This is to certify that the

dissertation entitled

INTERSPECIES GENE TRANSFER IN THE EVOLUTION OF
2,4-DICHLOROPHENOXYACETATE
DEGRADING BACTERIA

presented by

Catherine McGowan

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Microbiology

Major professor

Date 7/6/95

DATE DUE	DATE DUE	DATE DUE

INTERSPECIES GENE TRANSFER IN THE EVOLUTION OF 2,4-DICHLOROPHENOXYACETATE DEGRADING BACTERIA

By

Catherine McGowan

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Microbiology

1995

ABSTRACT

INTERSPECIES GENE TRANSFER IN THE EVOLUTION OF 2,4-DICHLOROPHENOXYACETATE DEGRADING BACTERIA

By

Catherine McGowan

A great diversity of 2,4-D degrading bacteria exists in nature, yet it is not known by what mechanisms this diversity evolved. This study has demonstrated that interspecies gene transfer plays an important role in the evolution of this diversity.

2,4-D degrading strains isolated from various parts of the world were analyzed for their phylogenetic diversity as inferred from their 16S ribosomal DNA sequences. By this analysis, of 23 strains tested, 18 unique 16S rDNA sequences were found which belonged to members of the alpha, beta and gamma *Proteobacteria.*

The genetic diversity of the genes in these strains was further examined by hybridization studies with *tfd*A, B, and C genes, which encode the first three enzymes in the degradation of 2,4-D. Hybridization patterns indicated fifteen different classes of 2,4-D degraders. These patterns also showed that some strains contained pathways which appear to be mosaics of *tfd* genes, which suggests interspecies transfer and recombination of *tfd* pathways. This hybridization analysis also showed that two or more *tfd* probes hybridized to all beta and gamma *Proteobacteria,* but not to members of the alpha *Proteobacteria.* Only *tfd*B could hybridize at low stringency to only some members of the alpha *Proteobacteria.*

To compare the phylogeny of *tfd* sequences with the phylogeny of 16S rDNA sequences, *tfdA* genes from the beta and gamma *Proteobacteria* were partially sequenced. Three classes of *tfdA*-like sequences were found in these strains, differing by up to 24 percent from the corresponding sequence for JMP134. Dendrograms derived from these *tfdA* genes differed dramatically from the dendrogram of the 16S rDNA sequences from the same organisms, providing compelling evidence of interspecies horizontal gene transfer.

To discover whether these strains could mobilize 2,4-D genes *in vitro,* they were mated with recipient strain *Burkholderia cepacia* D5. Several strains transferred and expressed the ability to degrade 2,4-D, whereas other strains did not. Those that rapidly transferred and expressed the 2,4-D phenotype included strains with *tfd*A genes greater than 99% similar to *tfd*A from JMP134. Those strains that did not transfer the ability to degrade 2,4-D, or did so only at extremely low frequencies, had *tfd*A gene sequences differing by almost 24 percent from JMP134. One of these non-conjugal 2,4-D strains, *Burkholderia* strain RASC, was found to transfer its 2,4-D genes from its chromosome into the chromosome of *B. cepacia* at an extremely low frequency and required a long incubation period.

ACKNOWLEDGEMENTS

This dissertation was completed with the help of many people, whose efforts I would like to acknowledge here. I thank my advisor, Dr. Jim Tiedje, for giving me freedom to pursue my ideas and for his teaching that in science, as often in life, there are no problems, only challenges. I also thank my committee members, Dr. Michael Bagdasarian, Dr. Wendy Champness, and Dr. Pat Oriel for their help and encouragement. I also thank Dr. Richard Lenski for agreeing to be on my committee at a late date and for encouraging me to compare phylogenies of *tfd*A with that of the 16S rDNA sequences. I also thank Dr. Lynn Margulis for fanning the flames of my curiosity about the evolution and diversity of life.

For providing the strains used in this study, I would like to thank Dr. Nancy Tonso Sasaki, Dr. Bill Holben, Dr. Jong-Ok Ka, and Dr. Roberta Fulthorpe. I would especially like to thank Dr. Fulthorpe for her help in growing about half the strains, extracting DNA from them, and checking for 2,4-D degradation. I thank Arturo Massol-Deya, for the rD1 and fD1 primers used in PCR amplification of the 16S rDNA genes. I also thank the Michigan State University DNA synthesis laboratory for synthesizing the 519R primer used in sequencing and Sue Lootens from the MSU molecular sequencing facility for sequencing the genes on the ABI automated sequencer. For strain I-18, I owe thanks to Dr. Olga Maltseva for preparation of this strain and its DNA and assistance in PCR amplification and sequencing of this strain. I owe

many thanks to Dr. Tom Schmidt and Dr. Jim Smith for the useful discussions about data analysis. I thank Randy Russell for assistance in programming a distance matrix algorithm for analyzing sequence data. I am grateful to Dr. Cindy Nakatsu for teaching me how to use GenBank and BLAST. I also thank Dr. Jim Cole, Amy Foxworthy, and Dr. Jizhong Zhou who shared their ideas on acquiring the sequence data.

I give special thanks to Dr. Roberta Fulthorpe who collaborated with me on the hybridization work and who will be first author on a publication resulting from this work. I thank Dr. Bill Holben who gave us DNA for making probes from *tfd*A, B, and C for the hybridization studies.

For the design and preparation of the *tfd*A primers, I owe thanks to Dr. Tatiana Vallaeys and Dr. Alice Wright. Dr. Fulthorpe, Dr. Wright and I together used these primers to PCR amplify the sequences. Dr. Wright will be first author on a publication about the *tfd*A sequencing. I owe thanks to Dr. Olga Maltseva for assistance in the preparation of *tfd*A sequence from strain I-18. For *tfd*A sequence information, I thank Dr. Yuichi Suwa for RASC, Dr. Grace Matheson for TFD6, Dr. Cindy Nakatsu for TFD41 and Dr. Fumiyasu Fukumori for TFD159.

I owe thanks to Dr. Yuichi Suwa for the preparation of a probe from RASC. I also thank Dr. Larry Forney for encouraging me to pursue the gene transfer experiments from strain RASC.

I especially want to thank Fly for his loving support and discussions about science. I thank Supai for her ever wagging tail.

TABLE OF CONTENTS

LIST OF TABLES

Table Page

Chapter One

Chapter Two

Chapter Three

Chapter Four

Chapter Five

LIST OF FIGURES

Figure Page

Chapter One

Chapter Two

Chapter Two (continued)

Chapter Three

Chapter Four

Chapter Four (continued)

Chapter 5

Chapter One

Introduction:

Gene Transfer in the Evolution of 2,4-D Degrading Bacteria

Chapter 1. Introduction: Gene transfer in the evolution of 2,4-D degrading bacteria

TABLE OF CONTENTS

OVERVIEW

Interspecies genetic transfer may play an important role in the evolution of diversity of 2,4-D degrading strains. Gene transfer has been shown to be involved in the evolution of xenobiotic degrading organisms *in vitro* (Kellogg et al. 1981; Latorre et al. 1984; Reinecke and Knackmuss, 1979), yet it is not known how important gene transfer is in nature for the evolution of strains capable of degrading xenobiotic compounds. Hypothetically, the diversity of organisms capable of 2,4-D degradation could have evolved in one of three ways either by 1) **divergence** of an existing strain into unique strains of 2,4-D degraders; 2) **convergence** or **parallel evolution** of two unrelated strains into 2,4-D degraders; or 3) **gene transfer,** in which the genes for 2,4-D degradation evolved once or more times and were subsequently transferred between unrelated strains (Figure 1). Of course, there may also be many permutations to these three basic themes. For example, only the upper pathway may have transferred to strains which had already evolved a lower catechol dioxygenase pathway. The goal of this project was to examine the hypothesis that gene transfer played an important role in the evolution of diversity of 2,4-D degraders.

Although no one argues that genetic transfer occurs in bacteria, and that interspecies gene transfer has been observed in the laboratory, the view is generally held that gene transfer in nature generally occurs within a species, (Dykhuizen and Green, 1991) and that the range of gene transfer even defines

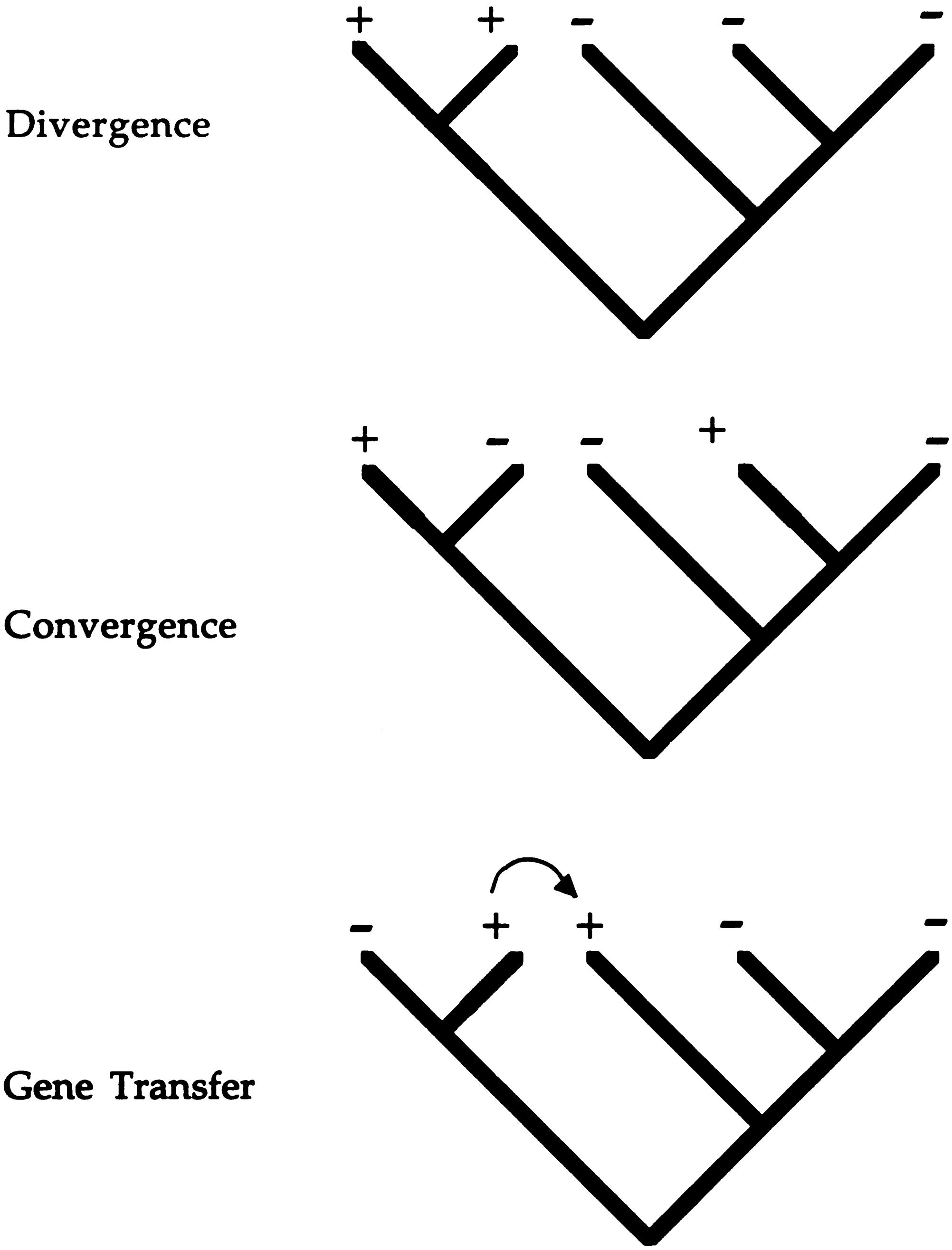


Figure 1. Three models diagraming possible evolution of 2,4-D degrading strains. Stippled lines and a "+" indicate the presence of 2,4-D pathway. Solid lines and a "- indicate strains that do not have the 2,4-D pathway.

a species, analogous to the definition of species in multicellular, eukaryotic organisms (Stackebrandt et al. 1991).

One way to determine whether gene transfer is responsible for the evolution of 2,4-D degrading strains is to apply a "phylogenetic congruency test" that predicts if the phylogenies of two separate genes are different (incongruent), then horizontal gene transfer has occurred within the group of organisms under investigation (Wilson et al. 1977). In other words, "recombination mixes the phylogenetic relationships of the strains" (Dykhuizen and Green, 1991) (Figure 2).

In this study, I compared the phylogeny derived from 16S gene sequences to the phylogeny derived from 2,4-D genes, in particular the *tfd*A gene, the first gene in the pathway of 2,4-D degradation. Then, to see whether transfer of the 2,4-D genes was observable *in vitro*, these strains were subjected to mating experiments. The gene transfer of one strain was then studied more in depth.

To accomplish these tasks, I performed the following experiments which are described in the next four chapters. In the second chapter, I examined the phylogeny of a group of 2,4-D degrading organisms based on partial sequences of 16S ribosomal DNA. In the third chapter, I describe work done by Dr. Roberta Fulthorpe and myself on the diversity of 2,4-D degrading genes from diverse bacteria that hybridize to the *tfdA*, *tfdB* and *tfdC* genes from plasmid pJP4. In the fourth chapter, I describe partial sequencing of the *tfdA* genes from a select group of 2,4-D degraders and comparison of the dendrogram derived from this information to that derived by sequencing of the 16S rDNA genes. Finally, in the fifth chapter, I describe an unusual 2,4-D

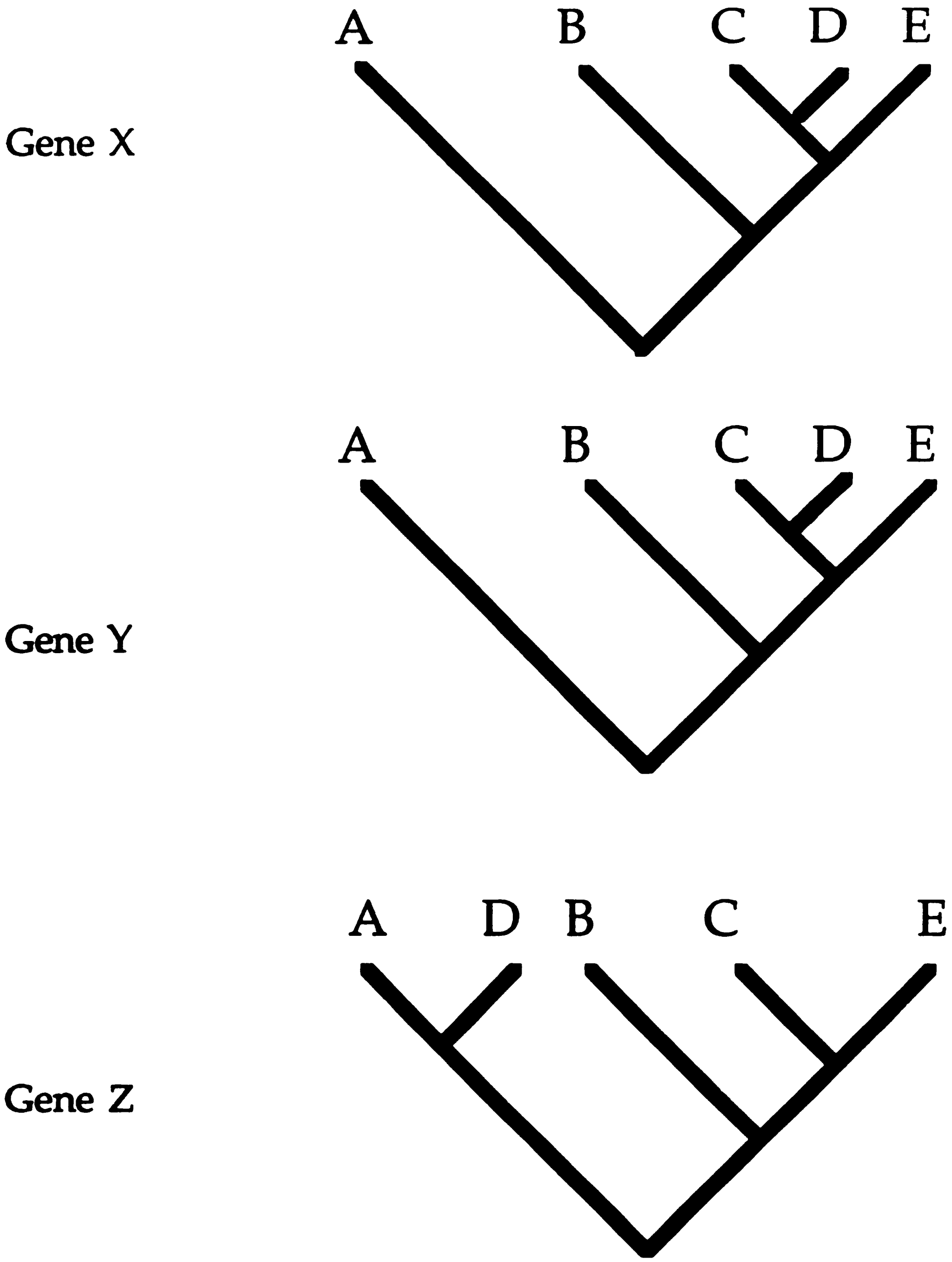


Figure 2. Phylogenetic congruency test. If phylogenies for two genes are identical, or congruent, as are gene X and gene Y in this diagram, then gene transfer has not necessarily occurred. However, if phylogenies for the two genes are different in the same group of organisms, then this is evidence of horizontal gene exchange, as is the case illustrated here where the phylogeny of gene Z differs from those of gene X and gene Z.

gene transfer dynamic in strain RASC.

Before describing these experiments, I use the rest of the introductory chapter to provide background information on the project. I describe the rationale for this project; give an overview of the evolution of xenobiotic degradation pathways; describe the 2,4-D model system, the diversity of 2,4-D degrading organisms, and their genes; and I describe mechanisms of gene transfer and discuss what is known about gene transfer in the environment.

RATIONALE

"The great aim of education is not knowledge, but action"
~Herbert Spencer (1820-1903)

Earth has entered a new geological era, that of the psychozoic, in which human influence on the biogeochemistry of the planet approaches the magnitude of previous abiotic (tectonic) and biotic (i.e. production of oxidizing atmosphere) forces (Piruzyan et al., 1985). Humans have influenced the biogeochemistry of the planet in two fundamental ways: by the generation of millions of axenic compounds and by an explosion in population growth which in turn has accelerated and altered many biogeochemical processes.

One of these influences is that humans synthesize most of the two hundred thousand novel substances added yearly to the list of over 5 million chemical compounds described in Chemical Abstracts (Piruzyan et al., 1985) and the United States alone produces approximately 100 million tons of synthetic organic chemicals annually (Cork and Kreuger, 1991). What is the fate of these xenobiotic compounds in nature? Some, usually analogs of natural products, may be degraded by extant microorganisms, while for some others, microbes may evolve novel pathways to degrade them (Allewell, 1989; Yano, 1980, Ornston and Parke, 1976).

One class of anthropogenic compounds is pesticides, used to combat plant pests which destroy nearly one-third of the world's agricultural output

(Davidson et al. 1991). In the United States approximately 200,000 tons of herbicides are applied annually at a cost of about $5 billion (Zilberman et al., 1991). The initial cost of pesticides, which have allowed a "Green Revolution" enabling farmers to grow food enough to feed the planet, does not include the potential hidden costs, such as contamination of the environment (Postel, 1988). The removal of unwanted pesticides in some cases may be achieved through biodegradation. Some pesticides are known to be degraded due to the evolution of microorganisms capable of metabolizing the compound as a substrate for growth. A factor contributing to this metabolic evolution is thought to be the transfer of pesticide degrading genes between various microbes (Fox, 1983; Kellogg et al., 1981; Yano, 1980).

To fully understand how microbes have evolved to degrade xenobiotic compounds will require a substantial effort by many research groups. Nonetheless, one potentially important component of microbial evolution, i. e., the role of gene transfer, was examined using the pathway for the degradation of 2,4-dichlorophenoxyacetic acid as a model system.

Bioremediation: The natural phenomenon of biodegradation is now being exploited by humans to remove toxic, manmade chemicals from chemical spills, aquifers, and Superfund sites (Kobayashi and Rittman, 1982). An estimated 20% of the 1245 Superfund sites, for which the U.S. federal government spends approximately $11 billion annually to clean up, might be detoxified biologically (Begley and Waldrop, 1989; Ghisalba, 1983, Ghosal et al. 1985, Gibson, 1984; Madsen, 1991; Reinecke and Knackmuss, 1988; Rochkind et al. 1986). Various strategies have been employed to facilitate biodegradation, including addition of nutrients or aeration to encourage growth of indigenous microbes, as done with the Exxon Valdez oil spill (Fox, 1989) or addition of exogenous microorganisms capable of utilizing the substrate (Roberts, 1987). In the future, genetically engineered microorganisms may be useful for some bioremediation. If the release of genetically-engineered, detoxifying microorganisms (GEMs) is approved, introduction of degradative genes on broad host range, conjugal plasmids or otherwise mobilizable genes may be an effective method for bioremediation, since the pathways effecting the metabolism of recalcitrant compounds may function best if naturally transferred into indigenous populations at the site instead of by adding a foreign strain which would be less likely to survive (Goldstein et al. 1985).

With more knowledge of how catabolic strains have evolved in nature, humans can use a more holistic approach to environmental bioremediation (Verstraete and Top, 1992).

EVOLUTION

> "The extent to which we can hope to understand ourselves and to plan our future depends in some measure on our ability to read the riddles of the past."
> ~ George Gaylord Simpson

When and how did 2,4-D degrading microbes evolve? If we can learn how 2,4-D pathways and organisms evolved we might develop better solutions for pollution management.

Learning about the mechanisms of evolution in a relatively simple system such as 2,4-D degrading bacteria can also shed light on evolutionary processes in general and add to our understanding of the evolutionary relatedness of all organisms (Figure 3). A firmer understanding of evolutionary processes may bolster the theory of evolution, which nearly fifty percent of the U. S. population doubts, in favor of a theory of creationism (Gallup, 1994, Taylor, 1994). An excellent model to understand the fundamental principles of evolution is in the evolution of the diversity of 2,4-D degrading bacteria.

Evolution of pathways for biodegradation of xenobiotic compounds: Gene transfer has been shown to be involved in the evolution of novel pathways by the recruitment of protein modules (Baron et al. 1991). Organisms with novel catabolic pathways appear to have evolved as the result of gene transfer in *vitro* (Kellogg et al. 1981; Latorre et al. 1984), yet it is not known how important gene transfer is in nature for the evolution of novel organisms.

It is surprising that so many exotic, anthropogenic organic compounds can be biodegraded since microbes were presumably not exposed to these compounds in any great quantity prior to the 1940's when they were first synthesized and therefore might be expected to lack enzymes capable of recognizing such compounds as substrates. Even though over 1500 naturally occurring chlorinated compounds (including 2,6-dichlorophenol, the sex pheromone of the female lone star tick) have been isolated (Suide and DeBernardis, 1973; Rhodes, 1994), no naturally occurring chlorinated phenoxyacetic acid has been reported. Have organisms evolved catabolic pathways *de novo* since the widespread introduction of these artificially synthesized compounds, or is this simply outgrowth of already existing pathways?

