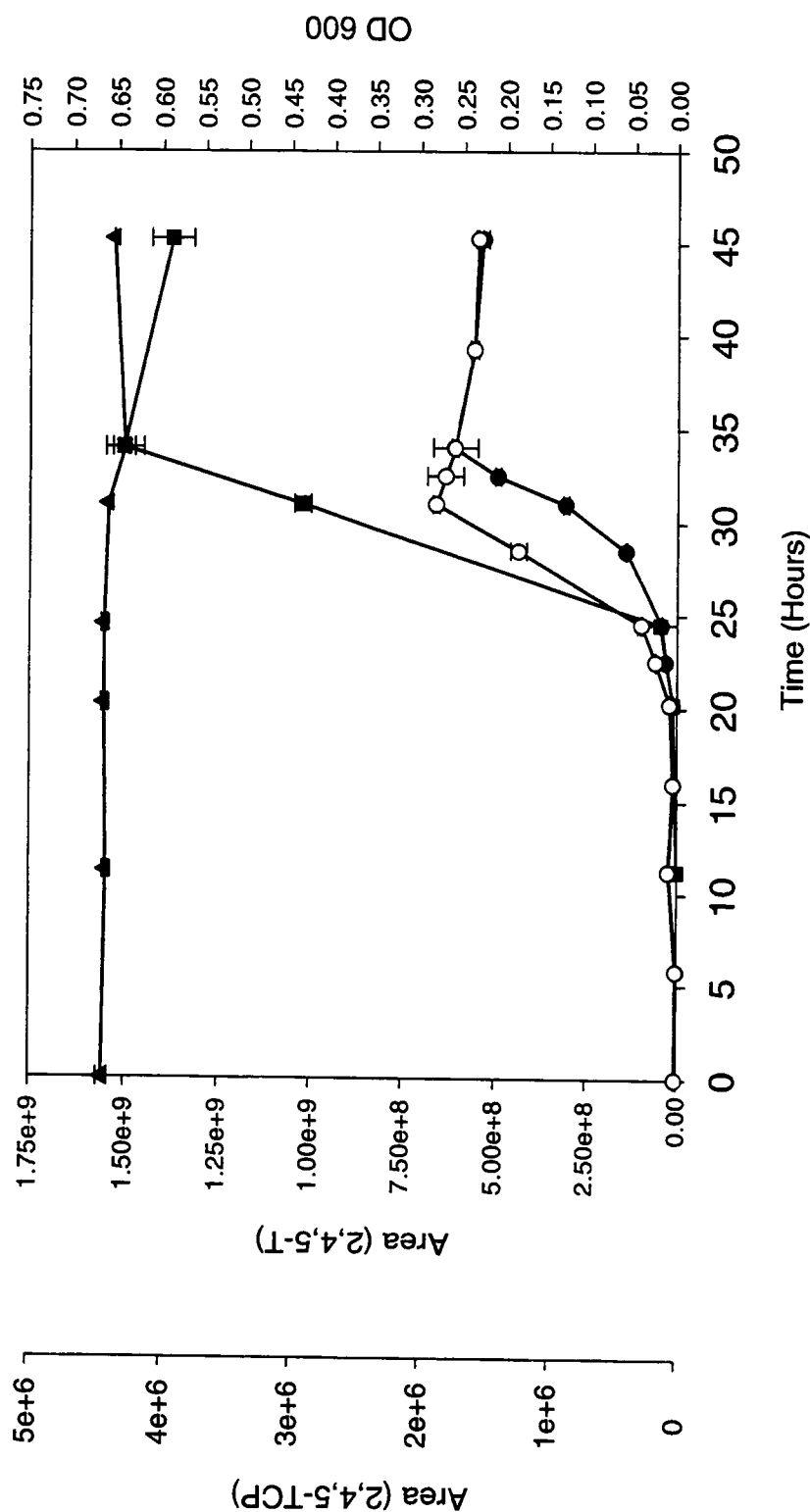


**Figure 4.21** Effects of glucose on 2,4,5-T mediated growth and degradation for Agent Orange enrichment culture JR7B. PAS cultures supplemented with 1000 mg/L 2,4,5-T and 5.6 mM glucose were the source of samples for HPLC analysis to determine 2,4,5-T concentrations (▲) and 2,4,5-TCP concentrations (■). The optical density of these cultures (●) was also determined spectrophotometrically (600 nm). The optical density of a control culture (○) containing only PAS amended with 3.7 mM glucose was included for growth comparisons. Data points represent the average of triplicate cultures. Error bars represent the standard deviation of among these cultures.

2,4,5-TCP was produced in correspondence with growth on glucose, and a slight decrease in 2,4,5-T concentrations was observed. In comparison, succinate addition did not result in a significant growth delay during succinate utilization and 2,4,5-TCP production was limited to 0.399 mg/L (Figure 4.22). Controls demonstrated no 2,4,5-T degradation and 2,4,5-TCP were maintained at background concentrations (Figure 4.23). These results describe the 1<sup>st</sup> 45 hours of growth during growing cell assays containing either PAS + 2,4,5-T, PAS + 2,4,5-T + glucose, and PAS + 2,4,5-T + succinate (appropriate controls were included for these cultures, specifically biotic, abiotic, glucose only, and succinate only). During this first 45 hours of growth, the reproducibility between replicates was high. However once cells had exhausted either glucose or succinate, respectively, reproducibility was diminished. Figures 4.24, 4.25, and 4.26 describe growth and degradation of 2,4,5-T after continued incubation for these cultures. Growth on 2,4,5-T as a sole carbon and energy source occurred slowly and 2,4,5-TCP concentrations were maintained at or below approximately 100 µg/L (Figure 4.24). From this growth experiment, it seems apparent that the inherent variation between three replicates was due to inconsistencies in initiation of growth on 2,4,5-T.

Initial 2,4,5-T degradation by glucose and succinate supplemented cultures proceeded more rapidly than cultures containing only 2,4,5-T (Figure 4.25 and 4.26). 2,4,5-TCP production after glucose and succinate mediated growth corresponded with 2,4,5-T degradation (Figure 4.25 and 4.26) reaching maximum average concentrations of 13.9 and 19.0, respectively. Succinate cultures appeared to have the most difficulty dealing with these high 2,4,5-TCP concentrations and the time of growth lag

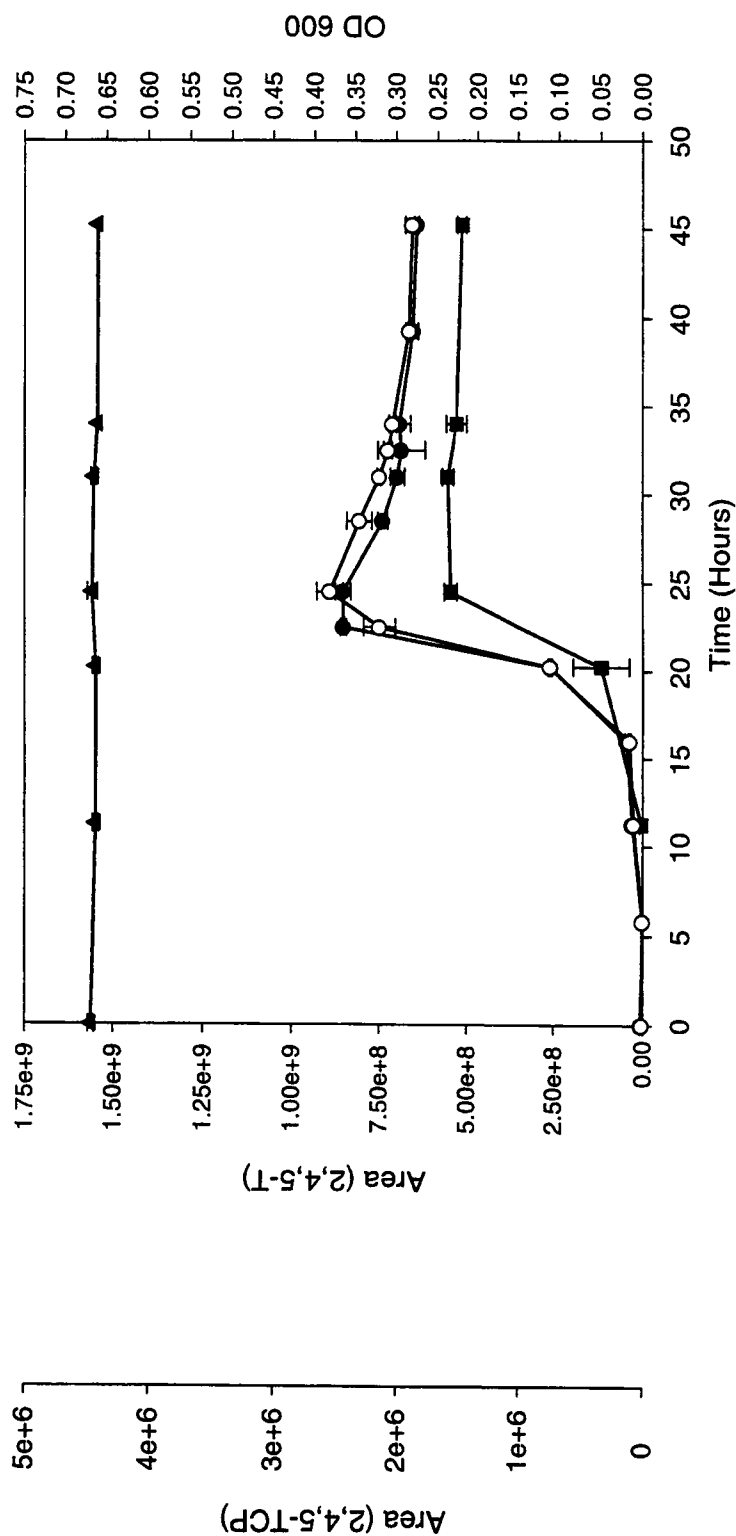


**Figure 4.22** Effects of succinate on 2,4,5-T mediated growth and degradation for Agent Orange enrichment culture JR7B. PAS cultures supplemented with 1000 mg/L 2,4,5-T and 3.7 mM succinate were the source of samples for HPLC analysis to determine 2,4,5-T concentrations (▲) and 2,4,5-TCP concentrations (■). The optical density of these cultures (●) was also determined spectrophotometrically (600 nm). The optical density of a control culture (O) containing only PAS amended with 3.7 mM succinate was included for growth comparisons. Data points represent the average of triplicate cultures. Error bars represent the standard deviation of among these cultures.

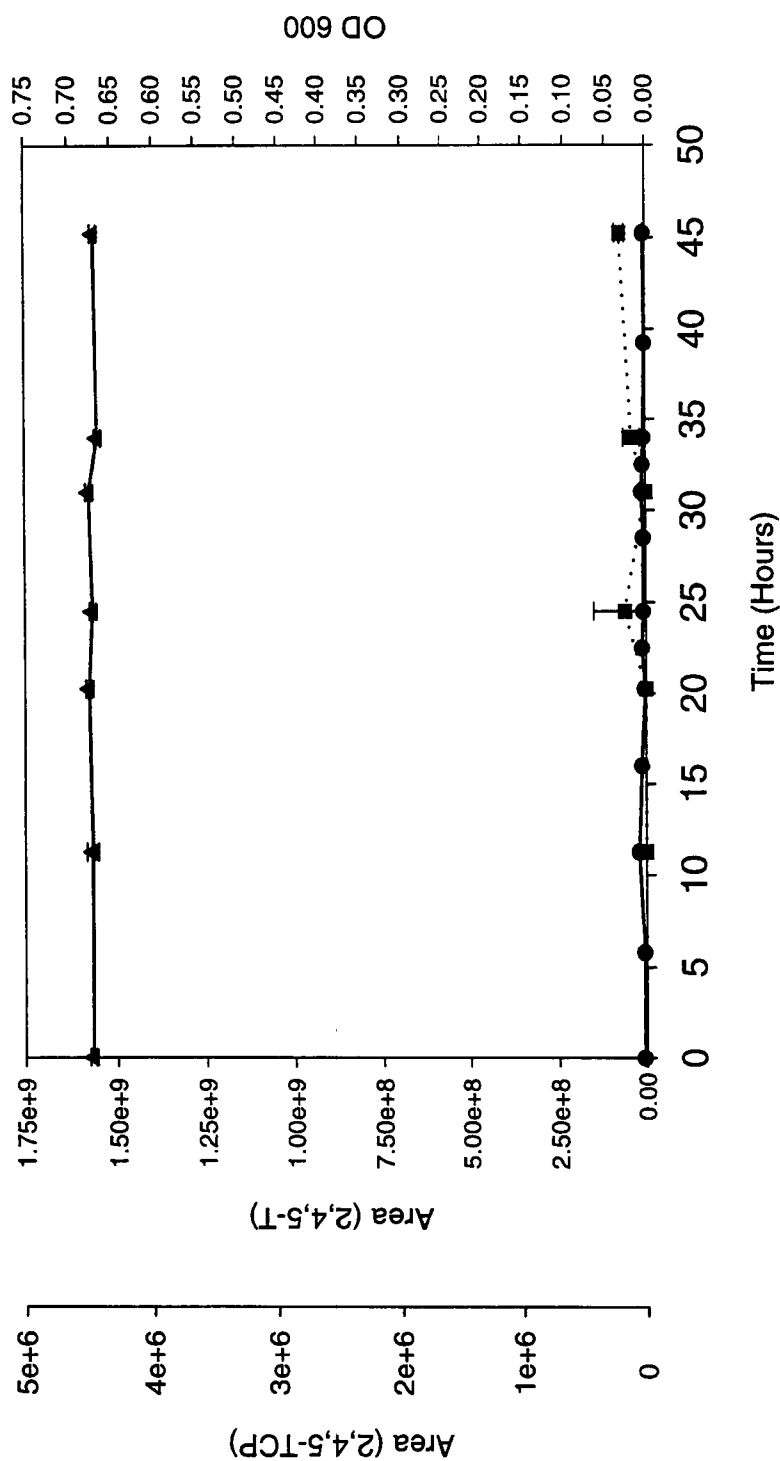


**Figure 4.23** Initial 2,4,5-T mediated growth and degradation curves for Agent Orange enrichment culture JR7B. PAS cultures supplemented with 1000 mg/L 2,4,5-T were the source of samples for HPLC analysis to determine 2,4,5-T concentrations ( $\blacktriangle$ ) and 2,4,5-TCP concentrations ( $\blacksquare$ ). The optical density of these cultures ( $\bullet$ ) was also determined spectrophotometrically at 600 nm. Data points represent the average of triplicate cultures. Error bars represent the standard deviation of among these cultures.

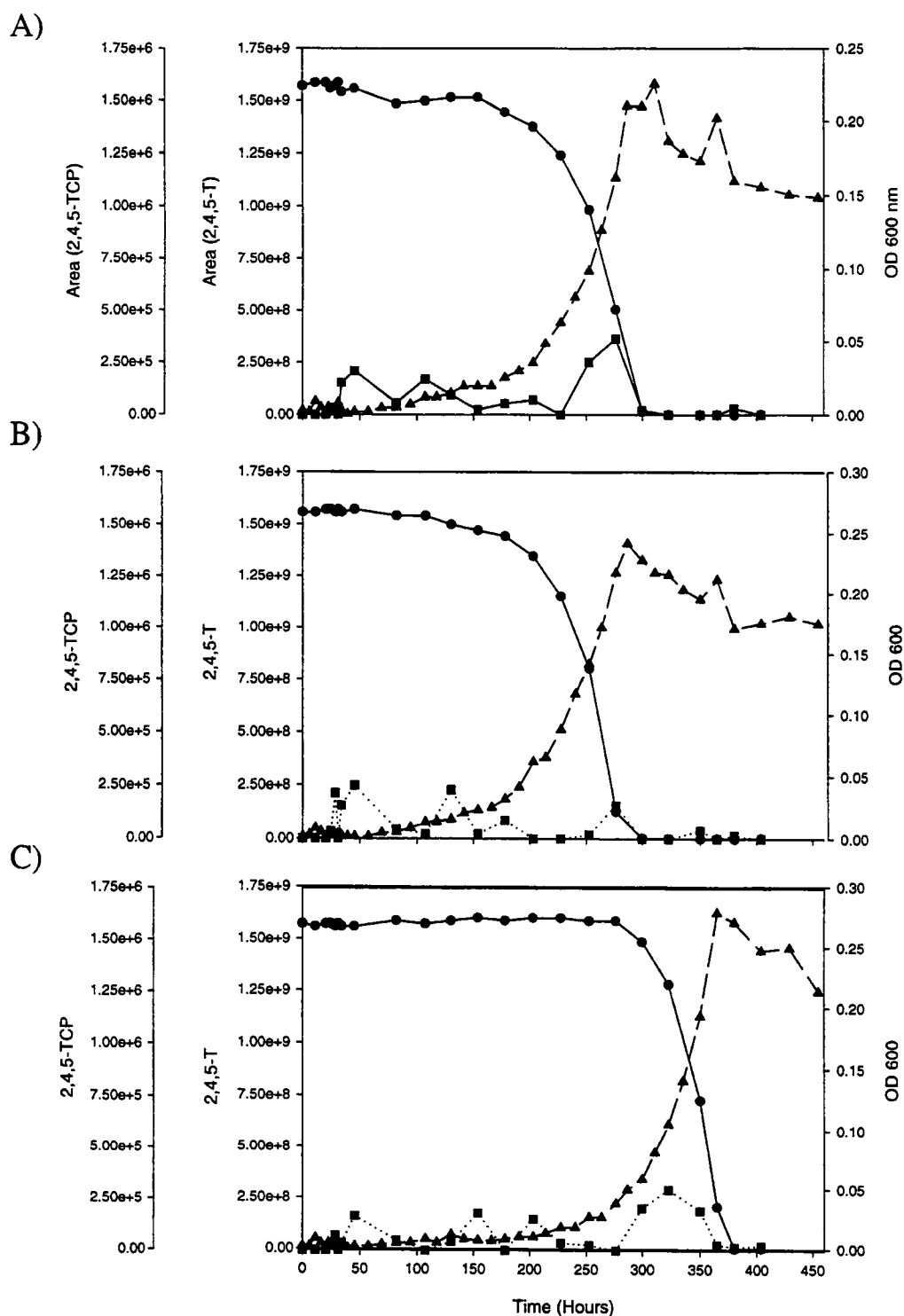


**Figure 4.24** 2,4,5-T mediated growth and degradation curves for Agent Orange enrichment cultures JR7B containing 1000 mg/L 2,4,5-T. A), B), and C) represent three separate cultures. All cultures were grown in PAS media amended with 1000 mg/L 2,4,5-T under identical growth conditions. HPLC was used to determine 2,4,5-T concentrations (●) and 2,4,5-TCP concentrations (■). The optical density of these cultures (▲) was determined spectrophotometrically at 600 nm.

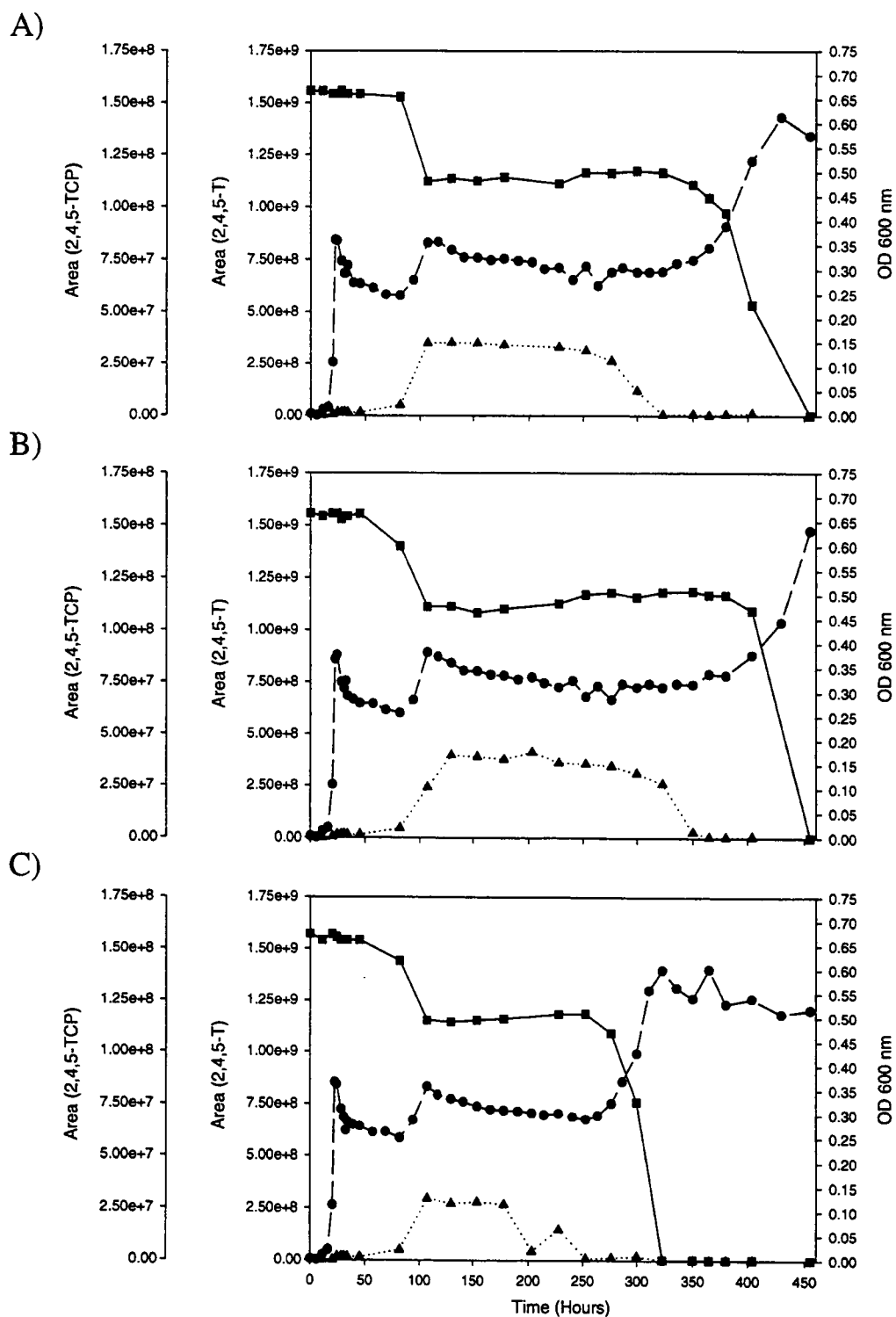
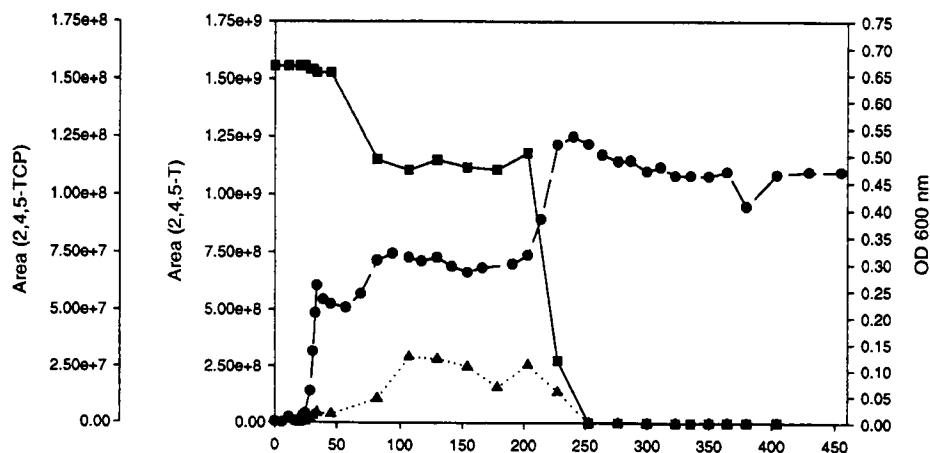
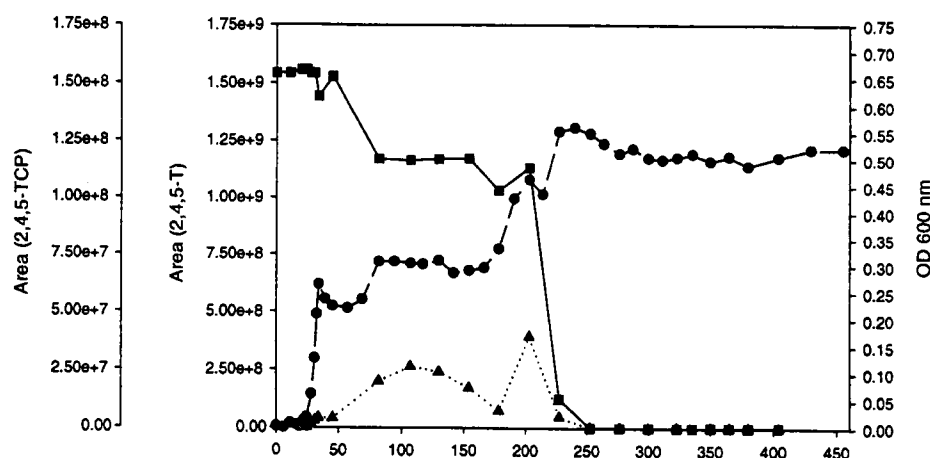


**Figure 4.25** 2,4,5-T mediated growth and degradation curves for Agent Orange enrichment cultures JR7B containing 1000 mg/L 2,4,5-T and 3.7 mM succinate A), B), and C) represent three separate cultures. All cultures were grown in PAS media amended with 1000 mg/L 2,4,5-T + 3.7 mM succinate under identical growth conditions. HPLC was used to determine 2,4,5-T concentrations (■) and 2,4,5-TCP concentrations (▲). The optical density of these cultures (●) was determined spectrophotometrically at 600 nm.

A)



B)



C)

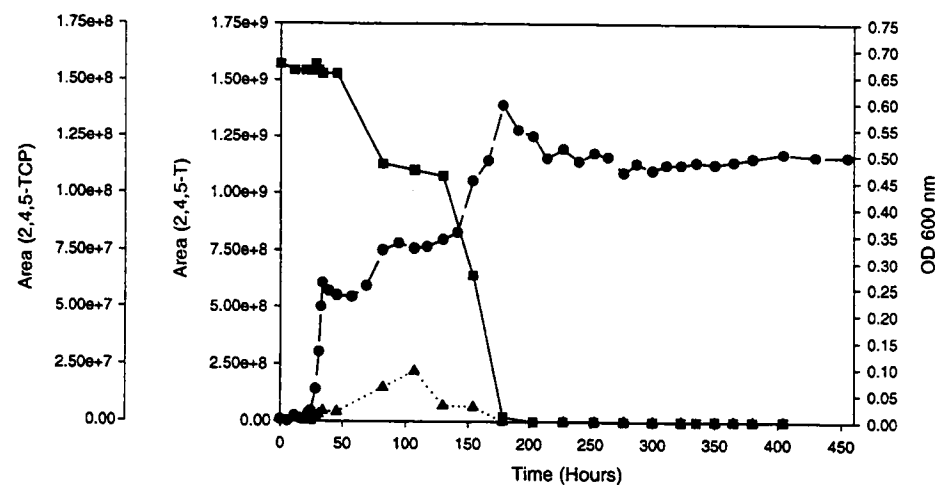


**Figure 4.26** 2,4,5-T mediated growth and degradation curves for Agent Orange enrichment cultures JR7B containing 1000 mg/L 2,4,5-T and 5.6 mM glucose. A), B), and C) represent three separate cultures. All cultures were grown in PAS media amended with 1000 mg/L 2,4,5-T + 5.6 mM glucose under identical growth conditions. HPLC was used to determine 2,4,5-T concentrations (■) and 2,4,5-TCP concentrations (▲). The optical density of these cultures (●) was determined spectrophotometrically at 600 nm.

corresponded with the concentration of 2,4,5-TCP that had accumulated in the culture medium (Figure 4.25). For glucose grown cells, generally less 2,4,5-TCP was produced, and these cells degraded all of the 2,4,5-T present within 250 hours compared to 350 - 450 hours for succinate cultures (Figure 4.26).

***4.4.5 Auxotrophic growth of Burkholderia species strain JR7B3 in pure culture containing 2,4,5-T and glucose resulted in toxic levels of 2,4,5-TCP which inhibited growth and degradation of 2,4,5-T.***

The presence of mixed inhibitory organic substances has been demonstrated to be problematic during aerobic batch cultures (Mungkarndee et al. 1997). These studies reported that 40 mg/L or greater 2,4-DCP inhibited degradation of 2,4-D and transformation of 4-chloro-2-methylphenol (4C2MP) was completely inhibited. It was also suggested that there were interactions among all three of these compounds, and that 4C2MP inhibited 2,4-D metabolism and resulted in accumulation of 2,4-DCP. Additional experiments in which exogenous 2,4-DCP was added showed that 2,4-D inhibited both 2,4-D and 4C2MP degradation. Furthermore it was suggested that utilization of 2,4-DCP was the rate-limiting step for 2,4-D degradation in their system. In light of the results from experiments using the 2,4,5-T degrading consortia isolated from Agent Orange contaminated soil, *Burkholderia* species strain JR7B3 was studied in pure culture to attempt to further identify the effects of glucose and succinate on bacterial catabolism of 2,4,5-T.

Growth in PAS medium containing 2,4,5-T, 0.01% yeast extract, and 2.5 mM glucose was severely limited for *Burkholderia* species strain JR7B3 (Figure 4.27). Under these growth conditions the optical density of the culture reached only 0.25 compared to glucose controls that reached an OD of 0.72. Also, 2,4,5-TCP accumulated to 20.5 mg/L and was maintained at this high level for the duration of these experiments (Figure 4.27). Further growth for these cultures was never observed. In comparison to glucose cultures, succinate cultures demonstrated only a slight delay in growth in the presence of 2,4,5-T and the initial biomass was approximately equivalent to succinate controls (Figure 4.28). Succinate cultures containing 2,4,5-T exhibited only slight accumulations of 2,4,5-TCP, 0.322 mg/L (Figure 4.28). These cultures sustained a higher level of biomass throughout the course of the experiment and removed greater than  $\frac{1}{2}$  of the 2,4,5-T present after 260 hours. Cultures containing only 2,4,5-T and 0.01% yeast extract exhibited an intermediate growth and degradation profile. For these cultures, initial degradation of 2,4,5-T was apparent and coincided with accumulation of 2,4,5-TCP (14.32 mg/L) and bacterial growth on residual carbon present in the yeast extract (Figure 4.29). For these cultures 2,4,5-T was completely removed after 260 hours and correlated well with diminishing levels of 2,4,5-TCP and increasing biomass (Figure 4.29). An analysis of *tftA* and *tftC* mRNA abundance for cells grown in PAS + Casamino acids (a general non-inducing carbon source) amended with succinate or glucose demonstrated that expression profiles for all conditions tested were similar after accounting for experimental variation (Figure 4.30, 4.31, 4.32, and 4.33). However, cultures amended with glucose, demonstrated increased *tftA* mRNA abundance (Figure

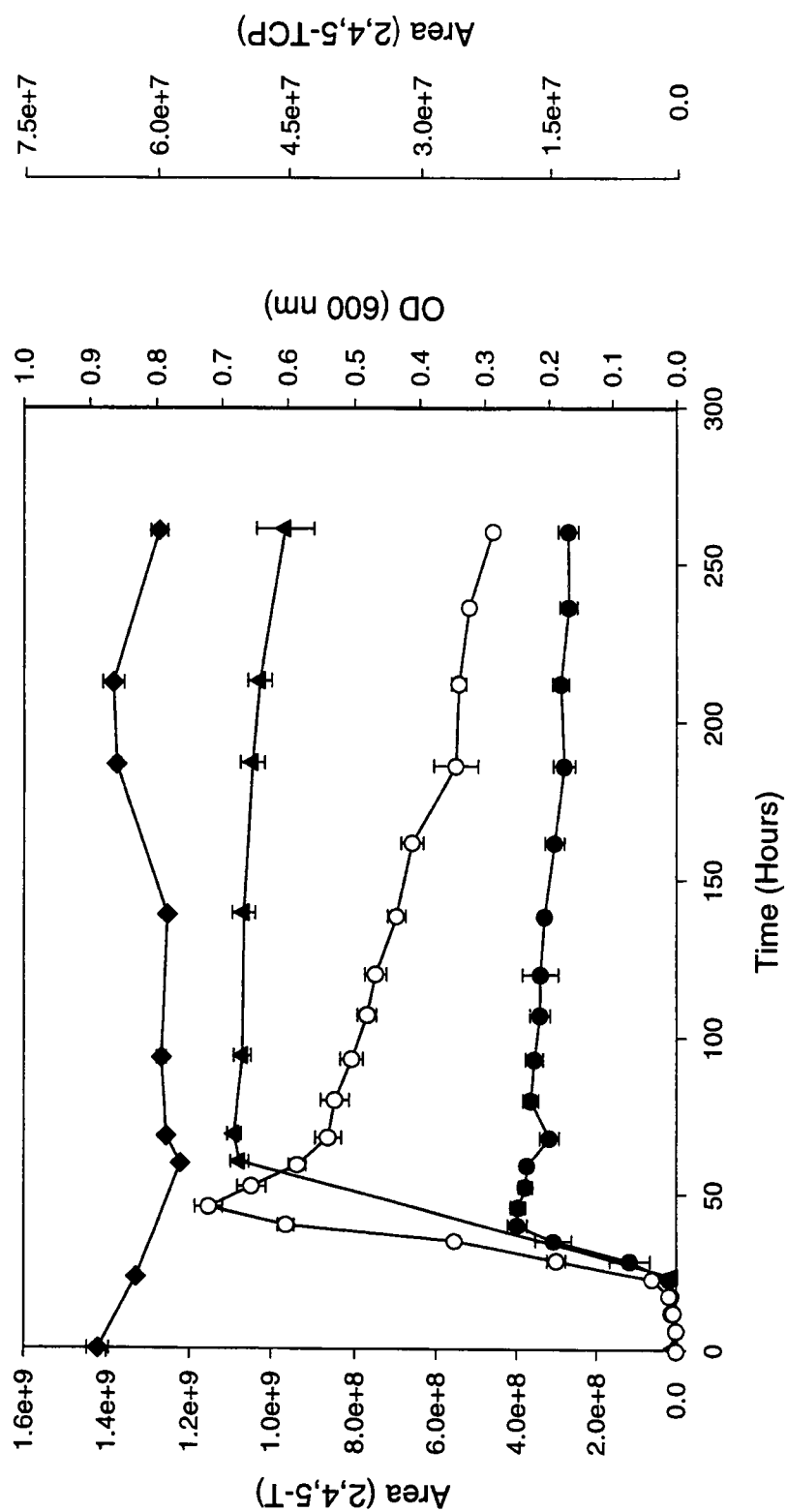


**Figure 4.27** 2,4,5-T mediated growth and degradation curves for *Burkholderia* species strain JR7B3 cultures containing 1000 mg/L 2,4,5-T and 2.5 mM glucose. PAS cultures supplemented with 2,4,5-T and glucose were the source of samples for HPLC analysis to determine 2,4,5-T concentrations (◆) and 2,4,5-TCP concentrations (▲). The optical density of these cultures (○) was also determined spectrophotometrically (600 nm). A control culture containing only PAS + 2.5 mM glucose (○) was also included in this experiment. Data points represent the average of triplicate cultures. Error bars represent the standard deviation of among these cultures.