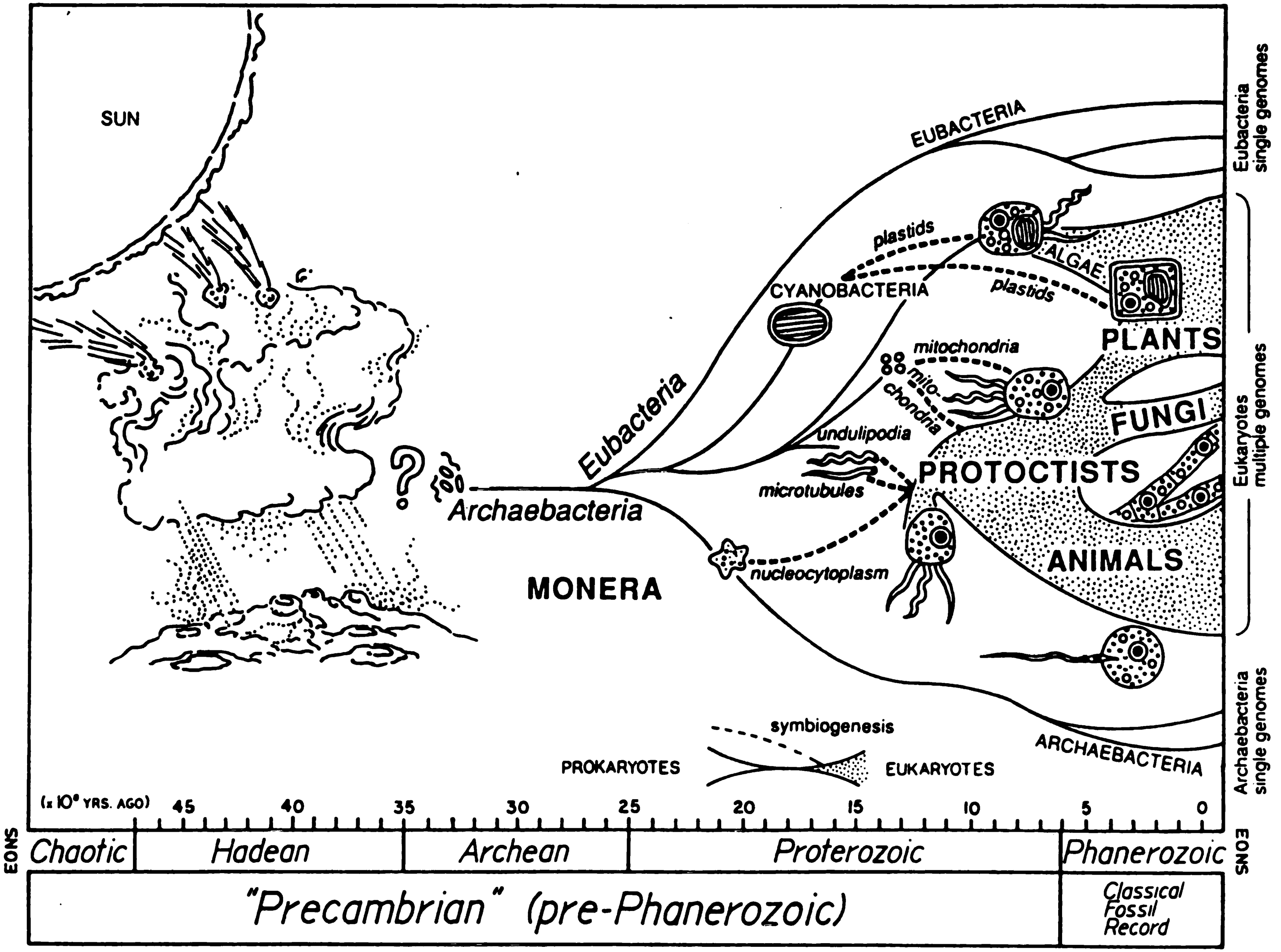


Figure 3. Systematic phylogeny of all life from origin to present. Drawing from Margulis, 1992.

The research described in this dissertation has explored the possibility that novel 2,4-D degrading organisms have evolved via gene rearrangement and recruitment facilitated by gene transfer. Several researchers have explored the evolution of metabolic function, which is reviewed by Mortlock (1992); Ornston and Parke (1976); and Schlömann et al. (1992). Evidence for evolution by gene exchange comes from laboratory experiments (Kellogg et al., 1981; Latorre et al., 1984) but is thought to occur in nature as well. Although scientists have described the prevalence and importance of gene transfer in the environment (for review see Coughter and Stewart, 1989; Fry and Day, 1989; Levy and Miller, 1989; Levy and Marshall, 1988; Stotzky, 1989; Stotzky and Babich, 1986; Trevors et al. 1986) it is still not known to what degree gene transfer in the environment facilitates the emergence of novel, xenobiotic-degrading strains.

2,4-D MODEL SYSTEM

2,4-D degradation: A useful model for studying evolution of catabolic pathways is the pathway for 2,4-dichlorophenoxyacetate degradation. One reason for its usefulness as a model is because 2,4-D was presumably not a substrate for bacteria since first being synthesized by humans in 1942 (it has never been detected in pristine environments). Other reasons for choosing 2,4-D biodegradation as a model system include its widespread use, that it is a synthetic compound of moderate recalcitrance, and because one pathway for its degradation has been characterized biochemically (Duxbury et al., 1970; Tiedje et al., 1969; Bollag et al., 1968; Loos et al., 1966; Don and Pemberton, 1991) and genetically (Perkins et al., 1990; Kaphammer et al.,1990; Don and Pemberton, 1985; Fukumori and Hausinger, 1993; Suwa et al. in prep; Matheson et al. 1994; Nakatsu et al. in prep; Kamagata, et al. in prep).

The herbicide 2,4-D is a functional analog of the plant hormone, auxin, that kills dicotyledonous plants by causing them to "grow to death" (Corbett, 1984). Over 60 million pounds of 2,4-D (sold as over 1500 different registered products including the bestselling lawn herbicide "Weed-B-Gone," (Ortho)) are applied annually for agriculture, in forests and on turf to control broadleaf weeds. This herbicide also was used as a defoliant during the military engagement in "Operation Ranchland" in Viet Nam. The Environmental Protection Agency (EPA) has concluded that 2,4-D poses no significant health hazards when used according to the label. This limited hazard is due, at least in part, to the fact that 2,4-D is readily degraded in the environment.

Known 2,4-D degradation pathway: The pathway of 2,4-D degradation encoded by plasmid pJP4 in *Alcaligenes eutrophus* JMP134 is the most well characterized of all 2,4-D pathways (Figure 4) and its enzymes are best understood (Table 1). The initial reaction is catalyzed by the product of the *tfdA* gene, 2,4-D dioxygenase, an alpha keto glutarate dependent dioxygenase (Fukumori, and Hausinger, 1993) which cleaves the ether linkage yielding glyoxylic acid and and 2,4-dichlorophenol. This latter compound is subsequently hydroxylated by 2,4-dichlorophenol hydroxylase, encoded by the *tfdB* gene, to form 3,5-dichlorocatechol (Perkins et al. 1990). This ring compound is then cleaved via a modified ortho pathway, to *cis,cis*-2,4-dichloromuconic acid, and then to 2-chloro-4-carboxymethylene *but*-2-enolide and finally to 2-chloromaleylacetic acid (Perkins et al. 1990). Chromosomally encoded enzymes further metabolize 2-chloromaleylacetic acid to succinate and acetyl CoA (Kukor, et al, 1989).

Although only one pathway for the degradation of 2,4-D has been well characterized, other pathways may exist (as have been shown for toluene degradation) but have not yet been described. An additional pathway for 2,4-D degradation has been partially characterized which includes the formation of a 3,5-dichlorohydroxyhydroquinone intermediate in *Nocardioides simplex* (Kozyreva et al. 1993).

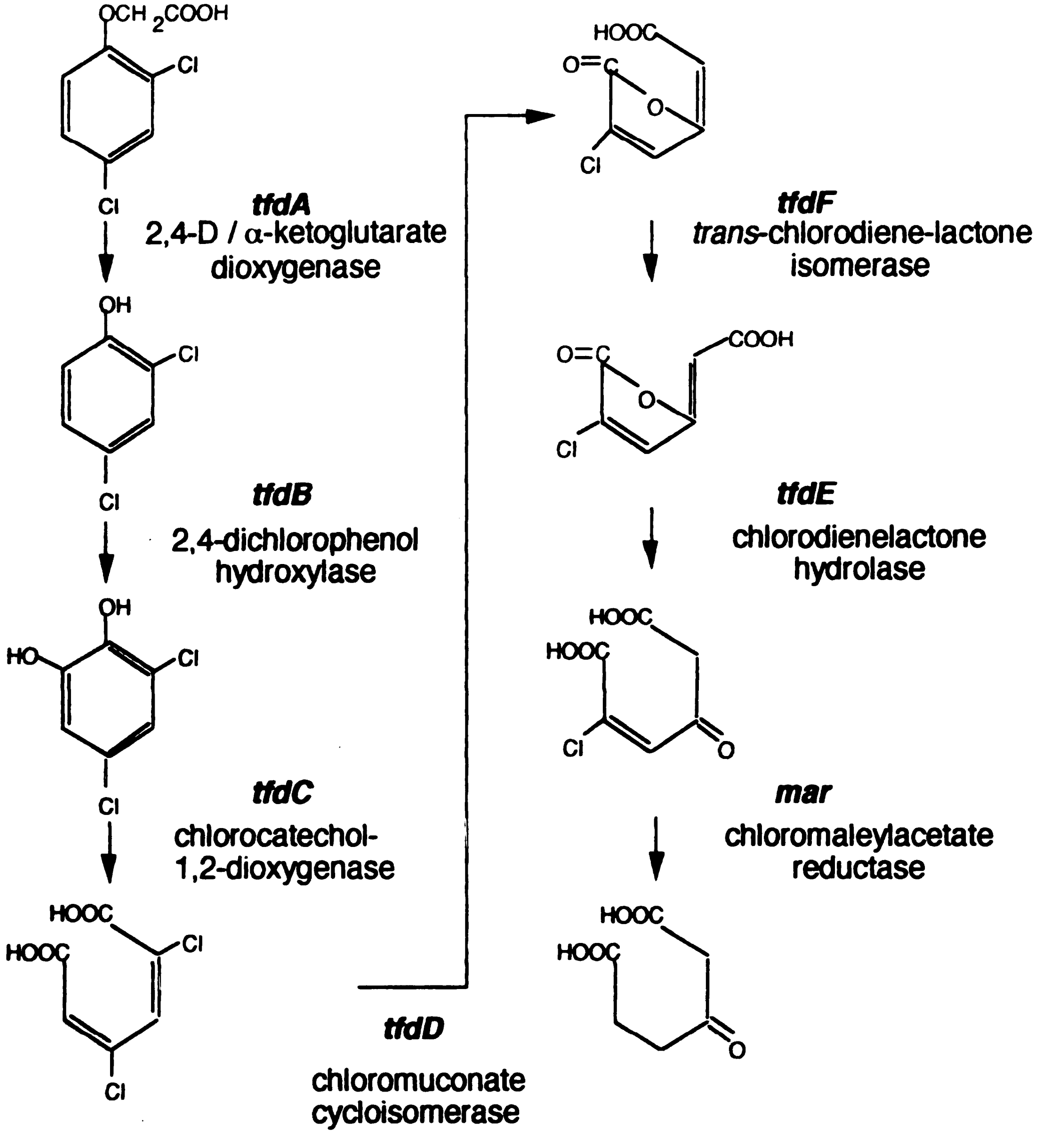


Figure 4. Pathway for 2,4-D degradation found in *Alcaligenes eutrophus* JMP134. (Diagram courtesy of Dr. Cindy Nakatsu).

Table 1. Genes from JMP134 involved in 2,4-D degradation.

Gene	Gene Product	bp	Start Codon	Notes	References
*tfd*A	2,4-D etherase = dioxygenase	861	GTA	Confers growth on PAA*	a, b
*tfd*B	2,4-dichlorophenol hydroxylase	1400	ATG	Not expressed in *E. coli*	b
*tfd*C	chlorocatechol 1,2 dioxygenase	654	GTG		a
*tfd*D	chloromuconate cycloisomerase	1113	ATG	Fortuitously dechlorinates	a
*tfd*E	dienelactone hydrolase		ATG		a
*tfd*F	transchlorodienelactone isomerase	1062	ATG		a
mar	maleylacetate reductase			Chromosomally encoded	c
*tfd*R	regulator of tfdA, tfdCD and tfdEF			Negative regulator	e
*tfd*S	regulator			Repressor activator of *tfd*B	d

From: a) Perkins, et al. 1990; b) Streber, et al., 1987; c) Kukor, et al., 1989; d)Kaphammer and Olsen, 1990; Harker, et al., 1989.

* PAA = phenoxyacetate

DIVERSITY OF 2,4-D DEGRADING ORGANISMS

Before examining the role of gene transfer in generating diversity of 2,4-D degraders, the range of different organisms capable of degrading 2,4-D needs to be presented. Understanding the diversity of bacteria is not a simple task, since there are different and sometimes conflicting measures of diversity, different schemes used to classify this diversity, and even different definitions for the word "species" used to describe this diversity. In this section I will discuss the diversity of 2,4-D degrading organisms already known. Then I will briefly discuss the methods used to characterize and classify the diversity of recently isolated 2,4-D degraders in an attempt to give them a species designation. In particular, I will emphasize the use of 16S rRNA as a tool in determining phylogenetic diversity and mention some of the methods used to infer phylogenies based on 16S rRNA sequences. Then I will discuss potential problems in defining diversity, including ambiguities in nomenclature and the definition of species.

Many organisms have been isolated that can grow on 2,4-D as sole carbon and energy source, including *Arthrobacter* (Loos et al., 1966; Tiedje et al. 1969); *Bordatella* (Greer et al. 1992); *Flavobacterium,* (Chaudry and Huang, 1988); *Alcaligenes, Pseudomonas cepacia* (Greer et al. 1990), and others (Bell, 1957) (Table 2). Many of these isolates that grow on 2,4-D as sole carbon and energy source include species putatively assigned to *Alcaligenes* (Amy et al.,1985), including *Alcaligenes eutrophus* JMP134, which contains the well characterized 2,4-D plasmid pJP4 (Don and Pemberton, 1981). In addition to

Table 2. Selected list of 2,4-dichlorophenoxyacetate degrading organisms.*

Organism	Pathway Location	Size	Origin	Reference
Alcaligenes eutrophus JMP134	pJP4	89kb	Australia	Don and Pemberton, 1981
Alcaligenes paradoxus JMP116	pJP1	58mD	Australia	Fisher, et al., 1978
Alcaligenes paradoxus	pJP2		Australia	Don and Pemberton, 1981
Alcaligenes paradoxus	pJP3	53kD	Australia	Don and Pemberton, 1981
Alcaligenes eutrophus	pJP9	37kD	Australia	Don.and Pemberton, 1981
Alcaligenes sp.	pEML159	56kD	Oregon	Amy, et al., 1985
Achromobacter	*			Steenson and Walker,1957
Arthrobacter sp.	*		New York State	Loos, et al., 1966;Tiedje, et al.,, 1969
Corynebacterium	*			Leather and Fox, 1977
Flavobacterium	pRC10	45 kb		Chaudry and Huang, 1989
F. peregrinum	*			Spicher, 1954; Steenson and Walker, 1957
Mycoplana	*			Walker and Newman, 1956
Nocardia	*			Loos, 1975
Pseudomonas sp.	*			Evans and Smith, 1954; Gamer and Gaunt, 1971
Sporocytophaga congregata (F. aquatile)		*		Jenson and Perterson, 1952
Streptomyces viridochromogenes (ring uncleaved)	*			Bounds and Colmer, 1965
Xanthobacter sp	*			Ditzelmüller, et al., 1989
Pseudomonas sp.	pEST4001	78	Estonia	Ausmees and Heinaru, 1990
?	pEST4003	8	Estonia	Ausmees and Heinaru, 1990
P. cepacia BRI6001	chromosome		Nova Scotia, peat	Greer, et al., 1990
Bordatella				Greer, et al., 1990
Phanerochaete crysosporium (fungus)				Yadav and Reddy, 199?
Strain RASC	chromosome		Oregon	Amy, et al., 1985
TFD6	chromosome		Michigan	Tonso, et al., 1994

---

* Plasmids encoding 2,4-D degradation have not yet been identified for these strains. This does not necessarily mean the 2,4-D pathway is chromosomally encoded.

Gram negative bacteria, several Gram positive 2,4-D degraders have been reported, including *Arthrobacter* (Loos et al. 1966); *Nocardioides simplex* (Kozyreva et al. 1993); *Streptomyces* sp. (Speicher, 1954); and various other unidentified Gram positive bacteria (Amy et al. 1985; Tonso et al. in press). In addition to bacteria, the fungus *Phanerochaete chrysosporium* has been shown to degrade 2,4-D, although not as a growth substrate (Yadav and Reddy, 1993).

In addition to the organisms described above, scores more strains capable of degrading 2,4-D have been recovered (Ka et al. 1994; Tonso et al., submitted). Because of the great numbers of 2,4-D degraders that have been isolated, researchers have sought new ways to quickly and easily characterize these strains to understand their diversity. The diversity of any group of organisms can be classified and organized in two ways: by evolutionary, or phylogenetic, classification, or by phenotypic classification.

Phenotypic classification: Until relatively recently, phenotypic characterization has been the only means available for classifying bacteria. Bergey's Manual of Determinative Bacteriology has been the definitive source of classification which was based solely on phenotypic classification. Some techniques for assessing phenotypic characteristics include classical microbiological techniques (such as the Gram stain), Biolog^{TM}, and fatty acid methyl ester (FAME) analysis (Tonso et al. 1992). Genetic characteristics have also been used to assess the diversity of 2,4-D degraders, including plasmid hybridization (Tonso et al. 1994) and a chromosome fingerprinting technique using PCR amplification of repetitive extragenomic palindromic (REP) sequences as primers (Fulthorpe et al. submitted; Ka et al. 1994; Tonso

(REP) sequences as primers (Fulthorpe et al. submitted; Ka et al. 1994; Tonso et al. in press).

Although non-molecular techniques, such a classical techniques like Gram stain and morphology, and newer techniques like FAME and Biolog provide information on the phenotypic diversity and REP and hybridization analyses indicate the genetic diversity of these organisms, these methods cannot be relied on to provide a true phylogeny of these organisms, and thus the ancestral lineage of these strains. Evidence has shown that phenotypic groupings do not necessarily reflect phylogenies of these strains. For example, it was originally thought that all purple, photosynthetic bacteria were closely related and formed a tight clade to the exclusion of other bacteria until Woese et al. (1980) discovered, using comparisons of 16S rRNA and cytochrome genes, that these purple photosynthetic bacteria were more related to other Gram negative bacteria than they were to each other. For this reason, I will use phylogenetic relatedness based on 16S rRNA as one indicator of the evolutionary relatedness of these strains. Once the ancestry of the strains and the relatedness of the genes involved in 2,4-D degradation is known, I can ask whether the diversity of 2,4-D strains arose by divergence or by gene transfer.

Phylogenetic classification: Another way to catalogue the diversity of 2,4-D degrading strains is using phylogenetic classification using 16S rDNA. This recently developed technique may fulfill Darwin's prophesy:

> "The time will come I believe, though I shall not live to see it, when we shall have very fairly true genealogical trees of each great kingdom of nature."
>
> ~ Charles Darwin (in Wheelis, 1992)

Studying phylogenies of bacteria has revitalized microbiology (Margulis, 1992; Olsen et al. 1994). Unlike eukaryotic phylogenies which can be based on morphological data, bacterial phylogenies were practically impossible to do before the advent of molecular sequencing. Molecules used in phylogenetic analysis are often highly conserved and include cytochromes, elongation factors, and small and large ribosomal subunits. "Molecular sequencing technology has brought biology into the era of global (universal) classification" in which all organisms can be compared to all others (Wheelis et al. 1992).

Phylogenetic analysis is growing in popularity as molecular techniques become increasingly widespread. Now that phylogenies based on molecular sequence data have become better understood, many phenotypic characteristics, such as biochemical markers, have been discovered to be phylogenetically relevant. For example, particular fatty acid methyl esters have been found to be indicators of alpha, beta, and all four gamma subgroups of *Proteobacteria*. In addition to these markers, one might ask if any of the 2,4-D genes are markers for particular phylogenetic groups. This research will address this question.

16S rRNA: Of all the molecules to be sequenced for phylogenetic analysis, 16S rRNA is the most popular, as indicated by the large numbers of sequences deposited in GenBank and the Ribosomal Database Project and the deeply rooted, extensive phylogenies produced with this molecule (Figure 5). This molecule, which is approximately 1500 nucleotides in length in bacteria, is highly conserved (Figure 6), and is therefore a useful molecule for doing phylogenetic studies. It is convenient to use this molecule because new sequences can be compared to large numbers of other 16S rRNA molecules. Since first sequenced in *Escherichia coli* (Brosius et al. 1978), over 2600 additional sequences of small ribosomal sequences have been added to the rapidly growing Ribosomal Database Project (RDP) and even more are accessible in Genbank. Comparing small ribosomal subunits has been extremely useful in creating a universal phylogeny (Woese, 1978; Fox et al. 1980). In addition to characterizing extant, cultured organisms, 16S rDNA sequencing has allowed researchers to characterize ancient organisms, such as Bacilli trapped in bee guts in amber 25-40 million years old (Cano, 1994) and organisms which have not been cultured (Amann et al. 1994; Schmidt et al. 1991).