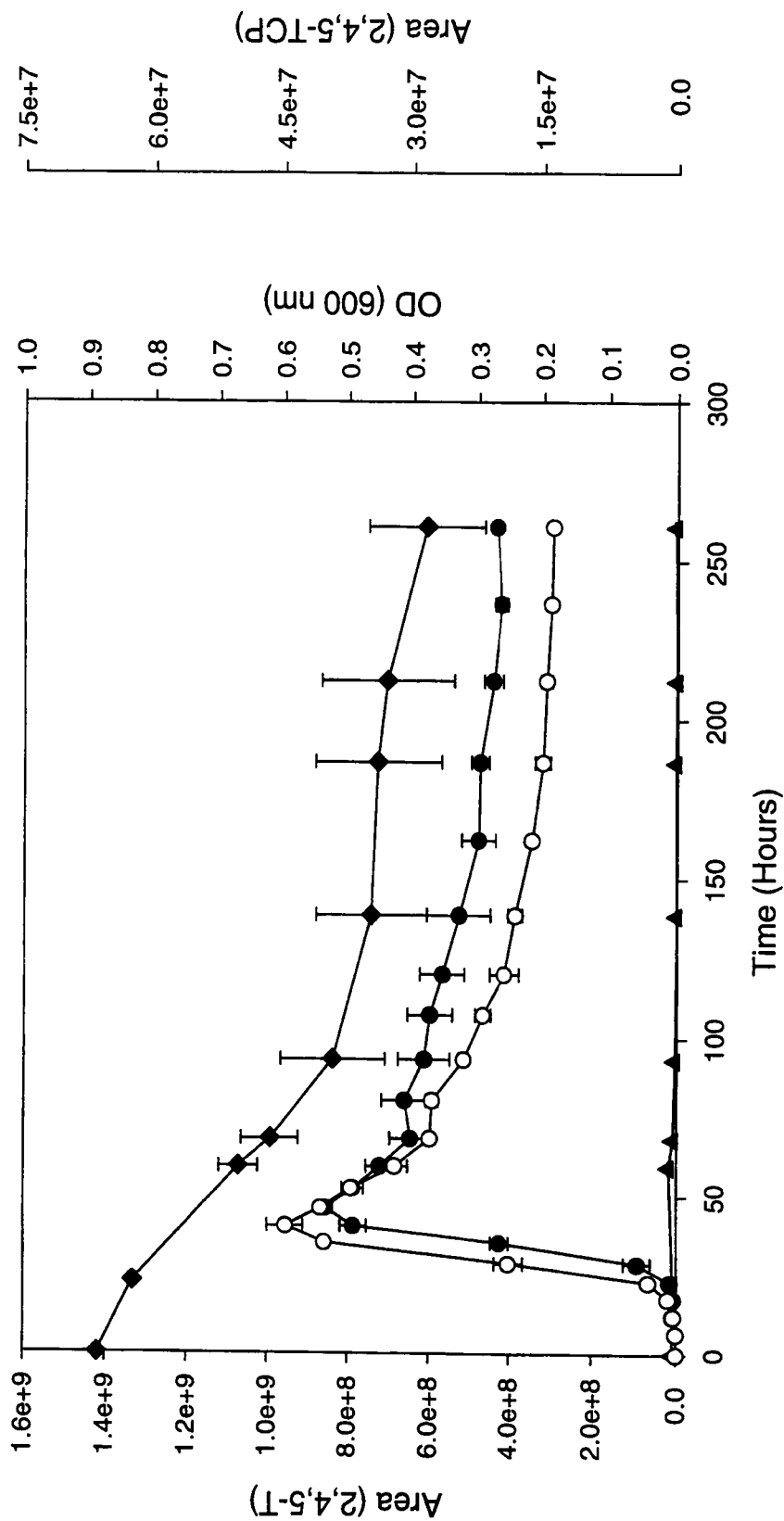


**Figure 4.28** 2,4,5-T mediated growth and degradation curves for *Burkholderia* species strain JR7B3 cultures containing 1000 mg/L 2,4,5-T and 3.7 mM succinate. PAS cultures supplemented with 2,4,5-T and succinate were the source of samples for HPLC analysis to determine 2,4,5-T concentrations (◆) and 2,4,5-TCP concentrations (▲). The optical density of these cultures (●) was also determined spectrophotometrically (600 nm). A control culture containing only PAS + 2.5 mM succinate (○) was also included in this experiment. Data points represent the average of triplicate cultures. Error bars represent the standard deviation of among these cultures.

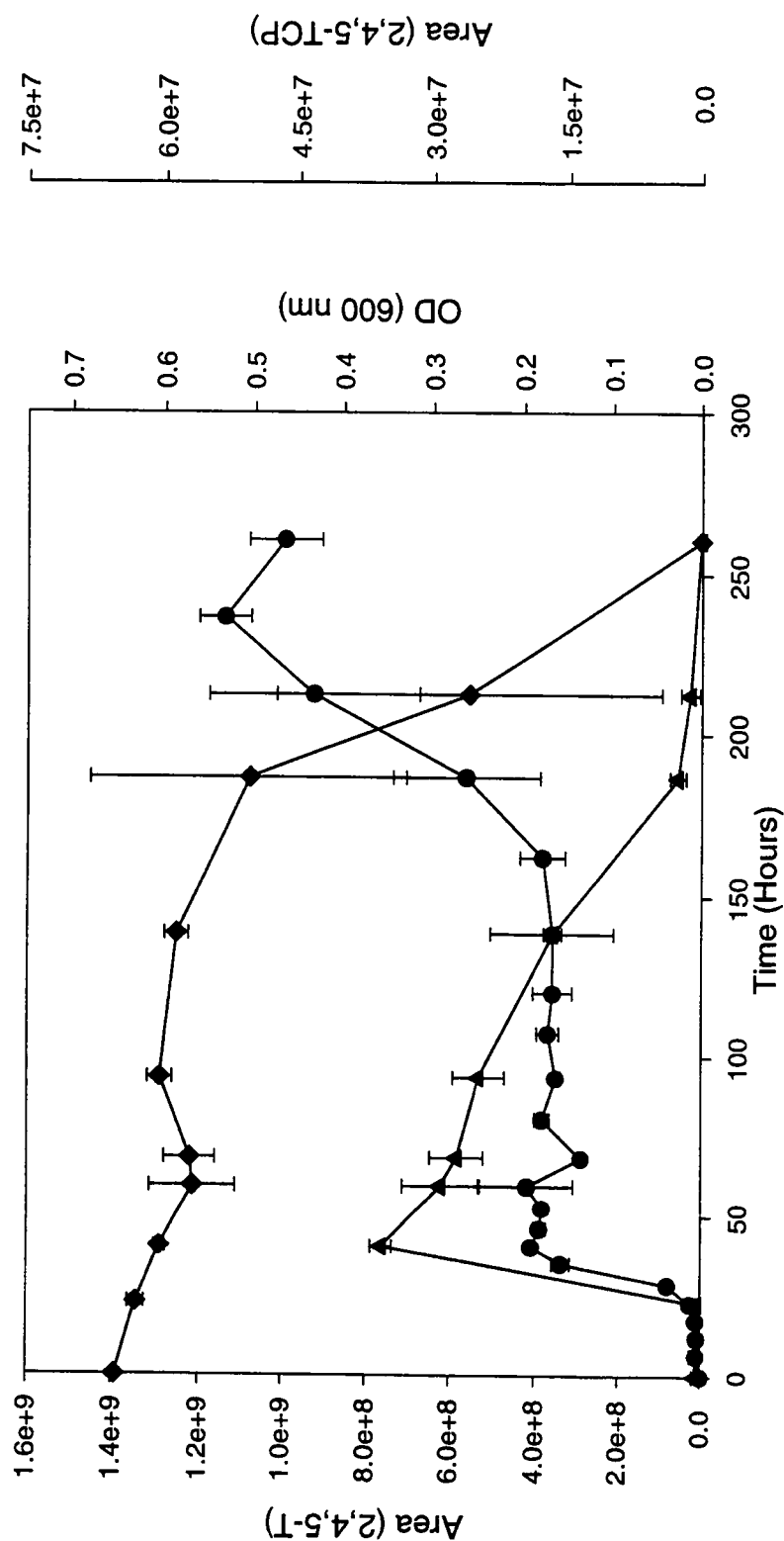
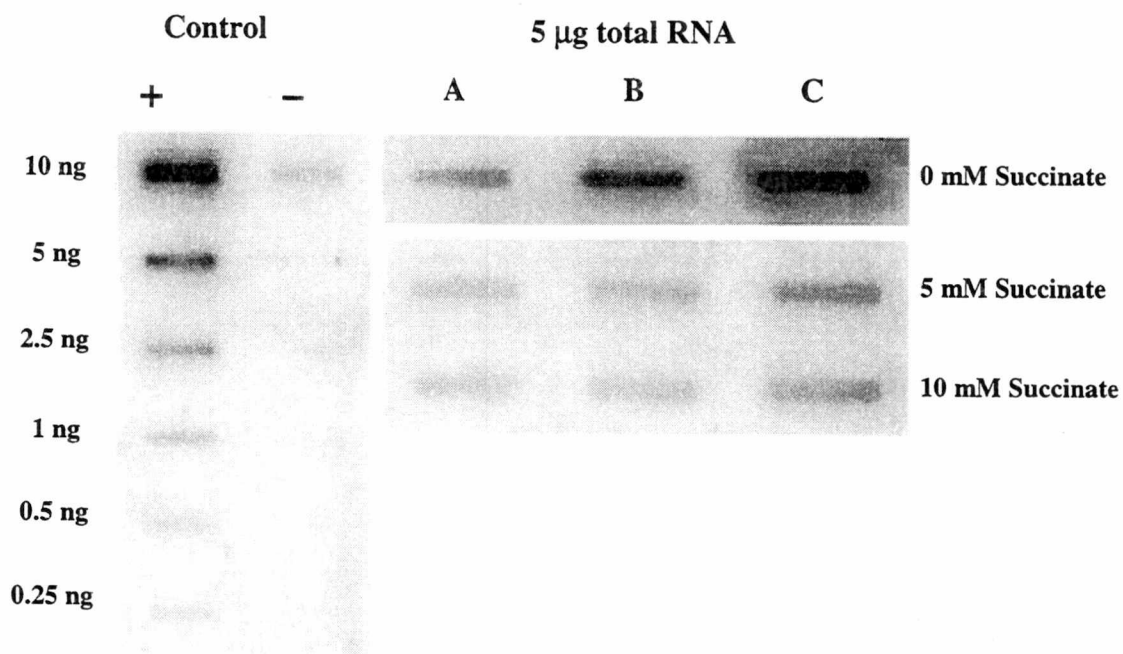


**Figure 4.29** 2,4,5-T mediated growth and degradation curves for *Burkholderia* species strain JR7B3 cultures containing 1000 mg/L 2,4,5-T. PAS cultures supplemented with 2,4,5-T were the source of samples for HPLC analysis to determine 2,4,5-T concentrations (♦) and 2,4,5-TCP concentrations (▲). The optical density of these cultures (●) was also determined spectrophotometrically (600 nm). Data points represent the average of triplicate cultures. Error bars represent the standard deviation of among these cultures.

A)



**B)**

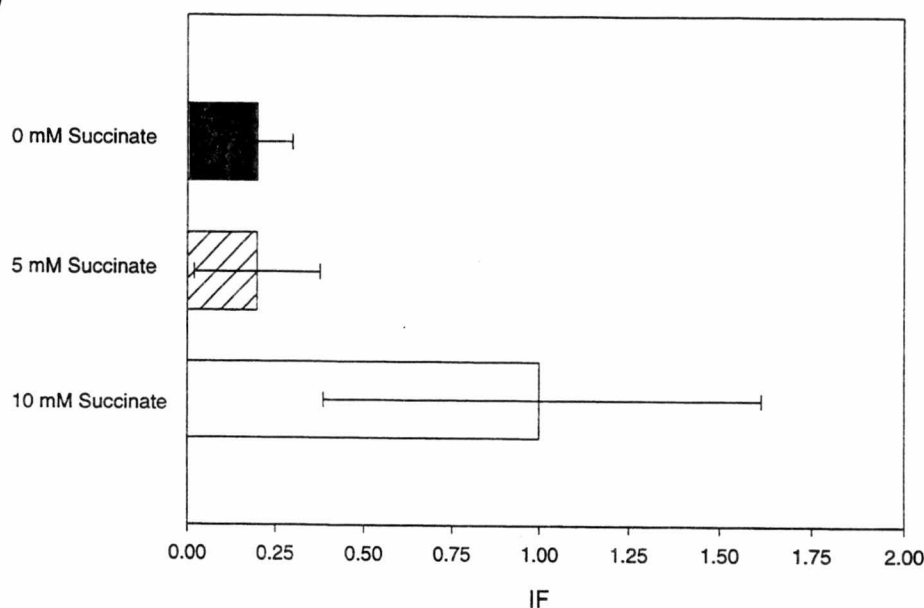
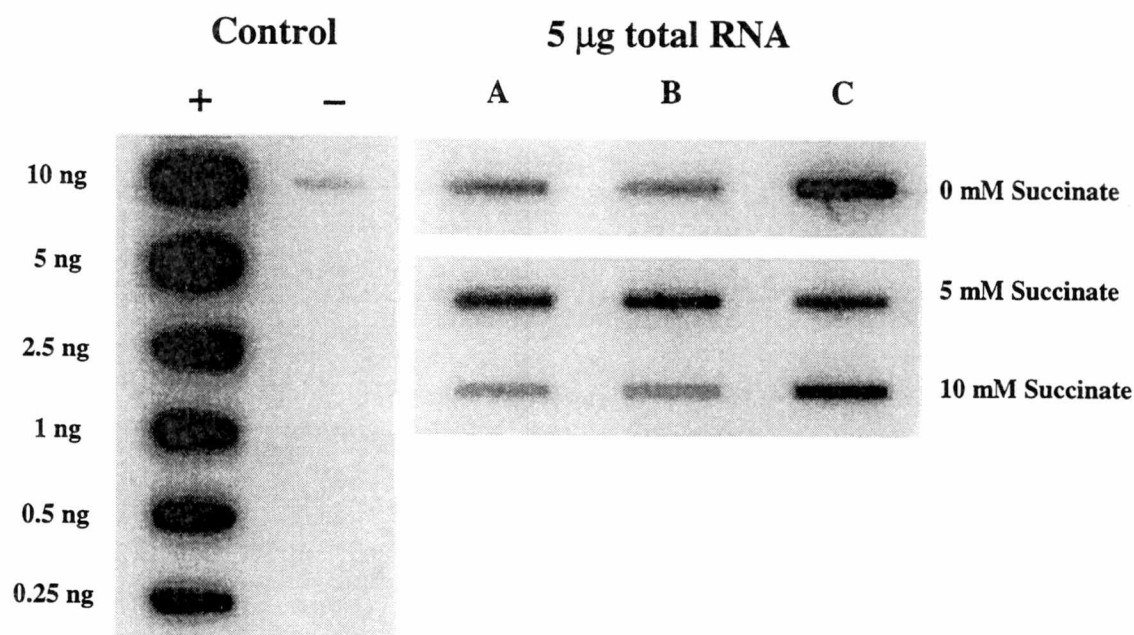


**Figure 4.30** Analysis of *tftA* mRNA expression in response to succinate addition for *Burkholderia* species strain JR7B3. A) RNA hybridization analysis. Positive controls are shown on the left side of the figure and cover a concentration range from 0.25 ng to 10 ng. A 10 ng negative control was blotted adjacent to the 10 ng positive control. Five  $\mu$ g of total RNA isolated from cultures amended with succinate was applied to the nylon membranes, and probed using a *tftA* specific,  $\alpha$ - $^{32}$ P-CTP labeled, probe. B) Graphical representation of Induction Factors (IF); IF values were determined by dividing results for each treatment condition by the control value and represent the ratio of condition mRNA/control mRNA. Error bars represent the standard deviations among three separate experiments.

A)



B)

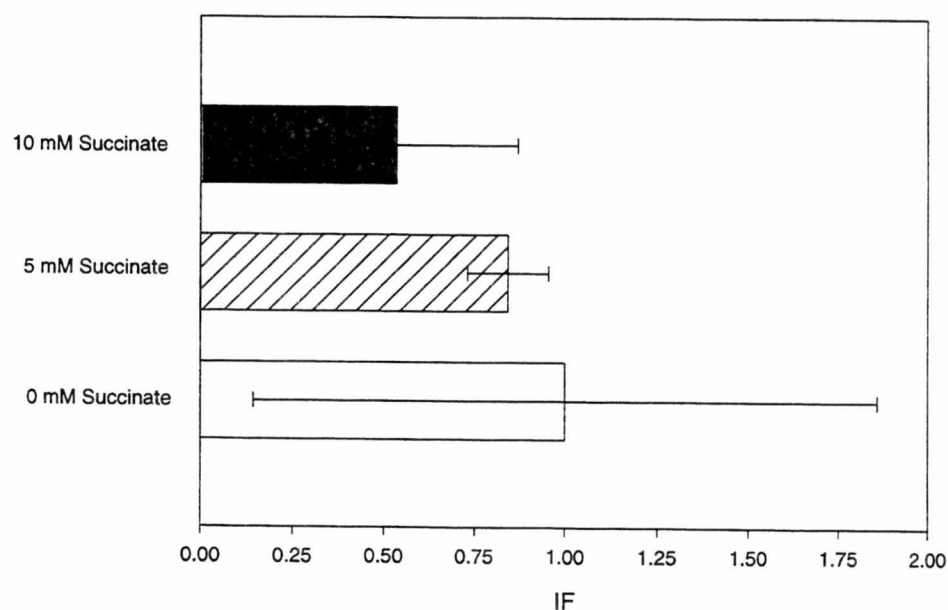


**Figure 4.31** Analysis of *tftC* mRNA expression in response to succinate addition for *Burkholderia* species strain JR7B3. A) RNA hybridization analysis. Positive controls are shown on the left side of the figure and cover a concentration range from 0.25 ng to 10 ng. A 10 ng negative control was blotted adjacent to the 10 ng positive control. Five  $\mu$ g of total RNA isolated from cultures amended with succinate was applied to the nylon membranes, and probed using a *tftC* specific,  $\alpha$ - $^{32}$ P-CTP labeled, probe. B) Graphical representation of Induction Factors (IF); IF values were determined by dividing results for each treatment condition by the control value and represent the ratio of condition mRNA/control mRNA. Error bars represent the standard deviations among three separate experiments.

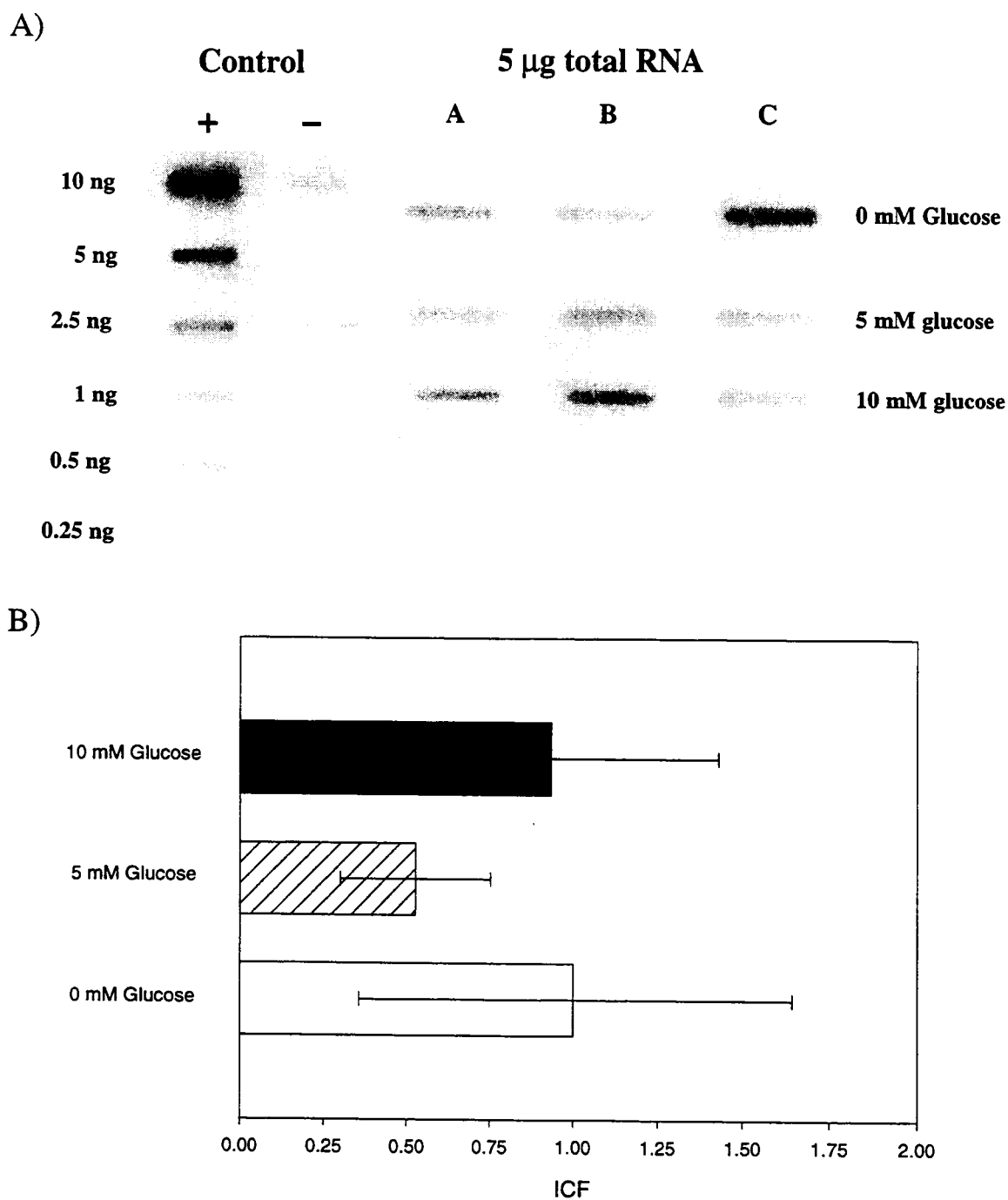
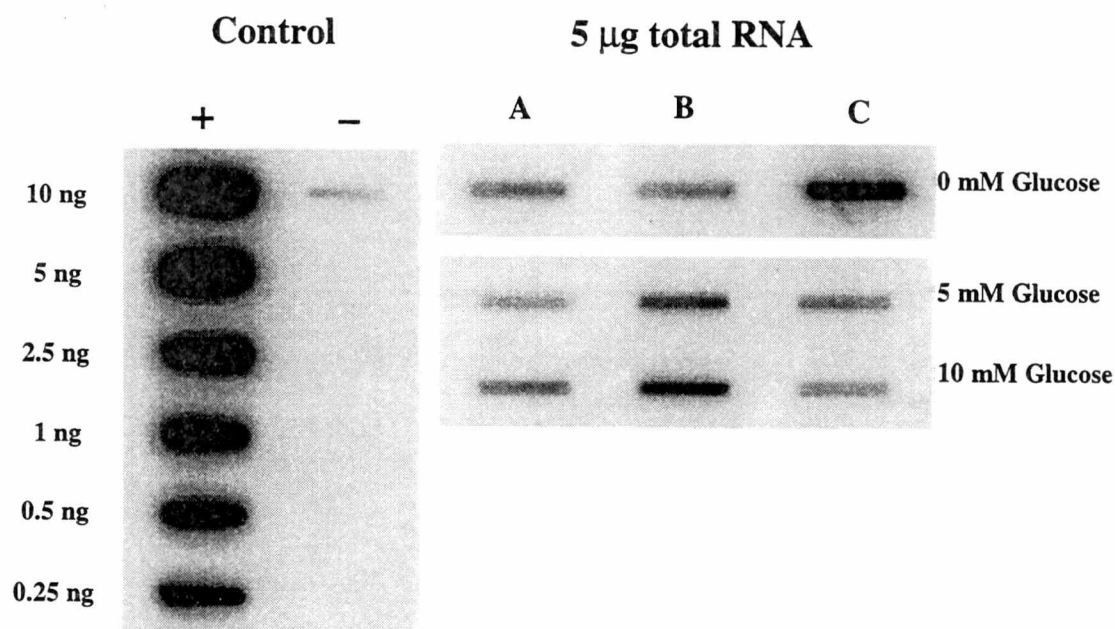


**Figure 4.32** Analysis of *tftA* mRNA expression in response to glucose addition for *Burkholderia* species strain JR7B3. A) RNA hybridization analysis. Positive controls are shown on the left side of the figure and cover a concentration range from 0.25 ng to 10 ng. A 10 ng negative control was blotted adjacent to the 10 ng positive control. Five  $\mu$ g of total RNA isolated from cultures amended with glucose was applied to the nylon membranes, and probed using a *tftA* specific,  $\alpha$ - $^{32}$ P-CTP labeled, probe. B) Graphical representation of Induction Factors (IF); IF values were determined by dividing results for each treatment condition by the control value and represent the ratio of condition mRNA/control mRNA. Error bars represent the standard deviations among three separate experiments.

A)



B)

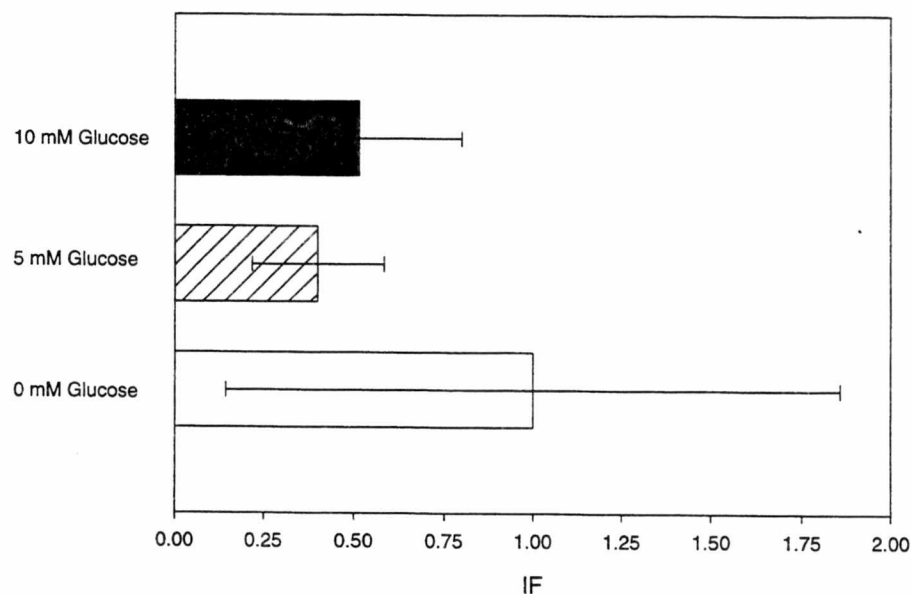


**Figure 4.33** Analysis of *tftC* mRNA expression in response to glucose addition for *Burkholderia* species strain JR7B3. A) RNA hybridization analysis. Positive controls are shown on the left side of the figure and cover a concentration range from 0.25 ng to 10 ng. A 10 ng negative control was blotted adjacent to the 10 ng positive control. Five µg of total RNA isolated from cultures amended with glucose was applied to the nylon membranes, and probed using a *tftC* specific,  $\alpha$ - $^{32}$ P-CTP labeled, probe. B) Graphical representation of Induction Factors (IF); IF values were determined by dividing results for each treatment condition by the control value and represent the ratio of condition mRNA/control mRNA. Error bars represent the standard deviations among three separate experiments.

4.32) and decreased *tftC* mRNA transcripts (Figure 4.33) compared to succinate cultures (Figure 4.33).

#### **4.4.6 2,4-DCP induced 2,4,5-T metabolism in *Burkholderia* species strain JR7B3.**

Based on evidence that 2,4-DCP and 2,4,5-TCP accumulated during growth on 2,4-D and 2,4,5-T by *Burkholderia* species strain JRB1 and JR7B3, respectively, experiments were conducted using strain JR7B3 designed to examine potential inhibition by 2,4-D and 2,4-DCP. Remarkably, degradation of 2,4,5-T by *Burkholderia* species strain JR7B3 inoculated into PAS medium containing 1000 mg/L 2,4,5-T, 0.01% yeast extract and 2,4-DCP was enhanced relative to control, glucose, and succinate cultures (Figure 4.34 and 4.35). Strain JR7B3 cultures containing 10 mg/L 2,4-DCP completely degraded 2,4,5-T within 150 hours. The reproducibility associated with these cultures was good. There was a slight red hue, which developed in these cultures, but was subsequently removed during cellular growth on 2,4,5-T. 2,4,5-TCP only accumulated to 7.0 mg/L compared with 14.3 (Figure 4.34) for control cultures without 2,4-DCP (Figure 4.29). Similar cultures containing 25 mg/L 2,4-DCP also demonstrated enhanced 2,4,5-T degradation and only accumulated 2,4-DCP to only 2.8 mg/L (Figure 4.35). For both 2,4-DCP treatments, a corresponding increase in biomass was apparent. An examination of *tftA* and *tftC* mRNA transcript abundance yielded similar data as the succinate treatments (Figure 4.36 and 4.37). Decreased *tftA* mRNA transcripts were observed (Figure 4.36) relative to glucose treated cultures (Figure 4.32). Continued incubation of these cultures in the presence of 10 mg/L 2,4-DCP demonstrated that these cells were

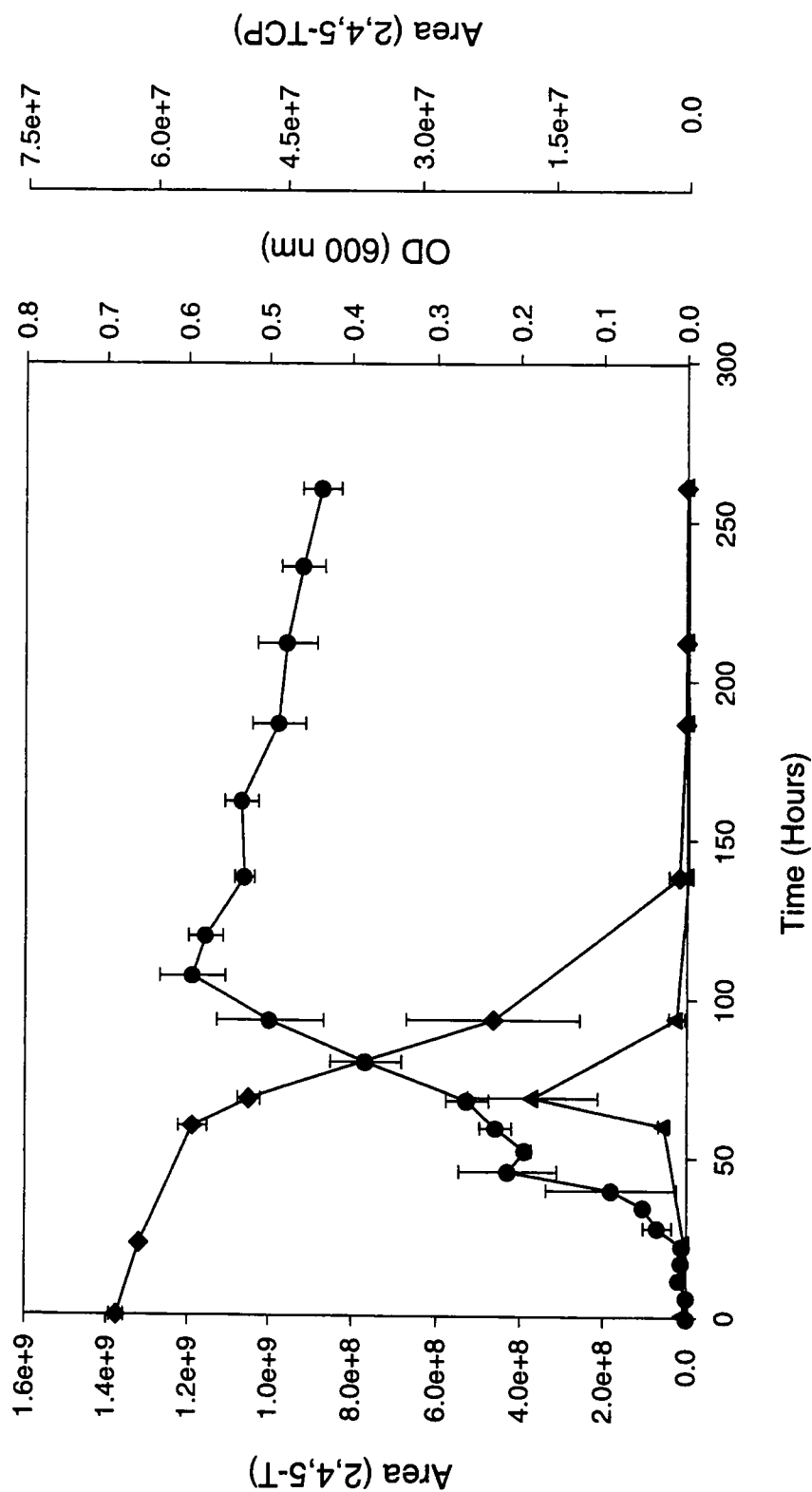


**Figure 4.34** Effects of 10 mg/L 2,4-DCP on 2,4,5-T mediated growth and degradation for *Burkholderia* species strain JR7B3. PAS cultures supplemented with 1000 mg/L 2,4,5-T and 10 mg/L 2,4-DCP were the source of samples for HPLC analysis to determine 2,4,5-T concentrations (◆) and 2,4,5-TCP concentrations (▲). The optical density of these cultures (●) was also determined spectrophotometrically (600 nm). Data points represent the average of triplicate cultures. Error bars represent the standard deviation of among these cultures.

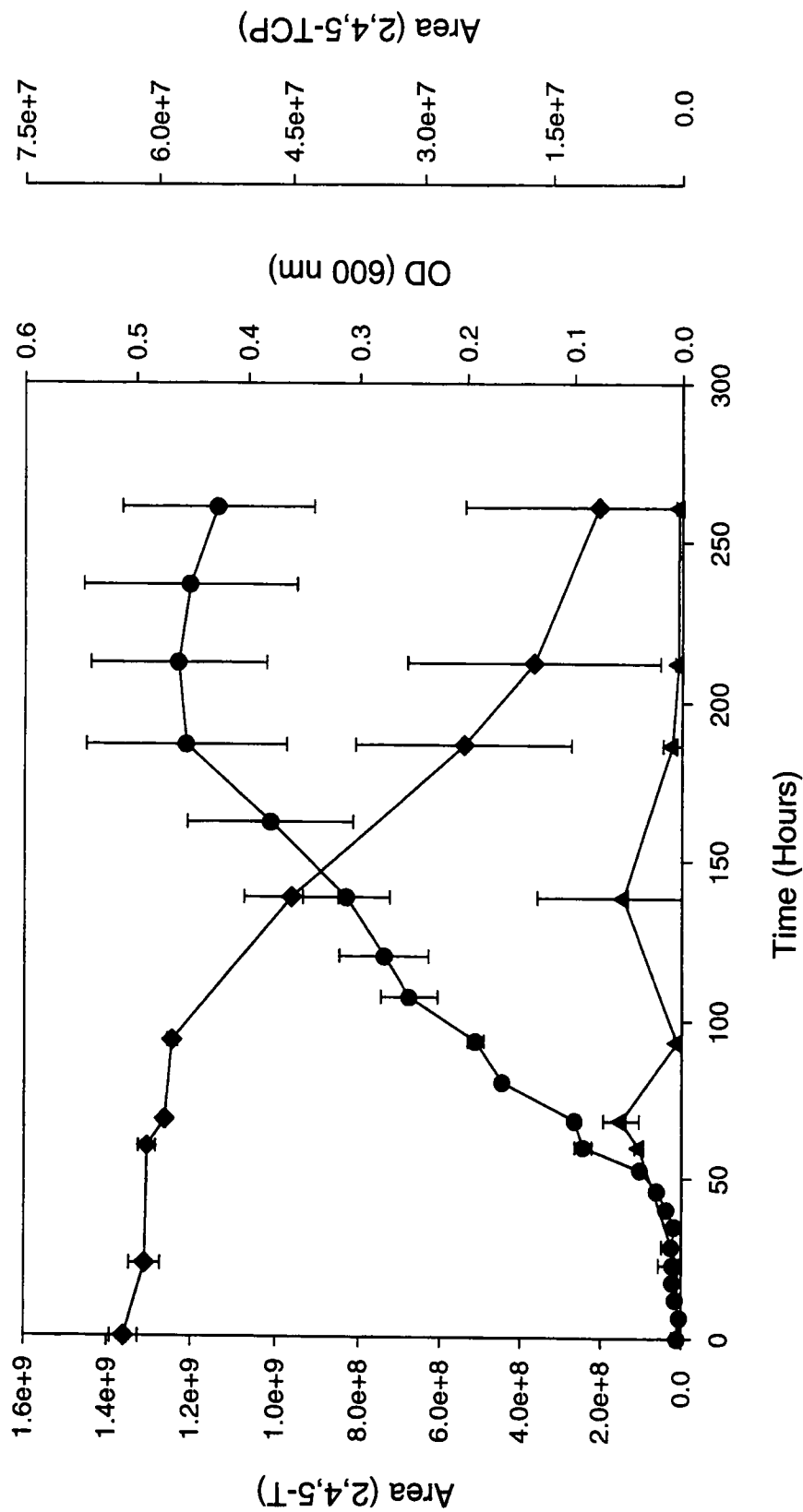


**Figure 4.35** Effects of 25 mg/L 2,4-DCP on 2,4,5-T mediated growth and degradation for *Burkholderia* species strain JR7B3. PAS cultures supplemented with 1000 mg/L 2,4,5-T and 25 mg/L 2,4-DCP were the source of samples for HPLC analysis to determine 2,4,5-T concentrations (◆) and 2,4,5-TCP concentrations (▲). The optical density of these cultures (●) was also determined spectrophotometrically (600 nm). Data points represent the average of triplicate cultures. Error bars represent the standard deviation of among these cultures.



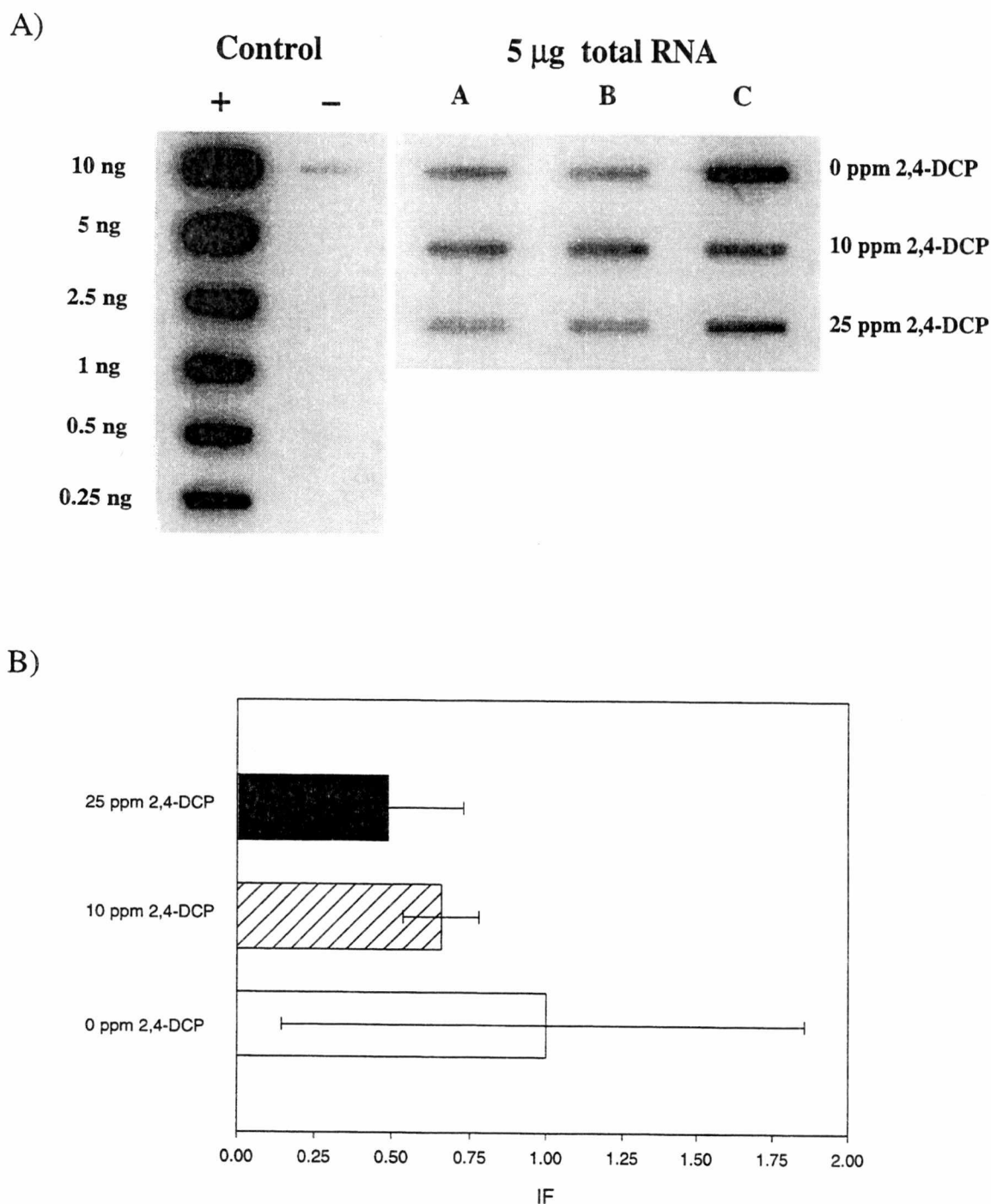


**Figure 4.37** Analysis of *tftC* mRNA expression in response to 2,4-DCP addition for *Burkholderia* species strain JR7B3. A) RNA hybridization analysis. Positive controls are shown on the left side of the figure and cover a concentration range from 0.25 ng to 10 ng. A 10 ng negative control was blotted adjacent to the 10 ng positive control. Five µg of total RNA isolated from cultures amended with 2,4-DCP was applied to the nylon membranes, and probed using a *tftC* specific,  $\alpha$ - $^{32}$ P-CTP labeled, probe. B) Graphical representation of Induction Factors (IF); IF values were determined by dividing results for each treatment condition by the control value and represent the ratio of condition mRNA/control mRNA. Error bars represent the standard deviations among three separate experiments.

better able to metabolize 2,4,5-T compared to controls without 2,4-DCP. The presence of 25 mg/L 2,4-DCP increased the time of 2,4,5-T degradation and growth relative to cultures containing 10 mg/L, but cultures having 25 mg/L still initiated 2,4,5-T degradation sooner than control cultures. Cultures containing 25 mg/L 2,4-DCP also produced the least amount of 2,4,5-TCP (2.8 mg/L).

2,4,5-T mediated growth and subsequent degradation was inhibited for strain JR7B3 cultures containing 500 ppm 2,4,5-T and 500 ppm 2,4-D (Figure 4.38). 2,4,5-T degradation and corresponding 2,4,5-TCP production was dramatically decreased and 2,4,5-TCP concentrations reached an upper concentration of only 0.6 ppm at about 40 hours after cell growth. 14.3 mg/L 2,4-DCP was produced in associated with 2,4,5-TCP production was gradually removed from the media during the course of the experiment (Figure 4.38). Despite the dramatic inhibition of 2,4,5-T degradation, a slow steady growth was still evident throughout the course of the experiment and both 2,4-D and 2,4,5-T concentrations were slightly diminished. These cultures had a striking red color associated with them that has been previously reported as a byproduct of 2,4-D degradation by the 2,4,5-T metabolic pathway, specifically a chlorohydroquinone (Haugland et al. 1990 and Zaborina et al. 1998). An interesting point of difference was the accumulation of high levels of 2,4,5-TCP in Dr. Haugland's previous experiments, compared to these results in which 2,4,5-TCP production was reduced. An examination of *tftA* and *tftC* expression in 2,4-D treated cultures demonstrated that there was no significant change in the mRNA abundance profiles (Figure 4.39 and 4.40).

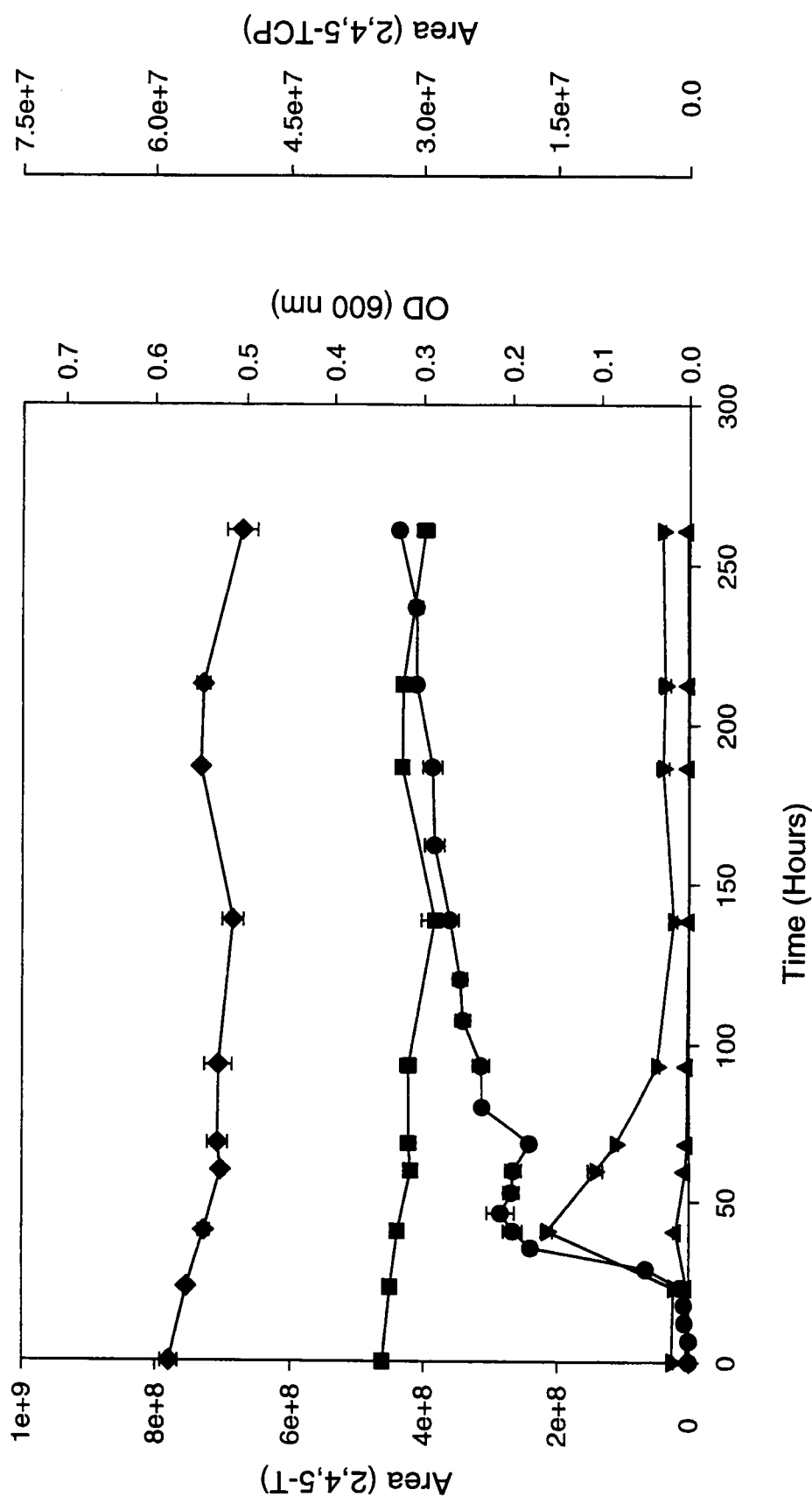


**Figure 4.38** Effects of 500 mg/L 2,4-D on 2,4,5-T mediated growth and degradation for *Burkholderia* species strain JR7B3. PAS cultures supplemented with 500 mg/L 2,4,5-T and 500 mg/L 2,4-D were the source of samples for HPLC analysis to determine 2,4,5-T concentrations (◆), 2,4-D concentrations (■), 2,4,5-TCP concentrations (▲), and 2,4-DCP concentrations (▼). The optical density of these cultures (●) was also determined spectrophotometrically (600 nm). Data points represent the average of triplicate cultures. Error bars represent the standard deviation among these cultures

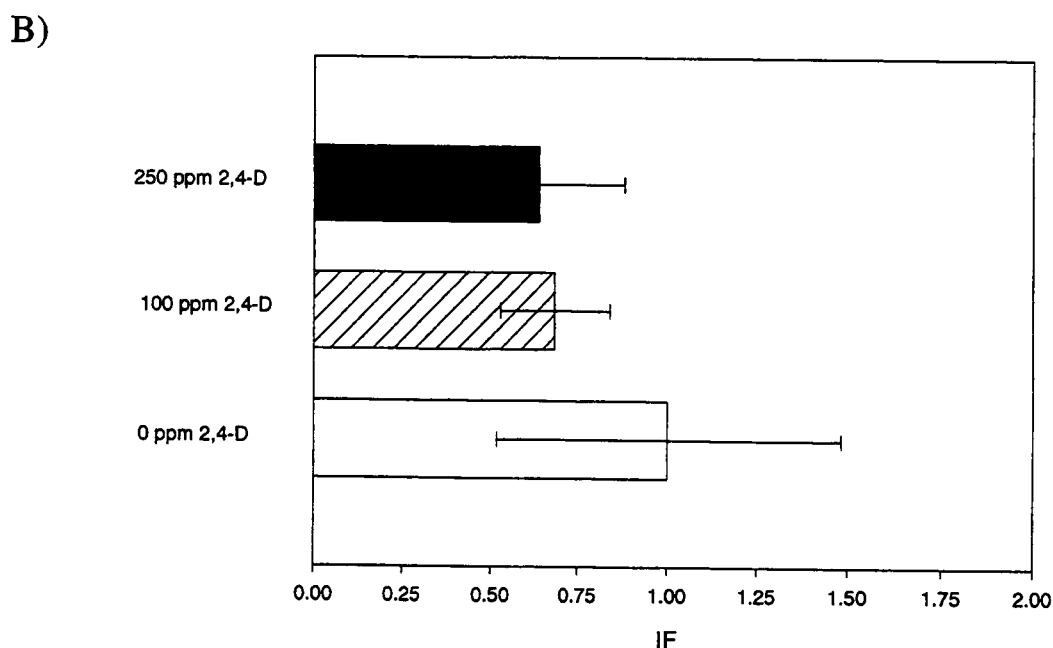
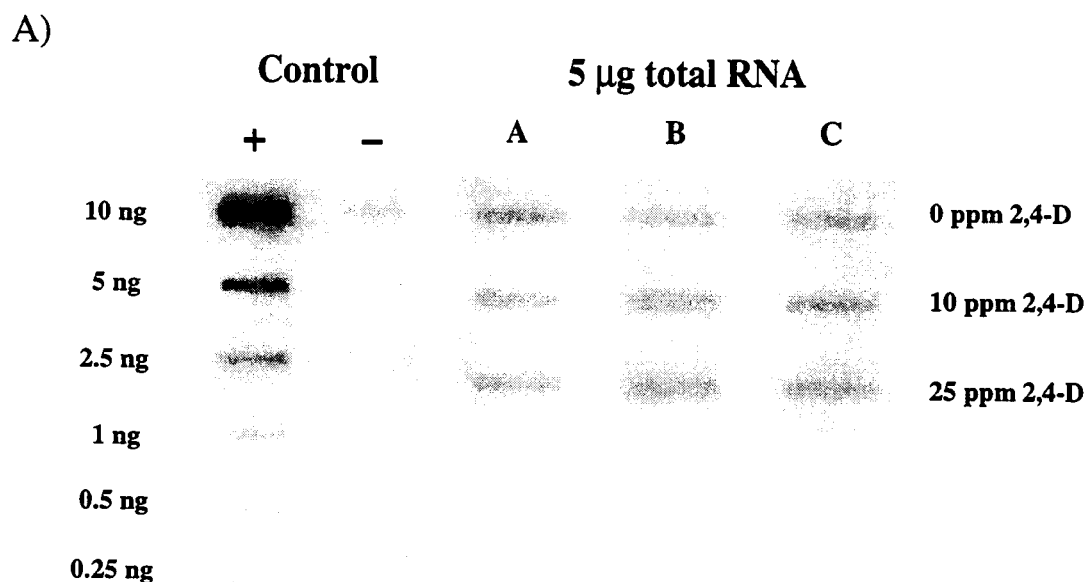


**Figure 4.39** Analysis of *tftA* mRNA expression in response to 2,4-D addition for *Burkholderia* species strain JR7B3. A) RNA hybridization analysis. Positive controls are shown on the left side of the figure and cover a concentration range from 0.25 ng to 10 ng. A 10 ng negative control was blotted adjacent to the 10 ng positive control. Five  $\mu$ g of total RNA isolated from cultures amended with 2,4-D was applied to the nylon membranes, and probed using a *tftA* specific,  $\alpha$ - $^{32}$ P-CTP labeled, probe. B) Graphical representation of Induction Factors (IF); IF values were determined by dividing results for each treatment condition by the control value and represent the ratio of condition mRNA/control mRNA. Error bars represent the standard deviations among three separate experiments.



## 4.5 DISCUSSION

Bacterial mediated degradation of Agent Orange potentially involves multiple metabolic pathways specific for 2,4-D or 2,4,5-T as well as tertiary contaminants such as chlorinated dibenzo-*p*-dioxins and dibenzofurans. Microbial catabolism of 2,4-D has been well studied among members of the  $\alpha$ ,  $\beta$ , and  $\gamma$ -*Proteobacteria*. In particular, *R. eutrophus* JMP 134 has been extensively examined and a complete degradation pathway elucidated. 2,4,5-T degradation has also been characterized in *B. cepacia* AC1100 and most of the metabolic pathway has been elucidated. Genes associated with each of the metabolic pathways present in these model organisms have been identified in two environmental bacteria isolated from Agent Orange contaminated soils (Rice, Part III). One of these microorganisms, *Burkholderia* species strain JRB1, is capable of growing on 2,4-D as a sole carbon and energy source and contains the *tfdA*, *B*, *C*, *E*, and *R* genes. A second organism, *Burkholderia* species strain JR7B3, is able to completely metabolize 2,4,5-T, during auxotrophic growth, and contains genes corresponding to the *tfdA*, *C*, and *E* genes. The presence of each of these degradation pathways in association with Agent Orange contaminated soils stimulates questions regarding the interactions of 2,4-D and 2,4,5-T pathways and the influences of their metabolic products. 2,4-DCP and 2,4,5-TCP are commonly detected as a consequence of incomplete metabolism of chlorinated organic pollutants, such as 2,4-D and the effects each of these chlorinated phenols have on cellular metabolism has been superficially examined. The primary aim of this research project was to study the consequences of 2,4-D and 2,4,5-T metabolic

intermediates on bacterial catabolism of 2,4-D and 2,4,5-T by environmental bacteria isolated from Agent Orange contaminated soil, specifically, *Burkholderia* species strain JRB1 and *Burkholderia* species strain JR7B3.

Carbon catabolite repression in Pseudomonads appears to be regulated differently than the model cAMP mediated mechanism previously characterized for *E. coli* which is associated with the phosphotransferase system (Staijen et al. 1999). The current paradigm for carbon catabolite repression for bacterial degradation of chlorocatechol is competitive inhibition of fumarate with the 2-chloro-*cis-cis*-muconate inducer molecule in association with the ClcR *lysR* transcriptional regulator in *Pseudomonas putida*. This model is based on recent examination of catabolite repression of the evolutionarily related *catBCA* and *clcABD* operons in *P. putida*, by McFall et al. (1997). The *catBCA* and *clcABD* operons are regulated by the LysR-type regulators CatR and ClcR respectively, and are 32.5% identical and 43% similar in their amino acid sequences. In vitro transcription analyses, using *clcR-AB-lacZ* and *catB-lacZ* transcriptional fusions, demonstrated that the *clcABD*, but not the *catBCA* was catabolite repressed when cells were grown in the presence of succinate, citrate, or fumarate (McFall et al. 1997). This repression was mediated specifically by fumarate, which most probably acts as an anti-inducer by competing for the 2-chloro-*cis-cis*-muconate binding site on the ClcR. Inhibition of the ClcR was reversible. McFall et al. (1997) suggested that the utilization of fumarate as a key signaling molecule would provide the cell with an elegant sensing mechanism to facilitate catabolism of a myriad of chloroaromatic in the absence of preferred carbon and energy sources.

The fact that chlorocatechol is an important metabolic intermediate in many catabolic pathways, including the 2,4-D degradation pathway, suggest that catabolite modulation may be involved during adaptation of bacterial cells to available carbon sources and potentially influence degradation of chlorinated aromatic compounds at contaminated sites. This idea is supported by the fact that metabolism of 2,4-D by *R. eutrophus* JMP 134 and *Burkholderia* species has previously been shown to involve interactions of 2,4-dichloromuconic acid with the TfdR *lysR* regulatory protein (Matrubutham and Harker 1994), and the *tfd* pathway has been shown to be evolutionarily related to the *clcABD* pathway (Ogawa and Miyashita 1999).

A goal of this research project was to examine the effects of exogenous glucose and succinate on growth and degradation of 2,4-D by *Burkholderia* species strain JRB1. *Burkholderia* species strain JRB1 grown in the presence of 2,4-D and succinate accumulated 24.2 mg/L 2,4-DCP. This increase in 2,4-DCP occurred during growth on succinate as an alternative carbon source, and resulted in a cell death phase and a substantial decrease in 2,4-D transformation. Prior to 2,4-DCP accumulation, 2,4-D cultures containing succinate had similar 2,4-D degradation rates as control cultures without 2,4-D. Succinate addition to 2,4-D degrading cultures resulted in decreased mRNA transcripts for the *tfdA*, *B*, and *C* genes. These results are in contrast to cultures treated with glucose, which did not accumulate 2,4-DCP and demonstrated an enhanced 2,4-D degradation profile. Addition of glucose to 2,4-D cultures did not result in a significant changes in *tfdA* and *tfdC* mRNA transcript abundance but did result in increased *tfdB* mRNA.

These results suggest that carbon catabolite repression of the TfdB occurred in response to succinate but not glucose. The most likely model supported by these data is the current repression model hypothesized for the *clcABD* genes (McFall et al. 1997). Based on this model succinate would be converted to fumarate and would compete with 2,4-dichloromuconate for the TfdR binding site, thereby resulting in decreased transcription associated with the *tfdB*, *tfdC* and *tfdA* operons. It is interesting that the *tfdB* seemed most susceptible to this repression and that glucose amendments increased *tfdB* mRNA only. Hypothetically, glucose could also result in increased fumarate concentrations and subsequent repression. The fact that initial growth of 2,4-D/glucose and 2,4-D cultures were the same suggest that 2,4-D is a preferred growth substrate for these cells, and growth profiles similar to glucose controls were observed only after 2,4-D concentrations were diminished. This phenomenon could also be the result of phenotypic lag since cultures used for the inoculum were acclimated to 2,4-D and not glucose growth. Growth patterns for succinate/2,4-D cultures were different than glucose/2,4-D cultures with an increasing optical density relative to 2,4-D controls observed as soon as 5 hours. This is not unexpected because succinate is an important intermediate during 2,4-D metabolism, and these cells were likely already poised for succinate metabolism. The fact that similar repression was not observed during glucose utilization indicates that accumulation of 2,4-DCP is not strictly proportional to the amount of cellular biomass and that under these conditions intracellular succinate concentration are maintained at a level low enough that repression does not occur. This implies that repression of these genes is fairly specific and that general growth enhancers

may not be detrimental to 2,4-D degradation processes. Succinate repression of upper pathway enzymes may provide these bacteria with an efficient sensing and regulatory mechanism to facilitate catabolism of 2,4-D.

In a related previous research study, a bacterial consortium was isolated which was able to utilize 2,4,5-T as a sole carbon and energy source (Rice, Part III). Phenotypic and genotypic characterization of this mixed culture identified two *Burkholderia* species, which were subsequently designated JR7B2 and JR7B3. Strain JR7B3 was shown to contain the *tftA*, *tftC*, and *tftE* genes, which have been shown to be essential for 2,4,5-T metabolism by *B. cepacia* AC1100. RT-PCR studies using this consortium demonstrated that these genes were expressed during growth on 2,4,5-T but were absent during growth on succinate alone (Rice, Part III). Specific goals of this research project were 1) to examine the effects of 2,4-D and 2,4,5-T metabolic intermediates on 2,4,5-T degradation, and 2) to determine whether or not alternative carbon sources potentially inhibit 2,4-D and 2,4,5-T degradative pathways and thereby result in accumulation of toxic catabolic intermediates such as 2,4-DCP and 2,4,5-TCP.

Glucose/2,4,5-T cultures demonstrated increased 2,4,5-T transformation and subsequent 2,4,5-TCP production relative to succinate/2,4,5-T cultures in which transformation of 2,4,5-T was negligible. Transformation of 2,4,5-T to 2,4,5-TCP coincided with a decrease in cellular growth compared to glucose controls. These results suggest that growth on glucose allowed increased metabolism of 2,4,5-T whereas succinate mediated growth does not. 2,4,5-T degradation by glucose and succinate grown cultures demonstrated an interesting trend. For glucose grown cultures 2,4,5-T

degradation was more productive having less 2,4,5-TCP accumulate and faster overall 2,4,5-T specific growth and subsequent removal. Succinate grown cultures, however, demonstrated a decreased ability to degrade 2,4,5-TCP and prolonged periods of adaptation were necessary prior to complete removal of 2,4,5-T. In the absence of succinate or glucose 2,4,5-T was slowly degraded and 2,4,5-TCP was maintained at minimal concentrations.

During these experiments, the apparent optical density of the succinate cultures was about 30% higher and could potentially account for the increase in 2,4,5-TCP production. However, during initial growth of glucose cultures, a decrease in growth rate and increase in 2,4,5-TCP production was observed, compared to succinate cultures in which the growth rate was not affected and 2,4,5-T conversion to 2,4,5-TCP was 79% less. This implies that glucose-containing cultures were better able to metabolize 2,4,5-T, and may have been able to adapt more quickly to 2,4,5-TCP utilization. This idea is supported by the fact that initial 2,4,5-T degradation proceeded more rapidly for glucose cultures despite a lower overall biomass. Another fact that must be considered during interpretation of the experimental results is that these cultures contain a bacterial consortium. The increase in 2,4,5-T degradation activity could be due to a numerical bias for the 2,4,5-T degrading bacteria during growth on glucose. In order to help address these issues pure culture analysis of strain JR7B3 was done.

Because these initial studies were complicated by the presence of a secondary non 2,4,5-T-degrading microorganism, *Burkholderia* species strain JR7B2, pure culture studies using *Burkholderia* species strain JR7B3 were conducted in hopes of elucidating

the underlying reason for these observations. These studies had similar results but the effects of glucose and succinate were magnified. Cultures containing glucose and 2,4,5-T accumulated toxic levels of 2,4,5-TCP, which prevented further metabolism of either glucose or 2,4,5-T. Succinate/2,4,5-T cultures demonstrated low levels of 2,4,5-TCP, and when compared to control cultures without succinate or glucose amendments it seems that succinate grown cells are better able to sustain 2,4,5-TCP metabolism thereby facilitating 2,4,5-T degradation. 2,4,5-T degradation associated with succinate/2,4,5-T cultures tapered off during the course of the experiment in contrast to control cultures that completely removed 2,4,5-T within 250 hours. An analysis of *tft* mRNA transcripts demonstrated that transcription levels appear to be similar regardless of whether glucose or succinate is present. The one exception was a slight increase in *tftA* transcripts relative to succinate treatments.

These data indicate that succinate is able to somehow curtail accumulation of toxic levels of 2,4,5-TCP and acclimate cells for slow degradation and growth on 2,4,5-T. This is in comparison to cultures without succinate in which 2,4,5-TCP accumulated to inhibitory concentrations and was slowly metabolized by bacterial cells until 2,4,5-T degradation capacity was dramatically increased at approximately 180 hours. The fact that 2,4,5-T disappearance for succinate cultures was higher than either glucose amended or control cultures suggest that these cells were better able to metabolize the 2,4,5-TCP produced. Furthermore, since glucose grown cultures and 2,4,5-T control cultures have identical biomass production and 2,4,5-T disappearance profiles, it appears that glucose

metabolism was inhibited by 2,4,5-TCP and that the increased trichlorophenol concentrations could be the result of a general increase in cell metabolism.

Herbicides containing a mixture of 2,4-D and 2,4,5-T pose a potential problem for 2,4-D and 2,4,5-T degrading bacteria. Although degradation of 2,4-D and 2,4,5-T by microorganisms has been demonstrated, several of the enzymes involved are quite different. 2,4-D is degraded by the activity of a  $\alpha$ -ketoglutarate dioxygenase and subsequent metabolism proceeds through a 3,5-DCC intermediate and requires the activity of a modified ortho-pathway containing a type II chlorocatechol dioxygenase. Subsequent ring cleavage of this 3,5-DCC produces 2,4-dichloromuconic acid, which is then dechlorinated by the action of several distinct reductases. 2,4,5-T metabolism is initiated by a 2,4,5-T oxygenase and then proceeds through a 2,5-dichloroquinone intermediate, which is then fully dechlorinated prior to ring cleavage. It has previously been proposed that 2,4,5-T inhibits the 2,4-dichlorophenoxyacetic acid/ $\alpha$ -ketoglutarate dioxygenase (TfdA, Haugland et al. 1990), and that conversion of 2,4,5-T to 2,4,5-TCP is minimal during growth on 2,4-D. To further assess the ramifications of 2,4-D degradation in mixtures of 2,4-D and 2,4,5-T *Burkholderia* species strain JRB1 was studied using growing cell assays and increasing concentrations of 2,4,5-T supplied as a co-contaminant. The specific goals of these studies were to determine if 2,4-D metabolism was significantly reduced in the presence of 2,4,5-T and if 2,4,5-T was co-metabolically transformed by this 2,4-D degrading bacterium. In the absence of complete mineralization, 2,4-D and 2,4,5-T can be incompletely transformed by abiotic and biotic processes generating 2,4-DCP and 2,4,5-TCP, respectively (Amy et al. 1985). The

presence of 2,4-DCP, 2,4,5-TCP, and 3,5-DCC in association with bacteria degrading 2,4-D and 2,4,5-T may cause repression of genes necessary for the cell's continued metabolic fitness. Compounds, such as 3-chlorocatechol, are known inhibitors of *meta*-ring cleavage enzymes (Klecka and Gibson 1981, and Bartels et al. 1984). These compounds form acyl halides, which bind to and inactivate *meta*-pyrocatechase type enzymes.

Initial 2,4-D degradation by *Burkholderia* species strain JRB1 in the presence of 2,4,5-T was not affected. During these experiments it was apparent that 2,4-D was preferentially degraded relative to 2,4,5-T in the culture medium as evidenced by decreasing 2,4-D concentrations without concomitant 2,4,5-T transformation. Low levels of 2,4,5-TCP were produced, however, and appeared to correspond to increasing 2,4,5-T/2,4-D ratios. These results suggest that at high concentrations of 2,4-D, 2,4,5-T is not a productive competitor for the 2,4-dichlorophenoxyacetic acid/ $\alpha$ -ketoglutarate dependent dioxygenase, but as 2,4-D concentrations decrease 2,4,5-T would be more likely to compete with 2,4-D at the TfdA binding site and subsequently enter the 2,4-D metabolic pathway.

During degradation of 2,4-D transformation of 2,4,5-T was minimal, but conversion of up to 1.4 mg/L of 2,4,5-TCP was apparent. This 2,4,5-TCP was removed in the case of all 2,4,5-T amendments. During degradation of 2,4-D in the presence of 2,4,5-T, 2,4-DCP accumulated to a small degree and was greatest in cultures containing 250 mg/L 2,4,5-T. A corresponding decrease in cellular growth and 2,4-D degradation was observed during peak production of 2,4,5-TCP. This supports the conclusion that

2,4,5-T does not interfere with degradation of 2,4-D. Rather, 2,4,5-TCP appears to be detrimental to 2,4-D degradation at even low concentrations. The mechanism of 2,4,5-TCP interference may or may not be due to the 2,4,5-TCP compound itself, which is known to be a potent inhibitor of cellular growth.

Studies using 10 mg/L and 25mg/L 2,4,5-TCP amendments demonstrated that 25 mg/L 2,4,5-TCP completely inhibited growth on either succinate or 2,4-D. Suggesting that at least at these concentrations a general toxicity of the trichlorophenol was responsible for the growth inhibition. 10 mg/L 2,4,5-TCP was also inhibitory to 2,4-D degradation and cell growth, but removal of 2,4,5-TCP from 2,4-D containing cultures was observed and corresponded with initiation of 2,4-D degradation. Complete removal of 2,4-D was observed in cultures treated with 10 mg/L 2,4,5-TCP, approximately 40 hours later than control cultures. 2,4,5-TCP treatment did not affect *tfdA* or *tfdC* mRNA expression. The fact that 2,4,5-TCP was metabolized by these cultures suggests that the TfdB may be able to non-specifically transform 2,4,5-TCP to a trichlorocatechol intermediate, which might prove to be a source of toxicity for these cells. However, experiments examining the effects of 2,4,5-TCP suggest that a general toxicity explanation rather than production of a non-metabolizable compound may be supported. It is possible that these cells have an additional enzyme that is capable of degrading 2,4,5-TCP after an adaptation phase.

*Burkholderia* species strain JR7B3 grown in the presence of 2,4-D and 2,4,5-T demonstrated a decreased ability to metabolize 2,4,5-T with higher concentrations of 2,4-DCP phenol being produced rather than 2,4,5-TCP. This suggests that 2,4-D was the

preferred substrate for the TftA enzyme and was competing with 2,4,5-T for the enzymes binding site. It has previously been hypothesized that 2,4-D metabolism by *B. cepacia* AC1100 results in a toxic chlorohydroquinone which acts to inhibit the TftCD two-component monooxygenase (Haugland et al. 1990). Other results of this study indicate however that cultures amended with 2,4-DCP are able to completely metabolize 2,4,5-T. Therefore the inhibition associated with 2,4-D addition is likely due to the 2,4-D itself and not a metabolic intermediate. This is supported by the slower but continued growth of these cultures and the presence of 2,4-DCP and 2,4,5-TCP at low concentrations through the experiment. A previous publication (Part III) has documented that this strain is capable of a low level of 2,4-D mineralization, and therefore it is possible that a low but significant amount of 2,4-D can be metabolized through the 2,4,5-T pathway or that another pathway capable of minimal 2,4-D degradation exists in this strain. Experiments examining the potential of 2,4-D to act as a carbon and energy source in liquid culture have been negative, but minimal growth is observed on solid media containing 2,4-D (control plates were negative for growth, data not shown).

It appears that the presence of 2,4-DCP in association with 2,4,5-T degradation results in a decreased conversion rate of 2,4,5-T to 2,4,5-TCP as evidenced by a decrease in cell growth and 2,4,5-T conversion during the first 50 hours of growth. This postulation was supported by a decrease in *tftA* mRNA transcripts in 2,4-DCP treated cultures. This suggests that conditions, which lower the metabolic activity of *tftA* either through specific interactions or more general stress, related responses allow for more efficient 2,4,5-T metabolism. The original 2,4,5-T-degrading consortium had a slow

growth rate with a mean generation time of greater than 30 hours. The implication of this data is that *tftA* activity must be carefully controlled during 2,4,5-T metabolism. Another possible mechanism by which 2,4-DCP may lower the metabolic activity of these cells is by acting as a competitive substrate for the TftCD enzyme. It has been demonstrated that *B. cepacia* AC1100 has two copies of the *tftCD* (Hubner et al. 1998) and it has been hypothesized that this allows the bacterium to cope with the formation of 2,4,5-TCP during 2,4,5-T metabolism. An alternative strategy that could be used to deal with 2,4,5-TCP accumulation problems during 2,4,5-T metabolism could be decreasing the rate at which cells metabolize 2,4,5-T either through a general decrease in growth rate or through modulation of the 2,4,5-T oxygenase.

#### 4.6 ACKNOWLEDGMENTS

This research project was supported by The United States Air Force Office of Scientific Research (AASERT Award) and the Dow Foundation (SPHERE Award).

## **PART V**

**MODULATION OF DIBENZOFURAN DEGRADATION  
BY METABOLIC INTERMEDIATES OF 2,4,5-T AND 2,4-D IN  
*SPHINGOMONAS* SPECIES STRAIN RW1.**

## 5.1 ABSTRACT

*Sphingomonas* species strain RW1 was chosen as a model bacterium to examine dibenzofuran (DBF) degradation in the presence of metabolic intermediates of 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). Bacterial growth experiments demonstrated that 2,4-dichlorophenol (2,4-DCP) and 2,4,5-trichlorophenol (2,4,5-TCP) inhibited growth when present at concentrations of 10 mg/L. Growing cell assays demonstrated that 500 mg/L 2,4-D and 500 mg/L 2,4,5-T delayed growth of *Sphingomonas* species strain RW1 on acetate, and bacterial cultures containing DBF and 2,4,5-T had a lower overall biomass relative to cultures grown with only DBF. Cultures containing acetate and 2,4-D or DBF and 2,4-D had increased overall biomass and metabolized all of the 2,4-D present. During growth on DBF/2,4-D and acetate/2,4-D 2,4-DCP concentrations were maintained below 1.0 mg/L. However, during growth on acetate/2,4,5-T, 2,4,5-TCP concentrations reached a maximum of 11.9 mg/L, although overall biomass relative to acetate controls was not significantly affected. During growth on DBF in the presence of 2,4,5-T 2,4,5-TCP concentrations were maintained at approximately 2.1 mg/L. Comparative mRNA fingerprint analysis using a modified Random Arbitrarily Primed Reverse Transcriptase - PCR (RAP-PCR) protocol identified an mRNA transcript, putatively designated *OBI*, which was upregulated in the presence of DBF. Bioinformatic analysis of this RNA transcript revealed that it corresponds to an open reading frame located 926 bp downstream and in frame with the *dxnB* gene. Furthermore, RNA hybridization studies showed that for acetate grown cells treated with 2,4-D or 2,4,5-T and dibenzofuran growing cells treated with 2,4-D resulted in decreased *dxnA1*, *dbfB* and *OBI* transcript abundance. Additionally, 2,4-DCP and 2,4,5-TCP

resulted in decreased *dxnA1*, *dbfB*, and *OBI* mRNA concentrations for both DBF and acetate grown cells.

## 5.2 INTRODUCTION

Chlorinated dibenzofurans (DBF) and dibenzo-*p*-dioxins (DD) are unintentionally produced during many industrial processes including the manufacture of herbicides such as Agent Orange, incineration of industrial and domestic wastes, and the bleaching of paper pulp (EPA 1995, Fortnagel et al. 1990, and Fortnagel et al. 1989). As a result of these processes, polychlorinated DBFs and DDs (PCDD/F) are now widespread contaminants in the environment. In fact, the Environmental Protection Agency has postulated that in 1970 up to one half of the environmental emissions of dioxins were due to the spraying of chlorophenolic herbicides and pesticides. Although the use of these herbicides and pesticides was discontinued in the 1980s, environmental sinks such as compost, sewage sludges, and sediments, may be a significant contributor to current dioxin deposition (EPA 1995). Agent Orange is composed of a mixture of 50% 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) and 50% 2,4-dichlorophenoxyacetic acid. During the synthesis of these phenoxyacetic acids, many chlorinated congeners of DD and DBF are generated as contaminants due to the chemical processes used (Thomas et al. 1996). These dioxins are found in most, if not all, Agent Orange samples from the environment and are responsible for many of the potential health risks associated with this herbicide.

The abundance of these PCDDs and PCDFs poses a significant contamination problem, because of their associated toxicity to both humans and animals and their

recalcitrance under environmental conditions. The extent to which these PCDD/Fs are biodegraded by environmental bacteria is still uncertain. Recently, several laboratories have begun to elucidate a degradation pathway in *Sphingomonas* sp. strain RW1, which allows this organism to co-metabolize several mono- and di-chlorinated DBFs and DDs, during the mineralization of unchlorinated DBFs and DDs (Halden et al. 1999 and Wilkes et al. 1996). Similar activities, by *Burkholderia* sp. strain LB400, have been observed for polychlorinated biphenyl (PCB) metabolism utilizing biphenyl as the growth substrate where PCB congeners containing up to six chlorine substituents are degraded.