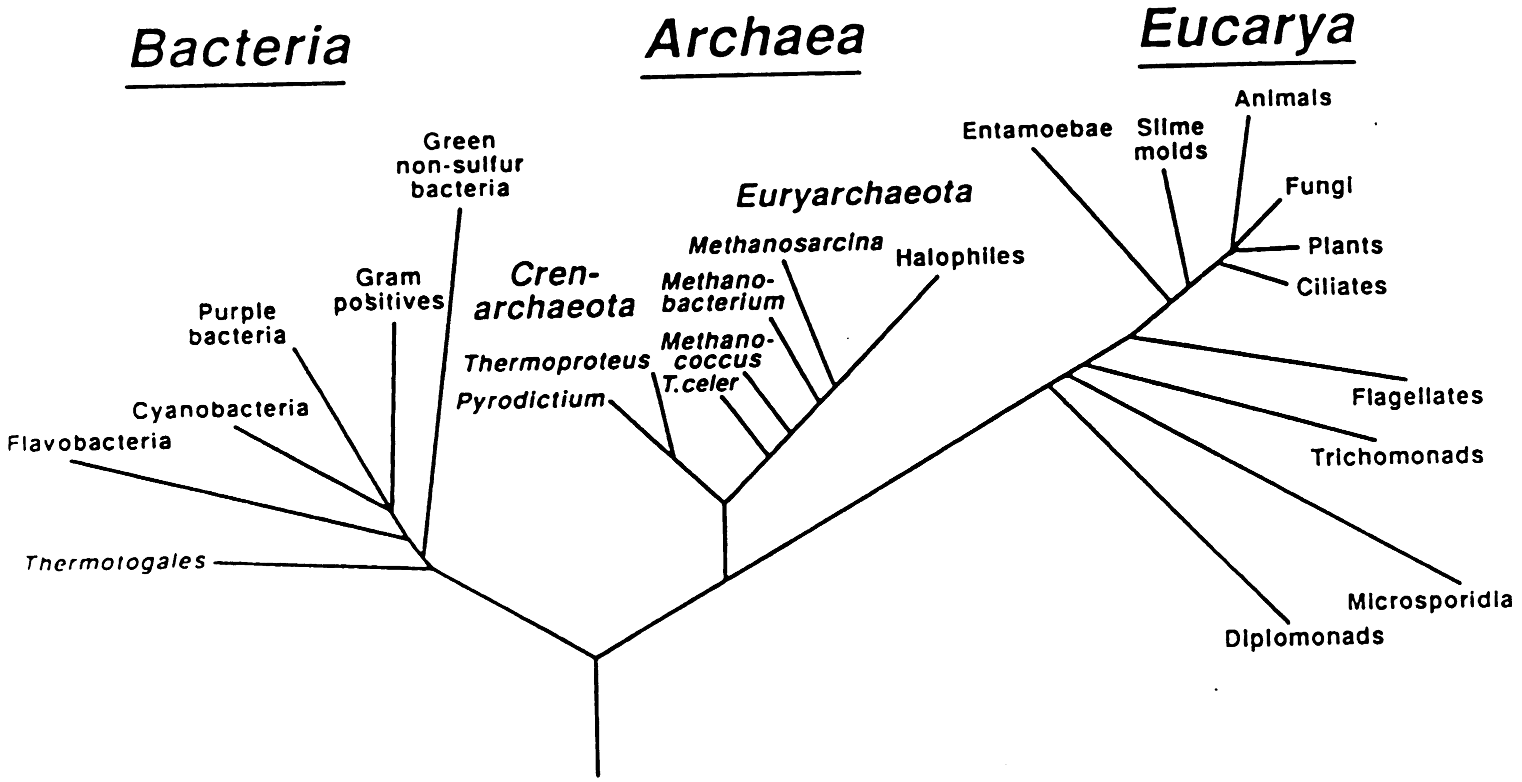


Figure 5. Rooted universal phylogenetic tree. (from Wheelis, et al., 1992).

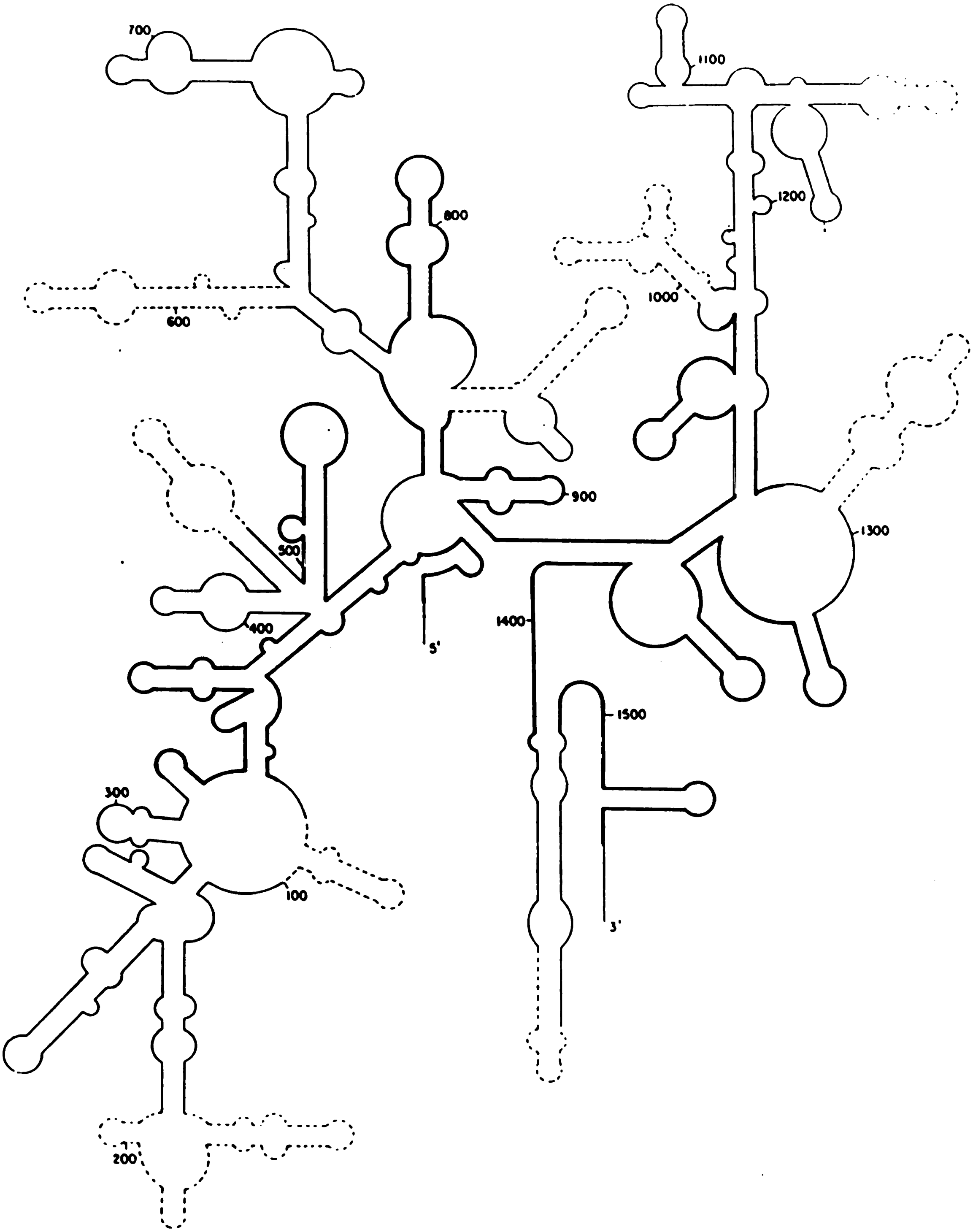


Figure 6. Diagram of 16S rRNA molecule. Heavy lines indicate highly conserved regions and dashed lines indicate hypervariable regions.

Inference of Phylogeny

Determining the diversity of strains based on sequence comparisons requires the reconstruction of a phylogeny. Reconstructing a true phylogeny is often a difficult task as illustrated by the fact that the number of possible unrooted, binary trees for N taxa is

$$(2N-5)!!$$

(that is, 1 x 3 x 5 . . . x (2N-5)) (Penny, 1991). So, for example, 10 taxa would yield 2,027,025 possible different trees (Penny, 1991). To assist in determining the correct tree from the huge number of possibilities, several methods can be used to infer a phylogeny from sequence data (reviewed by Doolittle, 1990; Felsenstein, 1988; Goodfellow, 1985; Kumar et al. 1993; Maddison and Maddison, 1992; Penny, 1991; and Swofford and Olsen, 1990) The three major categories of methods, including parsimony, distance matrix and maximum likelihood, are briefly outlined here.

Parsimony: Parsimony uses the least amount of convergence and reversal among the characters to determine a tree. The measure of evolutionary change is the treelength, which is calculated by the weighted sum of "steps, " or base pair changes. Programs designed to analyze trees using maximum parsimony include PAUP, PHYLIP and MacClade. MacClade lets one observe treelengths and other tree statistics, although it does not automatically calculate the shortest treelength. PAUP and a program in the PHYLIP package automatically calculates the most parsimonious trees using a variety of algorithms. Parsimony methods work well if there is little change over evolutionary time (Felsenstein, 1981; Stewart, 1993). However, parsimony methods can fail if there are large amounts of change or if some branches of the tree are much longer than other branches of the tree, since parsimony tends to group all long branches together, even if they are not related.

Distance Matrix: There are several distance matrix methods which build trees based on distances between pairs of strains. These techniques include unweighted pair group method of analysis, UPGMA, the neighbor joining method and the Fitch and Kitch least squares techniques (Felsenstein, 1993). The DeSoete Tree Fit package used by the RDP is an example of using the least squares fitting algorithm on a distance matrix. UPGMA tends to fail if a molecular clock is not obeyed, that is, if rates of evolution differ between different taxa being analyzed (Felsenstein, 1981). Another problem with these methods is that they fail to fully exploit all information in the original data.

Maximum Likelihood: This involves inferring phylogenies from a simple probabilistic model of evolution (Felsenstein, 1981). Maximum likelihood "involves finding that evolutionary tree which yields the highest probability of evolving the observed data" (Felsenstein, 1981, p 369). This is based on a function of the tree (the hypothesis) and not a function of the data (sequences). RESTML, which is part of the PHYLIP package, is software designed to estimate maximum-likelihoods from restriction site data (Felsenstein, 1992)

Confidence test for phylogenies: Because different methods often yield different trees, and sometimes more than one optimal tree is found per method, it is important to estimate the confidence in the tree (Felsenstein, 1985). The most widely used statistical tool for this task is the "Bootstrap" method, which Felsenstein was the first to apply to phylogenies. For this method to work, many iterations of the tree algorithm are performed, each one with resampled data. In each iteration of the bootstrap, nucleotides (or other character types) in a data matrix are resampled such that some nucleotides (characters) are counted one or more times and other nucleotides are not counted at all.

Phylogenetic congruency: How reliable is a single molecule, such as 16S rRNA, for determining the phylogeny of an entire organism? This question has not been extensively investigated and is not yet known how great an effect gene transfer and gene duplication has had on the evolution of organisms (Doolittle and Brown, 1994). However, in cases that have been tested, the deeper branches of the 16S phylogenetic tree have shown to be congruous with phylogenies derived from using cytochromes, elongation factors, and ATPases (Iwabe et al. 1989).

Species Definition: In discussing diversity, the term "species" is often invoked, although in bacteria this term is difficult to define. There are two ways to define species: 1) using a phenotypic definition or 2) using a biological species definition which says members of same species are inbreeding groups.

Adherents of the biological species concept at one extreme claim that there are no species of bacteria, since there is no genetic isolation among bacteria (Sonea, 1989). A more moderate view of the biological species is that of Dykhuizen and Green (1991) who define species as those strains which demonstrate a high degree of recombination.

The current working definition of bacterial species accepted by most microbiologists is phenetic definition. This definition was agreed to by a convention of bacterial systematists who decided that two strains that are at least 70% identical on the level of DNA:DNA hybridization are members of the same species (Wayne et al. 1987).

Because so many researchers are now using 16S rDNA to classify bacteria, Stackebrandt and Goebel (1994) have proposed that any two strains sharing less than 97% similarity of 16S rRNA sequences belong to different species. Stackebrandt and Goebel (1994) report that "organisms that have less than 97.0% sequence homology will not reassociate to more than 60%" which is below the 70% threshold for a species definition. It should be noted that the divergence of total genomic DNA is not necessarily reflected in the divergence of 16S rDNA (Table 4). For example, *Burkholderia cepacia* and *Alcaligenes eutrophus* differ by 4.3% at level of 16S rDNA sequences, whereas these differ by 93% at level of DNA-DNA similarity (Li, 1993).

Our definition and understanding of species may define our understanding of the diversity of 2,4-D degrading strains. For example, many 2,4-D degrading strains appear to be pseudomonads (Ka et al. 1994) and like other pseudomonads may share a high degree of sequence similarity at the level of 16S rDNA, yet may have diverged considerably at the level of total genomic DNA (Johnson and Palleroni, 1989). "In contrast, strains of different species of other genera may show in their DNAs a degree of conservatism approaching that of the ribosomal genes. It is tempting to attribute these peculiar properties of the pseudomonads to the ubiquitous occurrence of these organisms and the concomitant need for adjustment to the selective pressures of highly variable natural habitats." (Johnson, 1989). Perhaps these

Table 4. Similarity of strains comparing 16S sequence identity versus percent of DNA-DNA hybridization

Strain 1	Strain 2	%DNA-DNA Similarity	% 16S	Reference
Bacillus psychrophilus	*B. globisporus*	23.5	99.8	Fox, et al., 1992
Mycobacterium intracellulare serovar 12	*M. intracellulare* serovar 8	49	98.5	Baess, et al.,1983 Boddinghaus, et al.,1983
Serpula hyodysenteriae B78	*Serpula innocens* B256	41	99.5	Stanton, 1991
Alcaligenes eutrophus ATCC 17697	*Burkholderia cepacia* ATCC 25416	7	95.7	Johnson and Palleroni ,1989 Li, et al., 1993
B. andropogonis ATCC 23060	*B solanacearum* ATCC10692	1	95	Johnson and Palleroni,1989 Li, et al., 1993

pseudomonads adjust to their habitats by genetic exchange.

Determining the diversity of 2,4-D degrading strains could be confused not only by differences in species concepts, but also by the confusion brought on by changing species and genus names. For example, *Pseudomonas* strains have undergone various name changes in the past 10 years. Strains once belonging to the genus *Pseudomonas* have been reclassified into several groups such as *Burkholderia, Sphingomonas, Comamonas,* and *Xanthomonas* (de Ley et al. 1986; Stackebrandt et al. 1988; Yabuuchi et al., 1992). In order to help sort out changing names and classifications, Table 3 shows past and present names and classifications of selected strains of *Proteobacteria.*

Table 3. Nomenclature key for class *Proteobacteria* (Purple Bacteria)

Woese, 1987 Subclasses		Woese, 1984 R Group	Palleroni., *Pseudomonas* rRNA Homology Groups	Old Names	Revised names
Alpha	1	RI	Group IV	*Pseudomonas diminuta*	*Sphingomonas diminuta*
Alpha	2	RI	Group IV	*Rhodopseudomonas palustris*	*Rhodopseudomonas palustris*
Alpha	3	RI	Group IV		
Alpha	4	RI	Group IV	*Pseudomonas paucimobilus*	*Sphingomonas paucimobilus*
Beta	1	RII	Group III	*Pseudomonas testosteroni*	*Comamonas testosteroni*
Beta	2	RII	Group II	*Pseudomonas cepacia*	*Burkholderia cepacia*
Beta	2	RII	Group II	*Alcaligenes eutrophus*	*Alcaligenes eutrophus*
Beta	3	RII	Group II		
Gamma	1	RIII	Group I	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*
Gamma	2	RIII	Group I		
Gamma	3	RIII	Group V	*Pseudomonas maltophilia*	*Xanthomonas maltophilia*

DIVERSITY OF 2,4-D DEGRADING GENES

In the previous section I discussed methods and issues for assessing the diversity of bacteria. However, this knowledge reveals nothing of a strain's genetic constitution for degrading 2,4-D. In this section I will discuss what is known about the diversity of genes for degrading 2,4-D and related chloroaromatic compounds.

Gene Similarities: There has been little comparison of the genes responsible for degrading 2,4-D. The entire pathway, *tfd*A-*tfd*F, has been sequenced only in JMP134(pJP4) (Don et al. 1985; Perkins et al. 1990, Streber et al. 1987). The *tfdA* gene has subsequently been sequenced and found to be identical in strains EML159 and TFD38 (Fukumori, personal communication) and pCSV90 (Bhat et al. 1994) and found to differ by 23 percent in *Burkholderia* strain RASC (Suwa et al. 1994) and *Burkholderia* strain TFD6 (Matheson, 1994), the latter two of which are identical to each other.

It appears that alternate pathways and/or other genes involved in 2,4-D degradation differ substantially from those genes in the pathway of pJP4. This is suggested by 2,4-D organisms which do not hybridize to pJP4 in colony hybridization experiments. For example, Matheson et al., (1991) assessed the diversity of 2,4-D strains from different sources and found that pJP4 hybridized to only 16 of 52 (30%) of the 2,4-D degrading colonies isolated from three different locations. Furthermore, Holben et al., (1990) found that total bacterial DNA isolated from a Saskatchewan soil exposed to 2,4-D hybridized with genes *tfdA* and *tfdB*, but had no significant hybridization to *tfd*C, D, E, or F indicating a population of 2,4-D degraders different than that of pJP4. Ka et al. (1994) isolated 2,4-D degrading strains from a Michigan agricultural plot, some of which contain plasmids that hybridize to *tfd*A only, others to *tfd*A,B,C,D and still others which had no apparent similarity to pJP4.

Other chloroaromatic degradation genes: Other studies have examined the diversity of genes related to *tfdC,* a catechol dioxygenase, from other chloroaromatic degradative pathways such as the 3-chlorobenzoate (3CB) pathway and trichlorobenzene (TCB) pathways (for review see van der Meer, 1992 ; Harayama and Kok, 1992; and Fukuda, 1993; Ghosal and You, 1988; Ghosal et al. 1985). These studies showed that gene analogs of *tfd*C, D, E, F were in the same orders in operons found in the 2,4-D pathway of JMP134, in the 3CB pathway in plasmid pAC127, and in the TCB pathway of *Pseudomonas* P51 (Figure 7). Gene similarities can be determined by amino acid similarity, DNA sequence similarity or by gene probing. In contrast, hybridization analysis with various probes made from 2,4-D genes and 3-chlorobenzoate (3CB) genes (which have sequence similarity to the lower pathway of 2,4-D degradation in pJP4) offers a means of more efficiently surveying sequence similarity of the genes for 2,4-D degradation.

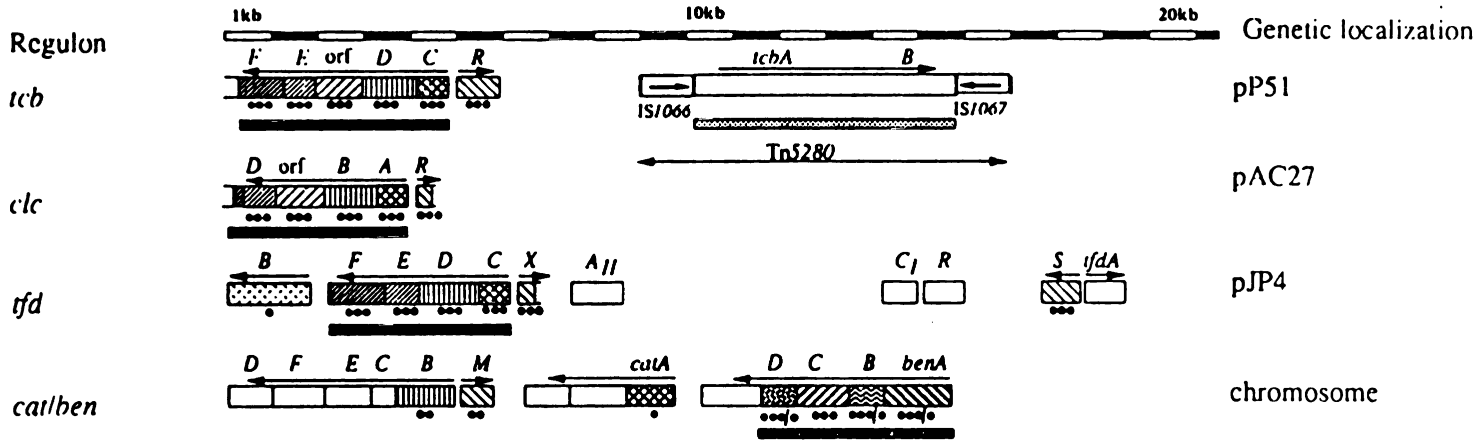


Figure 7. Comparison of genes for ortho-cleavage pathway of various aromatic compounds. (from Van der Meer, et al., 1992.)

GENE TRANSFER POTENTIAL

To understand the role of gene transfer in the evolution of 2,4-D degrading strains, it is useful to review some of the literature pertaining to gene transfer mechanisms in general, what is known about mobile 2,4-D degrading genetic elements in particular, and about gene transfer in nature.

Although conjugal gene transfer among certain types of 2,4-D degraders is documented (Don and Pemberton, 1981), it is not known to what extent gene transfer is responsible for the diversity of 2,4-D degraders observed. One aim of this research was to assess the ability of various 2,4-D degrading strains to transfer the 2,4-D phenotype. In addition, this project examines other modes of gene transfer aside from conjugation. Conjugation has been the only means of gene transfer reported for transfer of the 2,4-D phenotype.

Horizontal Gene Transfer: Interspecies horizontal gene transfer has been observed in a huge variety of strains, crossing all sorts of phylogenetic boundaries including species, genus, order, and even kingdom (Figure 8) (for review see Heinemann, 1991; Sikorski et al. 1990; Stachel and Zambryski, 1989). Transfer has been shown to occur by transformation (Frischer et al. 1990) transduction, conjugation of plasmids and transposons on these plasmids (Fulthorpe and Wyndham, 1991; Nakatsu, 1992) and retro-transfer (Top et al. 1994).

Perhaps mobile genetic elements are as important, or more important, than the chromosome itself in determining the persistence of a catabolic phenotype, particularly in pollution stressed environments. Different bacterial hosts may or may not survive in a stressed environment, but mobile

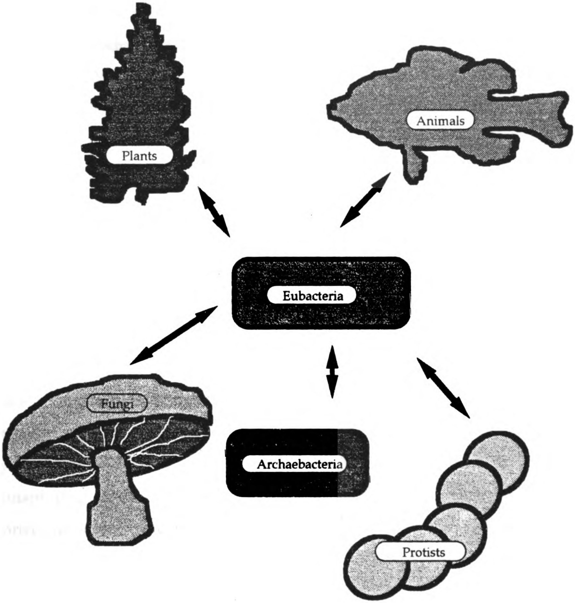


Figure 8. Diagram of interspecies gene transfer.

genetic elements might survive if transferred to an appropriate indigenous host. Populations of non-native organisms often are reduced to below detectable levels when introduced into an environment (Steffan et al.1989; Jain et al.,1987; McClure et al.,1989) thus minimizing their effectiveness in bioremediation. Perhaps the effectiveness of these introduced strains would be increased if the degradative genes were on mobile genetic elements.

Conjugation: Since the genes for 2,4-D degradation, like many other catabolic pathways, are often plasmid encoded, conjugation may play a major role in dispersing and evolving new catabolic pathways in the environment (Heinemann, 1991; Berg and Howe, 1990). For example, it has been hypothesized that "module's" of distinct parts of the 2,4-D pathway of pJP4 have been sequentially assembled on a plasmid (Streber et al.,1987).

Over 100 plasmids capable of catabolizing recalcitrant and manmade organic compounds have been isolated (Chakrabarty and Gunsalas, 1971; Mergeay et al., 1990; Sayler et al., 1990; Timmis and Pühler, 1979). Plasmids may be important for distributing genes involved in the degradation of pollutant compounds, (Sayler et al., 1990; Mergeay et al., 1990). It has been reported that when there is a high concentration of a contaminant, for which degradative genes are encoded on plasmids, the frequency of these plasmids increases in the population. For example, fewer than 5% of bacteria in pristine aquifers have been shown to contain plasmids, yet nearly 40% of the bacteria in contaminated aquifers contain plasmids (Wickham and Atlas, 1988).