*Sphingomonas* species strain RW1 is able to completely mineralize DBF and DD (Armengaud et al. 1998 and Happe et al. 1993). Initial dioxygenation is achieved through the activity of DxnA1A2, a three component angular extradiol dioxygenase, which introduces two hydroxyl groups at the 4 and 4a position between the two aromatic rings (Armengaud and Timmis 1997). It has been demonstrated that DxnA1A2 activity is modulated according to the available carbon source (Armengaud et al. 1998). Dihydroxylation of the biaryl either requires the interaction of a short electron transport chain, consisting of a 12 kDa ferredoxin and a monomeric flavoreductase, with the three component dioxin dioxygenase (Armengaud and Timmis 1998 and Butler and Mason 1997). This reaction requires both  $\text{NADH}_2$  and  $\text{O}_2$  and results in the formation of a diene-diol-hemiacetal, which is believed to spontaneously rearomatize to 2,2',3-trihydroxybiphenyl (Bunz and Cook 1993). 2,2',3-Trihydroxybiphenyl is then *meta*-cleaved by the product of the *dbfB* gene, 2,2',3-trihydroxybiphenyl dioxygenase, to form either 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate or the 6-(2-hydroxyphenyl) ester of 2-

hydroxymuconic acid, depending on whether DBF or DD was the initial substrate (Happe et al. 1993). This extradiol dioxygenase is active as a monomer and requires another molecule of dioxygen, but does not require an additional external reductant. 3-chlorocatechol inhibits this dioxygenase, as has been observed for other dioxygenases of this type (Happe et al. 1993).

Currently, it is thought that both DBF and DD metabolism proceeds using a single pathway for degradation rather than two separate pathways. 2-Hydroxy-6-oxo-6-phenylhexa-2,4-dienoate has been shown to be hydrolyzed by the H1 (DxnB) and/or H2 dibenzofuran hydrolase to generate 2-oxopentanoate and salicylic acid. These hydrolases were identified in strain RW1 using protein purification and subsequent enzyme assays (Bunz et al. 1993). However, the corresponding substituted dienoate from DD has not been demonstrated to be a substrate for these hydrolases, and no data has been presented speculating on whether or not these hydrolases are involved in DD mineralization. By whatever mechanism, the 6-(2-hydroxyphenyl) ester of 2-hydroxymuconic acid is postulated to be converted to 2,6-hydroxy-2,4-hexadiendioic acid and catechol (Wittich et al. 1992). The product of the H1/H2 hydrolases, salicylate, is believed to be converted to catechol via an unidentified mechanism (Happe et al. 1993). Catechols derived from DBF and DD are believed to be further metabolized through the activity of an unknown catechol-2,3-dioxygenase, which is distinct from the DbfB enzyme (Bunz et al. 1993). There has been no catechol-1,2-dioxygenase activity found in this organism to date (Bunz et al. 1993).

Previous studies addressing the biodegradability of Agent Orange in the environment have focused on 2,4-D and 2,4,5-T. Consequently, fundamental knowledge concerning the ability of environmental bacteria to mediate degradation of DBFs and DDs associated with Agent Orange contaminated soils is lacking. Since many of the toxicological effects of Agent Orange are believed to actually be the result of contaminating PCDD/Fs within this herbicide mixture, it is necessary to develop a more thorough understanding of how degradation of the "dioxin" class of compounds occurs in relationship with other Agent Orange constituents. The goal of this research project is to determine the extent to which intermediates of 2,4-D and 2,4,5-T metabolism modulate DBF degradation in *Sphingomonas* species strain RW1. This research project will further the understanding of the microbial processes surrounding DBF and DD metabolism and facilitate the development of future approaches for dealing with dioxins in the environment.

## 5.3 MATERIALS AND METHODS

### 5.3.1 Chemicals.

Chemicals used in this study were of the highest grade commercially available. Dibenzofuran (DBF, 99+%), 4-bromobenzoic acid, and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T, 97%) were obtained from Aldrich chemical Company, Inc. (Milwaukee, WI). 2,4-Dichlorophenol (2,4-DCP, 99%) and 2,4-dichlorophenoxyacetic acid (2,4-D, 99+%) were obtained from Acros Organics (Pittsburgh, PA). 2,4,5-Trichlorophenol (2,4,5-TCP,

99%) was purchased from Fluka Chemicals (Milwaukee, WI). Acetonitrile (HPLC grade) and water (HPLC grade) were obtained from Fischer Scientific. Dimethylformamide (DMF, 99+%) was purchased from Sigma chemical company (Milwaukee, WI). All other chemicals were of analytical grade.

### 5.3.2 Bacteria and media.

*Sphingomonas* species strain RW1 (Happe et al. 1993) was cultured in phosphate-buffered minimal salts medium (PAS), adjusted to pH 7.2, containing in grams per liter; 4.43 g  $K_2HPO_4$ , 1.72 g  $KH_2PO_4$ , 2.16 g  $NH_4Cl$ , 0.195 g  $MgSO_4 \cdot H_2O$ , 0.01 g  $FeSO_4 \cdot 2H_2O$ , and 0.003 g  $CaCl_2 \cdot 2H_2O$ . PAS media was supplemented with 50 mg of DBF (Stock solutions of DBF were prepared containing 500 mg of DBF dissolved in 1.0 ml of DMF). Addition of DBF in DMF solution resulted in finely dispersed DBF particulates. 12.0 mM acetate as an alternative carbon source was added prior to autoclaving and supplemented with 100  $\mu$ l DMF once cool. Solidified PAS medium was prepared by addition of 15 g/L of Agarose (Difco) added prior to autoclaving. 2,4-DCP and 2,4,5-TCP were solubilized in HPLC grade  $H_2O$  and added at appropriate concentrations whereas 2,4-D and 2,4,5-T were added to the growth media prior to autoclaving at concentrations up to but not exceeding 1000 mg/L. HPLC analysis of sterile PAS media containing 2,4-D or 2,4,5-T was used to verify the stability of these compounds during heating. These examinations did not detect significant concentrations of 2,4-DCP or 2,4,5-TCP in autoclaved media. Once prepared all media containing 2,4-D and 2,4,5-T were stored at 28°C in the dark. *Escherichia coli* strain DH5 $\alpha$  (Invitrogen,

San Diego, California) was grown in Luria-Bertani broth (10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract) and used in combination with the pCR 2.1 (Invitrogen, San Diego, California) plasmid vector for all cloning experiments as described by the manufacturer.

### ***5.3.3 Random arbitrarily primed reverse transcriptase - PCR (RAP-PCR)***

A modified comparative RAP-PCR protocol was used to identify potentially novel genetic elements, which were induced during growth on DBF. For these experiments *Sphingomonas* species strain RW1 was cultured using PAS + DBF. Starter cultures were grown to an approximate optical density (600 nm) of 0.5, and 0.5 ml was used to inoculate both test cultures. Mid-log phase cultures, approximately at optical density of 0.5, were then the source of a series of samples for RNA extractions. First, 6.0 ml of each culture was aliquoted into four Eppendorf tubes and immediately placed in an ice bath. Rifampicin was then added to each culture to a final concentration of 0.2 mg/ml. Rifampicin was added as a transcriptional inhibitor, thus allowing mRNA transcripts to be differentiated from rRNA during RT-PCR (Nagel et al. 1999). The cultures were then allowed to incubate for up to 64 min after adding rifampicin addition. 6.0 ml samples from the rifampicin treated cultures were then transferred to 1.5 ml Eppendorf tubes and placed in the ice bath at 0, 4, 8, 16, 32, and 64 min. Once samples were collected, they were centrifuged at 14,000 rpm in a 4°C cold room. Cell pellets were washed with ice cold 1X PAS media, re-centrifuged and stored at -80°C.

Total RNA was extracted using the hot phenol method, as previously described (Fleming et al. 1993), and stored at -80°C prior to reverse transcription. Reverse

transcription reaction components were as follows: 200  $\mu$ M dNTP, 5.0 mM DTT, 50 units of MMLV reverse transcriptase (Gibco BRL, Grand Island, New York), 1X MMLV reaction buffer, 0.4  $\mu$ M Shine-Dalgarno primer and 1.0  $\mu$ g of total RNA was used as the template. For these experiments, the Shine-Dalgarno primer was used for the reverse transcription step, in addition to the PCR. During the reverse transcription step, the Shine-Dalgarno primer acts only as a random, non-specific primer, whereas during the PCR step the Shine-Dalgarno targets the 5' end of the messenger RNA during amplification, as previously described (Fleming et al. 1998). Reaction conditions for the reverse transcription were: an initial ramping down from 55°C to 37°C for primer annealing, reverse transcription at 37°C for 60 min, and a final 5.0 min incubation at 75°C for enzyme inactivation. Reverse transcription reactions were stored at -20°C prior to PCR. Components for the PCR were as follows: 20.0  $\mu$ M dNTP, 1X PCR Buffer (Gibco BRL, Grand Island, New York), 0.3  $\mu$ l Taq Polymerase (Gibco BRL, Grand Island, New York), 1.8  $\mu$ l DMSO, 0.4  $\mu$ M Shine-Dalgarno primer, 0.3  $\mu$ l 10% TritonX-100, and 0.25  $\mu$ l  $\alpha$ -<sup>32</sup>P CTP (800  $\mu$ C/mM), 3.0  $\mu$ l reverse transcription reaction, and PCR reactions volumes were adjusted to 30.0  $\mu$ l using ultrapure H<sub>2</sub>O. Following an initial denaturation at 92°C for 2.0 min, 40 cycles of PCR consisted of: denaturation at 92°C for 15 sec, annealing at 40°C for 30 sec, and primer extension at 72°C for 30 sec.

PCR reactions were visualized on sequencing gels using a Genomix DNA sequencing apparatus (Genomix, Foster City, CA). Four  $\mu$ l of each PCR reaction were combined with 4.0  $\mu$ l of loading dye and incubated for 2.0 min at 100°C. Four  $\mu$ l of this mixture was electrophoresed using a 4.5% denaturing polyacrylamide gel for 2.0 hours at

2700 V. The polyacrylamide gel was then dried directly on the glass plate and urea residue was removed by washing with distilled H<sub>2</sub>O and subsequent drying according to manufacturer's directions. This washing procedure was repeated until no traces of urea were visible. After washing, the dried gel was exposed to autoradiography film (Genomix, Foster City, CA) for 12 – 96 hours.

Differential cDNA bands were eluted from the gel for further analysis. Using the autoradiogram as a template, cDNA bands were cut out using a razorblade and softened in 5.0 µl of HPLC grade water. These bands were diluted in 100 µl H<sub>2</sub>O, incubated at 68°C for 2.0 hours, and re-amplified using PCR (same conditions as above but omitting the radionucleotide). Reamplified PCR products were separated using a 1.0 % low melting point agarose (SeaPlaque<sup>®</sup> GTG) gel. Reamplified bands were excised from the agarose gel and used in subsequent studies.

Because of the presence of rRNAs in the RNA extractions, it was necessary to verify that the cDNA obtained through DDRT-PCR was actually the product of messenger RNA transcripts, prior to cloning. Total RNA from PAS + DBF cultures were vacuum blotted onto nylon membranes (ICN Biochemical, Cleveland, OH). The reamplified cDNAs were radiolabeled using T4 Kinase (Gibco BRL, Grand Island, New York) according to the manufacture's protocol (for a more detailed description see below). Membranes were pretreated in a solution (pH 7.3) containing, 0.5 M Na<sub>2</sub>HPO<sub>4</sub>, 1.0 mM EDTA, and 7.0 % SDS and incubated at 65°C in a shaking water bath. After 4.0 hours, the denatured cDNA probe was added to the solution and allowed to incubate with the membrane for up to 24 hours. Membranes were then washed four times with a high

stringency wash buffer containing, in g/L: 0.59 g NaCl, 3.15 g Tris-HCl, 0.37 g EDTA, and 5.0 g SDS. Hybridized blots were then exposed to Kodak Biomax<sup>TM</sup> MR film (Eastman Kodak, Rochester, NY) and allowed to incubate for 24 – 96 hours at -80°C or analyzed using the Storm<sup>TM</sup> 840 Phosphoimager (Molecular Dynamics, Sunnyvale, CA).

In addition to the RNA hybridization verification, standard RT-PCR was utilized to re-detect the band of interest and exclude the possibility of chimeric artifacts. Conditions for this RT-PCR were as follows: 200  $\mu$ M dNTP, 5.0 mM DTT, 50 units of MMLV reverse transcriptase (Gibco BRL, Grand Island, New York), 1X MMLV reaction buffer, 0.4  $\mu$ M random primer and 1.0  $\mu$ g of total RNA was used as the template. Reaction conditions for the reverse transcription were: an initial ramping down from 55°C to 40°C for primer annealing, reverse transcription at 37°C for 60 min, and a final 5.0 min incubation at 75°C for enzyme inactivation. Reverse transcription reactions were stored at -20°C prior to PCR. PCR beads (Amersham Pharmacia, Piscataway, New Jersey) were used for subsequent PCR amplification. 1 PCR bead was combined with 21  $\mu$ l HPLC grade H<sub>2</sub>O, 1.5  $\mu$ l 2.0  $\mu$ M primer A, 1.5  $\mu$ l 2.0  $\mu$ M primer B, and 1.0  $\mu$ l reverse transcription reaction. Primers A and B were specifically designed from the sequenced product of RAP-PCR analysis using the Primer3 software algorithm (Whitehead Institute for Biomedical Research, <http://www.genome.wi.mit.edu>). For band *OBI*, primer A was 5'-GCT CTA CCT TGG CAT TCT CG-3' and primer B was 5'-TGA AAA CAG TCC TGA CAT CG-3'. PCR was achieved using a modified touchdown protocol originally developed by Hecker et al. (1996), and consisted of an initial denaturation at 92°C for 4.0 min, 20 cycles of PCR including a denaturation at

92°C for 15 sec, annealing at (65 - 45 °C, 1°C degrees/cycle) for 30 sec, and primer extension at 72°C for 30 sec. Finally, a 7.0 min extension was followed by storage at 4°C until gel electrophoresis.

#### 5.3.4 Growing cell assays.

Growing cell assays were used to study the effects of 2,4-D, 2,4,5-T, 2,4-DCP, and 2,4,5-TCP on growth of *Sphingomonas* species strain RW1 in PAS + DBF and PAS + acetate. For these experiments, starter cultures containing 50 ml of PAS medium amended with 1 mg/ml DBF or 12.0 mM acetate, as described above, were inoculated *Sphingomonas* species strain SRW1 (0.5 ml from frozen freezer stocks was washed with 1X PAS and used to inoculate starter cultures). These cultures were then incubated at 28°C until mid-log growth phase was achieved. 0.1 ml from these starter cultures was then transferred to 50 ml sterile mineralization vials containing 10 ml of PAS medium amended with the appropriate concentration of 2,4-D, 2,4-DCP, 2,4,5-T, 2,4,5-TCP. During the course of the experiment, 200 µl samples were taken for optical density measurements and analytical determination of 2,4-D, 2,4,5-T, 2,4-DCP, and 2,4,5-TCP concentrations. Spectrophotometric monitoring was routinely done using a Beckman DU-70 spectrophotometer (Beckman, Fullerton, CA) at 600 nm. Analytical analysis was achieved using High Pressure Liquid Chromatography (HPLC) as described below.

### 5.3.5 HPLC analyses.

Analytical determination of 2,4-D, 2,4,5-T, 2,4-DCP, and 2,4,5-TCP concentrations in culture supernatants was determined using HPLC. Samples for analysis were obtained directly from culture supernatants. Aliquots, 100  $\mu$ l were taken from growing bacterial cultures and transferred to 1.5 ml microcentrifuge tubes. These were then microcentrifuged at 14,000 rpm to pellet bacterial cells, and the supernatant was frozen at -80°C for HPLC studies. Immediately prior to analysis, samples were warmed at 37°C for 5 min and 70  $\mu$ l was transferred to an 1.8 ml amber autosampler vial containing 730  $\mu$ l HPLC grade H<sub>2</sub>O and 50  $\mu$ g 4-bromobenzoic acid (4-BBA, internal standard) dissolved in 200  $\mu$ l acetonitrile. Sample injection was facilitated by a Perkin Elmer LC 600 autoinjector (Perkin Elmer, Foster City, CA) fitted with a 50  $\mu$ l injection loop. A Supelcosil LC-18-T reverse phase C<sub>18</sub> column (Supelco, Bellefonte, PA) was fitted onto a Perkin Elmer Binary LC 250 series pump (Perkin Elmer, Foster City, CA) and a Perkin Elmer model LC-235 diode array detector (Perkin Elmer, Foster City, CA). Data analysis was achieved using the Turbochrome version 4.1 software package (Perkin Elmer, PE Nelson Division, San Jose, CA). Conditions for HPLC analysis were optimized for detection of the above compounds in 20% acetonitrile:80% H<sub>2</sub>O. Samples were eluted at a flow rate of 1.0 ml/min with 0.025% H<sub>3</sub>PO<sub>4</sub>-acetonitrile gradient, as follows: 0% acetonitrile (isocratic, 5.0 min), 0 to 50% acetonitrile (linear, 5.0 min), 50% acetonitrile (isocratic, 20 min), 50 to 0% acetonitrile (linear, 5.0 min). Absorbance of eluted compounds was monitored at 210 nm. Sample retention times were compared to known standards prepared and analyzed using identical protocols. Retention times for

compounds of interest were 4-BBA (16.2), 2,4-D (17.1), 2,4-DCP (18.0), 2,4,5-T (19.2), and 2,4,5-TCP (21.5).

### 5.3.6 RNA hybridization.

RNA hybridization experiments were used to examine the effects of 2,4-D, 2,4,5-T, 2,4-DCP, and 2,4,5-TCP on expression of the *dxnA1*, *dbfB*, and *OBI* genes. For these experiments, *Sphingomonas* species strain RW1 was grown on PAS containing either DBF or acetate (as described above). These cultures were incubated at 28°C until mid-log phase was achieved. Aliquots from these cultures were then transferred into 20 ml sterile scintillation vials containing PAS media amended with the test compounds. The following treatments were tested: 0, 100, 250, and 500 mg/L 2,4-D or 2,4,5-T; 0, 10, and 25 mg/L 2,4,5-TCP or 2,4-DCP. Following amendments with these compounds, cultures were allowed to incubate for 1 generation time. Total RNA was extracted from these test cultures using the hot phenol method (Fleming et al. 1993). RNA hybridization analysis will be conducted as previously described (Sambrook et al. 1989). 2.5 µg of total RNA was applied to nylon membranes as described above.

### 5.3.7 Probe preparation.

Primers for PCR amplification of the *dxnA1* and *dbfB* genes were developed using nucleic acid sequences obtained from the Genbank sequence database (National Center for Biotechnological Information, NCBI, <http://www.ncbi.nlm.nih.gov>). Specific primer sequences were chosen with the help of the Primer3 software algorithm (Whitehead

Institute for Biomedical Research, <http://www.genome.wi.mit.edu>) and Blast 2.0 algorithms (NCBI). Primer sequences were as follows: *dxnA1*\_L1 (5'-GCG GCA ACG CCA AA), *dxnA1*\_R1 (5'-CGT CGC CGT CGT TCA-3'), *dbfB*\_L1 (5'-GGC GCT CAA CTC G-3'), and *dbfB*\_R1 (5'-TTC CGC ATC AGC C-3'). These primer sets produced 504 and 415 bp products, respectively, corresponding to the 299 - 802 bp region of the *dxnA1* and the 172 - 586 bp region of the *dbfB*. All primers were synthesized using a Beckman oligo 100 DNA synthesizer (Beckman Instruments, Fullerton, CA) according to the manufacture's directions.

PCR amplifications were achieved using PCR beads (Amersham Pharmacia, Piscataway, New Jersey), according to manufacture's recommendations coupled with a modified touchdown PCR protocol. Initial PCR analyses were conducted using whole bacterial cells from solid media. One small bacterial colony was transferred to a sterile 200 µl thin wall PCR tube (Perkin Elmer, PE Nelson Division, San Jose, CA) and combined with the following components: 1.5 µl Primer A (2.0 µM stock), 1.5 µl Primer B (2.0 µM stock), 23.5 µl ultrapure H<sub>2</sub>O, and 1 PCR bead. Amplification of cloned PCR products was achieved using 1.5 µl Primer A (2.0 µM stock), 1.5 µl Primer B (2.0 µM stock), 22.5 µl ultrapure H<sub>2</sub>O, 1.0 ul Template DNA (100 ng) and 1 PCR bead. All PCR products were stored at -20°C prior to cloning, sequencing, and future analyses. PCR products were cloned using Invitrogen's TA cloning kit (Invitrogen, San Diego, California). Freshly amplified PCR bands were ligated into the pCR 2.1 vector, overnight at 14°C, and transformed into *E. coli* DH5α cells (Invitrogen, San Diego, California). Plasmid DNA was extracted using the alkali lysis method as described by

Sambrook et al. (1989) or the BIO-RAD Quantum Prep<sup>®</sup> plasmid miniprep kit (BIO-RAD Laboratories, Hercules, CA) and characterized using *Eco*R1 endonuclease digestion followed by agarose gel electrophoresis. Plasmid inserts matching the molecular weight of the expected cDNA band were sequenced by the Department of Microbiology Molecular Biology Resource Facility (The University of Tennessee, Knoxville) using either T7 or M17 based sequencing primers. Sequences obtained were analyzed using the Blast 2.0 algorithm (NCBI) in order to determine similarities to published gene sequences.

Probes for RNA hybridization studies were generated using PCR, as described above, coupled with Stratagene's Prime-It<sup>®</sup> Random primer labeling kit. PCR products were electrophoresed using 1.0 % low melting point agarose (SeaPlaque<sup>®</sup> GTG). Bands were excised from the gel, combined with 250  $\mu$ l HPLC grade H<sub>2</sub>O, and incubated at 60°C to soften the agarose. A 10  $\mu$ l aliquot from the softened band was then combined with 10  $\mu$ l of random primer and 14  $\mu$ l H<sub>2</sub>O and incubated at 100°C for 5 min. To this mixture 10  $\mu$ l of 5X primer buffer, 5  $\mu$ l  $\alpha$ -<sup>32</sup>P-dCTP, and 1  $\mu$ l Klenow enzyme was added. The reaction was incubated at 37°C for 15 min, and 2  $\mu$ l of stop mix was added. The mixture was push column purified according to manufacturer's instructions (Stratagene, La Jolla, CA), and counted using a Beckman LS 5000 TD scintillation counter (Beckman, Fullerton, CA). Probes were then boiled for 10 min, cooled quickly to 4°C and added directly to the hybridization solutions. For these hybridizations, Ambion's Ultrasensitive hybridization buffer (Ambion, Austin, TX) was used as described by the manufacturer. The hybridization solution was preheated at 60°C, and 10

ml was added to hybridization bags and allowed to prehybridize in a 42°C water bath for 2 hours. Radiolabeled probes were then added directly to the hybridization bags and allowed to incubate 16 - 24 hours. These blots were then washed twice using 2 X SSC, 0.1% SDS at 40° for 15 min, and twice using a high stringency wash buffer containing: 0.1X SSC and 0.1% SDS at 42°C for 15 min. RNA blots were quantitated using a Storm<sup>TM</sup> 840 Phosphoimager (Molecular Dynamics, Sunnyvale, CA).

#### ***5.3.8 Data analysis***

Experiments for this study were done in triplicate. For Growing cell assays, one starter culture was used to inoculate three independent cultures. Each of these replicate cultures was then sampled throughout the time course of the experiment. These samples were analyzed to determine optical density and compound concentrations. For messenger RNA analyses, three bacterial cultures were independently treated with the test compound and allowed to incubate for one generation time. Total RNA from these cultures was then extracted, applied to nylon membranes, and hybridized to a radiolabeled probe. Numerical data was obtained after densitometry using a phosphoimager (described above). These data were then entered into Microsoft Excel 97 (Microsoft Corporation, Redmond, WA) and an average value was calculated using the AVERAGE function. A standard deviation was also calculated for these values using the STDDEVP function. This standard deviation value is a measure of how widely values are dispersed from the mean value. No further statistical analysis was utilized. Conclusions

from these experiments were based on separation of the average values by at least one standard deviation.

## 5. 4 RESULTS

### *5.4.1 Random arbitrarily primed reverse transcriptase - PCR (RAP-PCR) identified a novel genetic element, OB1, which is transcribed during growth of Sphingomonas species strain RW1 on dibenzofuran.*

Using a degenerative primer sequence designed to include the Shine-Dalgarno sequence located at the 5' end of the messenger RNA molecule during RT-PCR, a comparative RT-PCR protocol was used to facilitate identification of potentially important metabolic genes induced during growth on dibenzofuran. During these experiments, a 781 bp gene sequence (designated OB1) was identified using total RNA isolated from *Sphingomonas* species strain RW1 cells grown on dibenzofuran as a sole carbon and energy source (Figure 5.1). The identification of this *OB1* genetic element was greatly facilitated by treating Strain RW1 cultures with rifampicin as described in the Material and Methods. The band corresponding to *OB1* was clearly diminished in the rifampicin treatments. An additional secondary advantage of the rifampicin treatment is that treated samples serve as excellent controls for DNA contamination. RNA hybridization studies confirmed that this genetic element was the product of mRNA

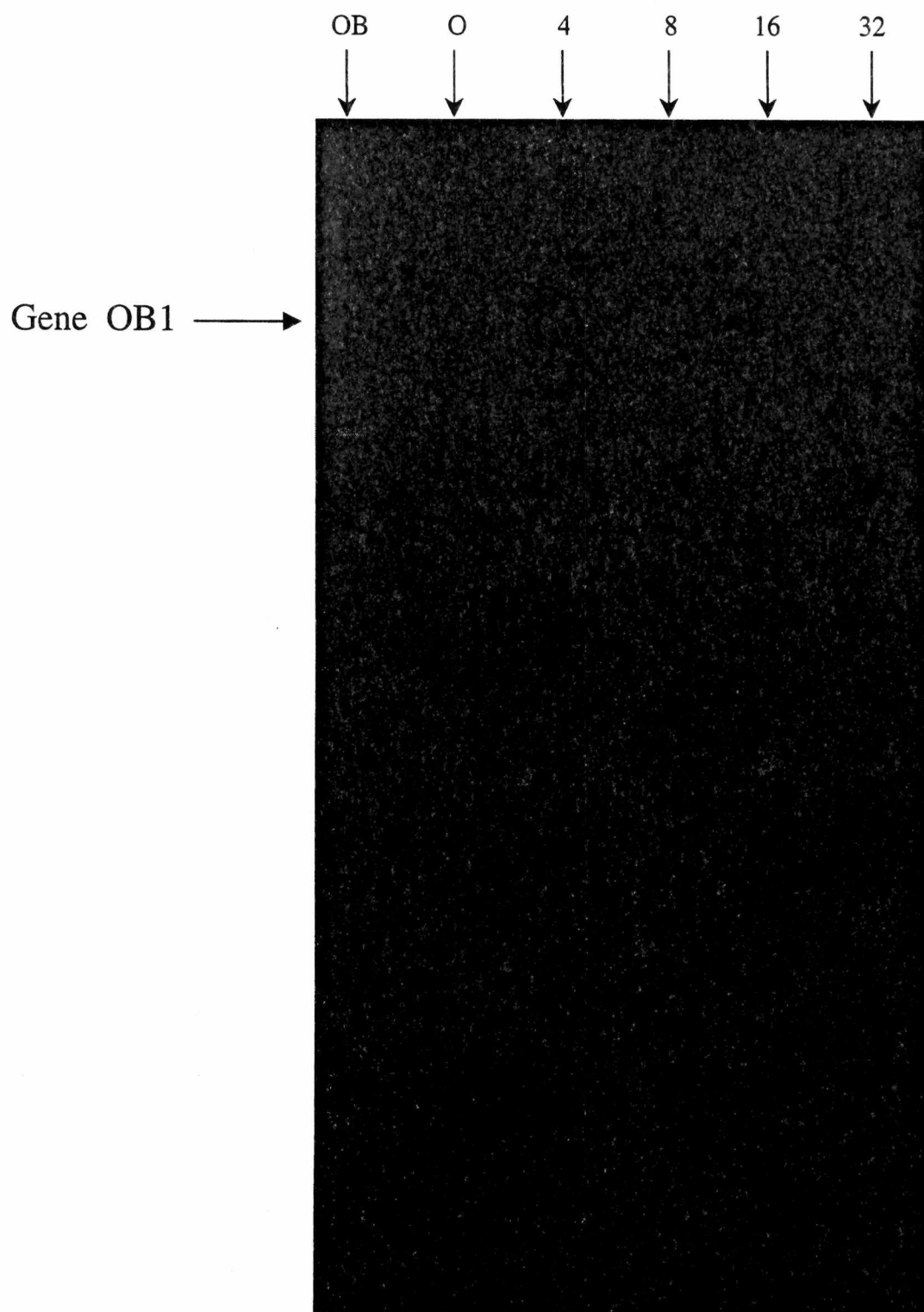


**Figure 5.1** RAP-PCR of dibenzofuran grown *Sphingomonas* species strain RW1 cells. A log phase culture was treated with 200  $\mu\text{g/ml}$  rifampicin and samples were taken at 0, 4, 8, 16 and 32min. The samples were the source for total RNA isolation and subsequent RAP-PCR. A sample was also obtained prior to rifampicin addition to serve as a background control. mRNA bands of importance are marked with arrows.

amplification and not rRNA (Figure 5.2). In addition to verification by RNA hybridization studies, standard, specific, RT-PCR, using primers designed from the *OBI* gene sequence, demonstrated that this genetic element was expressed during growth on dibenzofuran and not a chimeric artifact (Data not shown).

The sequence of this genetic element was found to be homologous to a region of DNA previously published by Dr. Jean Armengaud (accession number X72850), specifically corresponding to 12,673 - 13,454 bp in their sequence. An open reading frame search using this genetic element identified a 1106 bp open reading frame located between 12,808 and 13,914 base pairs. The ORF Finder algorithm located at the National Center for Biotechnological Information was used for this analysis. A blast search using the tblastn search protocol (NCBI) identified an unknown (possibly cryptic) amino acid sequence from *Sphingomonas aromaticivorans*, having 32% amino acid identity with the translation product from the *OBI* hypothetical open reading frame.

#### **5.4.2 *Sphingomonas* species strain RW1 is able to co-metabolize 2,4-D during growth on dibenzofuran or acetate.**

Growing cell assays were used to examine whether 2,4-D affected the growth of *Sphingomonas* species strain RW1. Growth of *Sphingomonas* species strain RW1 for cultures containing 1000 mg/L DBF and 500 mg/L 2,4-D was not significantly affected in regards to the initiation of growth, but initiation of growth for cultures containing 12.0

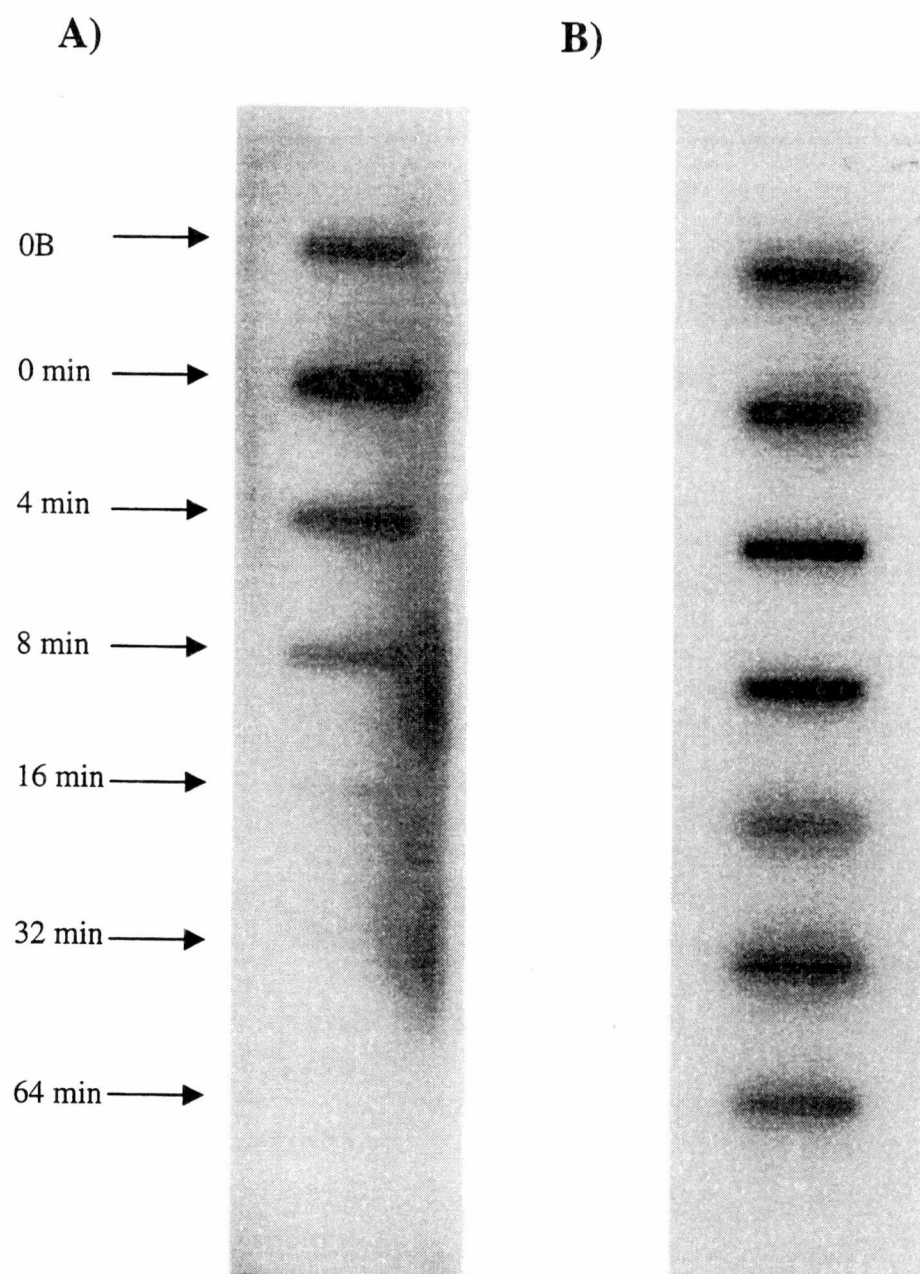
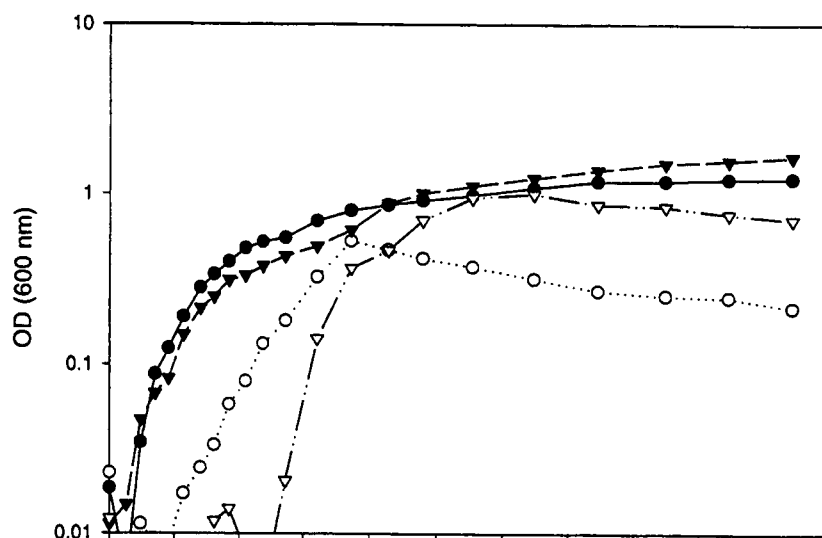


**Figure 5.2** RNA hybridization studies were used to verify that band *OBI* was of mRNA origin and not ribosomally derived from rRNA. The RAP-PCR Band was eluted from the gel and reamplified using PCR. This PCR product was then radiolabeled and used to probe total RNA isolated from *Sphingomonas* species strain RW1 grown on DBF (A). A 16S control probe was also labeled and used to probe an identical RNA blot (B).

mM acetate and 500 mg/L 2,4-D was delayed by approximately 30 hours (Figure 5.3A). For both DBF and acetate cultures amended with 2,4-D, overall growth was increase as evidenced by a significant increase in total cellular biomass (Figure 5.3B). It was observed that, for both these cultures, degradation of 2,4-D occurred with a corresponding low-level accumulation of 2,4-DCP in culture supernatants (Figure 5.4A,B). 2,4-DCP concentrations were maintained at ~1.0 ppm or less for the duration of the growing cell assays (Figure 5.4B). Complete biodegradation of 2,4-D was achieved after 140 hours for DBF + 2,4-D cultures and 160 hours for acetate + 2,4-D cultures. *Sphingomonas* species strain RW1 mediated 2,4-D removal was apparently not significantly affected by the carbon source used, even though DBF containing cultures maintained higher overall biomass throughout the course for the experiment (Figure 5.3B). The time of initiation for 2,4-D degradation was the same for both dibenzofuran and acetate grown cultures (Figure 5.4A) suggesting that 2,4-D degradation was mediated by a pathway that is distinct from the DBF degradation pathway. Growth experiments examining utilization of 2,4-D as a sole carbon and energy by *Sphingomonas* species strain RW1 were negative (data not shown).