Conjugation is a mode of horizontal gene transfer that allows exchange of genetic material within species, between species, between genera (Brisson-

Noel et al.,1988; Chatterjee and Chatterjee, 1987; Clewell, 1993; Datta and Hedges, 1972; Hardy, 1987; Mazodier et al., 1989; Natarajan and Oriel, 1991; Thomas, 1989; Trieu-Cout et al.,1987; Willets, 1985) and even between kingdoms (Heinemann and Sprague, 1989). Several plasmid incompatibility groups, including IncP1 (e. g. RP4), IncQ (e. g. RSF1010), IncN, and IncW can transfer to a wide range of species at relatively high frequencies (Bagdasarian et al., 1981; Frey and Bagdasarian, 1989; Simon et al,1981; Thomas, 1989). The features of these broad host range plasmids that allow them to be transferred to, and maintained in, a broad range of organisms are that they contain relatively few restriction sites and can replicate in a wide range of hosts by having special, largely self-contained replication machinery. Interspecies gene transfer is limited to some degree by the ability of plasmids to be transferred from one species to another, but is limited more by the ability of that plasmid to be maintained in various hosts (Sayler et al., 1990; Heineman, 1991).

Plasmid pJP4: The best studied plasmid for 2,4-D degradation, plasmid pJP4 (Don and Pemberton, 1981; Figure 9) is a broad host range, self transmissible plasmid of the IncP1ß incompatibility group. The 87.9 kb pJP4 has been shown to transfer freely to *E.coli, Rhodopseudomonas sphaeroides, Rhizobium* sp., *Agrobacterium tumefaciens, Pseudomonas putida, Pseudomonas fluorescens,* and *Acinetobacter calcoaceticus* at frequencies ranging from 10^{-1} to 10^{-7} (Don and Pemberton, 1981; Friedrich et al., 1985). Although pJP4 can be transferred to different species and genera of bacteria, the phenotype for 2,4-D degradation is observed only in *A. eutrophus,*

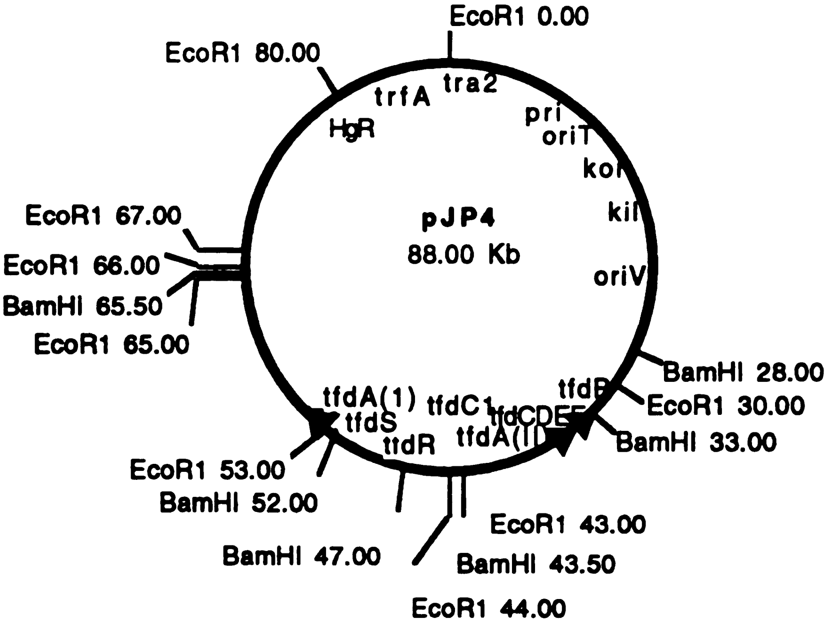


Figure 9. Diagram of plasmid pJP4 from *Alcaligenes eutrophus* JMP134.

A. paradoxus, P. cepacia and *P. putida* (Table 5). This phenotype is not detected in pJP4 transconjugants of *Agrobacterium, Rhizobium, E. coli, Rhodopseudomonas, Acinetobacter, P. fluorescens* and *P. aeruginosa.* One possible explanation for the limited host range of expression of genes for 2,4-D degradation is that the final gene in the pathway, maleylacetate reductase (*mar*) is chromosomally encoded, such that only strains with this gene can metabolize 2,4-D as their sole source of energy (Kukor et al.,1989).

Other 2,4-D plasmids: Other 2,4-D degraders have been found with 2,4-D genes located on plasmids (Amy et al.,1985; Chaudry and Huang, 1988; Don and Pemberton, 1981; Ausmees and Heinaru, 1989; Bhat et al. 1994). Pemberton et al., (1979), found that of 22 colonies isolated on 2,4-D, 17 produced 2,4-D negative segregants (tfd$^-$) after only 15 to 20 generations when cultured in non-selective broth, suggesting loss of a plasmid encoding genes for 2,4-D degradation. The remaining five strains all contained plasmids conferring ability to catabolize 2,4-D. Three of these were similar to pJP4 in size and restriction pattern and incompatibility group, whereas pJP2 and pJP9 differed in size, incompatibility group and had the ability to degrade phenoxyacetic acid (when not induced by 2,4-D), which pJP4 does not. Chaudry and Huang (1988) found another plasmid, pRC10, encoding a 2,4-D pathway in *Flavobacterium.* This 45 kb plasmid has sequences that hybridize to *tfdA* and *tfdB* of pJP4, but has no sequences that hybridize to the regions of pJP4 involved in conjugation, incompatibility, and maintenance. Amy and colleagues (1985) found plasmid pEML159 in Oregon sludge which was similar to pJP4 in size, restriction endonuclease fragment patterns, and ability

Table 5. Recipients of plasmid pJP4 from *Alcaligenes eutrophus* JMP134 indicating ability of recipients to express 2,4-D phenotype.

Recipients	*Proteobacteria* Subdivision	Frequency of transfer	Growth on 2,4-D	Ref*
Alcaligenes eutrophus	Beta	10-50%	+	1
Variovorax paradoxus	Beta	10-50%	+	1
Burkholderia cepacia	Beta	50%	+	2, 3
Pseudomonas oxalaticus		nd	+	2
Pseudomonas sp. PK01			+	4
Burkholderia pickettii	Beta		+	5
Burkholderia putida	Gamma	nd	-	2
Burkholderia putida	Gamma	nd	+	1
Escherichia coli	Gamma	4×10^{-4}	-	1
Pseudomonas fluorescens	Gamma	7×10^{-4}	-	1
Alcaligenes calcoaceticus		1×10^{-3}	-	1
Pseudomonas aeruginosa	Gamma		-	2
Rhodopseudomonas sphaeroides	Alpha	5×10^{-6}	-	1
Rhizobium	Alpha	1×10^{-6}	-	1
Agrobacterium	Alpha	2×10^{-2}	-	1

* References: 1= Don and Pemberton, 1981; 2=Harker,et al., 1989; 3=this lab;4=Kukor, et al, 1989; 5 = Friederich, et al., 1983.

to transfer the 2,4-D degrading phenotype to other bacteria. This similarity is perhaps surprising since pJP4 was isolated on the other side of the world in Australia. Ausmees and Heinaru (1989) found three other 2,4-D degrading strains in Estonia which contain plasmids for 2,4-D degradation.

In addition to pJP4, pRC10, pEML159 and the Estonian plasmids, many other plasmids have been found in 2,4-D degrading strains (Tonso et al. 1994 and Ka et al. 1994). It is still not known whether the 2,4-D degradative pathways in these strains are plasmid borne but presumably some of these plasmids are responsible for the 2,4-D phenotype since they hybridize to pJP4. Others plasmids which have no sequence similarity to pJP4 may or may not be responsible for the 2,4-D phenotype.

Some 2,4-D strains appear to be on plasmids which are capable of integrating into and excisizing from the chromosome. Ka and Tiedje showed that strain *P. pickettii* strain 2118 had variants with 2,4-D genes alternating between the plasmid and chromosome, and that under further selection for 2,4-D growth, a second, smaller version of the plasmid was excised which apparently left *tfd*A behind on the chromosome.

Chromosomally encoded 2,4-D genes: Although the best characterized genes for 2,4-D degradation are plasmid encoded (Fisher et al. 1978; Don and Pemberton, 1982; Friedrich et al. 1983; Chaudry and Huang, 1988; Ausmees et al. 1990; Amy et al. 1985; Bhat et al. 1994) there is evidence that many other 2,4-D degraders have chromosomally encoded 2,4-D genes, including RASC (chapter 4; Suwa, in prep); TFD6 (Matheson,et al., 1994) and BRI6001 (Greer et al. 1990) which appears to be identical to TFD6. Ka et al. 1994 also found that genes for 2,4-D degradation were chromosomally located. It is not known whether chromosomally located 2,4-D genes can be transferred.

Gene transfer in the environment: The first *in situ* observation of plasmid transfer in nature involved antibiotic resistant plasmids and introduced strains of *E. coli* as donor and recipient in sterile soil (Weinberg and Stotzky, 1972). Since then, many researchers have investigated plasmid transfer in nature and several review articles and books have been written on this topic (Levy and Marshall, 1988; Coughter and Stewart, 1989; Trevors et al.,1986; Stotzky,1989; Stotzky and Babich, 1986; Levy and Miller, 1989; Fry and Day, 1989). Most of these studies have focused on transfer of antibiotic resistance between strains of introduced bacteria. For the sake of brevity, I will discuss only selected studies, especially those which focus on transfer of catabolic plasmids and transfer to autochthonous soil microorganisms (Table 6).

To demonstrate that soils contained potential indigenous recipients of plasmids, Schlif and Klingmüller (1983) showed that 1.3% of 600 randomly isolated soil bacteria could be recipients of pRD1, a plasmid encoding resistance to carbenicillin, kanamycin and tetracycline, from *E. coli.*

In live soil, conjugal plasmid transfer has been demonstrated between introduced strains. Trevors and Oddie (1986) demonstrated transfer of tetracycline resistance of the R plasmid between strains of introduced *E. coli* in soil. Transfer of heavy metal resistance plasmids from *E. coli* to *Alcaligenes eutrophus* has been demonstrated in live soil amended with nutrients (Top et al., 1990). In addition, plasmid transfer has been shown to occur in soil between introduce strains of *Bacillus* (van Elsas et al., 1987) and between introduced strains of *Streptomyces* (Wellington et al., 1990).

Table 6. Gene exchange in nature.

Date	Researchers	Medium	Donor	Recipient	Plasmid	Marker
1972	Weinberg and Stotzky	Sterile soil	*E. coli*	*E. coli*	F	pro+; arg^{+},
1978	Graham and Istock	Soil	*B. subtilus*	Indigenous		
1983	Schilf and Klingmüller	*In vitro*	*E. coli*	Indigenous isolates	RP4	Cb,Tet,Kan
1984	Pertsova, et al.	Soil column	*Pseudomonas*	Indigenous	pBS2	3CB
1986	Trevors and Oddie	Live soil	*E. coli* MA527	*E. coli* K12	R plasmid	Tet^{R}, Sm^{R}
1987	van Elsas, et al.	Live and sterile soil	*B. subtilus*	*B. cereus*	pFT-30	
1988	Hirsch, et al.	Live soil	*R. leguminosarium*	*Rhizobium* spp	Sym plasmid	
1988	Bale, Fry and Day	River epilithon	*Indigenous*	*P. putida* KT2440	Indigenous	Hg^{R}
1990	McClure, et al.	Sludge microcosm	*P. putida*	Indigenous pseudomonads	pD10	3CB, kanR
1990	Wellington, et al.	Soil	*Streptomyces lividus*	*S. violaceticus*		
1990	Henschke and Schmidt	Live soil	*E. coli*	Indigenous	pFL119-2	kan^{R}
1990	Nuesslein	Sludge microcosm	*P. putida* KT2440	*P. putida* UWC1	pWWO-EB62	4-EB
1990	Henschke and Schmidt	Soil column	*E. coli*	Indigenous	pFL67-2	tobramycin^{R}
1991	Fulthorpe and Wyndham	Lake mesocosm	*Alcaligenes sp.* BR60	Indigenous	pBRC60	3CB
1991	Natarajan and Oriel	Live soil	*B. subtilis*	Indigenous	Tn916	TetM
1991	Brokamp and Schmidt	Soil column	*A. xylosoxidans* ABIV	Indigenous	pFL40	DCPA
1994	Top, et al.	Soil	Indigenous	*A. eutrophus* JMP228	pEMP1,3	2,4-D

The few studies demonstrating transfer to *indigenous* soil bacteria, generally have involved transfer of antibiotic resistance. For example, Henschke and Schmidt, (1990) demonstrated the transfer of the RSF1010-derived, kanamycin resistant recombinant plasmid pFL119-2, from *E. coli* into indigenous soil microorganisms. In their system, the kanamycin resistance gene was cloned behind a strong, broad host range *tac* promotor and in front of a strong terminator. The plasmid was mobilized by the transfer functions of RP4 cloned into the chromosome of the donor strain, *E. coli,* Although not necessarily resident on a plasmid, tetracycline resistance from the conjugative transposon Tn916 has been shown to transfer from *Bacillus subtilis* into indigenous soil actinomycetes (Natarajan and Oriel, 1991).

Transfer of catabolic plasmids has been demonstrated in various microcosm systems. For example, plasmid pWWO-EB62, a recombinant TOL plasmid with the ability to degrade ethylbenzoate, was shown to transfer from an introduced strain of *P. putida* KT2440 carrying the plasmid into the introduced recipient *P. putida* UWC1 in an activated sludge microcosm (Nüßlein et al., submitted). Transfer of the catabolic plasmid pBRC60, encoding 3-chlorobenzoate (3CB) degradation ability, into indigenous microorganisms has been demonstrated in a freshwater mesocosm (Fulthorpe and Wyndham, 1991). McClure et al., (1991) demonstrated transfer of the non self-conjugative 3CB recombinant plasmid, pD10, from *P. putida* UWC5.10, an indigenous strain with transfer functions, to 19 different morphotypic, gram negative, oxidase positive strains in an activated sludge bioreactor.

Transfer of catabolic genes to indigenous soil microorganisms has been demonstrated in two cases in irrigated soil columns. Pertsova et al., (1984), inoculated soil with 3.9 x 10^7 *P. putida* with a plasmid encoding enzymes for 3-chlorobenzoate degradation. A stream of mineral medium with 0.1% 3CB was run through 30 g of soil in column at a rate of 40-50 ml per day for 10 or 30 days. They found that the 3CB phenotype had transferred into strains in the inoculated soil column, but not in the control. Although the strains differed in various phenotypes from the introduced strain, no molecular or genetic analyses were employed to determine whether these were different strains or simply mutants of the original donor. Brokamp and Schmidt (1991) used a 40% soil/60% sand column to demonstrate the transfer of plasmid pFL40 (carrying genes for Dalapon (2,2-dichloropropionic acid) degradation) from *Alcaligenes xylosoxidans* ABIV into indigenous microorganisms. After 50 days in a soil column inoculated with the donor, various indigenous soil microorganisms were isolated which had received and expressed the plasmid, including a *Pseudomonas* species, *P. fluorescens*, *P. paucimobilis*, and *Alcaligenes* sp.

SUMMARY

In this chapter I have provided the background needed for assessing the role of gene transfer in the evolution of 2,4-D degrading strains. Three of these issues are: 1) the diversity of the strains; 2) the diversity of the genes involved in 2,4-D degradation; and 3) the transfer potential of these genes.

Here I have presented information on the diversity of bacteria capable of degrading 2,4-D and discussed various methods which have been used to analysis for evaluating the diversity of bacteria in general. Because it provides unambiguous data, and because a large database exists for sequence comparison, 16S rRNA sequence analysis is the method I have chosen to measure diversity of 2,4-D degrading strains.

The second issue to be addressed in understanding gene transfer in evolution of 2,4-D degraders is determining the diversity of 2,4-D degrading genes within these strains. In this chapter I have shown that one 2,4-D pathway has been extensively studied, yet other pathways known to exist have been only partially characterized or still remain unknown. Also, the distribution of the known pathways has been poorly studied. One way to examine the distribution of *tfd* genes is to hybridize the *tfd* genes of the well characterized plasmid pJP4 to various strains. This has been done by Ka et al. (1994). However, they used stringency hybridizations which would not detect *tfd* genes less than 90% identical to those of pJP4. In chapter 2, I describe low stringency hybridization experiments done collaboratively with Dr. Roberta Fulthorpe to detect additional, related 2,4-D degradation genes. In addition to hybridization analysis, the *tfd*A gene was sequenced to determine additional diversity of this gene.

The third issue in better understanding the role of gene exchange in generating the divert of 2,4-D degraders, is the potential for gene transfer potential. In the last part of this chapter I reviewed a small portion of the vast literature describing gene exchange as it occurs in the laboratory and where it has been shown in nature. In the last two chapters of this dissertation, I describe experiments investigating a somewhat unusual exchange of 2,4-D degrading genes in laboratory studies.

degrading genes in laboratory studies.

Taken together, these studies conclusively show that gene exchange has played an important role in the formation of the 2,4-D pathways found in 2,4-D degrading populations in nature.

Chapter Two

Phylogeny of 16S rDNA Genes from Diverse 2,4-Dichlorophenoxyacetate Degrading Bacteria

Phylogeny of 16S Ribosomal DNA from Diverse 2,4-Dichlorophenoxyacetate Degrading Bacteria

SUMMARY

Many 2,4-D degrading bacteria isolated from various sites in North America and Australia have been shown to be phenotypically and genotypically diverse, yet the phylogenetic relationship of these organisms was previously not known. The phylogenetic relationship of these strains was determined using two methods of 16S ribosomal DNA analysis. One method compared partially sequenced 16S genes and the other compared 16S ribosomal DNA using restriction fragment length polymorphisms (RFLPs) from PCR-amplified 16S rDNA. These fragments were digested with four-base cutting restriction enzymes and the resulting RFLP patterns were subjected to phylogenetic analysis. These strains were found to be phylogenetically distributed throughout the alpha, beta and gamma subgroups of the *Proteobacteria*. Of the 23 2,4-D degrading strains examined, 17 had unique 16S rDNA sequences, differing by up to 28 percent of their nucleotides. The majority of these strains were members of the beta subgroup of *Proteobacteria* and six of these were most closely related to the genus *Burkholderia*. Three 16S rDNA sequences were closely related to members of the beta subgroup of *Proteobacteria* most closely related to *Rhodoferax fermentens*. One salt tolerant strain was found to be closely related to the genus *Halomonas* in the gamma *Proteobacteria*. Five were members of the alpha *Proteobacteria* related to the genus *Sphingomonas*; and one was found to be most closely related to *Rhodopseudomonas palustris* from the alpha subgroup of *Proteobacteria*.

INTRODUCTION

> "Nothing in biology makes sense except in the light of evolution."
>
> - Theodosius Dobzhansky, 1973

This observation is particularly true for studying gene transfer since it is difficult to study gene flow without knowing how strains are related. This first part of the dissertation examines the phylogenetic relationships between 2,4-D degrading bacteria using 16S rDNA as a molecular marker.

The pesticide 2,4-D is quickly degraded in many environments due to a broad diversity of organisms capable of using 2,4-D (Alexander et al., 1969; Tiedje et al., 1969; Amy et al., 1985; Ka et al., 1993; Matheson et al., 1991; Vallaeys, personal communication; Mäe et al., 1993). Although these strains have tentatively been assigned to include species in the genera of *Arthrobacter* (Loos, et al, 1966; Tiedje et al., 1969) *Flavobacterium, Streptomyces* (Speicher, 1954), *Alcaligenes* (Bounds and Colmer, 1965), and *Pseudomonas* (Evans and Smith, 1954; Greer et al., 1990) the evolutionary relatedness of these strains remains in some cases uncertain due to ambiguities in bacterial nomenclature. For example, the genus designation *Pseudomonas* (despite its name meaning "false unit") continues to cause much confusion, since this "genus" comprises member species which are scattered throughout three of the four classes of the bacterial subdivision known as *Proteobacteria* (Woese, et al., 1984;Woese, 1994).

The diversity of 2,4-D degraders has been assessed by a variety of means, including classical bacteriological methods (Alexander et al., 1969;

Amy et al., 1987); by substrate utilization patterns using Biolog^{TM}; fatty acid methyl ester (FAME) (Ka and Tiedje, 1994; Tonso et al., 1992), plasmid hybridization (Matheson et al., 1991) and by genomic fingerprinting using PCR amplification of Repetitive Extragenomic Palindromic (REP) analyses (Ka, 1994; Tonso, et al., 1994; Fulthorpe, in prep) and by polyphasic taxonomic methods incorporating all of the above methods (Tonso et al., 1994). Classical methods, FAME, and Biolog provide information on the phenotypic diversity, and REP and hybridization analyses indicates the genetic diversity of these organisms, but none of these methods provides a rigorous phylogeny of these organisms. In contrast, it is hypothesized that 16S rDNA sequence analysis provides reliable bacterial phylogenies which in most cases have been confirmed by phylogenies derived from other sequence data such as cytochromes and elongation factors (Olsen et al., 1994; Woese, 1987). For this reason, comparative analyses of 16S rDNA sequences was used to assess the evolutionary relatedness of these strains.

In this study, we looked at the diversity of 23 2,4-D degrading strains isolated from various parts of the North America and Australia by two methods involving 16S rDNA. One method compared RFLP patterns derived from 16S rDNA digested with various 4-base cutting restriction enzymes, a method referred to as Amplified Ribosomal DNA Restriction Analysis (ARDRA) (Massol-Deya, 1994). Restriction pattern analysis of 16S genes has been successfully employed as a rapid system to analyze populations of bacteria from bioreactors degrading toluene (Massol-Deya, 1994); in estimating the diversity of strains from hydrothermal vent microbial mats (Moyer et al., 1994); in identifying various strains of *Rhizobia* (Laguerre et al., 1994) and symbionts of Flashlight fish (Wolfe and Haywood, 1991); and for sorting clones before sequencing (Giovanonni et al., 1991).

The second method used in analyzing these 2,4-D degrading strains involved comparing partial 16S rRNA sequences (Woese, 1981). A commonly used 16S primer, 519R, was used to obtain sequences from positions (corresponding to *E. coli*) of approximately 500 to 200. These sequences were compared to sequences in the Genbank database. Sequencing of 16S rDNA has been employed to infer the phylogenies of hundreds of strains including more than 2600 strains in the Ribosomal Database Project (Olsen et al., 1993, 1994), and for many smaller projects (e. g. Dewhirst et al., 1989; Giovanonni et al., 1990; Hiraishi et al., 1991; Li, et al., 1993; Olsen et al., 1993; Pace et al., 1986; Ward et al., 1993; Yabuuchi et al., 1992). More recently, several 16S rDNA sequencing studies have analyzed bacteria capable of degrading various xenobiotic compounds including toluene (Zhou et al., 1994); polychlorinated biphenyls (Tonso, et al., 1994; Williams, 1994) and other xenobiotic compounds (Busse et al., 1992).

MATERIALS AND METHODS

Media and growth conditions: Cultures were grown in MMO medium containing 500 or 250 ppm 2,4-D (Sigma) in a minimal mineral salts medium (Stanier, 1966). All cultures were grown at 30°C. Broth cultures were aerated by shaking on an Innova 4300 shaker (New Brunswick Scientific Co., New Brunswick, NJ) at 200 rpm.

Bacterial strains: Strains used in this study are listed in Table 1. Strains RASC and EML 159 were isolated from return activated sludge in Corvalis, Oregon, and EML 146 was isolated from the Willamette River in Corvalis, Oregon (Amy et al., 1985). *Alcaligenes eutrophus* JMP134(pJP4) (Don and Pemberton, 1981) was provided by Dr. R. H. Olson. Strains with prefix TFD were isolated by Holben et al., from the Kellogg Biological Station soils, Hickory Corners, Michigan, and from the Experimental Farm soils, Indian Head, Saskatchewan, Canada (Tonso et al., 1995; Holben et al., 1992). *Pseudomonas cepacia* strain BRI6001 was isolated from peat in Nova Scotia, Canada (Greer et al., 1990). Strain E2w1, isolated from soil in the El Loquillo forest of Puerto Rico, and those strains with prefix B6 (from Quebec) were from Dr. Roberta Fulthorpe. Strain 1443 and K712 were isolated from the Geneflow soil plots at the Kellogg Biological Station, Hickory Corners, Michigan (Ka et al., 1994). Strain M1 is an oligotrophic 2,4-D degrader isolated by Dr. Roberta Fulthorpe from the island of Moorea, French Polynesia. Strain 2,4-D1 was isolated by Robert Sanford from a herbicide wastewater facility in Montana.

2,4-D Biodegradation: Disappearance of 2,4-D from the culture medium was monitored by HPLC. The 2,4-D medium with cells was centrifuged at 14,000 rpm in a microfuge and filtered through 0.2 μ filters. These samples were then analyzed by HPLC on Lichrosorb Rp-18 column (Anspec Co., Ann Arbor) using 60% methanol and 40% 0.1% H_3PO_4 as the eluant. 2,4-D was detected by measuring light absorption at 230 nm.

Amplification of 16S genes: Prior to amplification by PCR, DNA was isolated using the DNA extraction procedure of Ausubel (1992). DNA from strains was subjected to PCR using primers to amplify most of the 16S rDNA of Eubacteria. These primers included fD1 (*E. coli* position 8-27F = 5'AGA GTT TGA TCC TGG CTC AG3') and rD1 (*E. coli* positions 1541-1524 = 5'-AAG GAG GTG ATC CAG CC A-3') (Weisburg et al., 1991) synthesized by the Macromolecular Sequencing Facility at Michigan State University. The PCR reaction mixtures were prepared according to the manufacturer's protocol (Perkins Elmer, Cetus). A master solution was prepared containing the following: 77.5 μl ddH_2O; 10 μl PCR Buffer; 2 μl each of 2 mM dCTP, dGTP, dATP and dTTP; 1 μl of 20 pmol/μl primer rD1; 1 μl of 20 pmol/μl primer fD1; 0.5 μl of 5U/μl Taq polymerase (Perkin Elmer, Cetus). Thermal cycling was done in a Perkin Elmer 9700 Thermal Cycler using the following conditions: melting at 92°C for 1 min; cycling 35 times at 92°C for 1 min, 10 s; 55°C for 30 s; 72°C for 2 min 10 s followed by a final extension of 6 min 10 s at 72°C.

Restriction Fragment Length Polymorphism Analysis: DNA was digested according to manufacturer's directions using restriction enzymes from Boehringer Mannheim and Gibco BRL (Gaithersburg, MD). The enzymes used were *Hae* III, *Rsa*I, *Alu*I, *Sau*A1 and *Msp*. Digested products were separated by electrophoresis on a 1.75% agarose gel (Boehringer Mannheim) at 38V for 12 hours. DNA molecular weight markers included bacteriophage lambda digested with *Hind*III (Boehringer Mannheim) and a 100 bp marker (Bethesda Research Laboratories, Gaithersburg, MD). Gels were stained with ethidium bromide and photographed with Polaroid positive negative film 55. The presence or absence of restriction fragments was entered into MacClade's data editor (Maddison and Maddison, 1992) and this data was used for the program RestML, a restriction fragment analysis program written by Felsenstein to determine a phylogenetic tree based on a maximum likelihood method (Felsenstein, 1992).

16S rDNA Partial Sequencing: PCR products were purified using the Gene Clean Kit (Bio 101, La Jolla, CA). Purified DNA was eluted from glass beads in 50 µl H_20 and quantitated using gel electrophoresis by comparison with a DNA mass ladder (Gibco, BRL, Gaithersburg, MD). Approximately 150 µl DNA template was added to 25 pmols primer and brought up to a volume of 20 µl with H_20. The sequencing primer used was 519R (5' GTA TTA CCG CGG CTG CTG G -3') synthesized by the Macromolecular Sequencing Facility at Michigan State University. This primer was chosen because it is a eubacterial version of one of the universal primers which has been shown to successfully amplify partial 16S DNA from every organism tested (Lane et al., 1985) and because many researchers have previously used this probe and thus there are a larger number of sequences in the database (Dewhirst,et al., 1992; Hiraishi et al., 1992; Lane et al., 1985; Paster et al., 1994; Schmidt et al., 1991). Sequencing, using fluorescently-labeled dye termination, was performed at Michigan State University Sequencing Facility using the Applied Biosystems

Model 373A automatic sequencer (Perkin Elmer Cetus).

Sequence Analysis: Sequences from 506 to 206 (according to *E. coli* numbering) were compared to sequences in databases from Genbank. Comparisons were made using the Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (Altschul, 1990). Those strains with highest maximal segment pair (MSP) scores were retrieved from Genbank (Table 2 lists accession numbers and ATCC designations) for building a phylogenetic tree. Sequences were aligned manually using the software SeqEd (Applied Biosystems) and with MacClade (Maddison and Maddison, 1992). A distance matrix was compiled based on the number of nucleotide changes per hundred base pairs using software programmed in HyperCard and with the Dnadist program from the PHYLIP package version 3.5 (Felsenstein, 1993) using the Jukes-Cantor model of base changes. Ambiguous nucleotides were deleted from the sequence alignments leaving 283 unambiguous sites before preparing the distance matrix. Phylogenetic trees were constructed using the neighbor joining distance matrix method (Felsenstein, 1993) and the maximum parsimony method using PAUP (Swofford, 1991).

RESULTS

Twenty three strains of 2,4-D degrading isolates and one non-2,4-D degrading strain of *Pseudomonas cepacia* strain D5 (aka *Burkholderia cepacia*) were selected (Table 1) from a larger collection of 2,4-D degraders on the basis of their uniqueness based on REP-PCR (Fulthorpe, personal communication), polyphasic characterization (Tonso et al., submitted) and hybridization patterns (next chapter). Two pairs of these strains, TFD41 and TFD38, and TFD6 and TFD20, appeared to be identical by all criteria examined. These four strains were isolated from Michigan samples. These were included to determine whether they contained identical 16S rDNA sequences.

Analysis of restriction patterns from 16S rDNA genes: After PCR amplification, DNA from individual strains was digested with each of five different restriction enzymes which recognize different four bp sites. Products were separated on a 1.75% agarose gel which allowed fragments as small as 50 nucleotides in size to be resolved. Of the 23 strains analyzed by this method, 15 had unique restriction patterns when digested with *Msp* I (Figure 1a), 13 when digested with *Hae*III (Figure 1b); six when digested with *Sau*3A (Figure 1c); 10 with *Alu*I (Figure 1d) and 11 types when cut with *Rsa*I (Figure 1e). In the latter three gels, *E. coli* was added to serve as an outgroup in phylogenetic analyses.

Several strains had identical 16S RFLP patterns, including TFD41 and TFD38; and TFD6, and TFD20. This was not surprising, since these appeared identical by all other measures.

Several other strains had nearly identical RFLP patterns, differing by

Table 1. 2,4-D degrading strains used for sequencing of 16S rDNA.

Strain	Origin	Reference	*Proteobacteria* most similar to*:	Subgroup
JMP134	Australia	Don and Pemberton, 1981	*Alcaligenes eutrophus*	Beta 2
EML159	Oregon	Amy, et al, 1985	*Burkholderia* sp.	Beta 2
TFD39	Saskatchewan	Tonso, et al., 1994	*Burkholderia* sp.	Beta 2
K712	Michigan	Ka, et al., 1994	*Burkholderia* sp.	Beta 2
TFD9	Saskatchewan	Tonso, et al., 1994	*Alcaligenes xylosoxidans*	Beta 2
TFD41	Michigan	Tonso, et al., 1994	*Alcaligenes eutrophus*	Beta 2
TFD38	Michigan	Tonso, et al., 1994	*Alcaligenes eutrophus*	Beta 2
TFD23	Michigan	Tonso, et al., 1994	*Rhodoferax fermentans*	Beta 2
DBO1(D5)		Walch and Ballou, 1983	*Burkholderia cepacia*	Beta 2
RASC	Oregon	Amy, et al, 1985	*Burkholderia* sp.	Beta 2
TFD6	Michigan	Tonso, et al., 1994	*Burkholderia* sp.	Beta 2
TFD20	Michigan	Tonso, et al., 1994	*Burkholderia* sp.	Beta 2
BRI6001	Quebec	Greer, et al., 1990	*Burkholderia* sp.	Beta 2
TFD2	Michigan	Tonso, et al., 1994	*Burkholderia* sp.	Beta 2
TFD31	Saskatchewan	Tonso, et al., 1994	*Rhodoferax fermentans*	Beta 1
B6-9	Ontario	Fulthorpe	*Rhodoferax fermentans*	Beta 1
I-18	Oregon	Malteseva	*Halomonas* sp.	Gamma
K1443	Michigan	Ka, et al., 1994	*Sphingomonas* sp.	Alpha 4
2,4-D1	Montana	Robert Sanford	*Sphingomonas* sp.	Alpha 4
B6-5	Ontario	Roberta Fulthorpe	*Sphingomonas* sp.	Alpha 4
B6-10	Ontario	Roberta Fulthorpe	*Sphingomonas* sp.	Alpha 4
EML146	Oregon	Amy, et al., 1985	*Sphingomonas* sp.	Alpha 4
M1	French Polynesia	Roberta Fulthorpe	*Rhodospeudomonas palustris*	Alpha 4

* Names refer to the genera or species most similar to the strain based on similarities of 16S rDNA (discussed in text).

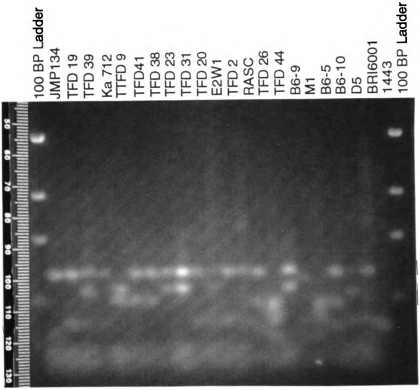


Figure 1. Amplified 16S ribosomal DNA restriction analysis (ARDRA) of 2,4-D degrading isolates. DNA was PCR amplified with 16S rDNA primers, digested with four-base cutting restriction enzyme, and size fractionated by electrophoresis on a 1.5% agarose gel. Figure 1a digested with *Msp*I; 1b with *Hae*II; 1c with *Sau*3A; 1d with *Alu*I; and 1e with *Rsa*I.

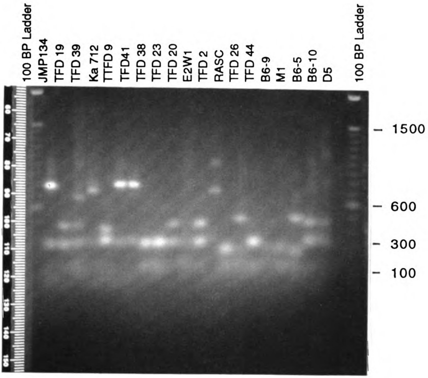


Figure 1b. Digested with *Hae*II.

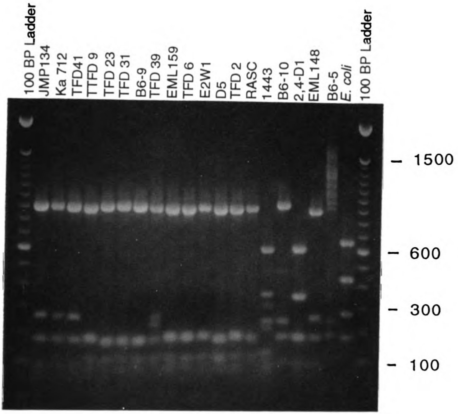


Figure 1c. Digested with *Sau*3A.

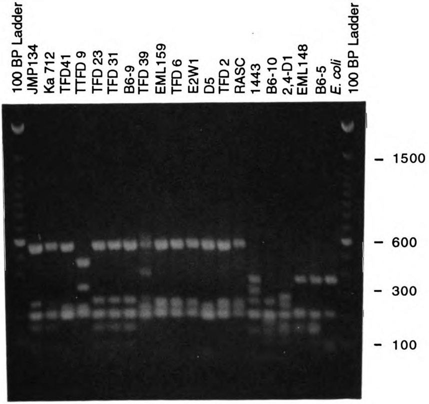


Figure 1d. Digested with *Alu*I.

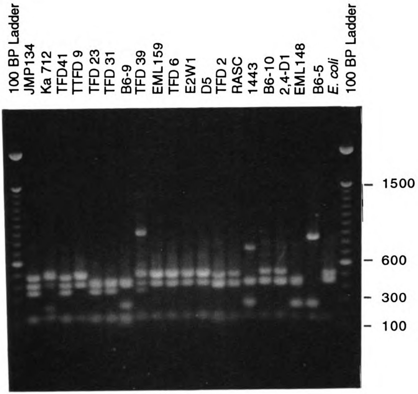


Figure 1e. Digested with *Rsa*I.

only one or two restriction fragments. For example, JMP134 was closely related to TFD41 and TFD38; D5 was nearly the same as TFD6; and B6-9 differed from TFD23 and TFD31 by only one *Alu*I band.

The fragment sizes resulting from these five restriction enzyme digestions were combined and 18 unique strain types could be distinguished (Table 2). The maximum likelihood method of Felsenstein (1992) using RestML program was used to estimate the phylogeny from restriction data. The resulting tree (Figure 2) contains a topology similar with some differences to that obtained by the sequence data (discussed below).

Diagnostic restriction fragment bands: These restriction fragment length polymorphisms were examined to see if any were diagnostic for particular lineages. We found several restriction fragments which may be diagnostic for different lineages including three for the *Proteobacteria,* or at least the alpha, beta and gamma subdivisions examined here; one band diagnostic of the beta subgroup; and three diagnostic restriction fragments for the alpha subgroup.

The several restriction fragments shared by all strains included a 150 bp *Rsa*I fragment; a 200 bp *Alu*II fragment; and a 100 bp *Msp*I fragment. Although it is tempting to speculate that these are signature fragment sizes for the *Proteobacteria,* more strains would have to be tested.

Only one band, a 350 bp *Hae*III fragment, appeared to be diagnostic for all of the beta *Proteobacteria* group 2,4-D degraders in this study, and was not found in any of the alphas, nor in *E. coli,* a member of the gamma *Proteobacteria.*

One restriction fragment, an 870 bp *Sau3A* fragment, although found in all beta strains tested here, was also found in two of the alpha strains,

Table 2. Presence, +, or absence, 0, of restriction fragments of amplified 16S rDNA.

	JMP	K712	TFD41	TFD9	TFD23	B6-9	TFD39	EML159	TFD6	E2W1	D5	TFD2	RASC	K1443	B610	24D1	EML148	B65	E. coli	M1
Msp550	+	+	+	0	+	+	+	+	+	+	+	+	+	+	+	0	+	0	?	0
Msp500	+	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	?	+
Msp450	0	0	0	+	+	+	+	0	0	+	+	0	0	0	0	0	0	0	?	0
Msp400	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	?	0
Msp375	0	0	0	0	0	0	I	0	0	0	0	0	0	0	0	0	0	0	?	+
Msp350	0	0	0	0	0	0	0	0	0	0	0	0	0	+	+	+	+	+	?	0
Msp325	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+	0	+	?	0
Msp300	0	0	0	0	0	0	0	+	+	+	0	+	+	0	0	+	0	+	?	0
Msp275	0	+	0	0	+	+	+	0	0	0	+	0	0	0	0	0	0	0	?	0
Msp250	0	0	0	0	0	0	0	0	0	0	0	0	0	+	+	0	+	0	?	0
Msp225	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+	0	+	?	+
Msp200	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	?	0
Msp150	0	+	+	+	+	+	+	+	+	+	+	+	0	0	0	0	0	0	?	0
Msp100	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	?	+
Msp50	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	?	0
Hae850	0	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	?	?
Hae800	+	0	0	0	0	0	0	0	0	0	0	0	+	0	0	0	0	0	?	?
Hae750	0	0	0	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	?	?
Hae550	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	?	?
Hae500	0	0	0	0	0	0	+	+	+	+	+	+	0	+	+	+	0	0	?	?
Hae450	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	?	?
Hae350	+	+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	0	0	?	?
Hae300	0	0	0	0	0	0	0	0	0	0	0	0	0	+	+	+	+	+	?	?
Hae200	0	+	0	+	+	+	?	+	+	+	+	+	+	0	0	+	0	+	?	?
Hae150	0	0	0	0	0	0	0	0	0	0	0	0	0	+	+	+	+	+	?	+
Hae100	+	?	?	0	?	?	?	0	?	?	0	?	?	?	?	?	?	?	?	?
Sau900	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+	0	0	?	0	?
Sau870	+	+	+	+	+	+	+	+	+	+	+	+	+	0	+	0	+	?	0	?
Sau650	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	?	+	?
Sau600	0	0	0	0	0	0	0	0	0	0	0	0	0	+	0	+	0	?	0	?
Sau430	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	?	+	?
Sau350	0	0	0	0	0	0	0	0	0	0	0	0	0	+	0	+	0	?	0	?
Sau300	0	0	0	0	0	0	0	0	0	0	0	0	0	+	0	0	0	?	0	?
Sau280	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+	?
Sau270	+	+	+	0	0	0	+	0	0	0	0	0	0	0	0	0	+	0	0	?
Sau250	0	0	0	0	0	0	0	0	0	0	0	0	0	+	+	0	0	+	0	?
Sau230	0	0	0	0	0	0	+	0	0	0	0	0	0	+	0	0	0	0	0	?
Sau180	+	+	+	+	0	0	+	+	+	+	0	+	+	0	+	+	+	+	+	?
Sau170	0	0	0	0	+	+	+	0	0	0	+	0	0	0	0	0	0	0	0	?
Sau120	0	0	0	+	+	+	+	+	+	+	+	+	+	+	0	0	0	0	0	?
Sau 90	+	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0	0	?
Alu550	+	+	+	0	+	+	+	+	+	+	+	+	+	0	0	0	0	0	0	?
Alu450	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	?
Alu400	0	0	0	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	?
Alu390	0	0	0	0	0	0	0	0	0	0	0	0	0	+	0	0	+	+	+	?
Alu300	0	0	0	0	+	+	+	0	0	0	0	0	0	+	+	0	0	0	0	?
Alu290	0	0	0	0	0	0	0	+	+	+	+	+	+	0	0	0	0	0	0	?
Alu200	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	?
Alu150	+	+	0	0	+	+	+	0	0	0	0	0	0	+	+	+	+	+	0	?
Alu100	+	+	0	0	+	+	0	0	0	0	0	0	0	0	+	+	+	+	+	?
Rsa900	0	0	0	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	?
Rsa850	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+	?
Rsa750	0	0	0	0	0	0	0	0	0	0	0	0	0	+	0	0	0	0	0	?
Rsa500	+	+	+	+	0	0	+	+	+	+	+	+	+	0	+	+	0	0	+	?
Rsa400	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0	+	?
Rsa350	+	0	+	0	+	0	+	0	0	0	0	0	0	0	0	0	0	0	0	?
Rsa250	0	+	0	0	0	0	+	0	0	0	0	0	0	+	0	0	0	+	+	?
Rsa150	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	?

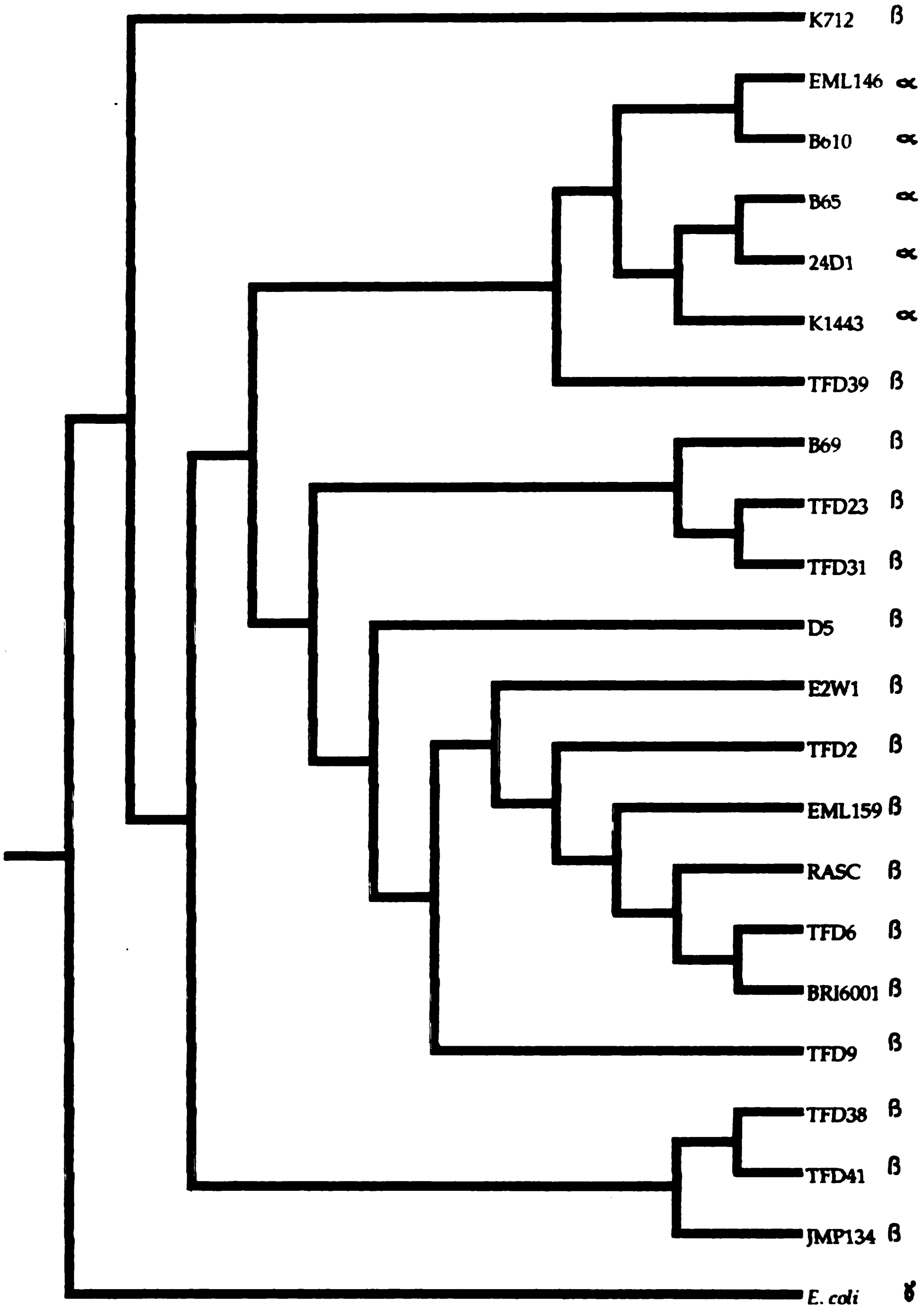


Figure 2. Dendrogram of 2,4-D degrading bacteria based on maximum likelihood analysis of restriction data using tetrameric restriction enzymes. Note that branch lengths are not to scale.

thereby lessening its usefulness as a diagnostic band.