***5.4.3 Metabolism of 2,4,5-trichlorophenoxy acetic acid during growth of Sphingomonas species strain RW1 on dibenzofuran resulted in accumulation of 2,4,5-trichlorophenol.***

A)



B)

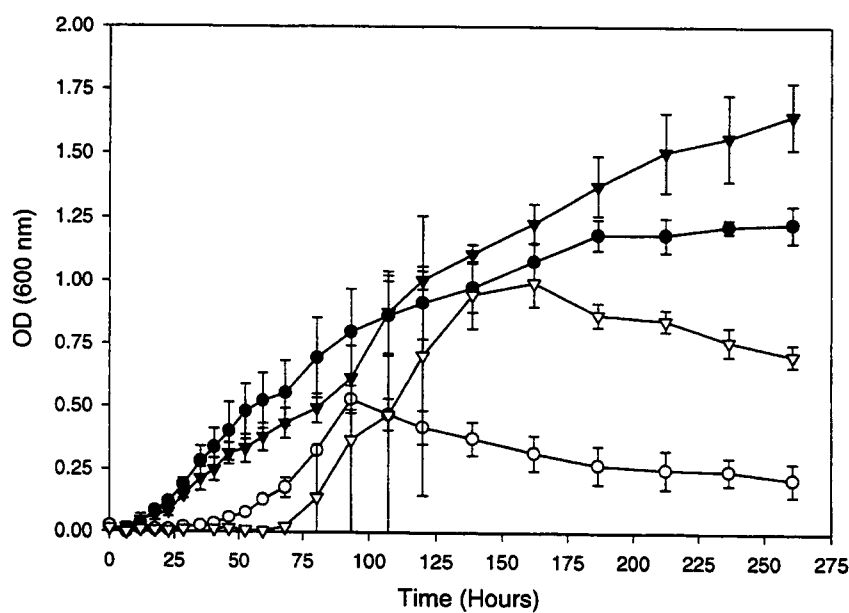
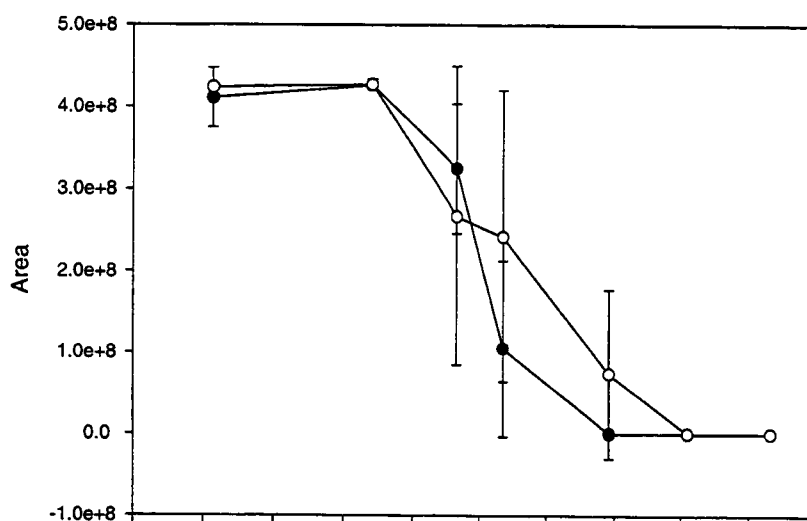


**Figure 5.3.** *Spingomonas* species strain RW1 growth experiment examining the effects of 2,4-D on DBF and acetate mediated growth. A) Log scale plot of the average of three separate experiments, and B) growth of Strain RW1 in PAS media amended with 0.1% DBF (●), 0.1% DBF + 500 mg/L 2,4-D (▼), 12.0 mM acetate (○), or 12.0 mM acetate + 500 mg/L 2,4-D (▽).

A)



B)

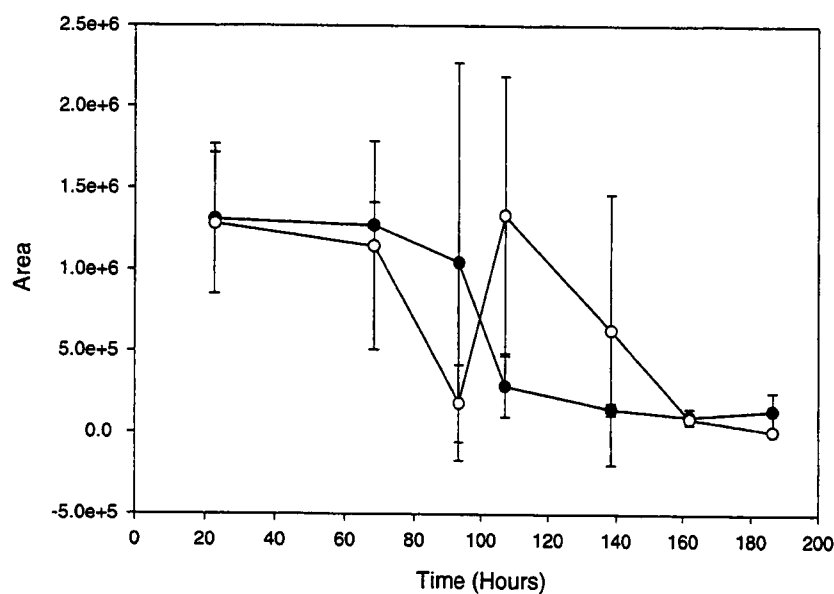


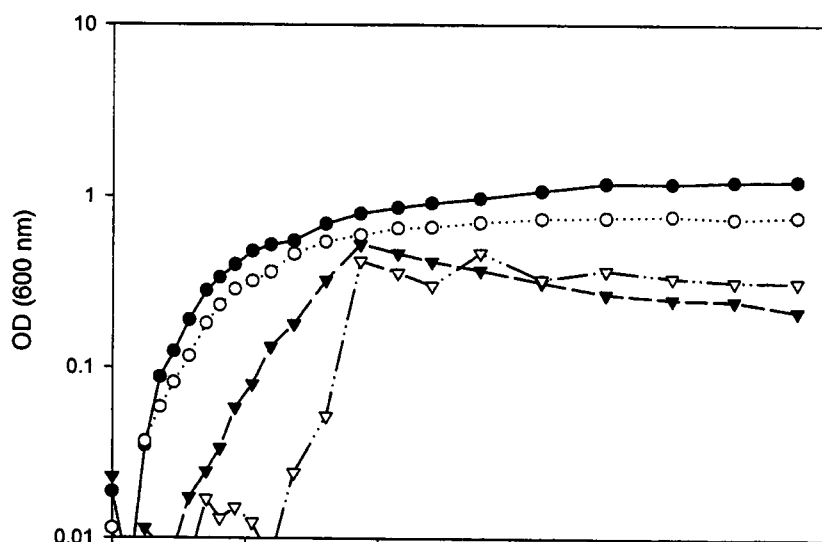
**Figure 5.4** *Sphingomonas* species strain RW1 growing cell assay experiment using PAS containing either dibenzofuran + 2,4-dichlorophenoxyacetic acid (●) or acetate + 2,4-dichlorophenoxyacetic acid (○). A) 2,4-dichlorophenoxyacetic acid concentrations, B) 2,4-dichlorophenol concentrations.

An examination of the effects of 2,4,5-T on growth of *Sphingomonas* species strain RW1 was facilitated by the use of growing cell assays. Addition of 500 mg/L 2,4,5-T into DBF cultures did not result in a delay in initiation of growth. Similarly to the acetate cultures supplemented with 2,4-D, acetate cultures amended with 2,4,5-T exhibited an approximately 30 hour lag in initiation of growth (Figure 5.5A). Unlike the effects seen when 2,4-D was added, addition of 2,4,5-T resulted in a decreased overall biomass for *Sphingomonas* species strain RW1 grown on DBF + 2,4,5-T (Figure 5.5B). During growth on DBF + 2,4,5-T, degradation of 2,4,5-T was minimal (Figure 5.6A), and 2,4,5-TCP concentrations were maintained at about 2.1 ppm (Figure 5.6B). This accumulation of 2,4,5-TCP seems to coincide with a decrease in cellular growth and suggests that 2,4,5-TCP is inhibitory to the cells. An unidentified metabolic intermediate was apparent during HPLC analysis of culture supernatants to access 2,4,5-T and 2,4,5-TCP concentrations, and had a retention time of approximately 14.5 min, (Data not shown). Cultures containing acetate and 2,4,5-T reached similar biomass levels as bacterial cells grown on acetate alone (Figure 5.5B). Unlike the DBF + 2,4,5-T cultures, these cultures accumulated 2,4,5-TCP up to 11.9 mg/L over the course of the experiment (Figure 5.6B), and 2,4,5-T concentrations were slightly decreased (Figure 5.6A).

#### ***5.4.4 2,4,5-T and 2,4-D repressed catabolic gene expression in *Sphingomonas* species strain RW1.***

Agent Orange is a mixture consisting of 50% 2,4-D and 50% 2,4,5-T and contains low levels of recalcitrant PCDDs and PCDFs compounds. Degradation of DD and DF in

A)



B)

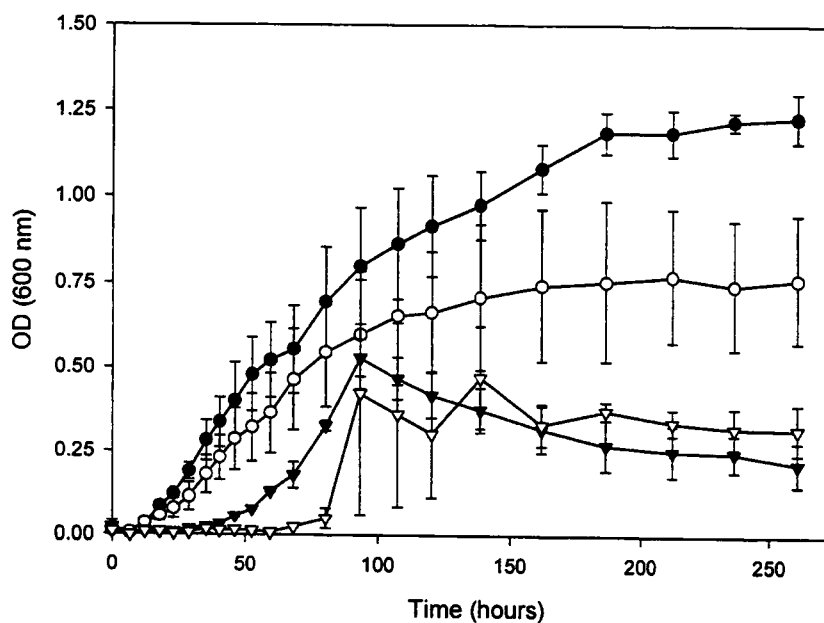
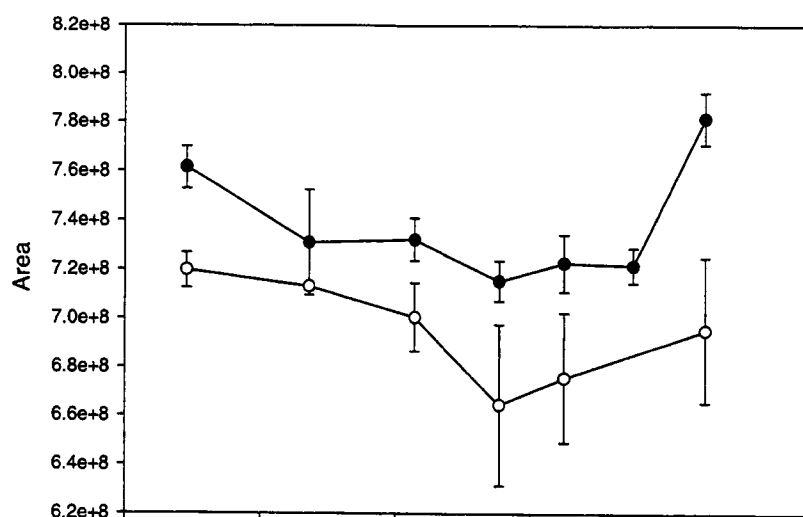


**Figure 5.5.** *SpHINGOMONAS* species strain RW1 growth experiment examining the effects of 2,4,5-T on DBF and acetate mediated growth. A) Log scale plot of the average of three separate experiments, and B) growth of Strain RW1 in PAS media amended with 0.1% DBF (●), 0.1% DBF + 500 mg/L 2,4,5-T (▼), 12.0 mM acetate (○), or 12.0 mM acetate + 500 mg/L 2,4,5-T (▽). Each Data point represents the average of three separate experiments. Error bars represent the standard deviation of this mean.

A)



B)

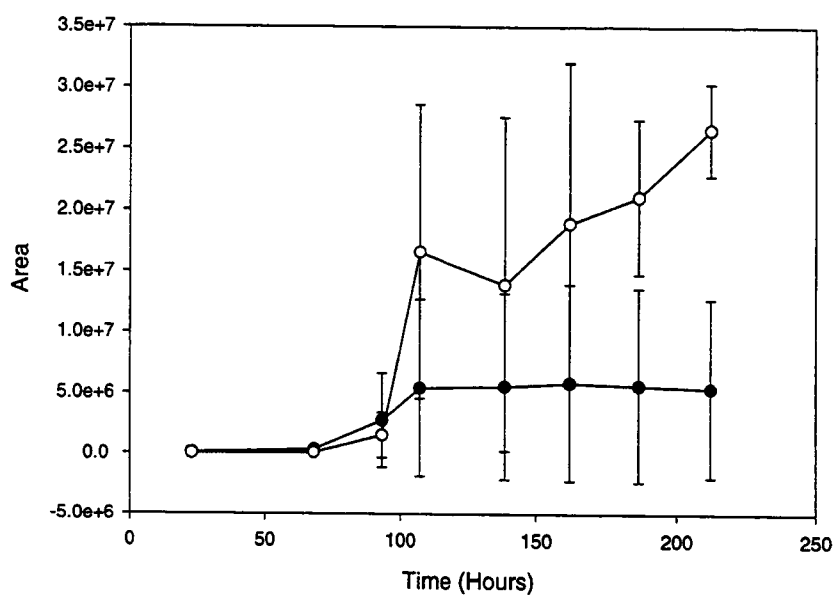


**Figure 5.6** *Spingomonas* species strain RW1 growing cell assay experiment using PAS containing either DBF + 2,4,5-trichlorophenoxyacetic acid (●) or acetate + 2,4,5-trichlorophenoxyacetic acid (○). A) 2,4,5-trichlorophenoxyacetic acid concentrations, B) 2,4,5-trichlorophenol concentrations. Each Data point represents the average of three separate experiments. Error bars represent the standard deviation of this mean.

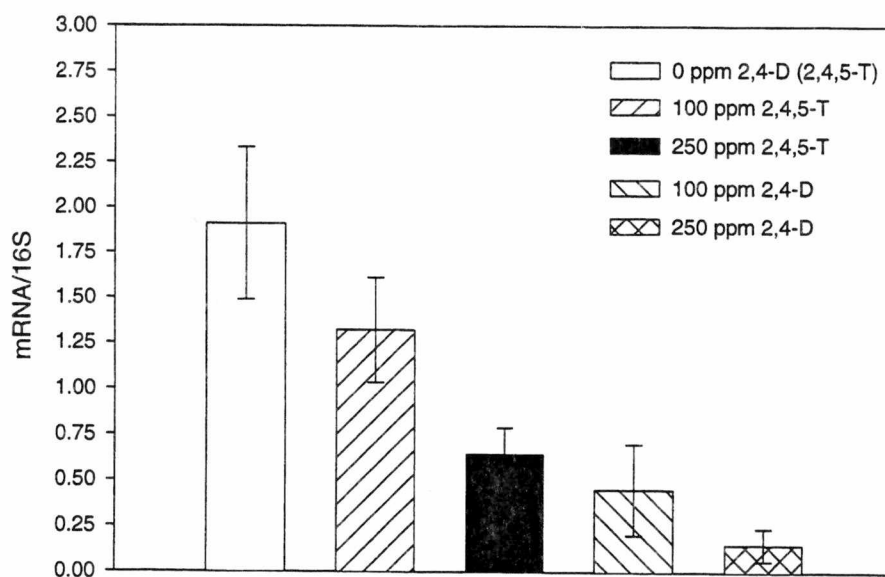
such a mixture is subject of this study. Growth and degradation experiments have demonstrated that both 2,4-D and 2,4,5-T delayed initiation of growth on acetate by *Sphingomonas* species strain RW1, suggesting that a detrimental effect is operating at some level. It was also observed that 2,4,5-T decreased the amount of cellular biomass present during growth on DBF whereas in the presence of 2,4-D an increased biomass was observed, potentially due to 2,4-D metabolism. During these experiments it was observed that both 2,4-DCP and 2,4,5-TCP were detected as metabolic intermediates. To further examine the interactions of these compounds, the catabolic gene expression of the *dxnA1*, *dbfB*, and gene *OBI* was examined in relationship with exogenous 2,4-D and 2,4,5-T. The goal of these studies was to elucidate whether 2,4-D and 2,4,5-T affected transcription of important degradative genes during growth on dibenzofuran or acetate.

The dibenzofuran-4,4a-dioxygenase, *DxnA1*, mRNA transcript was maximally induced in the absence of 2,4-D and 2,4,5-T (Table 5.1, Figure 5.7 and 5.8). Addition of 250 and 500 mg/L 2,4,5-T demonstrated little effect on the amount of transcript present for cell grown in DBF (Figure 5.7B). For acetate grown cells, 250 and 500 mg/L 2,4,5-T resulted in a 31 and 66 % decrease in *dxnA1* transcripts relative to cultures without 2,4,5-T (Figure 5.8A). 2,4-D amendments of 250 and 500 mg/L resulted in significant decreases in *dxnA1* mRNA abundance for both acetate and DBF grown cells (Table 5.1). Cells grown in DBF amended with 2,4-D contained 56 and 83 % less *dxnA1* mRNA compared to cultures without 2,4-D (Figure 5.8B), and corresponding acetate cultures demonstrated a 76 and 92 % decrease in *dxnA1* mRNA transcripts (Figure 5.8A).

**Table 5.1** Effects of 2,4-D and 2,4,5-T on mRNA expression of important dibenzofuran and dibenzo-*p*-dioxin degradative genes in *Sphingomonas* species strain RW1. Values represent the average of three experiments, and the numbers in parentheses are standard deviations based on these experiments.

|                        | <i>Dibenzofuran</i> |                 |                 | <i>Acetate</i>  |                 |                 |
|------------------------|---------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                        | <i>dxnAI</i>        | <i>dbfB</i>     | <i>OBI</i>      | <i>dxnAI</i>    | <i>dbfB</i>     | <i>OBI</i>      |
| <b>0 ppm 2,4,5-T</b>   | 0.32 +/- (0.12)     | 0.36 +/- (0.18) | 0.40 +/- (0.18) | 1.91 +/- (0.42) | 0.74 +/- (0.10) | 0.16 +/- (0.16) |
| <b>250 ppm 2,4,5-T</b> | 0.25 +/- (0.13)     | 0.21 +/- (0.07) | 0.21 +/- (0.16) | 1.32 +/- (0.29) | 0.59 +/- (0.12) | 0.07 +/- (0.07) |
| <b>500 ppm 2,4,5-T</b> | 0.27 +/- (0.17)     | 0.19 +/- (0.12) | 0.17 +/- (0.17) | 0.64 +/- (0.14) | 0.36 +/- (0.07) | 0.02 +/- (0.02) |
| <b>250 ppm 2,4-D</b>   | 0.14 +/- (0.01)     | 0.19 +/- (0.11) | 0.12 +/- (0.09) | 0.45 +/- (0.25) | 0.24 +/- (0.10) | 0.05 +/- (0.05) |
| <b>500 ppm 2,4-D</b>   | 0.05 +/- (0.01)     | 0.11 +/- (0.04) | 0.07 +/- (0.04) | 0.15 +/- (0.09) | 0.09 +/- (0.02) | 0.01 +/- (0.01) |

A)



B)

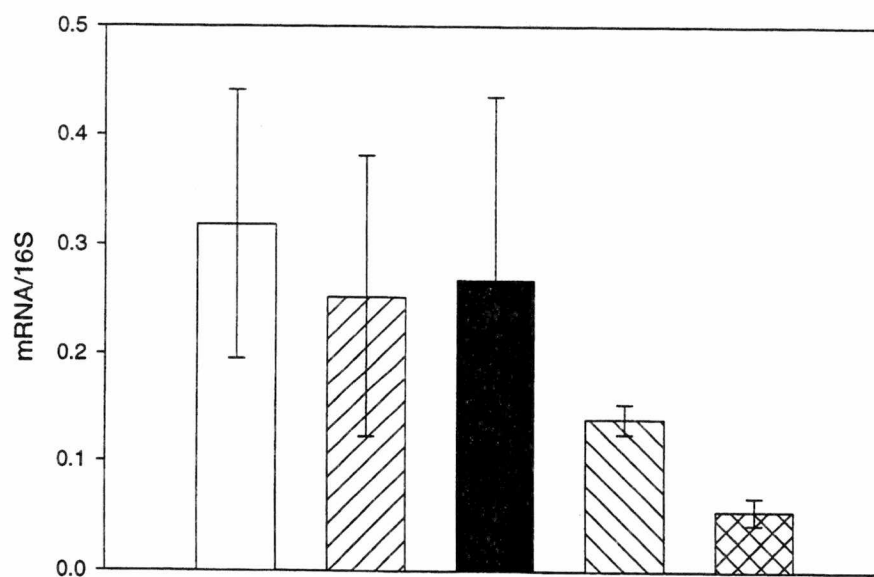
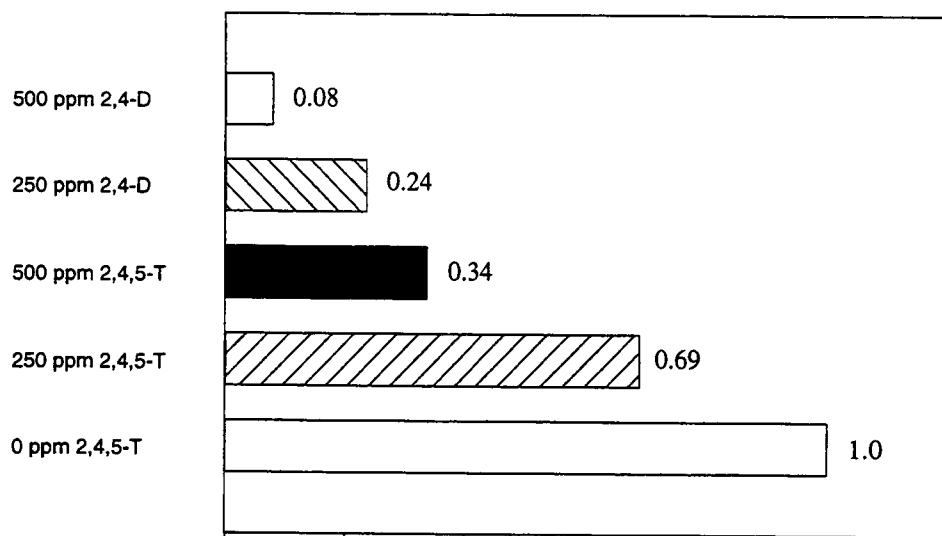


**Figure 5.7** Analysis of the effects of 2,4-D and 2,4,5-T on *dxnA1* mRNA expression in *Sphingomonas species* strain RW1 grown on A) acetate or B) dibenzofuran.

A)



B)

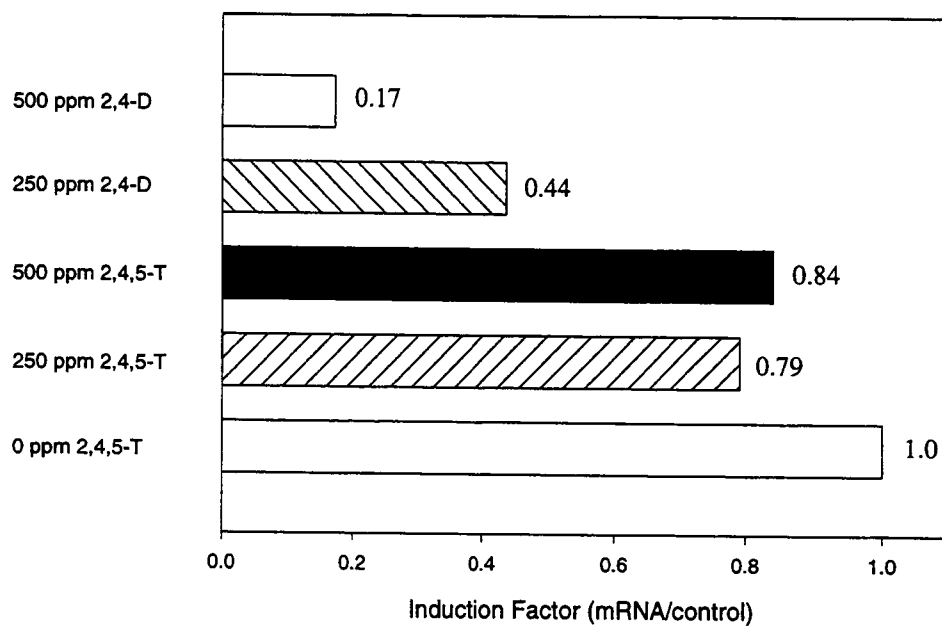


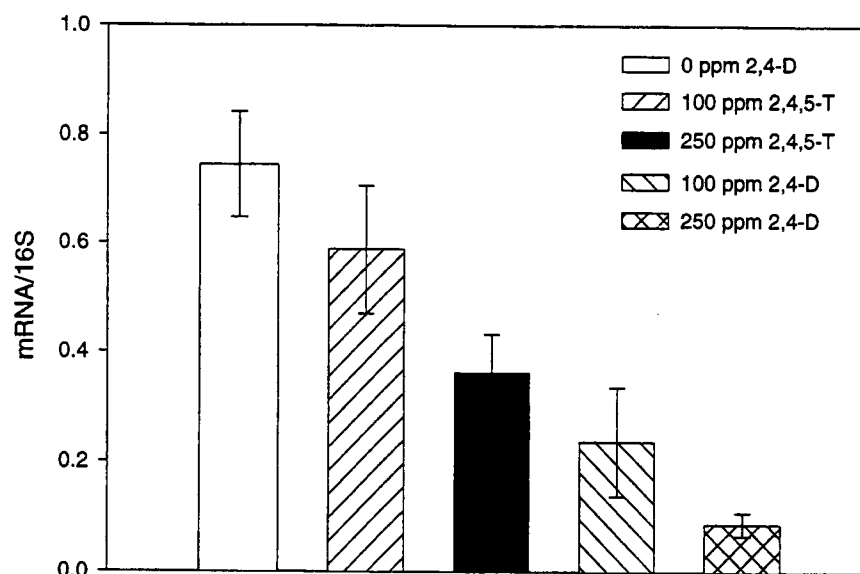
**Figure 5.8** *dxnA1* mRNA expression profiles for acetate (A) and dibenzofuran (B) grown *Sphingomonas* species strain RW1. Each bar represents the average of three separate experiments.

In addition to *dxnA1*, genes probes specific for the *dbfB* and *OBI* genes were utilized to examine mRNA transcript abundance in acetate and DBF grown cell subjected to 2,4-D and 2,4,5-T amendments. These studies had similar results to those obtained using the *dxnA1* gene probe. The *dbfB* and *OBI* were maximally transcribed during control conditions (Table 5.1, Figure 5.9 and 5.10). For acetate cultures, addition of 250 mg/L 2,4,5-T resulted in decreased mRNA abundance by 68 % and 64% (Figure 5.11A and Figure 5.12A), respectively, and in the presence of 500 mg/L a 88% reduction was apparent for both of these genes (Figure 5.11A and 5.12A). For DBF containing cultures the only significant reduction in mRNA transcript abundance was in the presence of 500 ppm 2,4-D. Under these conditions mRNA transcripts corresponding to the *dbfB* and *OBI* genes were reduced by 71 and 82 %, respectively (Figure 5.11B and 5.12B).

***5.4.5 2,4,5-trichlorophenol and 2,4-dichlorophenol inhibited normal growth of Sphingomonas species strain RW1 and resulted in decrease catabolic gene expression.***

During the course of this research project 2,4,5-TCP and 2,4-DCP have been implicated in potential metabolic inhibition for *Sphingomonas* species strain RW1 during growth on acetate and DBF. To further examine the potential effects of 2,4-DCP and 2,4,5-TCP growing cell assays coupled with mRNA analyses were used to examine influences of these compounds on metabolic processes. Growing cell assays demonstrated that 10 mg/L and 25 mg/L 2,4,5-TCP completely inhibited growth of *Sphingomonas* species strain RW1 on either acetate or DBF (Figure 5.13). 10 and 25 mg/L of 2,4-DCP also inhibited growth when acetate or DBF was supplied as a sole

A)



B)

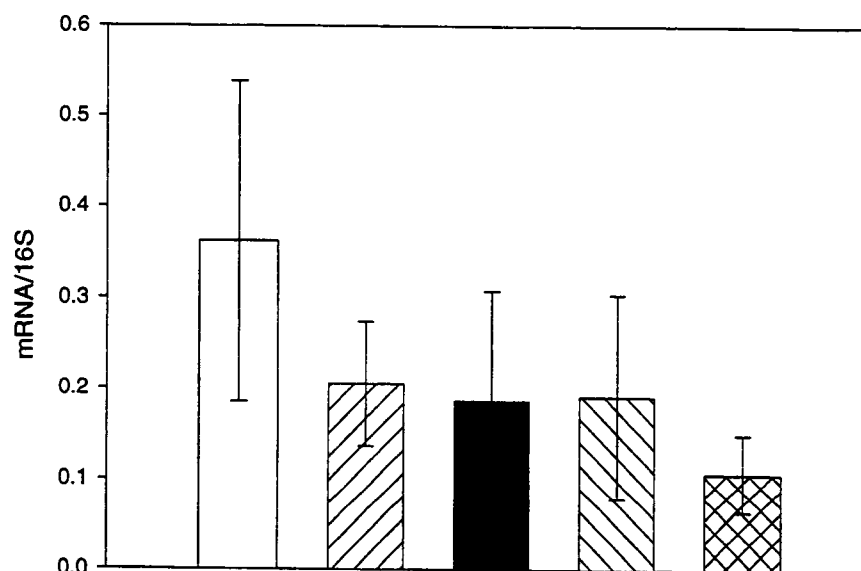
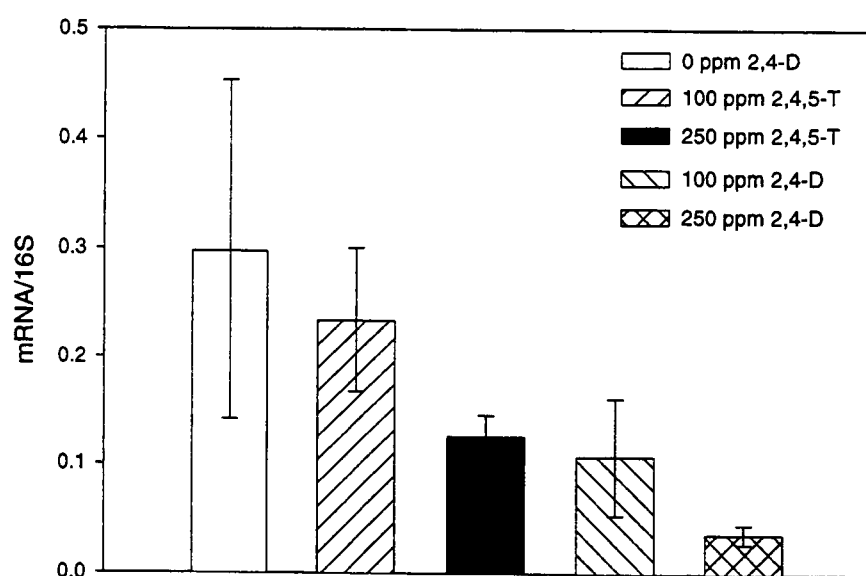


**Figure 5.9** Analysis of the effects of 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid on *dbfB* mRNA expression in *Spingomonas* species strain RW1 grown on A) acetate or B) dibenzofuran.

A)



B)

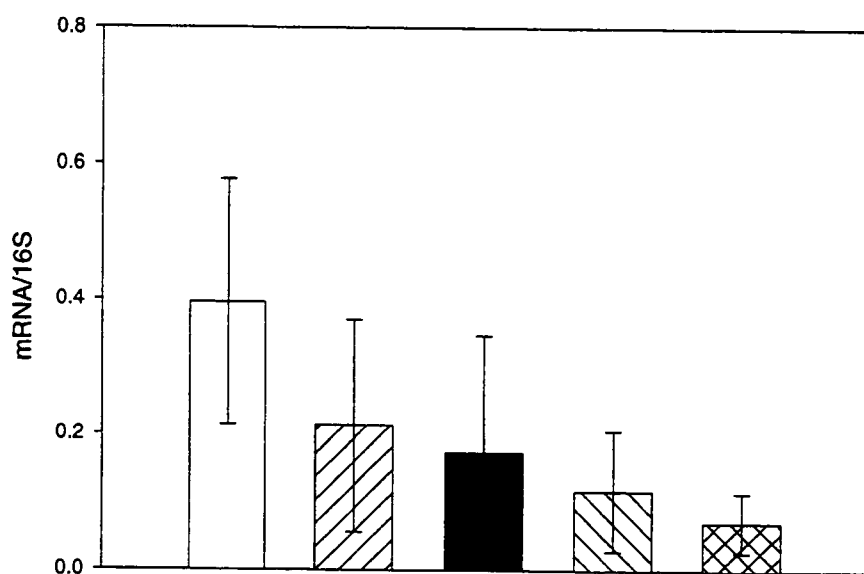
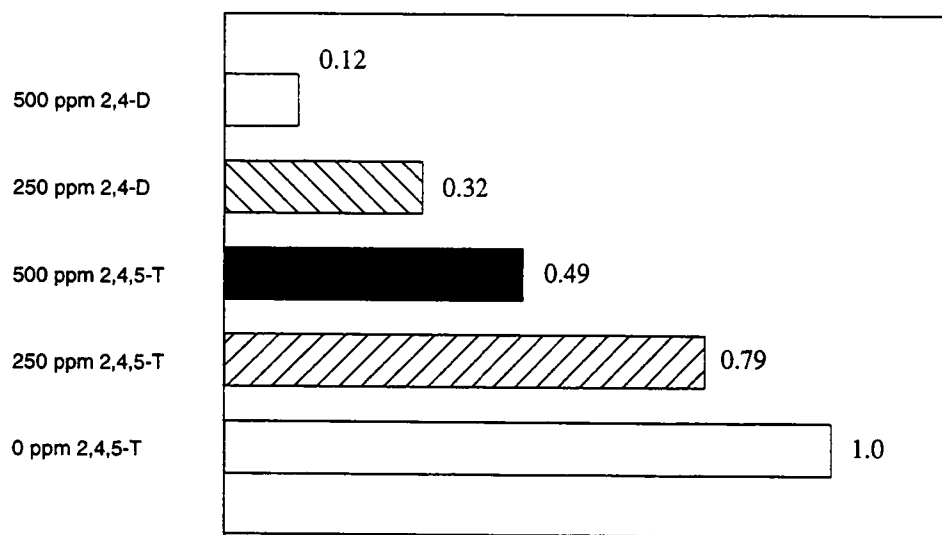


**Figure 5.10** Analysis of the effects of 2,4-D and 2,4,5-T on *OB1* mRNA expression in *Sphingomonas* species strain RW1 grown on A) acetate or B) dibenzofuran.

A)



B)

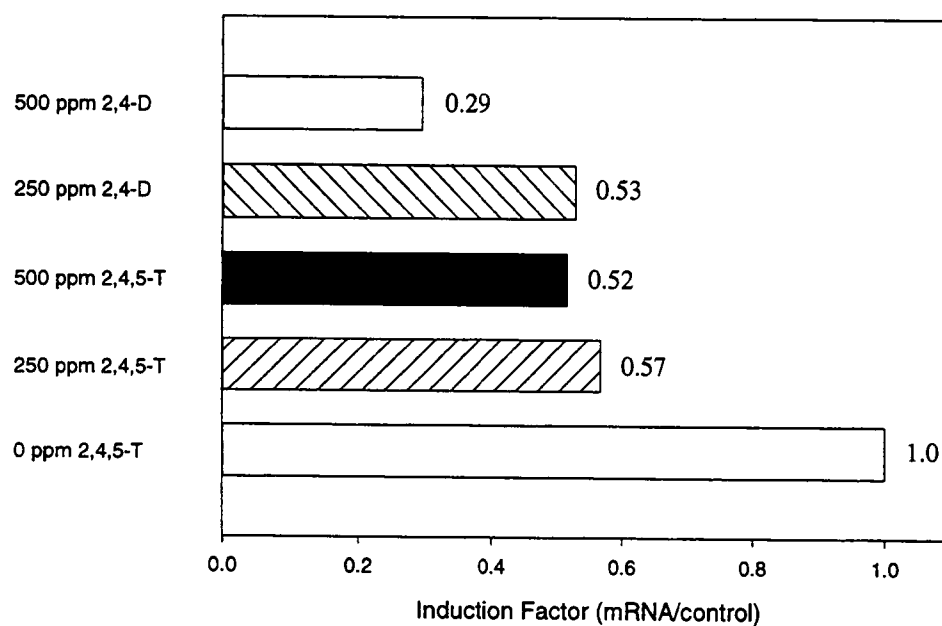
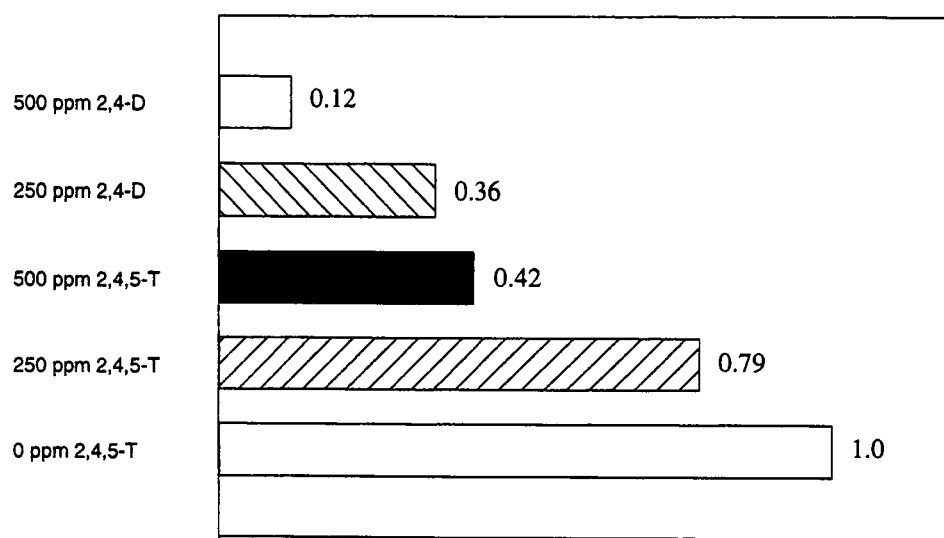


**Figure 5.11** *dbfB* mRNA expression profiles for acetate (A) and dibenzofuran (B) grown *Sphingomonas* species strain RW1.