Several bands were found in all of the alphas, but not in the betas nor in *E. coli*. These fragments included a 350 bp fragment from *MspI,* 150 and 300 bp fragments with *HaeIII,* and a 150 bp fragment from *Alu* I 150.

16S rDNA sequencing: A flowchart for acquiring and analyzing the 16S rDNA sequence data is depicted in Figure 3. Nucleotide sequences from approximately 510 to 0 (relative to *Escherichia coli)* were obtained for all strains. Since nucleotide resolution began to deteriorate in some strains from approximately 150 to 0 in the reverse direction, this data was not used in subsequent analyses. An example of the chromatogram obtained from the ABI sequencer depicts the typical data quality of sequences (Figure 4). The quality of the data for the first 300 nucleotides was high since the average numbers of ambiguous nucleotides averaged 1 per 300 nucleotides with a range of 0 to 3. As a control for sequencing accuracy, strain TFD6 was sequenced twice on different dates and yielded identical sequences on both occasions. In addition, strains TFD6 and TFD20, which were identical in every other respect (Tonso et al., 1994; Fulthorpe et al., 1994) also showed identical sequences, as did TFD38 and TFD41. In addition, some strains that had minor difference in REP patterns contained identical 16S sequences. For example, TFD23 and TFD31 had a few different REP bands, but shared identical 16S rDNA sequences. Also, E2w1 contained a REP pattern slightly different from that of TFD6, yet both had identical 16S rDNA sequences.

Sequence alignment: The 16S rDNA sequences were aligned manually using SeqEd and MacClade (Figure 5). Additional 16S rDNA reference sequences from the Ribosomal Database (Larsen, 1993) and from GenBank were added to

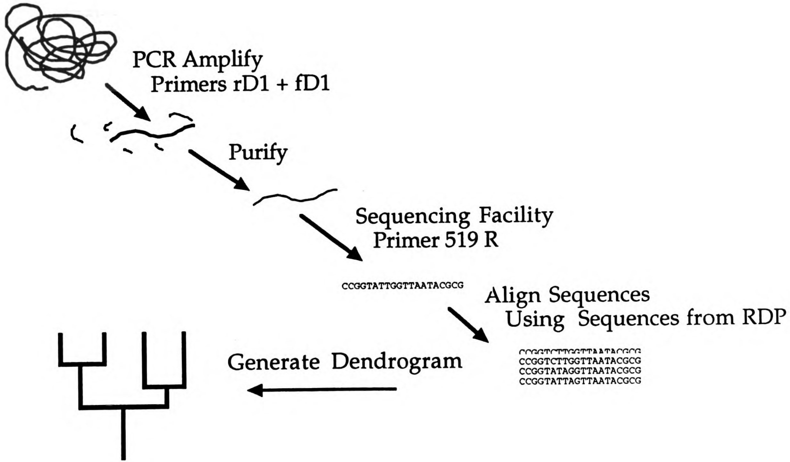


Figure 3. Flowchart indicating the collection and analysis of 16S sequence data.

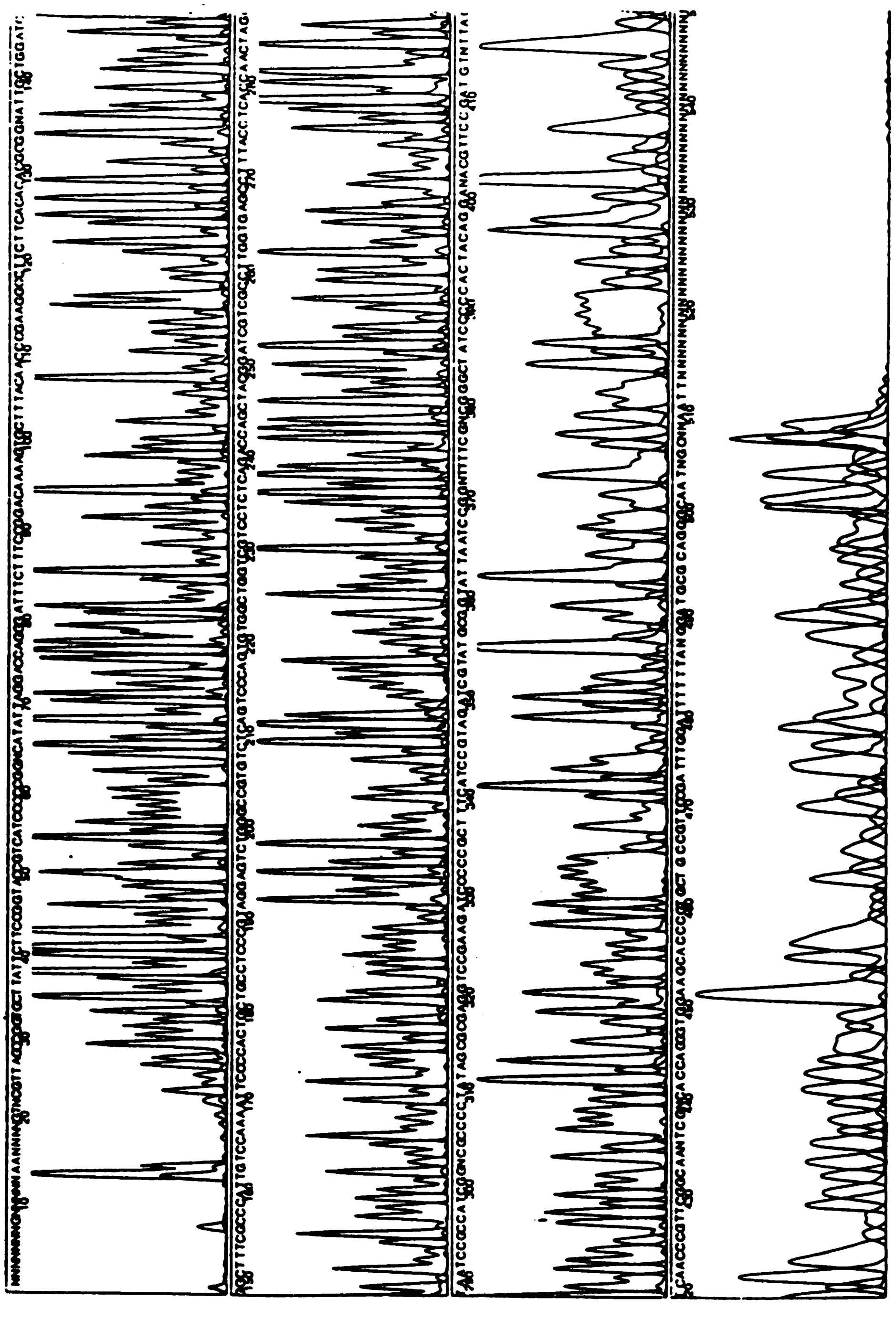


Figure 4. Example of chromatogram from Applied Biosystems model 373A automated sequencer.

```
[                                   1         2         3         4         5         6]
[                          123456789012345678901234567890123456789012345678901234567890]

Burkholderia cepacia       TCGGGCCTCGCGCTATAGGGTTGGCCGATGGCTGATTAGCTAGTTGGTGGGGTAAAGGCC  [60]
Burkholderia cepacia D5    ....?.......................................................  [60]
Burkholderia cepacia G4    ............................................................  [60]
Burkholderia mallei        ..................................................C.........  [60]
Burkholderia gladioli      ............................................................  [60]
TFD6                       AG..A......?...C....GC..........A...........................  [60]
BRI6001                    CG..A..........C....GC..........A...........................  [60]
EML159                     AG..A......?...C....GC..........A...........................  [60]
TFD2                       ....A.........CA....AC..........A...........................  [60]
Burkholderia caryophylli   ..............CA....GC..........G...........................  [60]
Burkholderia andropogonis  ....A...............GC..........A...........................  [60]
RASC                       A...A...............GC..........G................A.........T  [60]
K712                       ....A......?........?C........?..................A.........T  [60]
JMP134                     AAC.T........G.....AGC........T........................GA...  [60]
TFD41                      CAA.C......?.AGC...AGC.A......T.............................  [60]
TFD38                      CAA.C......?.AGC...AGC.A......T.............................  [60]
Alcaligenes eutrophus      ?AA............C...AGC........T.............................  [60]
Burkholderia solanacearum  CAA......AT........AGC........T.............................  [60]
TFD39                      ....?...T..?....CA.A..A.....?.T........................T....  [60]
Alcaligenes xylosoxidans   CAA.A...T..A....T..AGC.......AT.G......................C...T  [60]
TFD9                       CAA.A...T..A....TA.AGC.......AT.G....................GGC...T  [60]
B69                        CAA.A........G..T..AGC.......AT.A....................GG.A...  [60]
TFD23                      CAA.A........G..T..AGC.......AT.A....................G..A...  [60]
TFD31                      CAA.A........G..T..AGC.......AT.A....................G..A...  [60]
Rhodoferax fermentans      CAA.A........G..T..AGC.......AT.A................A.........T  [60]
Comamonas testostosteroni  ........T.T....CTA.AGC...T......A......G...................T  [60]
Halomonas elongata         ....A...T.......C..A.GA..TT...T.G........G.......A.....C...T  [60]
E. coli                    .........TT..C..C..A.GT...C.GATGG...........A..........C...T  [60]
Halomonas halmph           ....A...T.......C..A.GA..TT...T.G................A.....C...T  [60]
Del.hphila                 ....A...T.......C..A.GA...T...T.G................A.....T...T  [60]
'I-18'                     .G..A...T.......C..A.GA...A..?T.G...............A.....GC....  [60]
24D1                       AAA.ATA.AT.T.CCAG..A.GA...CGC.TAG......G...............T.T..  [60]
Sphingomonas adhaesiva     AAA.ATT.AT...CCAG..A.GA...CGC.T.G......G....................  [60]
Sphingomonas paucimobilus  AAA.ATT.AT...CTG...A.GA...CGC.TTG......G....................  [60]
Flavobacterium capsulatum  AAA.ATT.AT..GC.A...A.GA...CGC.TAG......G....................  [60]
B65                        AAA.ATA.AT.T.CCA...A.GA...CGC.TAG...........................  [60]
B610                       AAA.ATA.AT.T.CCAG..A.GA...CGC.TAG................A.........T  [60]
K1443                      AAA.ATA.AT.T.CCAG..A.GA...CGC.TAG................A.........T  [60]
EML146                     AAA.ATT.AT.T.CCAG..A.GA...CGC.TAG................A.........T  [60]
Sphingomonas yanoikuyae    AAA.ATT.AT...CCA...A.GA...CGC.TAG................A.........T  [60]
Rhodopseudomonas palustris AAA.ATT.AT...CGA.A.A.C....CGC.T..................A.....T...T  [60]
M1                         AAA.ATT.AT.T.CGA.A.A.C....CGC.T..................A.T...C...T  [60]
Desulfomonile tiedjii      -GAA...GATGC.A.-...A.G...TCGC..ACC.......T.......A.....C...T  [58]
```

Figure 5. Alignment of 16S sequences from positions 209-506 according to the *E. coli* numbering system.

```
[                                                                 1                    ]
[                          6        7         8         9         0         1         2]
[                          123456789012345678901234567890123456789012345678901234567890]

Burkholderia cepacia       TACCAAGGCGACGATCAGTAGCT?GTCTGAGAGGACGACCAGCCACACTGGGACTGAGAC   [120]
Burkholderia cepacia D5    .......?.................G....................................   [120]
Burkholderia cepacia G4    .......................G....................................   [120]
Burkholderia mallei        .......................G....................................   [120]
Burkholderia gladioli      C......................G....................................   [120]
TFD6                       .......C........T...T..G....................................   [120]
BRI6001                    .......C........T...T..G....................................   [120]
EML159                     .......C........T...T..G....................................   [120]
TFD2                       .......C........T...T..G....................................   [120]
Burkholderia caryophylli   ................C......G....................................   [120]
Burkholderia andropogonis  ................C......G....................................   [120]
RASC                       C...............C......G....................................   [120]
K712                       C......?........C......G....................................   [120]
JMP134                     C......................G..............T.....................   [120]
TFD41                      C......C............T..G..............T.....................   [120]
TFD38                      C......C............T..G..............T.....................   [120]
Alcaligenes eutrophus      .......................G..............T.....................   [120]
Burkholderia solanacearum  C......................G..............T.....................   [120]
TFD39                      C......C........C...A..G..............T.....................   [120]
Alcaligenes xylosoxidans   C...............C......G..T.................................   [120]
TFD9                       C......C........C......G..T.................................   [120]
B69                        .......C........T...T..G...........................A........   [120]
TFD23                      .......C........T...T..G...........................A........   [120]
TFD31                      .......C........T...T..G...........................A........   [120]
Rhodoferax fermentans      C...............T......G...........................A........   [120]
Comamonas testostosteroni  .......C.TG.....T......G....................................   [120]
Halomonas elongata         C...............C......G...........T..T........TC...........   [120]
E. coli                    C...T...........CC.....G...........T...............A........   [120]
Halomonas halmph           ................C......?...........T..T........TC...........   [120]
Del.hphila                 C........CG.....C......G...........T..T........TC...........   [120]
'I-18'                     .......C..?.....C...C..G...........T..T........TC..........?   [120]
24D1                       ......CC........CT.....G...........T..T.....................   [120]
Sphingomonas adhaesiva     .......C........C......G...........T..T.....................   [120]
Sphingomonas paucimobilus  .......C........CA.....G...........T..T.....................   [120]
Flavobacterium capsulatum  .......C........CT.....G...........T..T.....................   [120]
B65                        ................CT.....G...........T..T.....................   [120]
B610                       C......C........CT..T..G...........T..T.....................   [120]
K1443                      C......C........CT.....G...........T..T.....................   [120]
EML146                     C......C........CT.....G...........T..T.....................   [120]
Sphingomonas yanoikuyae    C...............CT.....G...........T..T.....................   [120]
Rhodopseudomonas palustris C......................G...........T..T........T............   [120]
M1                         C......C...............G...........T..T........T............   [120]
Desulfomonile tiedjii      C..?.....CGA...GGT.....?...........T..T............A........   [118]
```

Figure 5. (continued)

```
[                          1                                                           ]
[                          2        3         4         5         6         7         8]
[                          123456789012345678901234567890123456789012345678901234567890]

Burkholderia cepacia       ACGGCCC?GACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGCGAAAGCCTG   [180]
Burkholderia cepacia D5    .......A....................................................   [180]
Burkholderia cepacia G4    .......A....................................................   [180]
Burkholderia mallei        .......A............................................C.......   [180]
Burkholderia gladioli      .......A....................................................   [180]
TFD6                       .......A............................................C.......   [180]
BRI6001                    .......A............................................C.......   [180]
EML159                     .......A............................................C.......   [180]
TFD2                       .......A..........................................G.C..C....   [180]
Burkholderia caryophylli   .......A..........................................G.C..C....   [180]
Burkholderia andropogonis  .......A............................................C.......   [180]
RASC                       .......A....................................................   [180]
K712                       .......A............................................C..C....   [180]
JMP134                     .......A..........................................G.C..C....   [180]
TFD41                      .......A..........................................G.C..C....   [180]
TFD38                      .......A..........................................G.C..C....   [180]
Alcaligenes eutrophus      .......A..........................................G.C..C....   [180]
Burkholderia solanacearum  .......A..........................................G.C..C....   [180]
TFD39                      .......A.............................A......................   [180]
Alcaligenes xylosoxidans   .......A......?...................................G....C....   [180]
TFD9                       .......A..........................................G....C....   [180]
B69                        ....T..A............................................C.......   [180]
TFD23                      ....T..A............................................C.......   [180]
TFD31                      ....T..A............................................C.......   [180]
Rhodoferax fermentans      ....T..A............................................C.......   [180]
Comamonas testostosteroni  .......A....................................................   [180]
Halomonas elongata         .......GA............................A............G.C..C....   [180]
E. coli                    ....T..A.............................A...C..........C.......   [180]
Halomonas halmph           ......?GA............................A......................   [180]
Del.hphila                 .....???A....?.........?.?...........A......................   [180]
'I-18'                     ...?...GA.....?......................A............G.C..C....   [180]
24D1                       .......A.............................A......................   [180]
Sphingomonas adhaesiva     .......A.............................A......................   [180]
Sphingomonas paucimobilus  .......A.............................A......................   [180]
Flavobacterium capsulatum  .......A.............................A..............C.......   [180]
B65                        .......A......................A......A............GTC..C....   [180]
B610                       .......A...C..CC..C...............T..A.................A....   [180]
K1443                      .......A......................A......A............GTC..C....   [180]
EML146                     .......A......................A......A............G.C..C....   [180]
Sphingomonas yanoikuyae    .......A.............................A......................   [180]
Rhodopseudomonas palustris .......AA............................A............G....C....   [180]
M1                         .......AA............................A..............C.......   [180]
Desulfomonile tiedjii      ....T.??.?.....................A.....A...CG......AG....CT...   [178]
```

Figure 5. (continued)

```
[                           1                   2                                       ]
[                           8         9         0         1         2         3        4]
[                           123456789012345678901234567890123456789012345678901234567890]

Burkholderia cepacia        ATCCAGCAATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTTGTCCGGGA    [240]
Burkholderia cepacia D5     ..........C...............................................A.    [240]
Burkholderia cepacia G4     ..........................................................A.    [240]
Burkholderia mallei         ..........................................................A.    [240]
Burkholderia gladioli       ..........................................................A.    [240]
TFD6                        ..........C...............................................A.    [240]
BRI6001                     .....?....C..............................?................A.    [240]
EML159                      ..........C...............................................A.    [240]
TFD2                        ..........C...............................................A.    [240]
Burkholderia caryophylli    ....................C.....................................A.    [240]
Burkholderia andropogonis   ......................................................T.....    [240]
RASC                        ..........?...............................................A.    [240]
K712                        ..........C...............................................A.    [240]
JMP134                      ..........................................................A.    [240]
TFD41                       ..........C...............................................A.    [240]
TFD38                       ..........C...............................................A.    [240]
Alcaligenes eutrophus       ..........................................................A.    [240]
Burkholderia solanacearum   ..........................................................A.    [240]
TFD39                       .......C..C.................................................    [240]
Alcaligenes xylosoxidans    .......C..C.........C..T.....T.G.-...................G.A..A.    [239]
TFD9                        .......C..C.........C..T.............................G.A..A.    [240]
B69                         ..................CAG..T....................CTG.......A...A.    [240]
TFD23                       ..................CAG..T....................CTG.......A...A.    [240]
TFD31                       ..................CAG..T....................CTG.......A...A.    [240]
Rhodoferax fermentans       ..................CAG..T....................CTG.......A...AG    [240]
Comamonas testostosteroni   ..................CAG..T.......C............CTG.......A...A.    [240]
Halomonas elongata          .......C.......................C...................CAG.GA...    [240]
E. coli                     ..G....C...........A.........................T.....CAG.G....    [240]
Halomonas halmph            .......C......?................C...................CAGTGA...    [240]
Del.hphila                  .......C...........................................CAG.GA...    [240]
'I-18'                      .....?.C..C........................................CAG.GA...    [240]
24D1                        ..........?.......A....T.........A............T.....AC.A....    [240]
Sphingomonas adhaesiva      ..................A....T.........A............T.....AC......    [240]
Sphingomonas paucimobilus   ..................A....T.......C.A............T.....AC......    [240]
Flavobacterium capsulatum   ..................A....T..............C.......T.....AC.A...?    [240]
B65                         ..................A....T.......C.A............T.....AC...A..    [240]
B610                        ..........C..C....A....T.........A............T.....AC.....G    [240]
K1443                       ..................A....T.........A............T.....AC......    [240]
EML146                      ..................A....T.........A............T.....AC......    [240]
Sphingomonas yanoikuyae     ..................A....T.........A............T.....AC......    [240]
Rhodopseudomonas palustris  .......C..........A....T.......C.A............T.......G.....    [240]
M1                          .......C..........A....T.......C.A............T.......G.....    [240]
Desulfomonile tiedjii       .CG......C........G...........T...............C..G.CAGG.....    [238]
```

Figure 5. (continued)

```
[                          2                                                          ]
[                          4         5         6         7         8         9        ]
[                          12345678901234567890123456789012345678901234567890123456789]

Burkholderia cepacia       AG-AAATCCTTGGCTCTAATACAGCCGGGGGATGACGGTACCGGAAGAATAAGCACCGG   [298]
Burkholderia cepacia D5    ..-.....................T..................................   [298]
Burkholderia cepacia G4    ..-........................................................   [298]
Burkholderia mallei        ..-.....A..CTGG.......CCGGA.T..............................   [298]
Burkholderia gladioli      ..-.......GA.GG......TCCTTC................................   [298]
TFD6                       ..-...A...C.TGGT......CCGT.................................   [298]
BRI6001                    ..-...A...C.TGGT......CCGT.................................   [298]
EML159                     ..-...A...C.TGGT......CCGT.................................   [298]
TFD2                       ..-...A...CC.TC.......G.TG.................................   [298]
Burkholderia caryophylli   ..-.......GCCTGA......C.GGC................................   [298]
Burkholderia andropogonis  ..-...AGGCGA.GG......TCCTTT.CT.............................   [298]
RASC                       ..-......C...TC......TG?...................................   [298]
K712                       ..-......C...TC......TGC...................................   [298]
JMP134                     ..-...A..C.T..C.......G.TG.................................   [298]
TFD41                      ..-...A...CC..C.G.....G.TG.................................   [298]
TFD38                      ..-...A...CC..C.G.....G.TG.................................   [298]
Alcaligenes eutrophus      ..-....GGC.CTGGT......CCGG..TC.............................   [298]
Burkholderia solanacearum  ..-.....GCACTGGT......CTGGT.T........................G.....   [298]
TFD39                      ..-...G..A....C........TTG.T................C..............   [298]
Alcaligenes xylosoxidans   ..-...CGTCGT.GGT......CC.GC..CAC..........T.C..............   [297]
TFD9                       ..-...CGTCAT.GGT......CC.GT.AAAC..........T.C..............   [298]
B69                        C.-...CGG..CT...........GG..CTA............T...............   [298]
TFD23                      C.-...CGG..CT...........GG..CTA............T...............   [298]
TFD31                      C.-...CGG..CT...........GG..CTA............T...............   [298]
Rhodoferax fermentans      C.-...GG...CT.-.......GAGG..CTC............T...............   [297]
Comamonas testostosteroni  C.-...AG.C...GG......TCC.....TC............T...............   [298]
Halomonas elongata         ..-..TG.T.GTCGGT......CCGGCAA..GA...ATC..TC.C....G.........   [298]
E. coli                    T.-..GGGAG.AAAGT......CTTT.CTCAT.....T....C.C....G.........   [298]
Halomonas halmph           ..-..GG.T.GTCGGT......CCGGCA..A.G...ATC..TCAC....G....C....   [298]
Del.hphila                 ..-..CG.T.GT.GGT......CCTGCAA.A.G...ATC..TC.C....G.........   [298]
'I-18'                     ..-..GG...GCTG?.C.....CCAGCA..A.G...ATC..TC.C....G.........   [298]
24D1                       T--------------------------.ATA.....A.....T.G.........T....   [273]
Sphingomonas adhaesiva     T--------------------------.ATA.....A.......G.........T....   [273]
Sphingomonas paucimobilus  .--------------------------.ATA.....T.......G.........C....   [273]
Flavobacterium capsulatum  T--------------------------.ATA.....A.....T.G.........T....   [273]
B65                        T--------------------------.ATA.....A...T...G.........C....   [273]
B610                       .--------------------------.ATA.....T.......G.........T....   [273]
K1443                      T--------------------------.ATA.....A.......G.........C....   [273]
EML146                     T--------------------------.ATA.....A.......G.........C....   [273]
Sphingomonas yanoikuyae    T--------------------------.ATA.....A.......G.........T....   [273]
Rhodopseudomonas palustris .--------------------------.ATA............C..........C....   [273]
M1                         .--------------------------.ATA............C..........C....   [273]
Desulfomonile tiedjii      ..A..CCTT.GC.GA.G....GTC.GT.AAAC...........CC.A.GG.........   [297]
```

Figure 5. (continued)

the alignment. For the alignment of 16S rDNA sequences, only the nucleotides from 506R to 209 (using the *E. coli* numbering system) were used, and a mask was used to exclude any ambiguous nucleotides. This resulted in 283 aligned nucleotides which included 95 variable nucleotide positions.