A)



B)

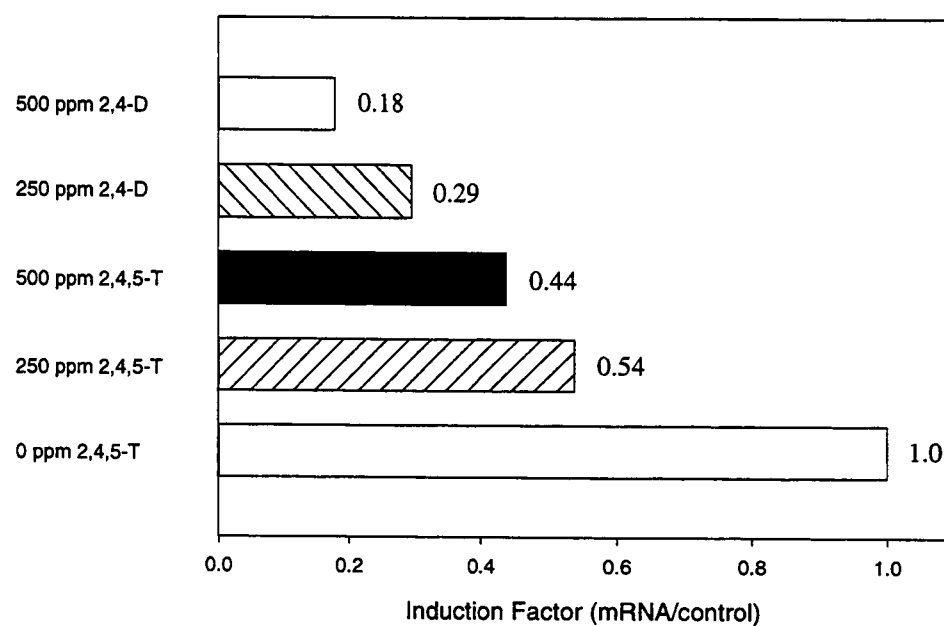


**Figure 5.12** *OBI* mRNA expression profiles for acetate (A) and dibenzofuran (B) grown *Sphingomonas* species strain RW1.

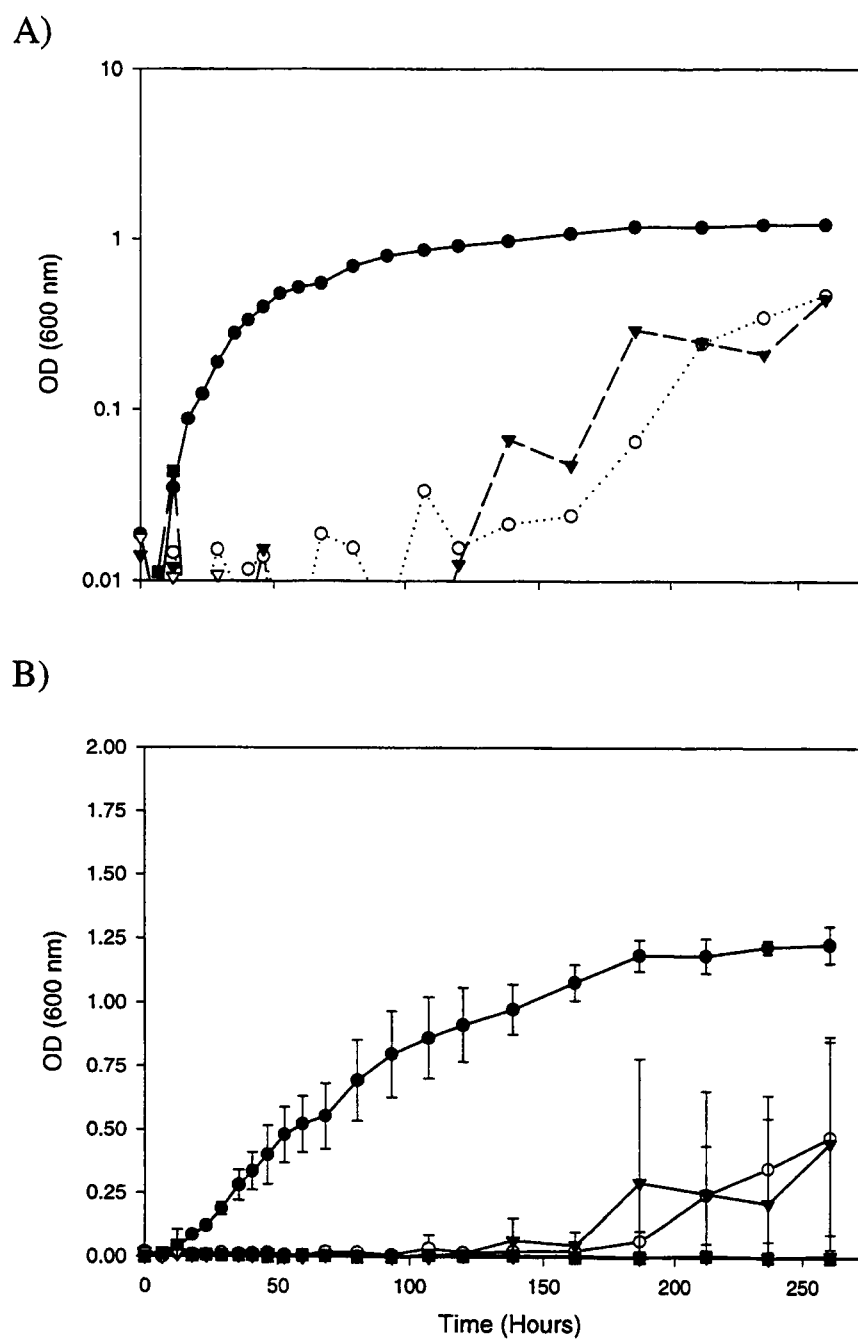


**Figure 5.13.** *Sphingomonas* species strain RW1 growth experiment examining the effects of 2,4-dichlorophenol (2,4-DCP) on dibenzofuran (DBF) and acetate mediated growth. A) Log scale plot of the average of three separate experiments, and B) growth of Strain RW1 in PAS media amended with 0.1% DBF (●), 0.1% DBF + 10 mg/L 2,4-DCP (▼), acetate + 10 mg/L 2,4-DCP (○), 0.1% DBF + 25 mg/L 2,4-DCP (■), or acetate + 25 mg/L 2,4-DCP (◆). Data points represent the average of triplicate cultures. Error bars represent the standard deviation among these cultures.

carbon and energy source, although, after approximately 100 hours growth was apparent for these cultures. Analysis of *dxnA1*, *dbfB*, and *OB1* metabolic gene expression yielded different patterns of inhibition compared to 2,4-D and 2,4,5-T experiments (Table 5.2, Figure 5.14, Figure 5.15, and Figure 5.16). Expression of *dxnA1* was inhibited by 10 and 25 mg/L 2,4,5-TCP by 47 and 44 % (Figure 5.17B), respectively, for dibenzofuran cultures, and 99 and 93 % for acetate cultures (Figure 5.17A). Repression of *dxnA1* by 10 and 25 mg/L 2,4-DCP was approximately 27 and 98 % (Figure 5.17A), respectively, for acetate grown cultures and 2 and 53 % for DBF grown cultures (figure 5.17B). Abundance of the *dbfB* gene was decreased in cultures containing 10 and 25 mg/L 2,4,5-TCP (77 and 80 %, respectively) and 10 and 25 mg/L 2,4-DCP (57 and 79 %, respectively) in DBF cultures (Figure 5.18B). For acetate cultures, the *dbfB* gene was repressed by 10 and 25 mg/L 2,4,5-TCP (97 and 80 %, respectively) and by 25 ppm DCP (95 %, Figure 5.18A). Similar trends were observed for the *OB1* gene, in which 10 and 25 mg/L 2,4,5-TCP (98 and 92 %) and 10 and 25 mg/L 2,4-DCP (61 and 89 %) repressed mRNA abundance during growth on acetate. Abundance of the *OB1* transcript was diminished by 67 and 75 % for 10 and 25 mg/L 2,4,5-TCP treatments (Figure 5.19A) and 41 and 78 % for 10 and 25 mg/L 2,4-DCP treatments (Figure 5.19B).

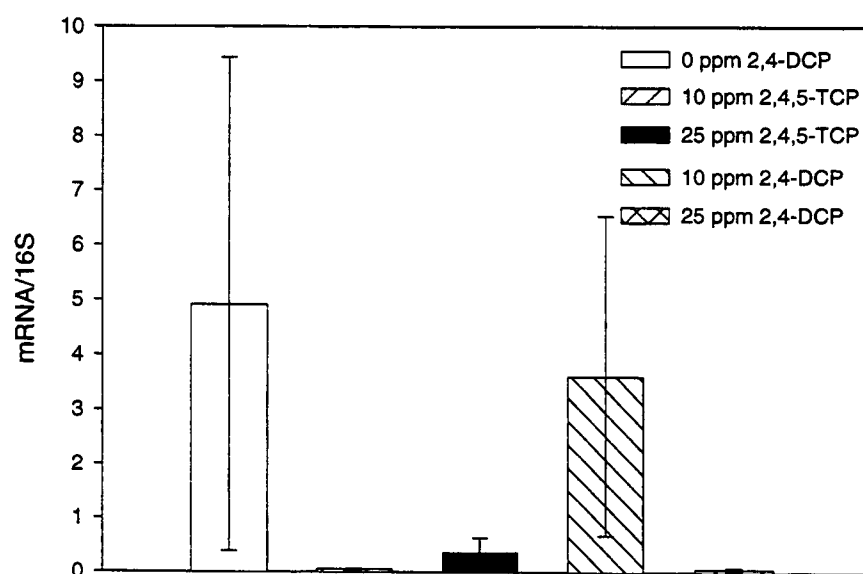
## 5.5 Discussion

Since its isolation from the river Elbe (Wittich et al., 1992), *Sphingomonas* species strain RW1 has been well characterized in terms of its DBF and DD

**Table 5.2** Effects of 2,4-DCP and 2,4,5-TCP on mRNA expression of important dibenzofuran and dibenzo-*p*-dioxin degradative genes in *Spingomonas* species strain RW1. Values represent the average of three experiments, and the numbers in parentheses are standard deviations based on these experiments.

|                         | <i>Dibenzofuran</i> |                |                | <i>Acetate</i> |                |                |
|-------------------------|---------------------|----------------|----------------|----------------|----------------|----------------|
|                         | <i>dxnA1</i>        | <i>dbfB</i>    | <i>OBI</i>     | <i>dxnA1</i>   | <i>dbfB</i>    | <i>OBI</i>     |
| <b>0 ppm 2,4,5-TCP</b>  | 0.11+/- (0.03)      | 1.32+/- (0.40) | 0.68+/- (0.08) | 4.91+/- (4.53) | 2.69+/- (0.83) | 2.67+/- (0.53) |
| <b>10 ppm 2,4,5-TCP</b> | 0.06+/- (0.01)      | 0.30+/- (0.12) | 0.16+/- (0.03) | 0.05+/- (0.01) | 0.19+/- (0.07) | 0.04+/- (0.01) |
| <b>25 ppm 2,4,5-TCP</b> | 0.07+/- (0.01)      | 0.27+/- (0.12) | 0.17+/- (0.03) | 0.36+/- (0.28) | 0.55+/- (0.21) | 0.22+/- (0.12) |
| <b>10 ppm 2,4-DCP</b>   | 0.12+/- (0.03)      | 0.57+/- (0.19) | 0.40+/- (0.07) | 3.60+/- (2.93) | 2.29+/- (0.94) | 1.03+/- (0.51) |
| <b>25 ppm 2,4-DCP</b>   | 0.08+/- (0.02)      | 0.34+/- (0.11) | 0.35+/- (0.15) | 0.05+/- (0.05) | 0.14+/- (0.05) | 0.29+/- (0.36) |

A)



B)

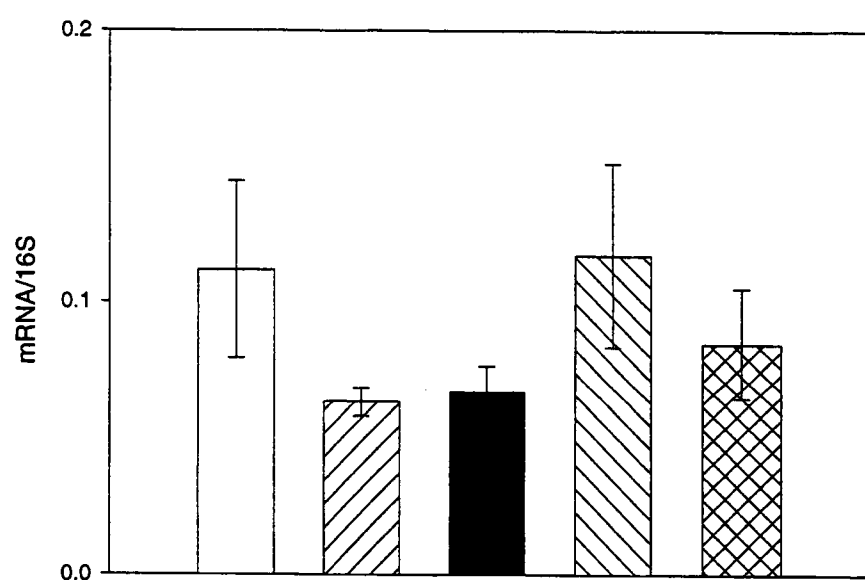
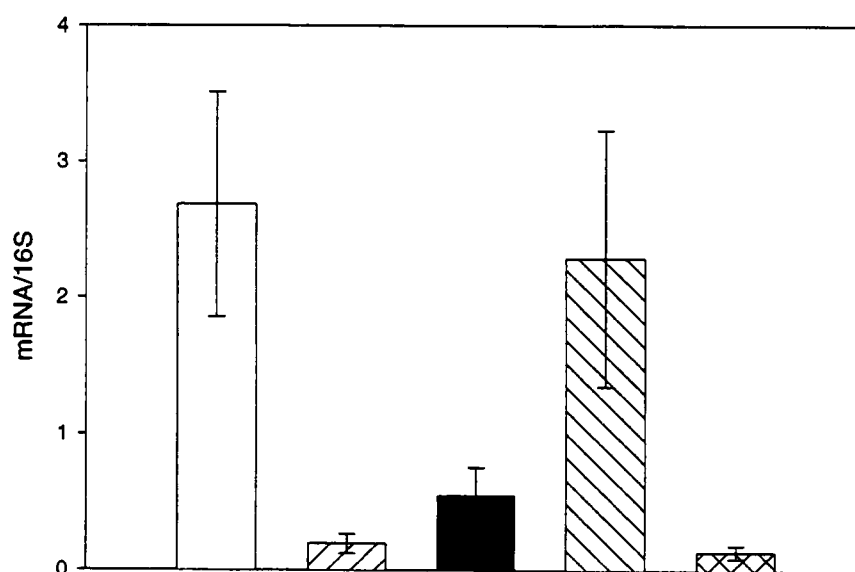


**Figure 5.14** Analysis of the effects of 2,4,5-trichlorophenol and 2,4-dichlorophenol on *dxnA1* mRNA expression in *Sphingomonas* species strain RW1 grown on A) acetate or B) dibenzofuran.

A)



B)

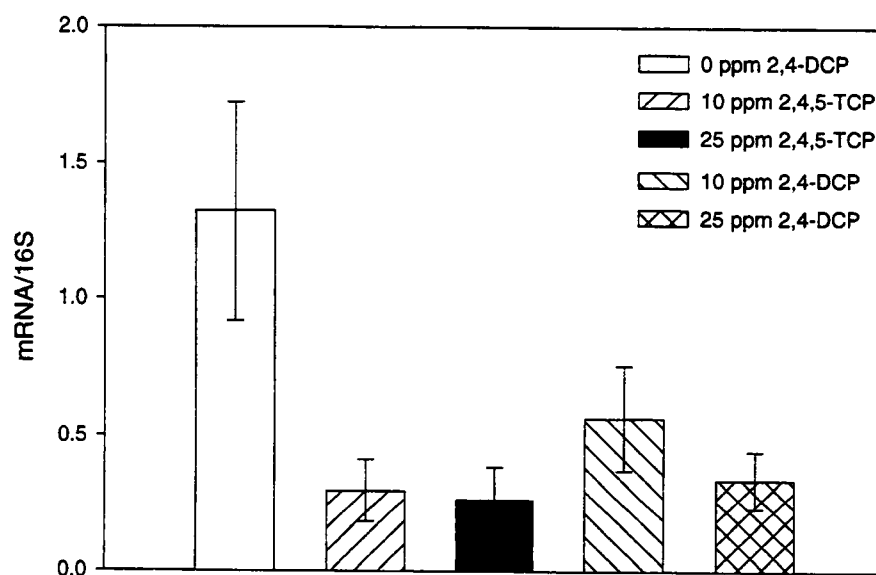
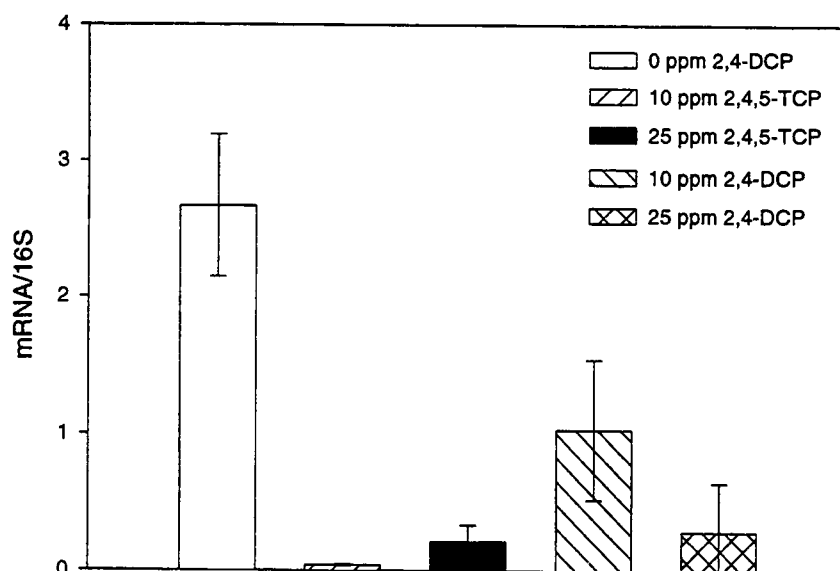


**Figure 5.15** Analysis of the effects of 2,4,5-trichlorophenol and 2,4-dichlorophenol on *dbfB* mRNA expression in *Spingomonas* species strain RW1 grown on A) acetate or B) dibenzofuran.

A)



B)

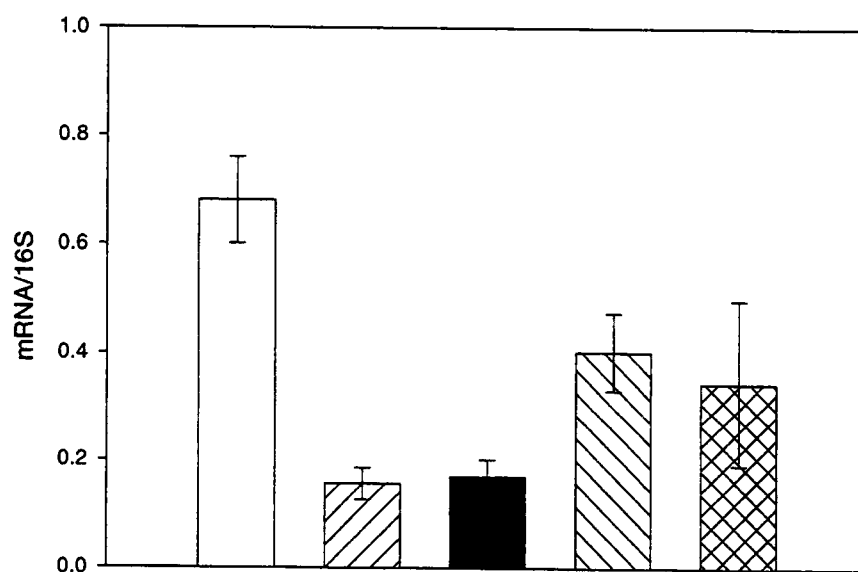
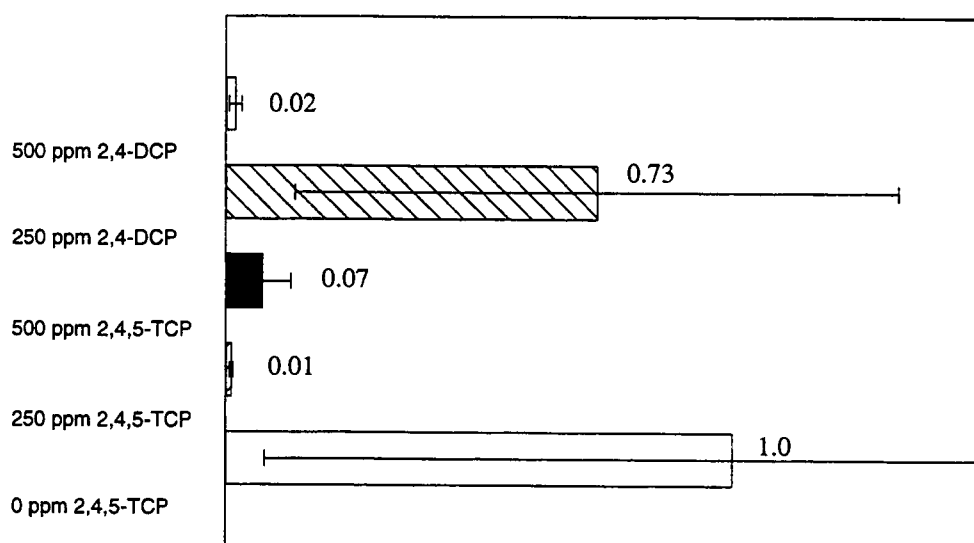


**Figure 5.16.** Analysis of the effects of 2,4,5-trichlorophenol and 2,4-dichlorophenol on *OB1* mRNA expression in *Spingomonas* species strain RW1 grown on A) acetate or B) dibenzofuran.

A)



B)

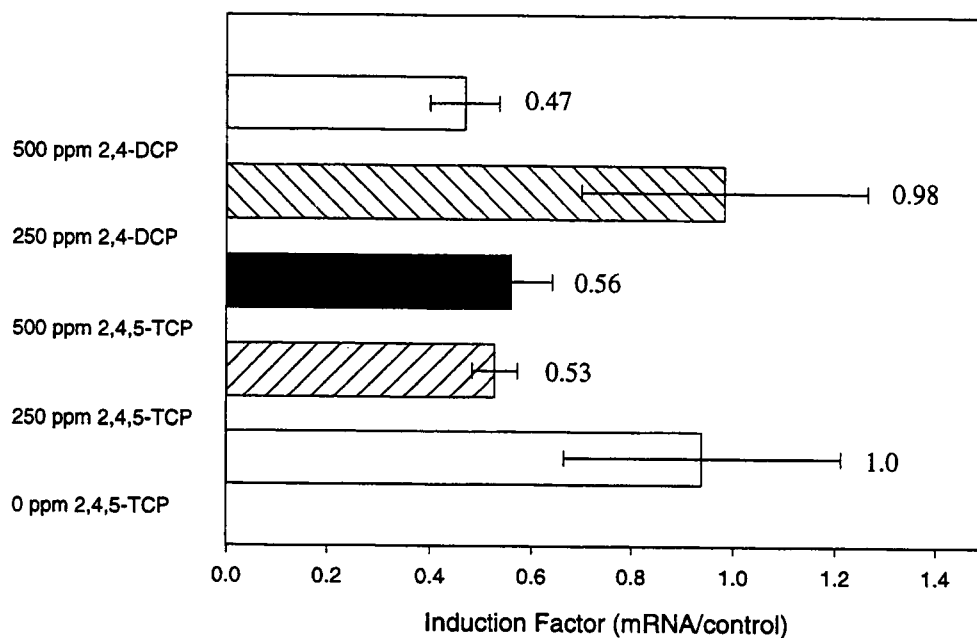
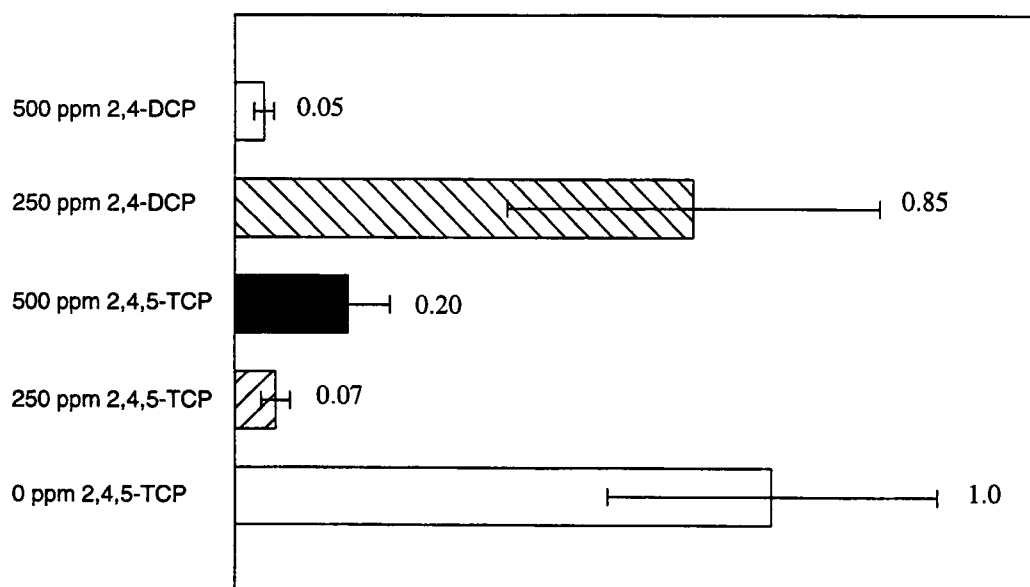


**Figure 5.17** *dnmA1* mRNA expression profiles for acetate (A) and dibenzofuran (B) grown *Sphingomonas* species strain RW1.

A)



B)

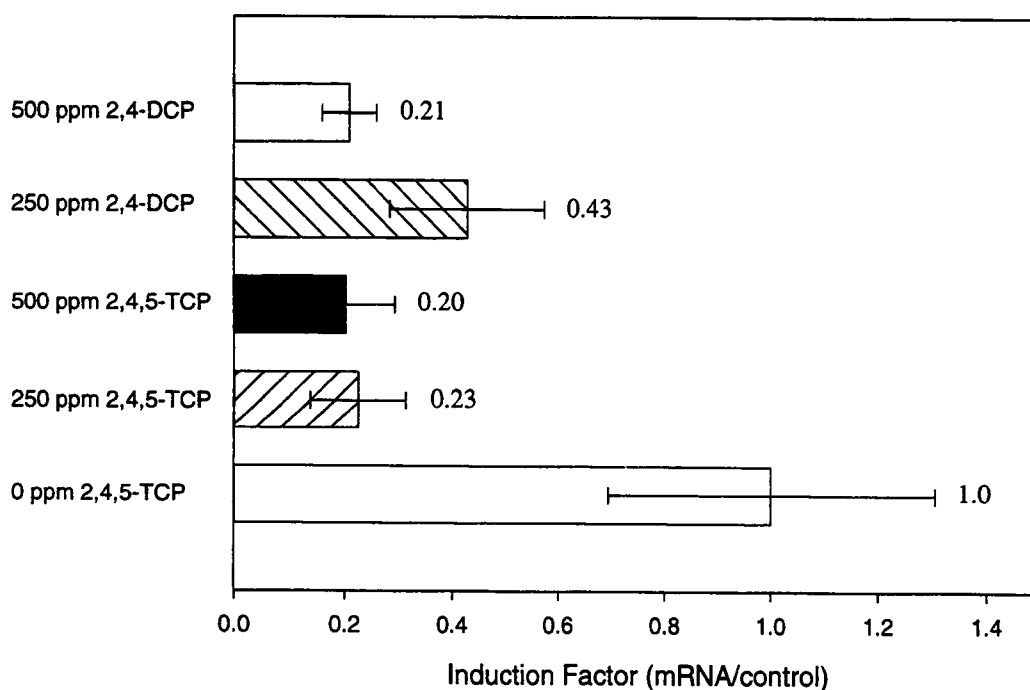
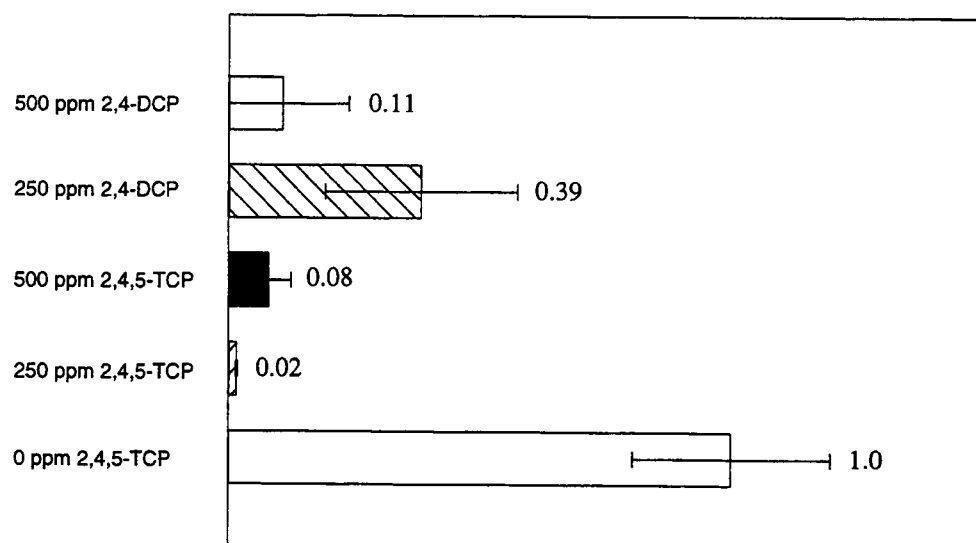


**Figure 5.18** *dbfB* mRNA expression profiles for acetate (A) and dibenzofuran (B) grown *Shingomonas* species strain RW1. Bars represent the average of three separate experiments. Error bars represent the standard deviation of this mean.

A)



B)

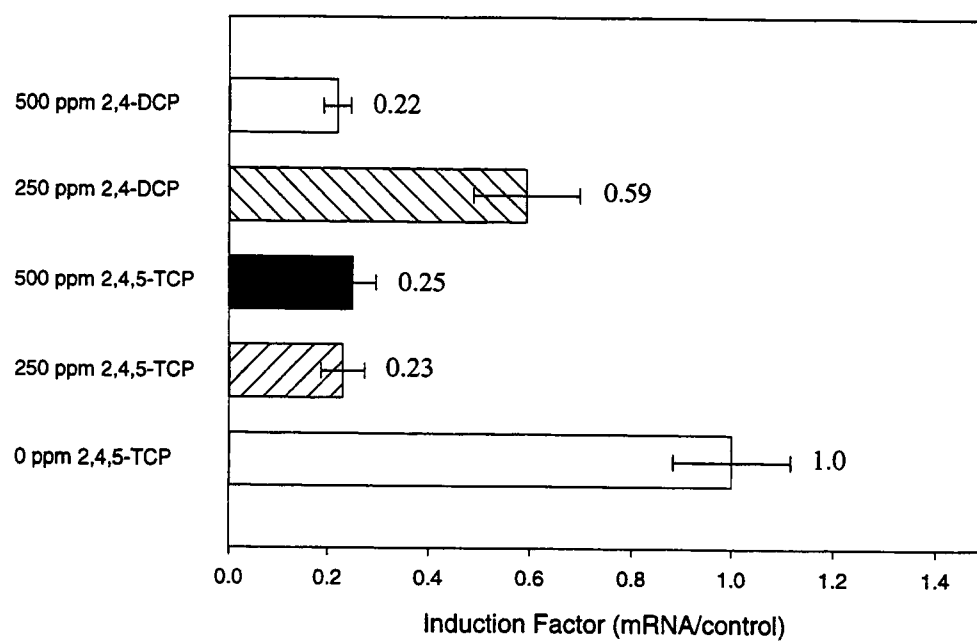


**Figure 5.19** *OBI* mRNA expression profiles for acetate (A) and dibenzofuran (B) grown *Sphingomonas* species strain RW1.

degrading capacity (Armengaud and Timmis 1998 and Happe et al. 1993). The genetic organization of its catabolic genes has been largely determined and found to consist of multiple cistrons containing the necessary genetic machinery for utilization of DBF and DD as sole carbon and energy sources (Armengaud et al. 1998). Many of these genes are localized on a large region of DNA, spanning approximately 20 kb (NCBI accession number X72850, Armengaud et al. 1998). These genes appear to be clustered in three distinct operon-like elements. The first of these elements contains the 2,2',3-trihydroxybiphenyl dioxygenase gene, *dbfB*, and is divergently transcribed from the second set of catabolic genes, which includes the *dxnA1*, *A2*, *B*, and putative *C*. The third and largest DNA segment contains the *fdx3*, *dxnD*, *dxnF*, *dxnG*, *dxnH*, and *dxnI*. These genes are postulated to encode the major electron transport system for this degradative pathway. Particular interesting is the identification of the genetic arrangement of the *dxnA1*, *A2*, *B*, *C* and the *fdx3*, *dxnD*, *F*, *G*, *H*, *I* genes. These operons containing these genes are separated by approximately 1610 bp. Current understanding is that each of these operons is distinct from each other and represents a novel organization of genes of this type.

Random arbitrarily primed reverse transcriptase-PCR mediated RNA fingerprinting studies have identified a novel genetic element, *OBI*, which is transcribed during growth of *Sphingomonas* species strain RW1 on DBF. The DNA sequence of this gene was found to be identical to the region of DNA separating the *dxnA1* and the *fdx3* operons, specifically corresponding to 12,673 - 13,454 bp. Open reading frame analysis identified an 1106 bp ORF encoding a hypothetical 368 amino acid. Previous conceptual

examination of this *Sphingomonas aromaticivorans* sequence has identified it as a putative inner membrane protein, containing a transmembrane-region (390 - 406) and a tyrosine kinase phosphorylation site signature (459 - 467). Furthermore, the hypothetical *OBI* gene sequence is only 68 bp upstream of the *fdx3* operon and 426 bp downstream of the *dxnA1* operon. The implications of this are that the *OBI* gene may be either the first gene in the *fdx3* operon or a link between the *dxnA1* and *fdx3* operon. The fact that it may be membrane bound and has a tyrosine kinase phosphorylation site may suggest its involvement in either regulation or transport of DBF/DD into the cell. It is important to note that the genbank accession sequence X72850 does not include the *dxnC* gene in its listing of known genes within this stretch of DNA sequence, but Armengaud et al. (1998) indicates that it falls within 12028 and 12372 bp in this region of DNA. In their manuscript the *dxnC* is described as a putative bacterial receptor protein. Perhaps the previously described *dxnC* is somehow related to the *OBI* gene sequence, through a corporation between the two proteins or overlapping gene sequences. Currently, no data is available to either support or refute this hypothesis.

Many of the toxicological effects associated with the herbicide Agent Orange are attributable to contaminating PCDDs and PCDFs. Degradation of these compounds in the environment is problematic because of their chemical stability, which leads to biochemical recalcitrance. The discovery and genetic characterization of a bacterial organism able to utilize DBF and DD as a primary carbon source will aid in the biological treatment and monitoring processes necessary for site maintenance and cleanup in polluted areas. However, the influences of co-pollutants in the environment

can not be adequately accessed without an understanding of the effects of these co-contaminants on biodegradation processes.

Growing cell assays demonstrated that *Sphingomonas* species strain RW1 acquired the ability to degrade 2,4-D during growth on acetate and DBF. The metabolic versatility of *Sphingomonas* species is well-documented (Wittich et al. 1992). In particular Kamagata et al. (1997), have demonstrated that some members of the  $\alpha$  - subdivision (mostly in the genus *Sphingomonas*) of the *Proteobacteria* contain 2,4-D degradative genes which are distinct from the extensively characterized *tfd*-type genes present in the  $\beta$  and  $\gamma$ -*Proteobacteria*. Currently, the mechanism by which the *Sphingomonads* mediate 2,4-D metabolism has not been genetically characterized. The data obtained from this research project suggest that 2,4-DCP is an intermediate in metabolism of 2,4-D in this strain. It is therefore possible that subsequent steps resemble the *tfd* type pathway, but this is purely speculative at this point. Additional research will be necessary to elucidate the structural and functional dynamics of this 2,4-D degradative pathway.

The implications of a 2,4-D degradation pathway in *Sphingomonas* species strain RW1 are numerous. Previous research by Wilkes et al. (1996) has shown that during resting cell assays 4-chlorocatechol and 4,5-dichlorocatechol are produced. These compounds are potent inhibitors of type I catechol-1,2-dioxygenases, and represent extremely recalcitrant intermediates which interfere with DBF and DD metabolism (Wilkes et al. 1996). Efforts to extend the capacity of Strain RW1 to include type II enzymes capable of degrading chlorinated catechols have recently been pursued by other

laboratories. Based on the results of this study, *Sphingomonas* species strain RW1 has a latent degradation pathway capable of degrading 2,4-D, potentially through a metabolic pathway including 3,5-dichlorocatechol. If conditions could be optimized to select for such a variant, a natural alternative to genetically engineered strains could be achieved and utilized for future biodegradation studies at sites contaminated with chlorinated mixtures of PCDDs and PCDFs. In fact research by Arfmann et al. 1997, has successfully demonstrated that *Sphingomonas* species strain RW1 grown in a consortium containing *Burkholderia* species strain JWS, which is able to degrade 3-chlorocatechol, is able to completely degrade 4-chlorodibenzofuran. Also, a 2,4-D adapted *Sphingomonas* species strain RW1 could potentially be applied for remediation of sites containing mixtures of the "dioxin" class of compounds and the herbicide 2,4-D.

Chemical mixtures, such as Agent Orange, are commonly found at hazardous waste sites (Haugland et al. 1990). The degradation of these pollutant mixtures by bacterial strains known to degrade specific environmental contaminants often results in production of toxic dead-end intermediates (Mungkarndee et al. 1997 and Amy et al. 1985). The generation of these intermediates is the result of non-specific transformations of secondary compounds due to relaxed substrate specificity of many of the upper pathway enzymes (Mungkarndee et al. 1997). Despite the apparent problems associated with contaminant mixtures most of the research to date addresses degradation of particular chemical pollutants. The herbicide Agent Orange contains many compounds, which may result in biological recalcitrance due to the misrouting of compounds through inappropriate pathways. Agent Orange as a pollutant mixture, contains 2,4-D, 2,4,5-T,

and many congeners of DBF and DD (EPA 1995). Whereas 2,4-D degrading bacteria are widespread in the environment, 2,4,5-T-degrading bacteria appear to be less common and as a consequence 2,4,5-T may be more recalcitrant in the environment. The persistence of 2,4,5-T in the environment may have implications for pollutant mixtures containing 2,4,5-T and DBF. Previously, research by Haugland et al.(1990) and Rice (Part III) have demonstrated that when 2,4-D and 2,4,5-T are both present as a chemical mixture the recalcitrance of 2,4,5-T is increased due to inhibition of important metabolic enzymes necessary for 2,4,5-T degradation by non-specific breakdown products of 2,4-DCP.

A primary purpose of this research was to examine whether 2,4-D, 2,4,5-T, 2,4-DCP, and 2,4,5-TCP interfered with metabolism of DBF by *Sphingomonas* species strain RW1. During growth on either acetate or DBF 2,4-D was degraded, and although 2,4-DCP was produced during this catabolism it was subsequently metabolized. The ability of *Sphingomonas* species strain RW1 to mediate 2,4-D degradation was realized only after a prolonged incubation suggesting that this pathway required some type of metabolic activation. Strain RW1 was also capable of mediating limited 2,4,5-T transformation to 2,4,5-TCP, which accumulated during growth on acetate. During growth on DBF, 2,4,5-TCP accumulation was substantially less than for acetate-grown cells, and did not exceed 2 mg/L. The appearance of 2,4,5-TCP in the culture medium corresponded with a decreased level of growth on DBF. These data suggest that *Sphingomonas* species strain RW1 is able to non-specifically metabolize 2,4,5-T to a minor degree and that the 2,4,5-TCP produced is inhibitory to DBF degrading cells. 2,4,5-T transformation during growth on DBF did not result in high levels of 2,4,5-TCP

implying that either the trichlorophenol was converted to a toxic intermediate, such as a trichlorocatechol, or that the trichlorophenol itself inhibits an important metabolic conversion. 2,4,5-T metabolism in the presence of acetate is most likely blocked after conversion of 2,4,5-T to 2,4,5-TCP and stationary phase cells accumulated toxic levels of 2,4,5-TCP. It is hypothesized that 2,4,5-TCP was non-specifically converted to 3,4,6-trichlorocatechol which interfered with further cell growth and metabolism using DBF as a carbon and energy substrate. This hypothesis is supported by the accumulation of 2,4,5-TCP in the absence of DBF. An analysis of *dxnA1*, *dbfB*, and *OBI* mRNA expression indicated that the level of transcription was decreased when high concentrations of 2,4-D were present. This may indicate that an increase in intracellular 2,4-DCP concentrations has detrimental effects on cellular metabolism. When high concentrations of 2,4,5-T were examined the transcription level of these genes was not affected to the same degree. The only significant repression was observed for the *dxnA1* and *dbfB* genes during growth on acetate, and the level of transcript modulation was about the same as the low concentration of 2,4-D. These data seem to infer that 2,4-D/2,4,5-T needs to be metabolized to attain optimal inhibitory activity, and that 2,4-DCP and 2,4,5-TCP are the actual inhibitory compounds.

The toxicity of these compounds has been well studied, and both 2,4-DCP and 2,4,5-TCP are potent uncouplers of the proton motive force (PMF) in bacterial cells (Schultz and Cronin 1997 and Schultz et al. 1996). In addition to acting directly to de-energize bacteria cells, 2,4-DCP and 2,4,5-TCP could be theoretically cleaved to yield corresponding di- and trichlorocatechols which have been documented to be detrimental

for aromatic degradation pathways. The fact that *Sphingomonas* species strain RW1 may contain a latent 2,4-D degradation pathway may indicate a metabolic potential for non-specific transformation of 2,4,5-TCP to a 3,4,6-trichlorocatechol which could interfere with the catechol-2,3-dioxygenase necessary for DBF degradation.

Examination of the influences 2,4-DCP and 2,4,5-TCP demonstrated that both of these compounds inhibited growth on acetate and DBF. 2,4-DCP containing cultures eventually recovered and growth was apparent for both carbon sources. No growth for 2,4,5-TCP treated cultures was observed. Analysis of *dxnA1*, *dbfB*, and *OBI* mRNA expression demonstrated that 2,4,5-TCP and high levels of 2,4-DCP reduced mRNA abundance for all genes tested. Low concentrations of 2,4-DCP were substantially less inhibitory to these genes. These results lend credibility to the idea that 2,4-DCP and 2,4,5-TCP are the actual compounds resulting in inhibition of growth on acetate or DBF. It appears that there are two mechanisms of inhibition in operation for growth on DBF and acetate by *Sphingomonas* species strain RW1. One is a general toxicity response mediated by deenergizing the proton motive force, and the second is a more specific toxic intermediate mediated repression. It appears that the more metabolizable compound, 2,4-D, has a greater impact on transcription of important DBF catabolic genes compared to 2,4,5-T. Since 2,4,5-T has a greater toxicity than 2,4-D, the supposition that an intermediate of 2,4-D breakdown is responsible for the inhibition of *dxnA1*, *dbfB*, and *OBI* is supported. 2,4,5-T did demonstrate an inhibitory profile during the mRNA analyses, and was more detrimental to catabolic gene abundance during growth on acetate. Possibly, the physiology of *Sphingomonas* species strain RW1 during growth on

acetate is more conducive to 2,4,5-T degradation to 2,4,5-TCP and that the generation of this compound accounts for the increased toxicity of 2,4,5-T. Alternatively, dibenzofuran grown cells may be capable of removing low levels of 2,4,5-TCP and thereby provide cells with moderate protection during the time course of the mRNA experiments.

Based on these results, 2,4,5-TCP inhibits growth of *Sphingomonas* species strain RW1 on both acetate and DBF. This inhibition appears to be related to a decrease in catabolic mRNA transcripts associated with increasing concentrations of 2,4,5-TCP. It appears that the major mechanism of inhibition in these cultures is a general toxicity response rather than interference with metabolic enzymes, because of the absence of growth and the similar patterns of repression observed for the 25 mg/L 2,4-DCP treatments. Production of 3,5-dichlorocatechol and 3,4,6-trichlorocatechol may still account for some measure of toxicity for these cultures and in co-operation with a more general toxicity response limit growth of *Sphingomonas* species strain RW1 on DBF and acetate in the presence of chlorinated phenols. The research reported here provides valuable information regarding the degradation of DBF in the presence 2,4,5-T, 2,4-D and metabolic intermediates produced during their degradation.

## 5.6 ACKNOWLEDGEMENTS

We gratefully thank Rolf-Michael Wittich for his generously supplying *Sphingomonas* species strain RW1 for this research project.

This research project was supported by The United States Air Force Office of Scientific Research (AASERT Award) and the Dow Foundation (SPHERE Award).

PART VI

DISCUSSION

Bacterial mediated degradation of Agent Orange potentially involves multiple metabolic pathways specific for 2,4-dichlorophenoxyacetic acid or 2,4,5-trichlorophenoxyacetic acid as well as tertiary contaminants such as chlorinated dibenzo-*p*-dioxins and dibenzofurans. Bacterial catabolism of these individual constituents have been shown to exist in many members of the  $\alpha$ ,  $\beta$ , and  $\gamma$ -*Proteobacteria* (McGowen et al. 1998 and Kamagata et al. 1997). In particular the metabolic pathway necessary for 2,4-D metabolism by *Ralstonia eutrophus* JMP 134 has been almost completely characterized, and shown to be readily transferred among members of the  $\gamma$  and  $\beta$ -*Proteobacteria* (McGowen et al. 1998). A 2,4,5-T degradative pathway has also been characterized for *Burkholderia cepacia* AC1100 and most of the metabolic genes necessary for transformation of this compound have been elucidated (Zaborina et al. 1998). Interestingly this pathway has not been identified in any other bacterial strain to date and the results from this study represent the first confirmation that this pathway is present in geographically distinct locations. Likewise, a DBF/DD degradative pathway has also been identified in *Sphingomonas* species strain RW1 and has been the subject of recent studies to describe the potential of this strain for remediation of PCDBF/PCDD contaminated sites (Wilkes et al. 1996).

Agent Orange contaminated soil samples were obtained for studies examining the interaction among 2,4-D, 2,4,5-T, and DBF/DD degrading bacteria. These soils originated from a loading/unloading area and were historically heavily contaminated with Herbicide Orange. Current 2,4-D and 2,4,5-T concentrations were approximately 22.4 and 73.8 mg/kg of soil, and it is therefore possible that much of the chlorophenoxy-

herbicide remaining in these soils is recalcitrant to bacterial degradation due to sorption to the soil organic matter. Isolation of 2,4-D and 2,4,5-T degrading bacterial cultures from these soils required very little enrichment culturing, and growth was apparent after the 1<sup>st</sup> week for 2,4-D degrading cultures and after only 3 weeks for 2,4,5-T degrading cultures. Growth of 2,4-D degrading bacteria was fairly rapid on PAS minimal media plates containing 1000 mg/L 2,4-D with visible colonies appearing with 2 - 3 days. Growth of 2,4,5-T degrading bacteria was much slower with visible colonies appearing only after 1 - 1 ½ weeks. Additionally, the Agent Orange enrichment culture JR7B was able to form minuet colonies on 2,4-D plates after 2 - 2 ½ weeks.

During the course of this research project, two *Burkholderia* species capable of mediating mineralization of 2,4-D and 2,4,5-T were isolated. *Burkholderia* species strain JRB1 was capable of mineralizing 2,4-D, and *Burkholderia* species strain JR7B3 was capable of mineralizing 2,4-D (32.1%) and 2,4,5-T (75.7%). Strain JR7B3 was originally isolated in association with a third organism, *Burkholderia* species strain JR7B2, whose 16S sequence most closely, resembles *Burkholderia caribensis* (99%). The 16S sequence of the 2,4-D degrading bacterium, *Burkholderia* species strain JRB1, is 97% similar to *Burkholderia glathei*. The 16S sequence of *Burkholderia* species strain JR7B3 was found to be 97% similar to *Burkholderia graminis*. *Burkholderia* species strain JRB1 was shown to completely degrade 2,4-D during growth on this compound as a sole carbon and energy source. *Burkholderia* species strain JR7B3 was able to utilize 2,4,5-T as a carbon and energy source in the presence of additional growth nutrients or in association with a consortium containing *Burkholderia* species strain JR7B2. Differences

in the growth characteristics for Strain JRB1 and Strain JR7B3 were very apparent with each having doubling times of hours versus days. Transmission electron micrographs of these bacterial strains demonstrated similar cell morphologies as those observed for *Burkholderia cepacia* AC1100. One notable difference was the presence of exopolysaccharide in association with *B. Cepacia* AC1100 cells, and during growth on PAS containing 1000 mg/L.

Genetic analysis of these bacterial strains using PCR identified 2,4-D and 2,4,5-T degradative genes. *Burkholderia* species strain JRB1 was shown to contain genes that were similar to previously published 2,4-D degradative genes. Gene sequences identified in this strain had regions of high similarity to the *tfdA* (96%), *tfdB* (99%), *tfdC* (98%), *tfdE* (98%), and *tfdR* (100%). Genetic elements similar to published sequences were also found in the 2,4,5-T degrading *Burkholderia* species strain JR7B3. Specifically, these included geneic regions similar to the *tftA* (98%), *tftC* (99%), and *tftE* (98%) genes. In addition to these previously reported genetic sequences, an apparently novel gene sequence was identified in *Burkholderia* species strain JR7B2. This genetic element was identified using DDRT-PCR applied to 2,4,5-T versus succinate grown minimal media cultures inoculated with the Agent Orange enrichment culture JR7B. No significant match for this gene was found in the current databases available for web based sequence analyses. It is interesting that this gene is induced in the presence of 2,4,5-T in the non-degrading *Burkholderia* species strain JR7B2. One implication of this finding is that this organism is responding to changing growth conditions as would be expected during acclimation of the microbial consortium for growth on different carbon sources. Another

possibility is that this gene is involved in adaptation of this organism to cellular stresses that may result during metabolism of 2,4,5-T by Strain JR7B3.

The presence of each of these degradation pathways in association with Agent Orange contaminated soils stimulates questions regarding the interactions of 2,4-D and 2,4,5-T pathways and the influences of their metabolic products. 2,4-DCP and 2,4,5-TCP are commonly detected as a consequence of incomplete metabolism of chlorinated organic pollutants, such as 2,4-D and the effects each of these chlorinated phenols have on cellular metabolism has been superficially examined. A primary aim of this research project was to study the consequences of 2,4-D and 2,4,5-T metabolic intermediates on bacterial catabolism of 2,4-D and 2,4,5-T by environmental bacteria isolated from Agent Orange contaminated soil, specifically, *Burkholderia* species strain JRB1 and *Burkholderia* species strain JR7B3.

*Burkholderia* species strain JRB1 grown in the presence of 2,4-D and succinate accumulated 24.2 mg/L 2,4-DCP. This increase in 2,4-DCP resulted in a cell death phase and a substantial decrease in 2,4-D transformation. Succinate addition to 2,4-D degrading cultures resulted in decreased mRNA transcripts for the *tfdA*, *B*, and *C* genes. These results are in contrast to cultures treated with glucose, which did not accumulate 2,4-DCP and demonstrated an enhanced 2,4-D degradation profile. Addition of glucose to 2,4-D cultures did not result in a significant changes in *tfdA* and *tfdC* mRNA transcript abundance but did result in increased *tfdB* mRNA. These results suggest that carbon catabolite repression of the TfdB occurs in response to succinate but not glucose. The most likely model supported by these data is the current repression model hypothesized

for the *clcABD* genes (McFall et al. 1997). Based on this model succinate would be converted to fumarate and would compete with 2,4-dichloromuconate for the TfdR binding site, thereby resulting in decreased transcription associated with the *tfdB*, *tfdC* and *tfdA* operons. It is interesting that the *tfdB* seemed most susceptible to this repression and that glucose amendments increased *tfdB* mRNA only. Hypothetically, glucose could also result in increased fumarate concentrations and subsequent repression. The fact that initial growth of 2,4-D/glucose and 2,4-D cultures were the same suggest that 2,4-D is a preferred growth substrate for these cells, and growth profiles similar to glucose controls were observed only after 2,4-D concentrations were diminished. This phenomenon could also be the result of phenotypic lag since cultures used for the inoculum were acclimated to 2,4-D and not glucose growth. Growth patterns for succinate/2,4-D cultures were different than glucose/2,4-D cultures with an increasing biomass relative to 2,4-D controls observed as soon as 5 hours. This is not unexpected because succinate is an important intermediate during 2,4-D metabolism, and these cells were likely already poised for succinate metabolism. Succinate repression of upper pathway enzymes may provide these bacteria with an efficient sensing and regulatory mechanism to facilitate catabolism of 2,4-D.

Similar studies using the 2,4,5-T degrading bacterium *Burkholderia* species strain JR7B3 demonstrated different results. A microbial consortium containing strain JR7B3 was utilized in initial experiments. Glucose/2,4,5-T cultures demonstrated increase 2,4,5-T transformation and subsequent 2,4,5-TCP production relative to succinate/2,4,5-T cultures in which transformation of 2,4,5-T was negligible. Transformation of 2,4,5-T to

2,4,5-TCP coincided with a decrease in cellular growth compared to glucose controls. These results suggest that growth on glucose allowed increased metabolism of 2,4,5-T whereas succinate mediated growth does not. 2,4,5-T degradation by glucose and succinate grown cultures demonstrated an interesting trend. For glucose grown cultures 2,4,5-T degradation was more productive having less 2,4,5-TCP accumulate and faster overall 2,4,5-T specific growth and subsequent removal. Succinate grown cultures, however, demonstrated a decreased ability to degrade 2,4,5-TCP and prolonged periods of adaptation were necessary prior to complete removal of 2,4,5-T. In the absence of succinate or glucose 2,4,5-T was slowly degraded and 2,4,5-TCP was maintained at minimal concentrations.

Because these initial studies were complicated by the presence of a secondary non 2,4,5-T-degrading microorganism, *Burkholderia* species strain JR7B2, pure culture studies using *Burkholderia* species strain JR7B3 were conducted in hopes of elucidating the underlying reason for these observations. These studies had similar results but the effects of glucose and succinate were magnified. Cultures containing glucose and 2,4,5-T accumulated toxic levels of 2,4,5-TCP, which prevented further metabolism of either glucose or 2,4,5-T. Succinate/2,4,5-T cultures demonstrated low levels of 2,4,5-TCP, and when compared to control cultures without succinate or glucose amendments it seems that succinate grown cells are better able to sustain 2,4,5-TCP metabolism thereby facilitating 2,4,5-T degradation. 2,4,5-T degradation associated with succinate/2,4,5-T cultures tapered off during the course of the experiment in contrast to control cultures that completely removed 2,4,5-T within 250 hours. An analysis of *tft* mRNA transcripts

demonstrated that transcription levels appear to be similar regardless of whether glucose or succinate is present. The one exception was a slight increase in *tftA* transcripts relative to succinate treatments.

2,4-D and 2,4,5-T mixtures were used to study potential inhibitory phenomena associated with metabolic conversion of these phenoxyacetic acid for both isolates obtained from Agent Orange enrichment cultures. Initial 2,4-D degradation by *Burkholderia* species strain JRB1 in the presence of 2,4,5-T was not affected. During these experiments it was apparent that 2,4-D was preferentially degraded relative to 2,4,5-T in the culture medium as evidenced by decreasing 2,4-D concentrations without concomitant 2,4,5-T transformation. Low levels of 2,4,5-TCP were produced, however, and appeared to correspond to increasing 2,4,5-T/2,4-D ratios. These strain were able to metabolize the 2,4,5-TCP produced but this corresponded to a decrease in cell growth and 2,4-D metabolism suggesting that a downstream intermediate is interfering with productive metabolism of 2,4-D.

Similar studies using 10 mg/L and 25mg/L 2,4,5-TCP amendments demonstrated that 25 mg/L 2,4,5-TCP completely inhibited growth on either succinate or 2,4-D. Suggesting that at least at these concentrations a general toxicity of the trichlorophenol was responsible for the growth inhibition. 10 mg/L 2,4,5-TCP was also inhibitory to 2,4-D degradation and cell growth, but removal of 2,4,5-TCP from 2,4-D containing cultures was observed and corresponded with initiation of 2,4-D degradation. Complete removal of 2,4-D was observed in cultures treated with 10 mg/L 2,4,5-TCP, approximately 40

hours later than control cultures. 2,4,5-TCP treatment did not affect *tfdA* or *tfdC* mRNA expression.

Based on the results it appears that when 2,4-D concentrations are high 2,4,5-T is not be a productive competitor for the 2,4-dichlorophenoxyacetic acid/ $\alpha$ -ketoglutarate dependent dioxygenase. As 2,4-D concentrations decrease 2,4,5-T would be more likely to compete with 2,4-D at the TfdA binding site and subsequently enter the 2,4-D metabolic pathway. This results in increased production of 2,4,5-TCP. 2,4,5-TCP then inhibits 2,4-D metabolism either by a general toxicity response or via non-specific metabolism of 2,4,5-TCP to a toxic metabolic intermediate such as 3,4,6-trichlorocatechol, which may serve as an additional source of toxicity for these cells.

*Burkholderia* species strain JR7B3 grown in the presence of 2,4-D and 2,4,5-T demonstrated a decreased ability to metabolize 2,4,5-T with higher concentrations of 2,4-DCP phenol being produced rather than 2,4,5-TCP. This suggests that 2,4-D was the preferred substrate for the TftA enzyme and was competing with 2,4,5-T for the enzyme's binding site. It has previously been hypothesized that 2,4-D metabolism by *B. cepacia* AC1100 results in a toxic chlorohydroquinone which acts to inhibit the TftCD two-component monooxygenase (Haugland et al. 1990). Results of this study indicate however that cultures amended with 2,4-DCP are able to completely metabolize 2,4,5-T. Therefore the inhibition associated with 2,4-D addition is likely due to the 2,4-D itself and not a metabolic intermediate. This is supported by the slower but continued growth of these cultures and presence of 2,4-DCP and 2,4,5-TCP at low concentrations through the experiment. Because this strain may be capable of a low level of 2,4-D

mineralization, and it is possible that a low but significant amount of 2,4-D can be metabolized through the 2,4,5-T pathway. Another possibility is that another pathway capable of minimal 2,4-D degradation exists in this strain. Experiments examining the potential of 2,4-D to act as a carbon and energy source in liquid culture have been negative, but minimal growth is observed on solid media containing 2,4-D.

These observations suggest that conditions, which lower the metabolic activity of the *tftA* either through specific interactions or more general stress related responses allow for more efficient 2,4,5-T metabolism. The original 2,4,5-T-degrading consortium had a slow growth rate with a mean generation time of greater than 30 hours. The implication of this data is that *tftA* activity must be carefully controlled during 2,4,5-T metabolism. It has been demonstrated that *Burkholderia cepacia* AC1100 has two copies of the *tftCD* (Hubner et al. 1998) and it has been hypothesized that this allows the bacterium to cope with the formation of 2,4,5-TCP during 2,4,5-T metabolism. An alternative strategy that could be used to deal with 2,4,5-TCP accumulation problems during 2,4,5-T metabolism could be decreasing the rate at which cell metabolize 2,4,5-T either through a general decrease in growth rate or through modulation of the 2,4,5-T dioxygenase.

Many of the toxicological effects associated with Agent Orange are believed to result from low concentrations of "dioxins". Studies of bacterially mediated biodegradation of these diaryl ethers have provided only one well-defined bacterial organism. This organism, *Sphingomonas* species strain RW1, was isolated from the river Elbe (Wittich et al., 1992), and has been well characterized in terms of its dibenzofuran and dibenzo-*p*-dioxin degrading capacity (Armengaud and Timmis 1998). The genetic

organization of its catabolic genes has been largely determined and found to consist of multiple cistrons containing the necessary genetic machinery for utilization of DBF and DD as sole carbon and energy sources (Armengaud et al. 1998). Particular interesting is the identification of the genetic arrangement of the *dxnA1*, *A2*, *B*, *C* and the *fdx3*, *dxn D*, *F*, *G*, *H*, *I* genes. These genes are found within two operons, respectively, and are separated by approximately 1610 bp. Current understanding is that each of these operons is distinct from each other and represents a novel organization of genes of this type.

Random arbitrarily primed reverse transcriptase-PCR mediated RNA fingerprinting studies have identified a novel genetic element, *OBI*, which is transcribed during growth of *Sphingomonas* species strain RW1 on dibenzofuran. The DNA sequence of this gene was found to be identical to the region of DNA separating the *dxnA1* and the *fdx3* operons, specifically corresponding to 12, 673 - 13,454 bp. Open reading frame analysis identified a 1106 bp ORF encoding a hypothetical 368 amino acid protein sharing homology with a putative inner membrane protein which has been hypothesized to contain a transmembrane region and a tyrosine kinase phosphorylation site. Furthermore, the hypothetical *OBI* gene sequence is only 68 bp upstream of the *fdx3* operon and 426 bp downstream of the *dxnA1* operon. The implications of this are that the *OBI* gene may be either the first gene in the *fdx3* operon or a link between the *dxnA1* and *fdx3* operon. The fact that it may be membrane bound and has a tyrosine kinase phosphorylation site may suggest its involvement in either regulation or transport of dibenzofuran/dibenzo-*p*-dioxin into the cell. It is important to note that the genbank accession sequence X72850 does not include the *dxnC* gene in its listing of known genes

within this stretch of DNA sequence, but Armengaud et al. (1998) indicates that it falls within 12028 and 12372 bp in this region of DNA. In their manuscript the *dxnC* is described as a putative bacterial receptor protein. Perhaps the previously described *dxnC* is somehow related to the *OBI* gene sequence, through a corporation between the two proteins or gene sequences. Currently, no data is available to either support or refute this hypothesis.

Growing cell assays demonstrated that *Sphingomonas* species strain RW1 acquired the ability to degrade 2,4-D during growth on acetate and dibenzofuran. The metabolic versatility of *Sphingomonas* species is well documented. In particular Kamagata et al. (1997), have demonstrated that some members of the  $\alpha$ -subdivision (mostly in the genus *Sphingomonas*) of the *Proteobacteria* contain 2,4-D degradative genes which are distinct from the extensively characterized *tfd*-type genes present in the  $\beta$  and  $\gamma$ -*Proteobacteria*. Currently, the mechanism by which the Sphingomonads mediate 2,4-D metabolism has not been genetically characterized. The data obtained from this research project suggest that 2,4-DCP is an intermediate in metabolism of 2,4-D in this strain. It is therefore possible that subsequent steps resemble the *tfd* type pathway, but this is purely speculative at this point. Additional research will be necessary to elucidate the structural and functional dynamics of this 2,4-D degradative pathway.

The implications of a 2,4-D degradation pathway in *Sphingomonas* species strain RW1 are numerous. Pervious research by Wilkes et al. (1996) have shown that during resting cell assays 4-chlorocatechol and 4,5-dichlorocatechol are produced. These compounds are potent inhibitors of type I catechol-1,2-dioxygenases, and represent

extremely recalcitrant intermediates which interfere with dibenzofuran and dibenzo-*p*-dioxin metabolism (Wilkes et al. 1996). Efforts to extend the capacity of strain RW1 to include type II enzymes capable of degrading chlorinated catechols have recently been pursued by other laboratories (Wilkes et al. 1996). Based on the results of this study, *Sphingomonas* species strain RW1 has a latent degradation pathway capable of degrading 2,4-D, potentially through a metabolic pathway including 3,5-dichlorocatechol. If conditions could be optimized to select for such a variant a natural alternative to genetically engineered strains could be achieved and utilized for future biodegradation studies at sites contaminated with chlorinated mixtures of PCDDs and PCDFs. In fact research by Arfmann et al., 1997, has successfully demonstrated that *Sphingomonas* species strain RW1 grown in a consortium containing *Burkholderia* species strain JWS, which is able to degrade 3-chlorocatechol, and allows complete degradation of 4-chlorodibenzofuran. Also, a 2,4-D adapted *Sphingomonas* species strain RW1 could potentially be applied for remediation of sites containing mixtures of the "dioxin" class of compounds and the herbicide 2,4-D.

Chemical mixtures, such as Agent Orange, are commonly found at hazardous waste sites (Haugland et al., 1990). The degradation of these pollutant mixtures by bacterial strains known to degrade specific environmental contaminants often results in production of toxic dead-end intermediates (Amy et al. 1985). The generation of these intermediates is the result of non-specific transformations of secondary compounds due to relaxed substrate specificity for many of the upper pathway enzymes. Despite the apparent problems associated with contaminant mixtures most of the research to date

addresses degradation of particular chemical pollutants. The herbicide Agent Orange contains many compounds, which may result in biological recalcitrance due to the misrouting of compounds through inappropriate pathways. Agent Orange as a pollutant mixture, contains 2,4-D, 2,4,5-T, and many congeners of dibenzofuran and dibenzo-*p*-dioxin (EPA 1995). Previous, research by Haugland et al. (1990) and Rice (Part III) have demonstrated that when 2,4-D and 2,4,5-T are both present as a chemical mixture the recalcitrance of 2,4,5-T is increased due to inhibition of important metabolic enzymes necessary for 2,4,5-T degradation by non-specific breakdown products of 2,4-DCP.

A primary purpose of this research was to examine whether 2,4-D, 2,4,5-T, 2,4-DCP, and 2,4,5-TCP interfered with metabolism of dibenzofuran by *Sphingomonas* species strain RW1. During growth on either acetate or dibenzofuran 2,4-D was degraded, and although 2,4-DCP was produced during this catabolism it was subsequently metabolized. The ability of *Sphingomonas* species strain RW1 to mediate 2,4-D degradation was realized only after a prolonged incubation suggesting that this pathway required some type of metabolic activation. Strain RW1 was also capable of mediating limited 2,4,5-T transformation to 2,4,5-TCP, which accumulated during growth on acetate. During growth on dibenzofuran, 2,4,5-TCP accumulation was substantially less than for acetate-grown cell, and did not exceed 2 mg/L. The appearance of 2,4,5-TCP in the culture medium corresponded with a decreased level of growth on dibenzofuran. It is hypothesized that 2,4,5-TCP was non-specifically converted to 3,4,6-trichlorocatechol which interfered with further cell growth and metabolism using dibenzofuran as a carbon and energy substrate. This hypothesis is

supported by the accumulation of 2,4,5-TCP in the absence of dibenzofuran. An analysis of *dxnA1*, *dbfB*, and *OBI* mRNA expression indicated that the level of transcription was decreased when high concentrations of 2,4-D were present. This may indicate that an increase in intracellular 2,4-DCP concentrations has detrimental effects on cellular metabolism. When high concentrations of 2,4,5-T were examined the transcription level of these genes was not affected to the same degree. The only significant repression was observed for the *dxnA1* and *dbfB* genes during growth on acetate, and the level of transcript modulation was about the same as the low concentration of 2,4-D. These data seem to infer that 2,4-D/2,4,5-T needs to be metabolized to attain optimal inhibitory activity, and that 2,4-DCP and 2,4,5-TCP are the actual inhibitory compounds. Examination of the influences 2,4-DCP and 2,4,5-TCP demonstrated that both of these compounds inhibited growth on acetate and dibenzofuran. 2,4-DCP containing cultures eventually recovered and growth was apparent for both carbon sources. No growth for 2,4,5-TCP treated cultures was observed. Analysis of *dxnA1*, *dbfB*, and *OBI* mRNA expression demonstrated that 2,4,5-TCP and high levels of 2,4-DCP reduced mRNA abundance for all genes tested. Low concentrations of 2,4-DCP were substantially less inhibitory to these genes. These results lend credibility to the idea that 2,4-DCP and 2,4,5-TCP are the actual compounds resulting in inhibition of growth on acetate or dibenzofuran. It appears that there are two mechanisms of inhibition in operation for growth on dibenzofuran and acetate by *Sphingomonas* species strain RW1. One is a general toxicity response mediated by deenergizing the proton motif force, and the second is a more specific chlorocatechol mediated repression. The research reported here

provides valuable information regarding the degradation of DBF in the presence 2,4,5-T, 2,4-D and metabolic intermediates produced during their degradation.

This study is the first to demonstrate that 2,4-D and 2,4,5-T degrading bacteria can co-exist in Agent Orange contaminate soils. Previous studies have relied on prolonged selective conditions within the laboratory, such as plasmid assisted molecular breeding (Kilbane et al. 1982), to allow bacterial organisms to adapt to growth on 2,4,5-T as a sole carbon and energy source. It has been postulated that microbial consortia may be more widely spread in association with 2,4-D breakdown in the environments than has previously been thought (Tiedje et al. 1997). Based on these experiments, this certainly seems to be likely for 2,4,5-T degradation as well. It is interesting, however, that most of the important metabolic potential necessary for 2,4,5-T transformation exists in a single microorganism. Further analysis of these bacterial strains will allow the potential dynamic interaction of 2,4-D and 2,4,5-T degradation pathways to be studied. Results from such studies have the potential for facilitating bioremediation and bioaugmentation efforts at sites contaminated 2,4-D and 2,4,5-T mixtures.