Phylogenetic inference: Two methods were used to infer the phylogeny of the strains. Once the sequences were aligned and ambiguous sites eliminated, percent differences in nucleotides between strains were used to make a pairwise matrix of distances (Table 4) between strains sequenced for this study and sequences from Genbank (Table 3) used as references. This matrix was then used in the Neighbor Joining distance matrix program of Felsenstein to create the tree depicted in Figure 6.

In addition to using a distance matrix method for creating a tree, a parsimony method was also employed using PAUP (Swofford, 1993) (figure 7). In this analysis, the beta group contained 25 taxa with 68 phylogenetically informative characters. The alpha subgroup contained 10 taxa which had 34 phylogenetically informative characters. Within that, the eight *Sphingomonas*-like strains had 17 phylogenetically informative characters.

Confidence level for tree: The validity of using partial 16S sequences for making reliable trees has been established by Lane et al. (1985) who found that fewer than 350 nucleotides of aligned sequence data yielded the same tree as did using the entire 16S sequence, for which only about 950 nucleotides were aligned unambiguously (Lane et al., 1985). In this study, strains which differed by more than 5% could be unambiguously grouped. It was also found that some of the uncertainty of the phylogeny of selected beta *Proteobacteria* was the same using either 300 or 1400 nucleotides (data not

Table. 4. Distance matrix indicates percent sequence similarity based on 283 aligned nucleotide sequences.

Strain #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37
1. TFD6		95	93	92	88	93	93	93	94	93	93	93	91	92	93	91	86	85	88	88	87	75	76	77	74	77	75	75	75	76	77	76	79	82	81	81	71
2. TFD2			93	93	88	93	94	94	93	95	92	93	93	94	91	90	85	84	86	87	86	75	75	77	74	76	76	76	76	76	76	77	78	82	80	80	72
3. RASC				98	89	95	95	95	92	92	93	94	92	90	90	89	87	87	88	85	87	75	76	78	76	77	75	77	77	79	78	78	80	80	81	82	73
4. K712					89	94	94	95	93	92	93	94	92	90	91	90	87	86	88	84	87	74	75	76	75	76	75	77	77	77	78	78	80	81	82	82	73
5. TFD39						92	93	92	89	87	88	90	90	88	87	87	84	85	84	83	83	76	76	78	75	77	75	76	76	77	77	78	82	84	83	86	72
6. *Burkholderia cepacia*							99	100	95	93	92	96	92	91	91	90	84	84	88	86	86	76	76	78	74	78	75	75	75	77	77	77	80	82	80	82	73
7. *Burkholderia cepacia.D5*								99	95	93	92	96	93	92	91	90	84	84	87	87	86	75	76	78	74	77	75	75	75	77	77	77	80	81	80	82	72
8. *Burkholderia cepacia* G4									96	93	92	96	92	91	91	90	85	84	88	87	86	75	76	78	74	77	75	75	75	77	77	77	80	81	80	81	73
9. *Burkholderia mallei*										94	93	96	90	90	94	93	84	84	86	87	86	75	76	77	73	77	75	75	75	76	77	76	81	83	82	82	73
10. *Burkholderia caryophyli*											92	93	91	91	92	92	87	86	85	86	85	75	76	77	74	76	77	76	77	76	76	76	78	83	82	81	72
11. *Burkholderia andropogonis*											94	90	88	91	90	86	86	88	87	86	76	77	78	74	78	76	76	77	77	77	81	81	81	82	73		
12. *Burkholderia gladioli*													91	90	91	91	86	85	87	84	85	75	76	77	74	77	75	75	75	77	77	77	81	82	82	83	74
13. JMP134														95	93	93	87	87	84	87	87	75	76	77	74	77	76	77	77	77	78	79	78	81	81	80	72
14. TFD41															93	92	86	85	83	87	87	75	76	77	75	77	76	77	77	77	78	79	77	81	79	79	73
15. *Alcaligenes eutrophus*																96	88	88	86	88	87	76	77	78	75	78	77	77	77	77	78	79	80	82	81	80	73
16. *Burkholderia solanacearum*																	87	88	83	86	86	75	76	77	74	77	76	77	77	77	78	78	80	81	81	80	73
17. *Alcaligenes xylosoxidans*																		96	84	83	84	75	75	76	74	76	75	76	76	76	76	77	80	82	83	83	73
18. TFD9																			84	84	84	75	75	76	74	75	74	76	76	76	76	77	79	82	83	83	74
19. *Comamonas testosteroni*																				88	89	72	72	75	70	74	71	71	72	73	75	75	77	75	75	76	70
20. TFD23																					96	73	75	76	72	75	73	73	74	75	75	75	78	76	74	75	70
21. *Rhodoferax fermentans*																						73	74	75	74	74	73	75	75	76	77	76	79	76	76	76	71
22. 24D1																							96	97	93	95	94	95	95	96	88	89	74	74	72	75	69
23. *Flavobacteria capsulata*																								96	92	95	94	94	94	96	89	88	75	75	73	74	69
24. *Sphingomonas adhaesaeva*																									94	97	95	95	96	98	91	91	75	75	74	76	70
25. B610																										93	92	95	95	96	89	89	72	72	72	73	70
26. *Sphingomonas paucimobilus*																											95	94	94	96	91	91	74	74	73	75	70
27. B65																												98	97	95	89	90	73	74	73	72	70
28. K1443																													99	97	90	91	74	74	75	75	71
29. EML146																														98	91	92	75	75	75	75	72
30. *Sphingomonas yanoikuyuae*																															92	92	75	74	75	76	72
31. K1																																98	72	73	74	74	70
32. *Rhodopseudomonas palustris*																																	71	73	75	75	71
33. E.coli																																		81	82	83	73
34. I-18																																			92	92	72
35. *Halomonas elongata*																																				94	73
36. *Del.halophilum*																																					73
37. *Desulfomonile tiedje*																																					

Table 3. Reference strains used in building phylogenies.

Strain	GenBank Accession Numbers
Burkholderia cepacia G4	L28675
Burkholderia gladioli	X67038
Burkholderia solanacearum	X67041
Burkholderia mallei	S55000
Burkholderia cepacia	M22518
Burkholderia andropogonis	X67037
Alcaligenes xylosoxidans	M22509
Alcaligenes eutrophus	M32021
Rhodoferax fermentans	D16211
Burkholderia caryophylli	X67039
Sphingomonas sp.	D16148
Sphingomonas capsulata	D16147
Sphingomonas yanoikuyae	D13782
Sphingomonas paucimobilus	D16144
Sphingomonas adhasiva	D16146
Sphingomonas parapaucimobilus	X72723
Flavobacterium capsulatum	M59296
Blastobacter	Z23156
Rhodopseudomonas palustris	D25312
Desulfomonile tiedjii	RDP*
Halomonas elongata	RDP*
E. coli	RDP*

* 16S sequences taken from Ribosomal Database Project

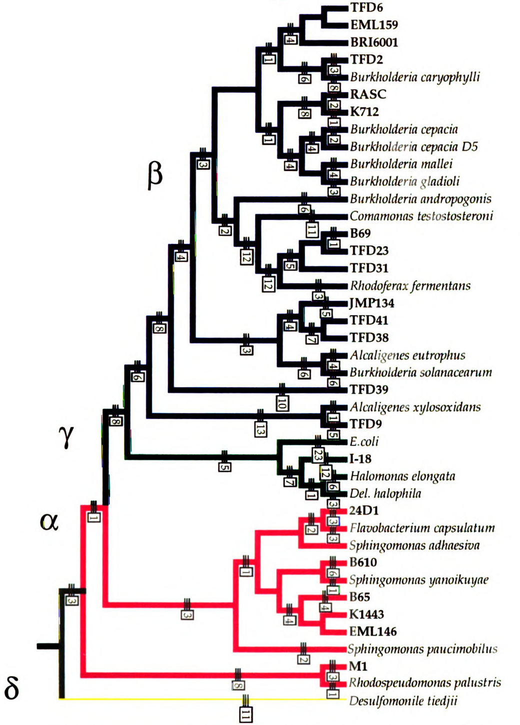


Figure 6. Dendrogram of 2,4-D degrading strains (bold type) and reference strains (italics) based on Neighbor Joining Method of PHYLIP. Subgroups of *Proteobacteria* are indicated in color: blue indicates betas; green gammas; red are alphas; and black indicates delta *Proteobacteria*. Numbers along branches indicate unambiguous nucleotide base changes between branches.

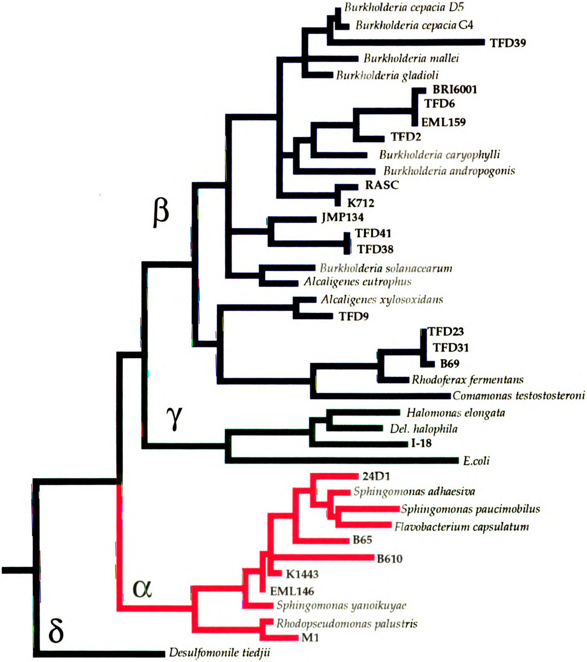


Figure 7. Phylogeny, based on maximum parsimony, of 16S rDNA from 2,4-D degrading bacteria, indicated in bold letters, and reference strains, indicated in italics. Colored lines indicate phylogenetic groups of *Proteobacteria*: blue are beta, green are gamma, red are alpha, and black is delta *Proteobacteria*.

shown).

There has been discussion as to whether or not 300 nucleotides is sufficient data to adequately resolve a phylogeny for a group of molecules. According to Stewart (1993), as a general principle, phylogenetic analysis works well only when there are more phylogenetically informative characters than taxa (strains) to test. This was certainly the case in this study, since the 23 strains under investigation had 95 variable characters.

The 300 nucleotides of aligned partial sequences used here yielded a tree with branching patterns similar to several trees published previously. For example, the tree produced by maximum parsimony correlated well with the same major branches as the recently released tree in the RDP, version 4, and with the previously published tree of Woese, 1984, which used oligonucleotide cataloging to build a tree which included members of the alpha, beta and gamma *Proteobacteria* (or group R_I, R_{II} and R_{III} as they were then designated). The most parsimonious tree presented here also agrees well with that of Yabuuchi et al., depicting a group of beta 2 *Proteobacteria*, which is now referred to as the genus *Burkholderia* (Yabuuchi et al., 1992; IJSB, 1992). The tree presented here also has a similar topology to that of the beta subgroup published by DeWhirst et al., 1989. Our tree also had a similar branching order as that for the the *Sphingomonas* and *Rhizomonas* group of alpha 4 (rRNA Superfamily IV) *Proteobacteria* (270 aligned nucleotides, positions 50-350) determined by Van Bruggen et al., 1993.

DISCUSSION

This study shows that the 2,4-D degraders investigated here represent diverse members of the *Proteobacteria* (Stackebrandt et al., 1988) which encompasses a large proportion of all Gram-negative bacteria (Murray et al., 1990). *Proteobacteria* aptly describes this diverse group of strains, since the term "proteo" is from the Greek sea god, Proteus, who had the ability to assume many diverse shapes and the *Proteobacteria* have members with many diverse metabolic capabilities.

Two methods based on comparisons of 16S ribosomal genes were employed to examine the diversity of these strains. The RFLP method was found to be useful for predicting minor differences in closely related strains and the 16S sequencing method was found useful in placing these strains among the burgeoning branches of the 16S phylogenetic tree (Woese, 1994; Larsen, 1993).

RFLP analysis of amplified 16S rDNA

This study compared 16S rDNA RFLPs to 16S partial sequence data. The trees derived from the two sets of data correlated well at the "twigs" of the trees, but not at deeper branches. The diversity of strains observed by sequence data generally reflected the diversity of strains determined by ARDRA data. For example, strains TFD23, TFD31 and B6-9 shared identical sequence similarity over all 300 nucleotides. However, B6-9 lacked one restriction band (a 350 bp *Rsa*I fragment) that was shared by TFD23 and TFD31. This difference is probably due to base pair differences in sequence outside the 300 nucleotides that were sequenced.

For initial quick and inexpensive genomic screening of many strains, where sequencing is too expensive, ARDRA has been shown to predict similar 16S genes and is therefore useful in eliminating duplicate strains before sequencing. ARDRA is better technique than REP-PCR for this purpose, because ARDRA data is reproducible under all conditions, whereas REP PCR can give variable results depending on the concentration of DNA, of magnesium, or of primer and on a variety of other conditions (Ausubel et al., 1992). ARDRA has the additional benefit over REP-PCR in that a database of ARDRA fragments can be made with which to compare test strains. Because REP fragments can be elements involved in recombination (Higgins et al., 1988; Stern et al., 1984; Gilson et al., 1987), at least some fragments may be subject to rearrangement within a few generations of laboratory cultivation (Nakatsu, personal communication) making REP-PCR suitable for strain identification at a more refined level than that provided by 16S rDNA RFLP analysis. This was observed in strains TFD23 and TFD31 which have identical 16S rDNA sequences, but showed different REP patterns (Tonso, 1994; Fulthorpe, personal communication).

Although ARDRA provides an inexpensive initial screen for choosing which strains to characterize more fully by sequencing, the information it yields is not suitable for phylogenetic analysis for five reasons: 1) the data cannot be compared to reference data outside this study because there is no large database of RFLP data as there is for 16S sequence data; 2) more than five restriction enzymes are required for proper resolution of deeper phylogenetic branches and eight restriction enzymes may not be enough (Tiedje and Moyer, personal communication); 3) data is prone to error due to band doublets; and 4) ARDRA has fewer phylogenetically informative characters and 5) bands of similar size which are not necessarily homologous could possibility be mistaken for convergent bands.

When used in conjunction with ARDRA to eliminate related strains, analysis of 16S rDNA sequences gives a reproducible, reliable and increasingly inexpensive means of determining the phylogenetic placement of strains. Advantages of 16S rDNA sequencing over FAME and BIOLOG, two of the most popular and widely used methods of identifying bacteria, include 1) largest strain database (2600 strains in the Ribosomal Database Project versus 1100 for BIOLOG and 1300 for MIDI in 1994); 2) more reproducibility independent of the strain's metabolic condition; 3) better software programs for phylogenetic comparison; and 4) characters of 16S rDNA are independent, unlike phenotypic characters of FAME or Biolog in which a single genetic change could influence a whole range of genetic characters. These benefits may justify the higher cost of sequencing especially since the cost of sequencing is declining.

In this study, we found that the most appropriate use of ARDRA is for determining the relatedness of strains to reduce replicates before sequencing. For this purpose, no more than two restriction enzymes would be necessary, making this technique a cost and time effective initial screen.

Diversity of 2,4-D degraders

Thirteen unique species of bacteria were observed in this study. These unique strains all differed by more than 3% from each other and from the strains in GenBank. Sequences diverging by more than 2.5% at the level of 16S rDNA sequences have been found to correspond to total DNA:DNA sequence differences greater than 30% (Stackebrandt and Goebel, 1994), which is the maximum amount of DNA divergence for two bacteria to still be considered members of the same species (Wayne et al., 1987).

The 2,4-D degraders in this study clustered into six relatively deeply branched groups which included eight strains related to the genus *Burkholderia,* three related to *Alcaligenes eutrophus,* three related to *Rhodoferax fermentans* (which is close to the class *Comamonadaceae*) one related to *A. xylosoxidans* five related to the genus *Sphingomonas,* and one related to *Rhodopseudomonas palustris.*

The majority of the bacteria surveyed here were most closely related to those of the genus *Burkholderia* (Yabuuchi, et al.1990) in the beta II subgroup of *Proteobacteria.* The genus Burkholderia was named after W. H. Burkholder. Burkholder discovered that onion rot was caused by *Burkholderia cepacia* (formerly *Pseudomonas cepacia*), the genus type strain (Yabuuchi et al., 1992). All strains in this group, which include TFD2, TFD39, TFD20, TFD6, BRI6001, EML159, RASC and K712 shared at least 92% sequence similarity with each other.

Within the *Burkholderia* group there were several tightly clustered groups including RASC and K712, which differed by only 1.7%, (five of 300 nucleotides). Strains TFD6, TFD20, and EML159 were identical. Strain BRI6001, found in Canadian peat, differed from these strains by one nucleotide. One strain, TFD39, was most closely related to this *Burkholderia* group, and *Burkholderia cepacia* in particular (93% similarity) although parsimony analysis showed it had diverged more than other strains within this group, making it the most difficult strain to place phylogenetically.

A group of 2,4-D degraders closely related to the genus *Burkholderia* included strains similar to *Alcaligenes eutrophus*, which differed by seven percent from strain JMP134 and by seven percent from strains TFD41 and TFD38. We found that strain TFD41 was most closely related to *A. eutrophus* as determined by 16S sequencing, in contrast to its previous designation to the genus *Comamonas*, using Biolog and FAME (Tonso et al., 1994). Perhaps this was due, in the case of Biolog, to the under-utilization of substrates, since *Alcaligenes eutrophus* strains typically use 47 of 95 substrates whereas *Comamonas testosteroni* typically only uses 25 substrates. Another possibility to account for these conflicting results is that through gene exchange, TFD41 contains DNA from both *A. eutrophus* and *Comamonas testosteroni*-like parents.

Another cluster of strains found within the beta subgroup of *Proteobacteria* included three strains that were most closely related to *Rhodoferax fermentans*. *R. fermentans*, previously known as *Rhodocyclus gelatinosa* (Hiraishi et al., 1991) is closely related to the family *Comamonadaceae*, members of which have been shown to degrade 3-chlorobenzoate (Nakatsu, 1992). Three of the 2,4-D degrading strains, TFD31 and TFD23, both isolated from the Kellogg Biological Station, and B6-9, isolated in Quebec, shared identical sequence and RFLP patterns, except that B6-9 16S rDNA differed in RFLP patterns when digested with *RsaI*.

Strain TFD9 stood apart among these beta *Proteobacteria*, and was most closely related to *Alcaligenes xylosoxidans*.

One strain, I-18, was found to be closely related to the genus *Halomonas*, in the gamma subgroup of *Proteobacteria*. This strains has been characterized by Dr. Olga Maltseva and found to be a salt tolerant 2,4-D degrading strain.

The remaining six strains all clustered in the alpha branch of *Proteobacteria*. It is interesting that none of these strains hybridized to the 2,4-D degradative gene probes, *tfdA* and *tfdC* from JMP134, as did all of the beta *Proteobacteria* (see chapters 3 and 4 for more discussion). This suggests a deep, natural division in degradative genes for these two branches of bacteria.

One lone strain, M1, which is the most oligotrophic and slow growing of all the strains used in this study, is 97% similar to *Rhodopseudomonas palustris*, a member of the alpha 2 group of *Proteobacteria* (Wong et al., 1994). Although M1 does not contain purple photo pigments as does the facultative, anoxygenic, phototrophic *R. palustris*, M1 may share the degradative abilities of *R. palustris*, which is known to grow on a diverse range of aromatic and heterocyclic compounds (Sasikala et al., 1994). The slow growing character of strain M1 correlates well with its placement near the slow growing *Bradyrhizobium*, which is 98.3% similar to *R. palustris* (Wong et al.1994),

The remaining strains all clustered together and were most closely related to various members of the genus *Sphingomonas* in the alpha 4 subgroup of *Proteobacteria*. All but one of the alpha strains were found to be closely related to the genus of *Sphingomonas*, named after the lipid "sphingosine" and sphingoglycolipids which these strains possess. The genus *Sphingomonas* was previously classified as Group IV *Pseudomonads* according to the classification of Paleroni, et al., (1973). Several members of the genus *Sphingomonas* were previously called *Pseudomonas* (*P. adhaesiva* and *P. paucimobilus*) and *Flavobacterium* (i.e. *F. capsulatum*.) All

All the *Sphingomonas*-like 2,4-D degrading strains had colonies with varying shades of the color of egg yolks, which is the colony color of *Flavobacteria* , whereas the beta, and gamma *Proteobacteria* observed in this study have off white to beige colonies.

Relationship to other xenobiotic degrading strains

Comparing the 16S rDNA sequences of these strains to the 16S rDNA sequences of other known xenobiotic degrading strains may shed some light on the degradative pathways of these organisms. Several of the strains sequenced in this study were found to be similar to a species of *Sphingomonas* isolated from the the Elbe River downstream from Hamburg, Germany. This strain of *Sphingomonas* has been show to grow on dibenzo-p-dioxin as the sole carbon and energy source (Moore et al., 1993) and polyethylene glycol (Takeuchi et al., 1993.) Perhaps the genes involved in dioxin degradation, especially those of the lower pathway, are related to those for 2,4-D degradation in these strains. Though the *Sphingomonas*-like strains in this study have been shown to use the *ortho* pathway for intradiol cleavage of catechol like JMP134 (Olga Maltseva, personal communication), these strains contain 2,4-D genes which are quite different than those of the well characterized JMP134 (Perkins, 1990) and strains TFD6 and RASC (Matheson, 1994; Suwa, et al, 1994).

Perhaps these 2,4-D degrading strains contain central metabolic pathways for degrading aromatic compounds like toluene, 2,4,5-trichlorophenoxyacetic acid and other aromatic compounds, since similar strains contain these pathways. For example, several of the 2,4-D degrading strains in this study were shown to be greater than 95% similar to

Pseudomonas cepacia (a.k.a. *Burkholderia cepacia*) strain G4, an isolate capable of growth on toluene as sole carbon and energy source (Zhou, Weller and Tiedje, in Genbank). Another strain, identified as *P. cepacia,* was found that could detoxify 2,4,5-trichlorophenoxyacetic acid (Kilbane et al., 1983).