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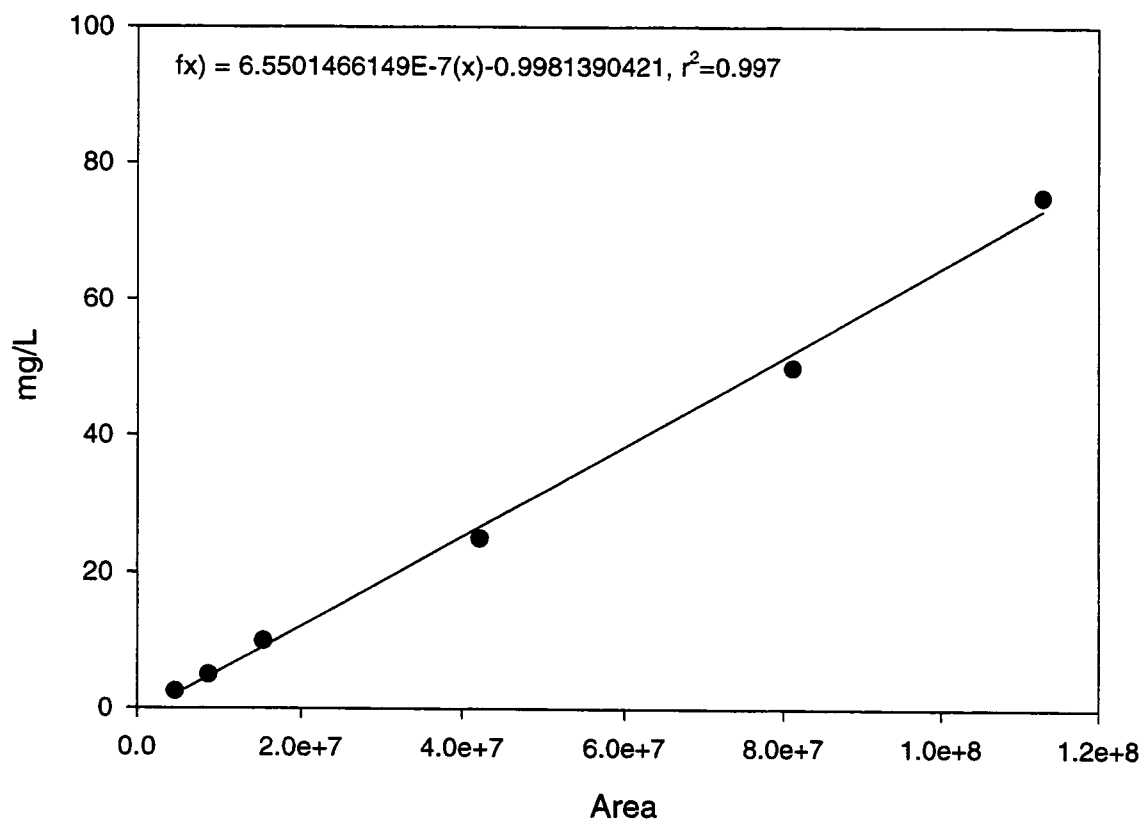
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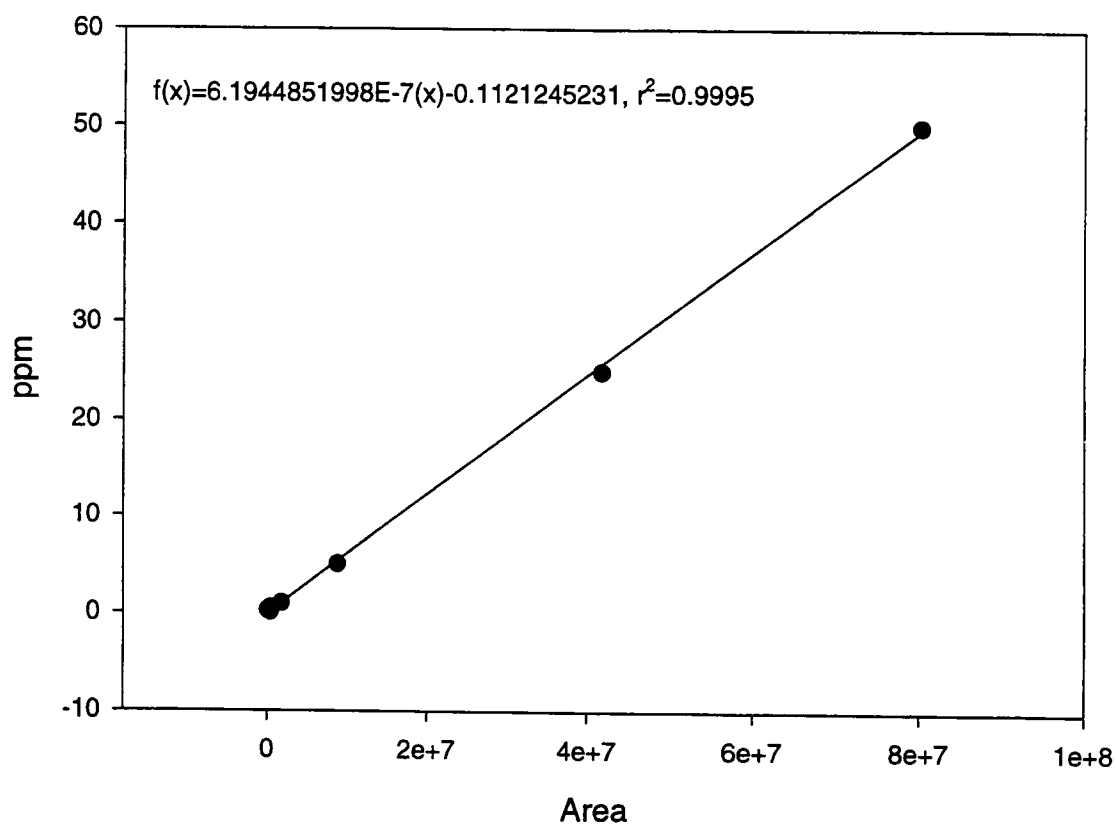
# **Appendix**

### 2,4,5-T Standard Curve

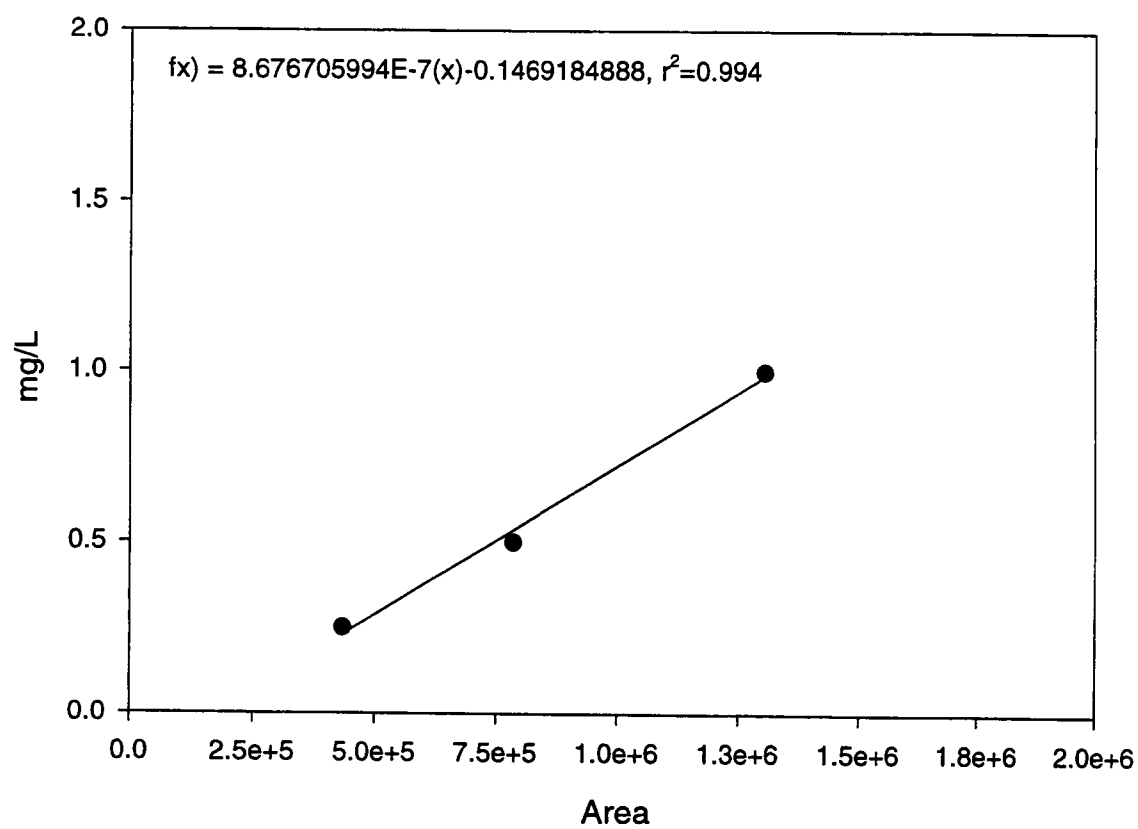


2,4,5-Trichlorophenoxyacetic acid standard curve 2.5 mg/L - 75 mg/L.

2,4,5-T Std Curve  
0.1 ppm - 75 ppm



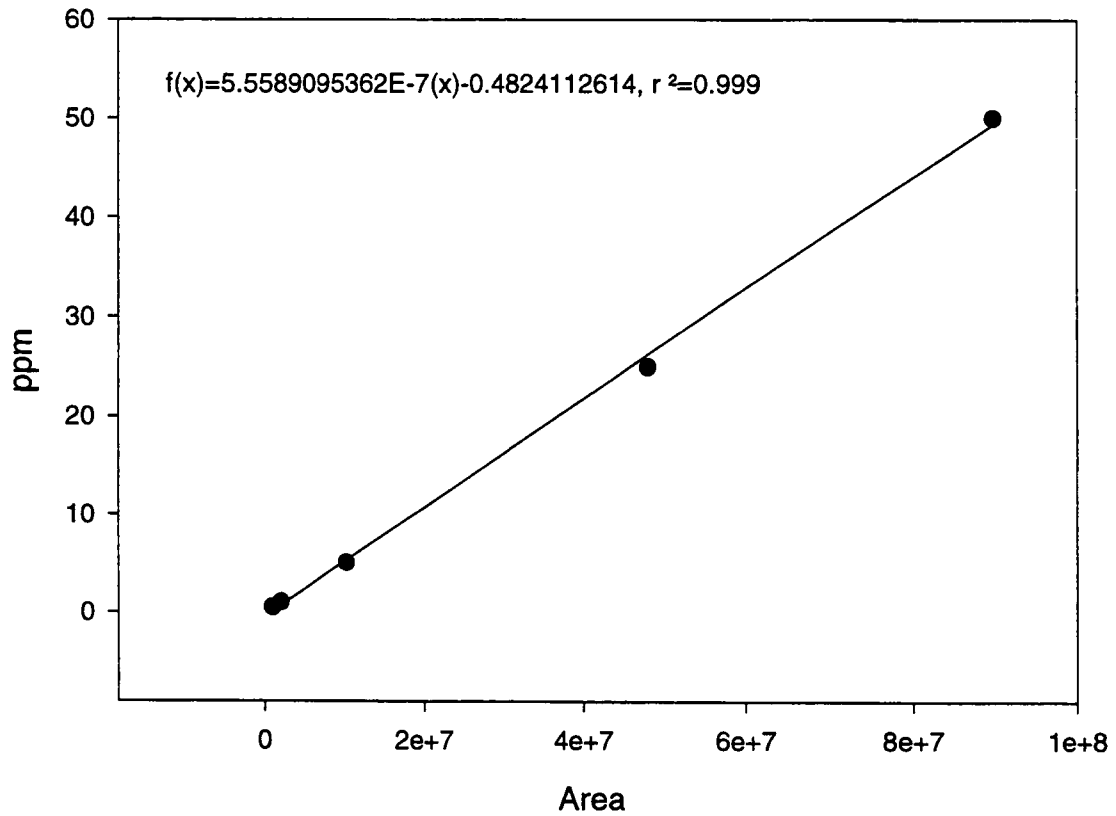
### 2,4,5-T Standard Curve



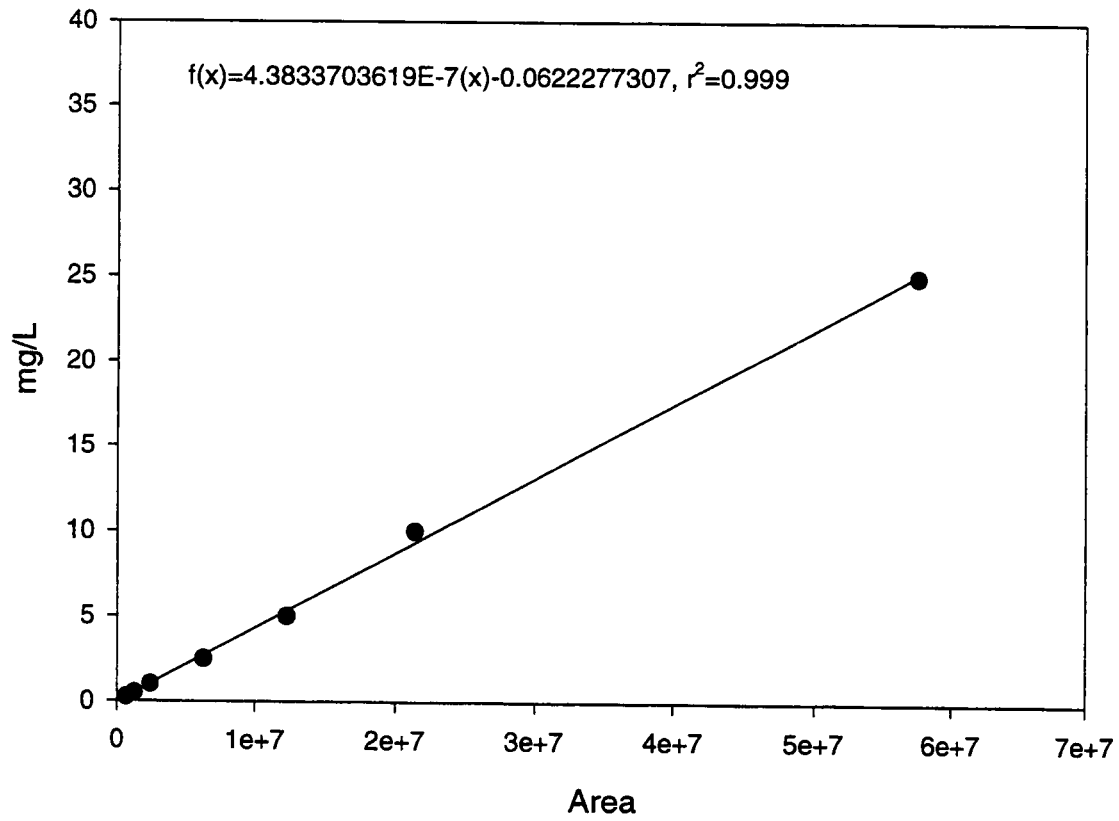
2,4,5-Trichlorophenoxyacetic acid standard curve 0.25 mg/L - 1.0 mg/L.

## 2,4,5-TCP Std Curve

0.5 ppm to 50 ppm

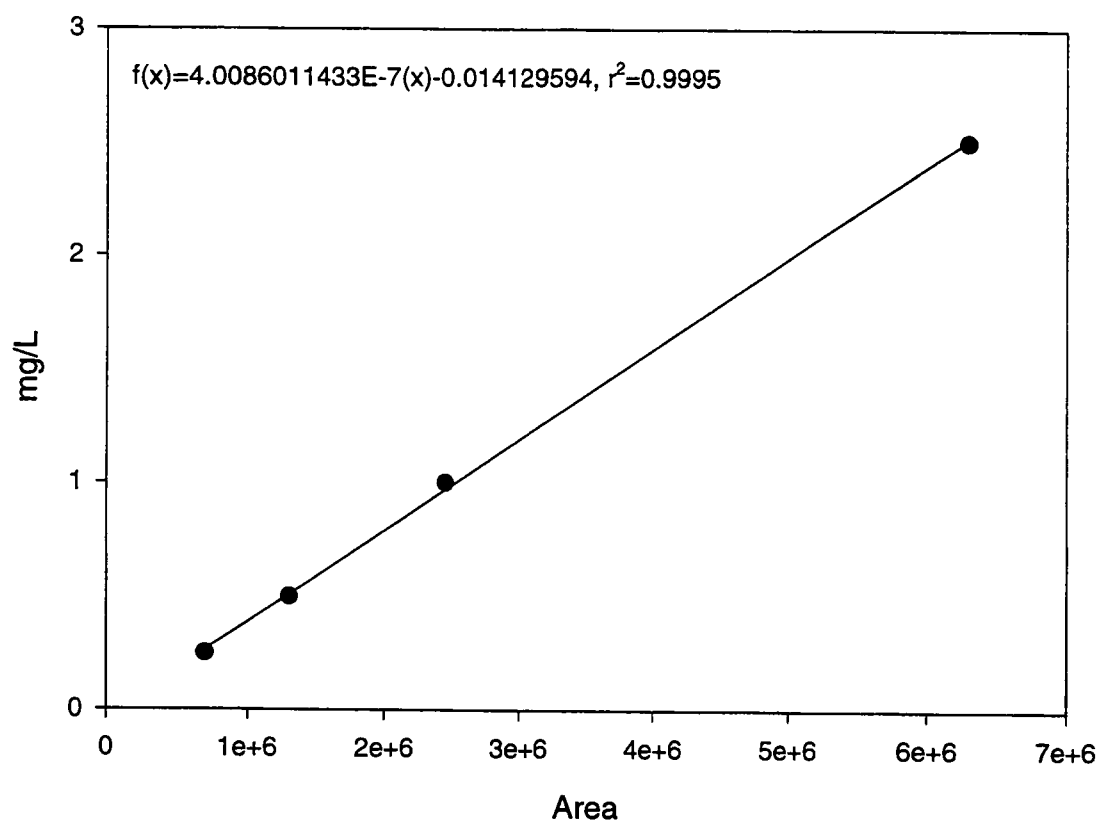


## 2,4,5-Trichlorophenol



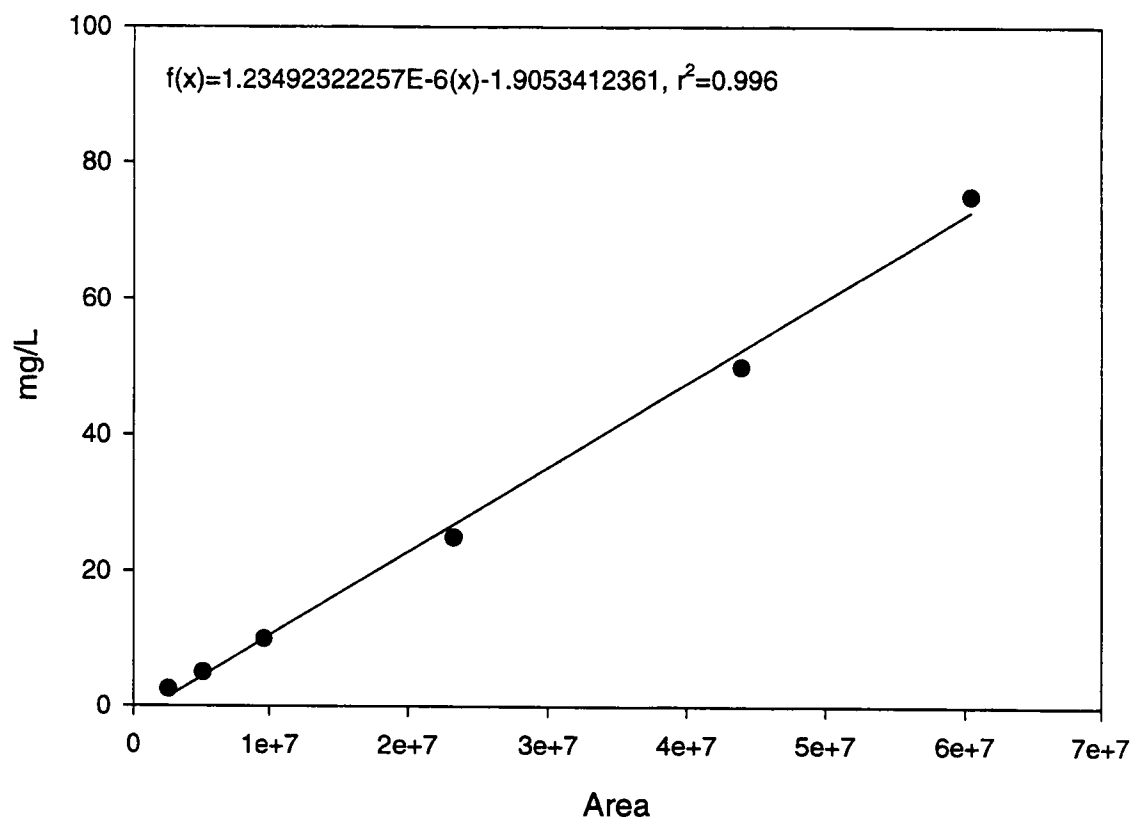
2,4,5-Trichlorophenol standard curve 0.25 mg/L - 25 mg/L.

## 2,4,5-TCP



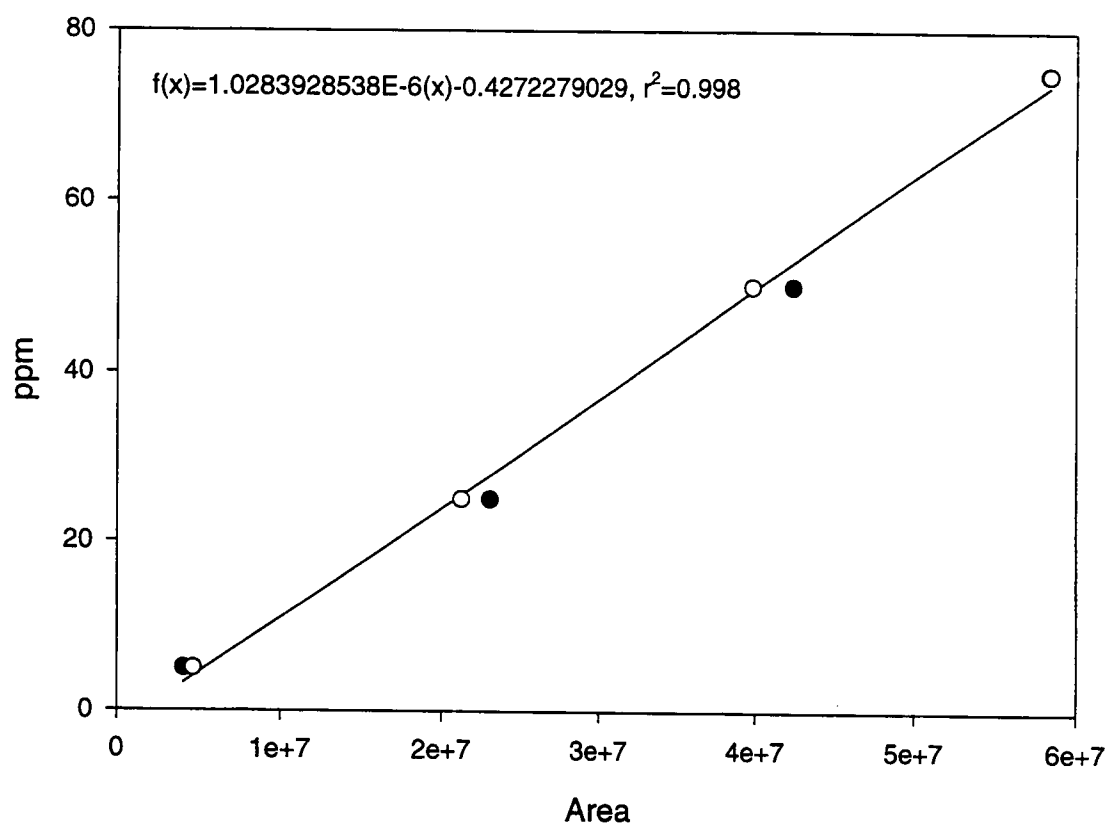
2,4,5-Trichlorophenol standard curve 0.25 mg/L - 2,5 mg/L.

## 2,4-Dichlorophenoxyacetic acid

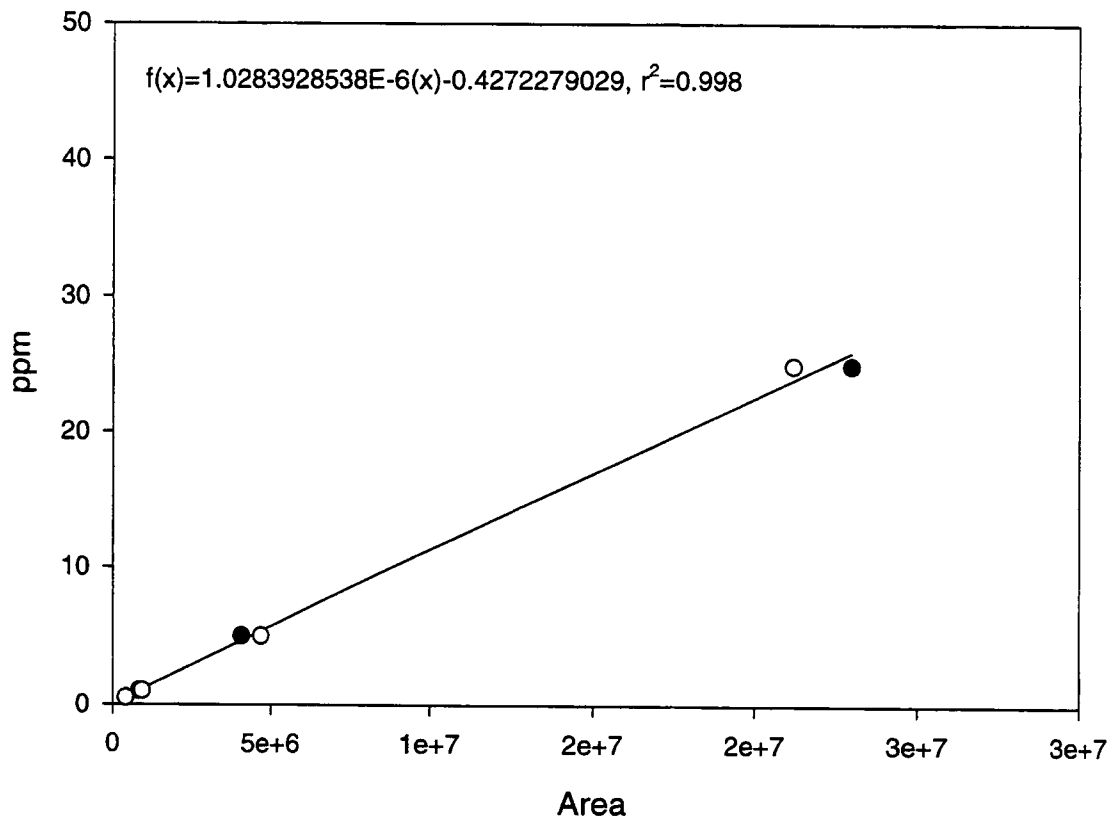


2,4-Dichlorophenoxyacetic acid standard curve med - high

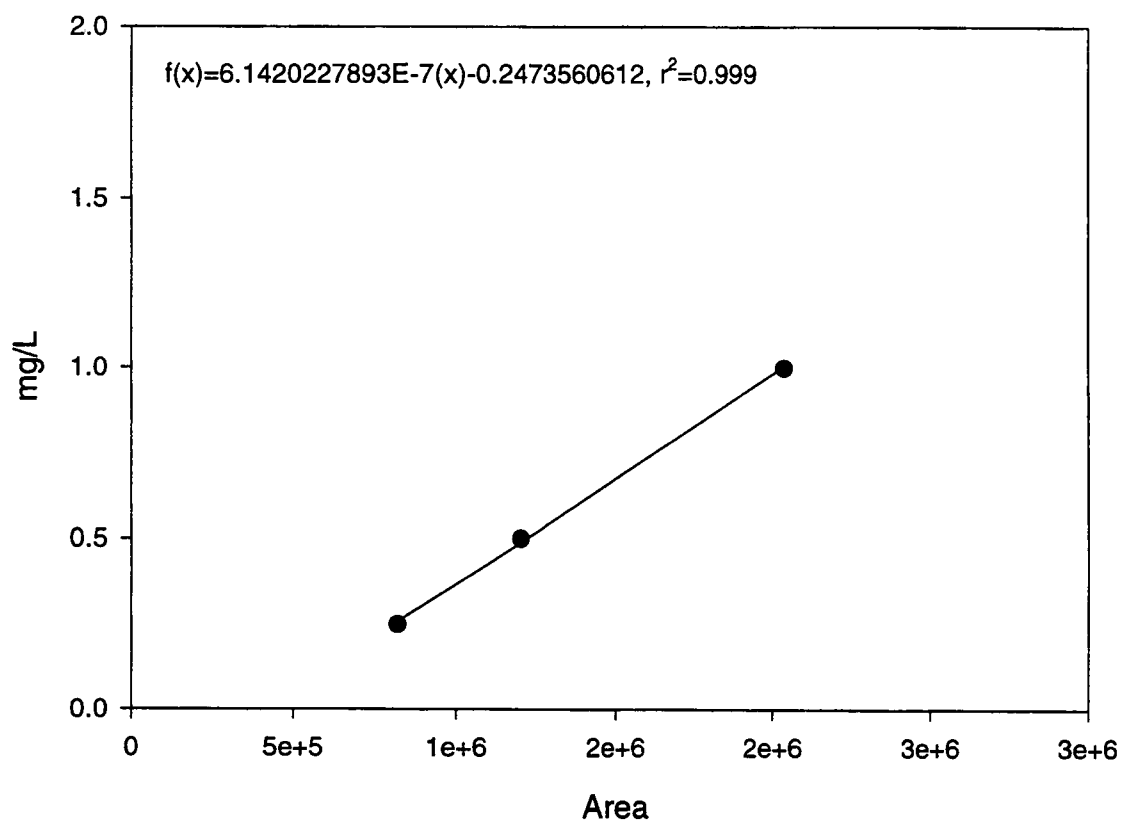
2,4-D standard curve  
5 ppm - 75 ppm



2,4-D standard curve  
(0.5 ppm - 25 ppm)

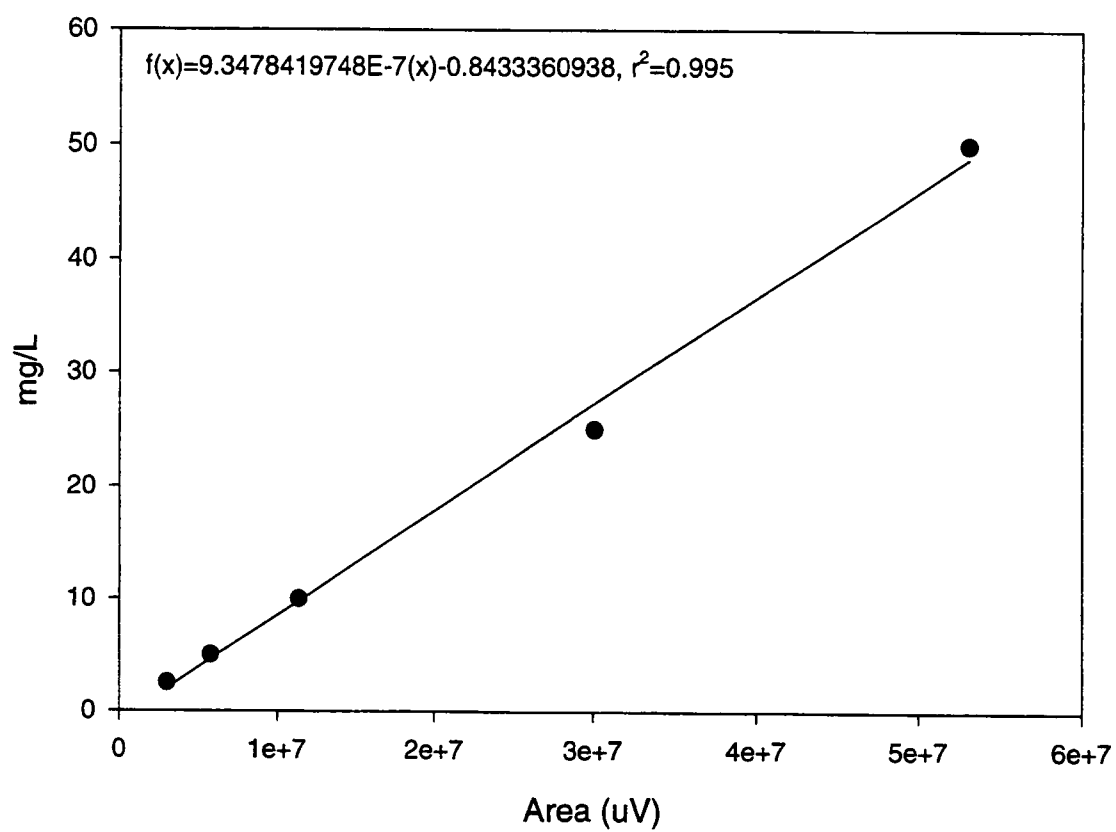


## 2,4-Dichlorophenoxyacetic acid



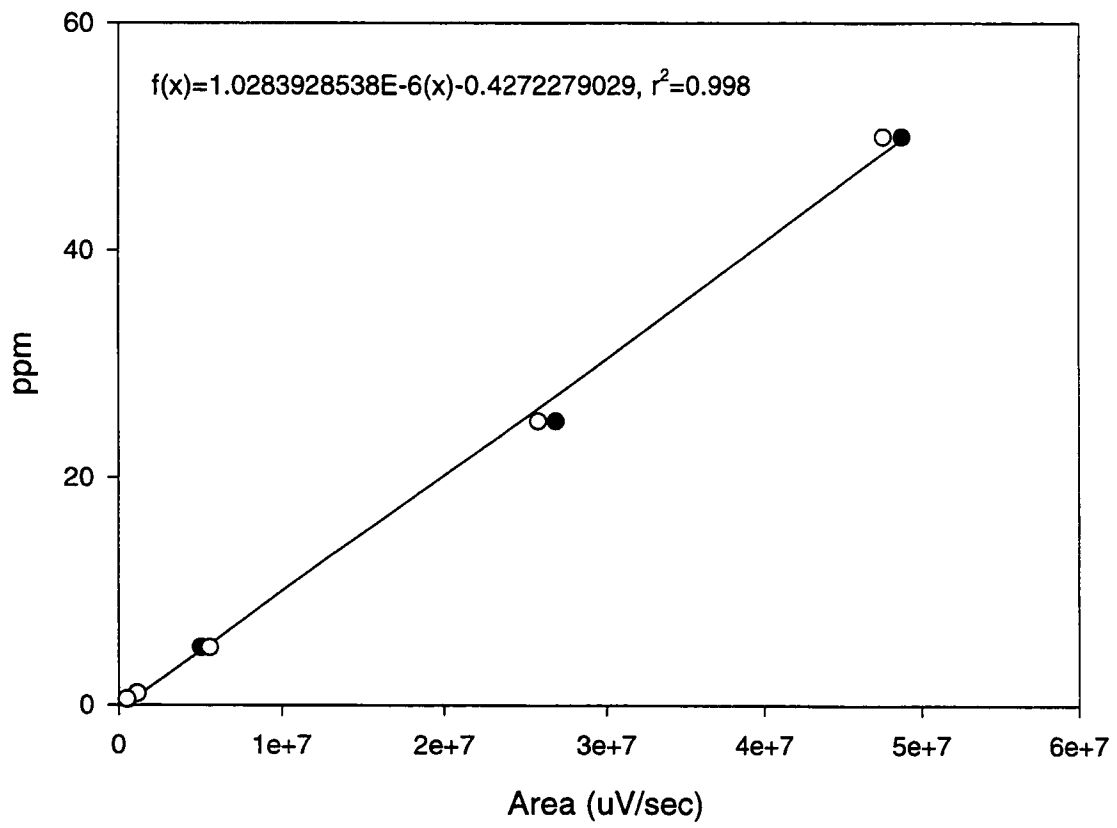
2,4-dichlorophenoxy acetic acid standard curve 0.25 ppm - 1 ppm.

## 2,4-Dichlorophenol

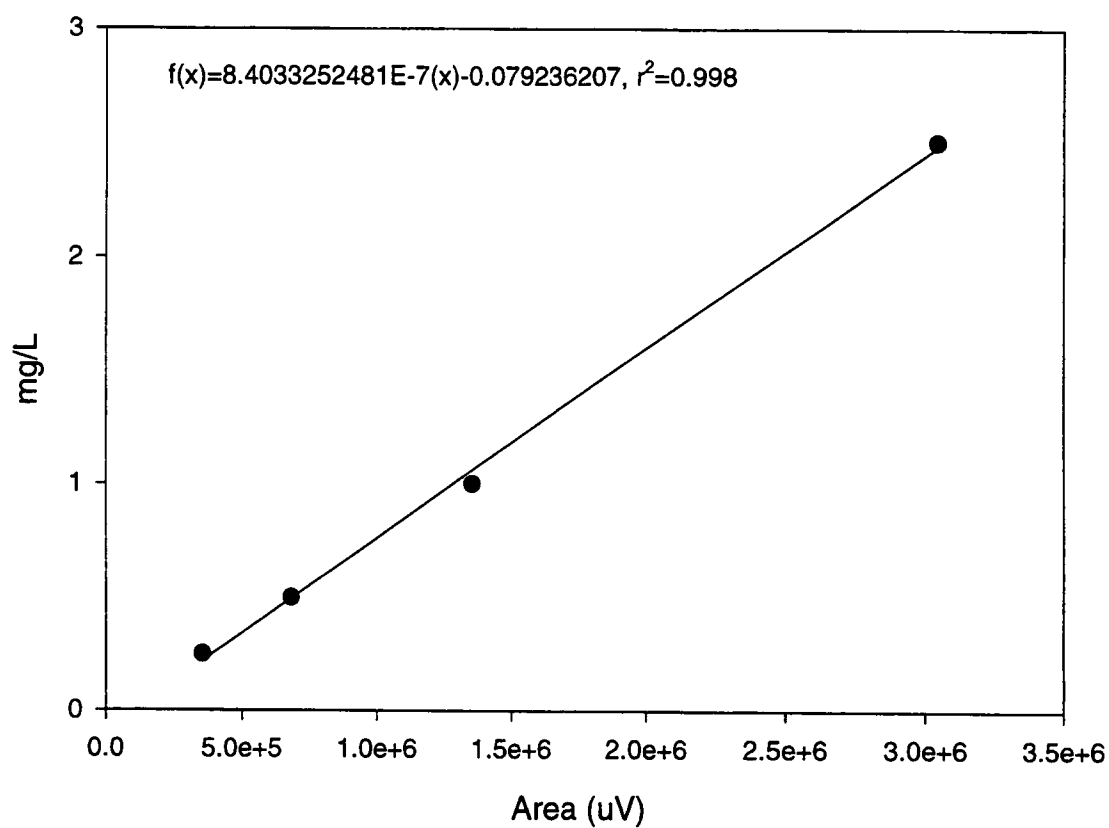


2,4-Dichlorophenol standard curve range 2.5 mg/L - 50 mg/L.

# 2,4-DCP standard curve



## 2,4-Dichlorophenol



2,4-Dichlorophenol standard curve. 250 µg/L - 2.5 mg/L range.

## VITA

James Franklin Rice, Jr. was born in Knoxville, Tennessee on November 12, 1968 at Fort Sander's Hospital. He has lived most of his life thus far in the city of Knoxville, TN. He attended South Knoxville Elementary School, South Knoxville Middle School, and graduated from South Young High School in May 1987. He enrolled at the University of Tennessee, Knoxville in August of 1987 and began his collegiate studies in the College of Engineering. Following two years of study he decided to pursue an interest in biology. During his high school studies the natural sciences had held his interest the most profoundly with high marks in all of his biologically related classes. Initially he switched to the College of Liberal Arts and changed his major to Biology. The following semester after an extremely interesting class taught by Dr. Jeffery M. Becker, he switched his major to Cell Biology. He thereafter discovered an interest in Microbiology after working as a laboratory assistant at the Center for Environmental Biotechnology founded by Dr. Gary S. Sayler. He received his Bachelor of Arts degree in Microbiology on May 23, 1993. In the fall of 1993, he enrolled as a graduate student in the department of Microbiology at the University of Tennessee. After 3 ½ years of academic study he presented his Master Thesis entitled, "Use of a Bioluminescent Reporter to Monitor Exopolysaccharide Production by Environmental Bacteria from Corroded Metal Surfaces," and received his Master of Science degree in December of 1995. He then decided to continue his scholarly studies under the guidance of Dr. Gary S. Sayler, and enrolled as a doctorate student in January 1996.