It is interesting that so many of the 2,4-D degrading strains investigated in this study were related to plant pathogens. Perhaps 2,4-D degradation phenotype evolved in bacteria known to be plant pathogens. For example, many 2,4-D degrading bacteria are related to members of the beta group of *Proteobacteria* which are all close relatives of plant pathogens, including *B. solanacearum* (from potato), *B. cepacia* (from onion), and *P. maltophilia* (barley). Since the plant hosts of these strains produce various aromatic compounds, including auxin, a functional analog of 2,4-D in plants, it is possible that the 2,4-D phenotype evolved in organisms associated with plants.

A group of recently isolated 2,4-D degraders appears to be related to *Xanthomonas* (Dunbar, personal communication), a plant pathogen which contains the yellow pigment xanthomonadin, a brominated poly-aryl ester. Perhaps the genes involved in the biosynthesis or biodegradation of this halogenated aromatic compound are related to genes responsible for the degradation of 2,4-D.

The phylogenetic diversity of 2,4-D degrading organisms is probably much greater than that indicated here. There are reports of 2,4-D degradation by strains which are not Gram negative, including such strains as *Arthrobacter* (Loos, 1969) and *Streptomyces* (Speicher, 1954) and other unidentified gram positive strains (Amy et al., 1987; Holben, personal communication); and even fungi such as *Phanaerochaete* (Yadav and Reddy, 1994).

Geographic distribution

This study showed that some strains with identical 16S genes are found in geographically distant places (Figure 8). For example, strain TFD2 and EML159 contain identical 16S rDNA sequences, yet the former was originally isolated from Michigan and the latter from Oregon. It is perhaps not surprising that the same sequence of DNA can come from places separated by several thousand miles, since the time required for divergence in any nucleotides may be much longer that the time for dispersal of a microbe. This wide dispersal of identical DNA in bacteria has been observed by others, including Sergei Bezborodnikov who found identical napthalene degrading genes in strains from Pennsylvania, the United Kingdom and the Ukraine.

Although this study includes a dendrogram based on partial sequence from 16S rDNA, this does not necessarily reflect the true phylogeny of these strains, but rather only the phylogeny of the 16S rDNA segments contained within these strains. 16S rDNA sequence analysis has become a useful tool for understanding the origin and evolution of 16S rDNA molecule, but it must be remembered that it accounts for only less than 0.2 percent of an entire typical bacterial genome. However, this 16S rDNA data when used with other information is important in understanding the evolution of these 2,4-D degrading bacteria.

Remembering Dobzhansky's words which opened this chapter we find that knowing the evolution of 16S rDNA genes from these 2,4-D degrading bacteria can shed light on the biology of these strains. This knowledge

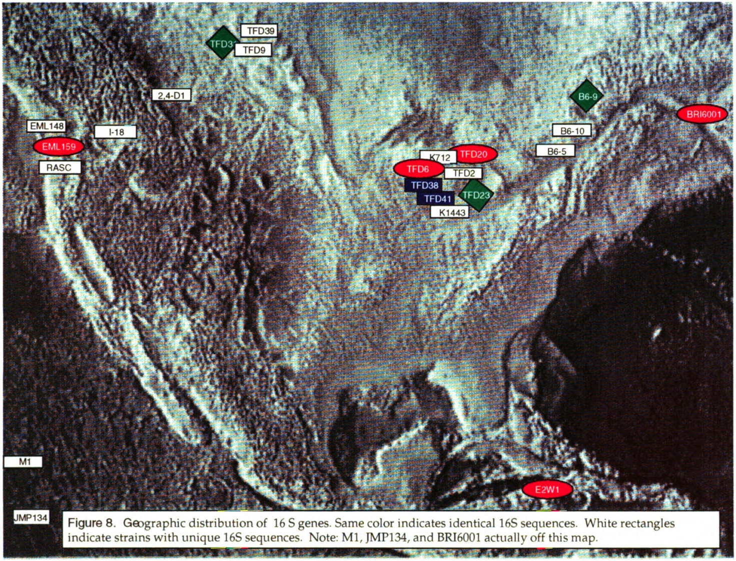


Figure 8. Geographic distribution of 16 S genes. Same color indicates identical 16S sequences. White rectangles indicate strains with unique 16S sequences. Note: M1, JMP134, and BRI6001 actually off this map.

provides a framework from which to evaluate the 2,4-D degradative pathways of these strains and whether gene exchange has occurred between them, topics which will be addressed in the following chapters.

ACKNOWLEDGEMENTS

I wish to thank Dr. Olga Maltseva for use of strain I-18 and for her assistance in sequencing the 16S rDNA sequence from that strain. I also thank Dr. James Cole, Amy Foxworthy, Dr. Arturo Massol-Deya, and Dr. Jizhong Zhou for the primers and information about sequencing. I also thank Dr. Tom Schmidt, Dr. Bonnie Bratina and Dr. Jim Smith for advice and assistance with sequence analysis. I also thank the people in the Research on Microbial Evolution Laboratory for discussion. This work was supported by the National Science Foundation grant number BIR912006.

Chapter Three

Diversity of 2,4-Dichlorophenoxyacetate Degrading Bacteria Determined by Gene Probe Hybridization Analysis

Diversity of 2,4-Dichlorophenoxyacetate Degrading Bacteria Determined by Gene Probe Hybridization Analysis

SUMMARY

Many species of 2,4-D degrading organisms exist yet the genetic diversity of the 2,4-D pathways for these strains is not known. The diversity of 32 strains of 2,4-D degrading bacteria isolated from seven different sites in the United States, Canada, French Polynesia and Australia were analyzed using gene probes made from internal DNA fragments of the first three genes in the 2,4-D pathway from *Alcaligenes eutrophus* JMP134, and using the *tfdA* gene isolated from strain RASC, which is 73% similar in sequence to *tfdA* from JMP134. Based on hybridization signal intensities under conditions of high, medium, and low stringencies using *tfd*A, B and C from pJP4, eight hybridization classes were found. On the basis of restriction length polymorphisms, fourteen unique classes could be distinguished. Hybridization patterns suggest that some strains contained pathways that are mosaics of *tfd* genes recruited from different pathways, a finding that suggests gene transfer and recombination. Nearly identical *tfd* genes were sometimes found in phylogenetically diverse host strains, indicating that gene transfer had occurred. A phylogenetic barrier appeared to separate two major types of 2,4-D degrading bacteria since the *tfd*A and *tfd*C genes from pJP4 hybridized only to the beta and gamma *Proteobacteria*, but not to members of the alpha *Proteobacteria*.

INTRODUCTION

If we understand the diversity of genes involved in the degradation of 2,4-D, then perhaps we will better understand how these individual genes and collective pathways evolved.

A broad diversity of organisms capable of using 2,4-D has been isolated (Alexander et al. 1969; Don and Pemberton, 1981; Amy et al. 1985; Ausmees and Heinaru, 1990; Matheson et al. 1991; Bhat et al. 1994; Ka et al. 1994; Tonso et al. 1994). Several studies have attempted to systematically characterize these strains to gain an insight into the extent of their diversity and elucidate patterns creating this diversity. Most of these studies have used classical strain characterization techniques, FAME, Biolog or REP PCR for assessing the phenotypic and genotypic diversity of the strains. These techniques, however, reveal nothing of a strain's genetic constitution for degrading 2,4-D.

The genetic similarities of 2,4-D degradation genes can be deduced from DNA sequences, from amino acid sequences, or by hybridization analysis using gene probes. The advantage of the former two techniques is that the data are relatively unambiguous as compared to hybridization analysis. The disadvantage is that these techniques are time consuming and costly, whereas many strains may be quickly analyzed using hybridizations studies.

Hybridization studies of 2,4-D degrading genes that appear after 2,4-D addition to soil have been performed using community DNA extracted from soil (Holben et al., 1992; and Ka et al., 1994). Ka et al. examined 47 strains of 2,4-D degrading isolates from Michigan agricultural soil that had been treated with different concentrations of 2,4-D. They performed hybridization analyses under high stringency conditions using the *tfdA, tfdB, tfdC* genes from pJP4 as well as a probe (*spa*) that tracked a 2,4-D degrading strain of *Sphingomonas* which did not hybridize to the tfd genes from JMP134. Using these techniques, Ka et al. found the 2,4-D degraders could be classified into four groups: 1) those that hybridized to the *tfdA, tfdB, tfdC* probes; 2) those that hybridized only to the *tfdA* probe; 3) those that hybridized only to the *spa* probe; and 4) those that hybridized to none of the probes. However, because they performed their hybridizations under conditions of high stringency, they may have missed some strains that have less, but still significant, similarity to pJP4.

This study builds upon that of Ka et al., and uses hybridizations performed at high, medium and low stringency to detect genes which are more distantly related to the 2,4-D genes known so far. We also used strains from more geographically diverse regions, including Australia, Oregon, Saskatchewan, Ontario, Montana, Polynesia, as well as Michigan. We used five gene probes: three from the first three steps of 2,4-D degradation in JMP134, including *tfdA, tfdB,* and *tfdC* from pJP4; the *tfdA* probe from strain RASC which contains a gene 73% similar to the *tfdA* probe from JMP134; and a probe which included genes of the 3-chlorobenzoate pathway from pAC27, which has 60% sequence similarity to the lower pathway of 2,4-D degradation in pJP4 (van der Meer, 1992).

MATERIALS AND METHODS

Medium and growth conditions: 2,4-D MMO containing 500 ppm 2,4-D (Sigma) in a mineral salts medium (Stanier et al. 1966) was used to grow the 2,4-D-degrading strains. All cultures were grown at 30°C. Broth cultures were aerated by shaking at 200 rpm.

Bacterial strains. Strains used in this study are listed in Table 1. Strains RASC, EML148, EML159, and EML 146 were isolated from Oregon (Amy et al. 1985). *Alcaligenes eutrophus* JMP134(pJP4) (Don and Pemberton, 1981) was provided by Dr. Ron Olson. Strains with prefix TFD were isolated from Hickory Corners, Michigan, and Saskatchewan soils (Holben et al. 1993; Tonso et al. 1994). Strain BRI6001 was isolated from Nova Scotian peat (Greer et al. 1990). Strain E2w1 was isolated from forest soil in Puerto Rico, and those strains with prefix B6 were isolated from Quebec by Dr. Roberta Fulthorpe. Strain 1443 and K712 were isolated from Hickory Corners, Michigan (Ka et al. 1994). Strain M1 is an oligotrophic 2,4-D degrader isolated by Dr. Fulthorpe from the Island of Moorea, French Polynesia. Strain 2,4-D1 was isolated by Rob Sanford from Montana.

2,4-D Biodegradation. Disappearance of 2,4-D was monitored by HPLC. Cultures of 2,4-D medium were centrifuged at 14,000 rpm and filtered through 0.2 µ filters. These were then analyzed by HPLC on a Lichrosorb Rp-18 column (Anspec Co., Ann Arbor) using 60% methanol and 40% 0.1% H_3PO_4 as eluant. 2,4-D was detected with a deuterium lamp (Hewlett Packard Series 1050) set for detecting wavelengths of 230 nm.

DNA preparation and restriction digestion. DNA was isolated using the procedure of Ausubel (1992). DNA was digested according to manufacturer's directions using *EcoRI* from Boehringer Mannheim (Indianapolis, IN). DNA molecular weight markers included bacteriophage lambda digested with *Hind*III (Boehringer Mannheim). After separation by gel electrophoresis, DNA was transferred to a nylon membrane (Amersham Hybond-N^{TM}) and covalently linked to the membrane with ultraviolet radiation using Stratalinker^{TM} 1800 (Stratagene, La Jolla, CA).

Hybridization. DNA probes were made from internal fragments of the *tfdA, tfdB,* and *tfdC* genes from plasmid pJP4 (Holben, et al, 1993). The clc probe was made from a fragment of the the 3-chlorobenzoate degrading plasmid, pAC27. The *tfd*A probe from strain RASC (here called *rsc*A to differentiate it from *tfd*A from pJP4) was provided courtesy of Dr. Yuichi Suwa. Plasmid vectors containing the genes were digested with appropriate restriction enzymes, purified by gel electrophoresis, excised from agarose, and labeled with digoxygenin-11-dUTP from (Boehringer Mannheim) using the Random Primers Labeling Kit (Boehringer Mannheim) according to manufacturer's instructions. All hybridizations were carried out at 62°C. High, medium, and low stringencies were achieved by varying formamide concentrations in the hybridization buffer from 50%, 20% and 0%, respectively (Wahl et al. 1987). After hybridization, blots were washed in double strength wash buffer at room temperature for 5 min, then subjected to colorimetric detection using alkaline phosphatase with X-phosphate as substrate according to manufacturer's directions. Photographs of blots were made using Polaroid 55 positive negative film.

RESULTS

The diversity of 2,4-D genes from 32 bacterial strains of 2,4-D degraders (Table 1) was assessed using gene probe analysis. DNA from each of these strains was isolated and digested with *EcoRI*, separated by gel electrophoresis, and transferred to a nylon membrane. We used the technique of Wahl et al. (1987) for optimizing hybridization conditions for detecting genes with sequence similarities in the ranges of high (90-100%); medium (75-90%) and low(55-75%) sequence similarities (Figure 1).

The calculated similarity required for hybridization was confirmed experimentally by finding that the *tfdA* probe from pJP4 hybridized to strain RASC only under conditions of low stringency, which agrees with the 73% sequence similarity found for that *tfd* gene in this strain.

Nylon membranes containing DNA from each of the strains were hybridized at high, medium or low stringencies to each of the probes, *tfd*A, *tfd*B, *tfd*C, *clc*, and the *rsc*A probes (Table 2). The *tfdA* probe from RASC was used to hybridize the blots under low stringency conditions only.

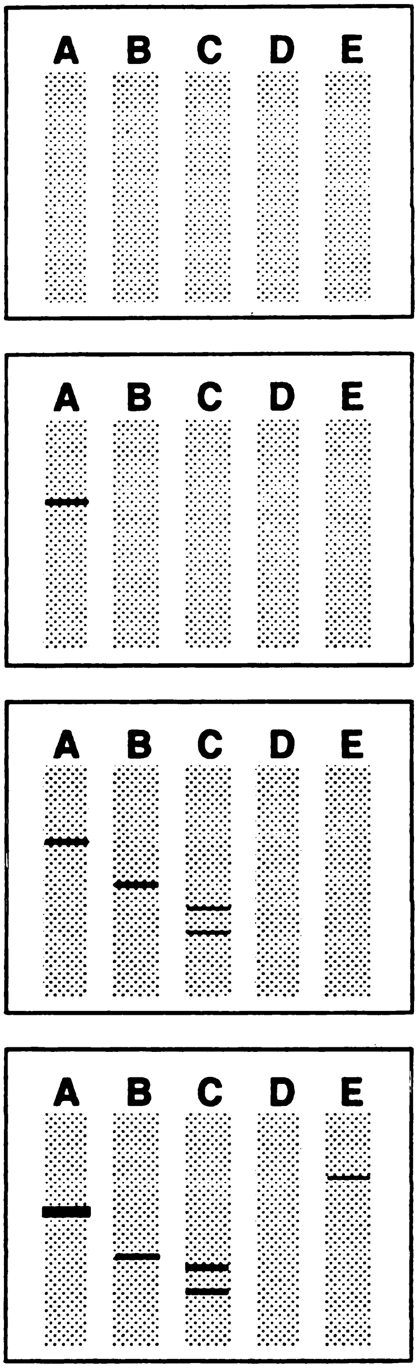
These strains could be grouped in two ways: either on the basis of hybridization stringency when hybridized with the probes, which indicates degree of similarity of the genes, or a more refined grouping based on restriction patterns, which may indicate a gene's similarity and location in the host genome. When restriction fragment sizes were considered along with the hybridization stringency patterns, at least 14 distinct 2,4-D gene patterns could be distinguished (Table 2). When grouped solely on the basis of hybridization intensity using the *tfd*A, B and C probes, eight unique groups

Table 1. 2,4-D degrading strains, their origins, references and REP groups.

Strain	Location	Source or Reference	*Proteobacteria* Most Similar To*:	Subgroup	REP**
JMP134	Australia	Don and Pemberton, 1981	*Alcaligenes eutrophus*	beta 2	U
EML159	Oregon	Amy et al, 1985	*Burkholderia* sp.	beta 2	U
EML157	Oregon	Amy et al, 1985			U
TFD39	Saskatchewan	Tonso et al., 1995	*Burkholderia* sp.	beta 2	U
K712	Michigan	Ka et al., 1994	*Burkholderia* sp.	beta 2	U
TFD9	Saskatchewan	Tonso et al., 1995	*Alcaligenes xylosoxidans*	beta 2	U
TFD41	Michigan	Tonso et al., 1995	*Alcaligenes eutrophus*	beta 2	5
TFD38	Michigan	Tonso et al., 1995	*Alcaligenes eutrophus*	beta 2	5
TFD23	Michigan	Tonso et al., 1995	*Rhodoferax fermentans*	beta 2	7a
RASC	Oregon	Amy et al, 1985	*Burkholderia* sp.	beta 2	U
TFD6	Michigan	Tonso et al., 1995	*Burkholderia* sp.	beta 2	1
TFD20	Michigan	Tonso et al., 1995	*Burkholderia* sp.	beta 2	1
BRI6001	Michigan	Greer 1990	*Burkholderia* sp.	beta 2	1
E2w1	Puerto Rico	Roberta Fulthorpe			1
TFD13	Michigan	Tonso et al., 1995	*Burkholderia* sp.	beta 2	1
TFD6	Michigan	Tonso et al., 1995			1
TFD18	Michigan	Tonso et al., 1995			1
TFD17	Oregon	Tonso et al., 1995			1
TFD21	Michigan	Tonso et al., 1995			1
TFD14	Michigan	Tonso et al., 1995			1
TFD15	Michigan	Tonso et al., 1995			1
TFD27	Michigan	Tonso et al., 1995			1
TFD2	Michigan	Tonso et al., 1995	*Burkholderia* sp.	beta 2	2
TFD4	Michigan	Tonso et al., 1995			2
TFD18	Saskatchewan	Tonso et al., 1995			2
TFD7	Michigan	Tonso et al., 1995			2
TFD31	Saskatchewan	Tonso et al., 1995	*Rhodoferax fermentans*	beta 1	7b
B6-9	Ontario	Fulthorpe	*Rhodoferax fermentans*	beta 1	U
2,4-D1	Montana	Robert Sanford	*Sphingomonas* sp.	alpha 4	U
B6-5	Ontario	Fulthorpe	*Sphingomonas* sp.	alpha 4	U
B6-10	Ontario	Fulthorpe	*Sphingomonas* sp	alpha 4	U
EML146	Oregon	Amy, et al., 1985	*Sphingomonas* sp.	alpha 4	U
K1443	Michigan	Ka, et al., 1994	*Sphingomonas* sp.	alpha 4	U
M1	French Polynesia	Roberta Fulthorpe	*Rhodopseudomonas paulustris*	alpha 1	U

* Strains related on basis of 16S rDNA analyses (chapter 2).

** Same number of REP class refers to strains with identical REP pattern. "U" indicates strains with unique REP patterns .



Total Genomic Digestion with *Eco*RI

High Stringency Hybrdization

Strain A > 90% Similar

Medium Stringency Hybrdization

Strains B and C 75 - 90% Similar

Low Stringency Hybrdization

Strain E 60 - 75% Similar
Strain D < 60% Similar

Figure 1. Strategy for hybridization at high, medium, and low stringencies.

Table 2. Hybridization patterns of 2,4-D degraders. Number(s) indicate restriction fragment sizes (sizes indicated only once for each group of strains). Fragments marked with *** hybridize at high stringency, those ** at medium stringency and those with * at low stringency. The symbol "ø" means no hybridization.

				*Eco*RI Fragment Sizes				
Strain	**Location**	**REP***	**Subgroup**	**tfdA**	**rscA**	**tfdB**	**tfdC**	**clc**
JMP134	Australia	U	beta 2	12***	12***	16***	16***	16***
EML159	Oregon	U	beta 2	8***	8***			8***
EML157	Oregon	U	nd					
TFD39	Saskatchewan	U	beta 2					
K712	Michigan	U	beta 2	6.3***	3.5***	3.5**	6*	6*
TFD9	Saskatchewan	U	beta 2	3.5***				
TFD41	Michigan	5	beta 2	4.5***	4.5***	20, 16***	20***	6*
TFD38	Michigan	5	beta 2	2.6***		4.1***	16, 6***	
TFD23	Michigan	7a	beta 2	7.2, 3.5***	nd	3.5*	6.3*	ø
TFD31	Saskatchewan	7b	beta 1	18*	14*	ø	ø	ø
B6-9	Ontario	U	beta 1	20, 15* 2.3*	20, 15*	ø	20*	20***
TFD6	Michigan	1	beta 2	9.5*	9.5***	9.5***	9.5***	9.5*
TFD20	Michigan	1	beta 2					
BRI6001	Quebec	1	beta 2					
TFD13	Michigan	1	beta 2					
TFD6	Michigan	1	nd					
TFD18	Michigan	1	nd					
TFD17	Oregon	1	nd					
TFD21	Michigan	1	nd					
TFD14	Michigan	1	nd					
TFD15	Michigan	1	nd					
TFD27	Michigan	1	nd					
E2w1	Puerto Rico	1	nd	11.3*	9.5***	9.5***	9.5***	ø
RASC	Oregon	U	beta 2	7*	7***	7, 3.5*	ø	ø
TFD2	Michigan	2	beta 2	9.5*	9.5*	9.5*	ø	ø
TFD4	Michigan	2	nd					
TFD18	Saskatchewan	2	nd					
TFD7	Michigan	2	nd					
2,4-D1	Montana	U	alpha 4	ø	nd	> 23, 8*	ø	ø
EML146	Oregon	U	alpha 4					
B6-5	Ontario	U	alpha 4	ø	nd	ø	ø	ø
B6-10	Ontario	U	alpha 4					
K1443	Michigan	U	alpha 4			8*		
M1	French Polynesia	U	alpha 1	ø	nd	12*	ø	ø

---

* REP group designation of each strain is indicated by number or by "U" which indicates unique REP group (Fulthorpe et al., submitted).

could be distinguished (Figure 2). Three of the strains did not hybridize to any of the probes.

Different *tfd* genes: Differences in hybridization stringencies of gene probes gives us an insight into differences in DNA sequences for these genes. For example, in the strains examined here with the *tfdA* probe from pJP4, three classes of gene sequences could be distinguished One class, hybridizing at high stringency, included strains JMP134, EML159, TFD39, TFD41, TFD 38, K712, TFD9 and TFD23. Another class of *tfdA* strains included TFD2, RASC, E2w1, TFD31, B6-9 and TFD6 and their siblings. These strains hybridized to *tfdA* from pJP4 only under low stringency conditions, indicating a theoretical sequence similarity between 55-73% (Wahl et al. 1987). Actual sequence data for the *tfd*A genes from strains TFD2, RASC, E2w1 and TFD6 showed that they are identical to each other and 73% similar to *tfd*A from pJP4 (chapter 4). The third class of strains, including strains EML148, 2,4-D1, B6-5, B6-10, K1443, and M1 did not hybridize to *tfdA* from pJP4 at all. None of the strains hybridized at medium stringency to *tfd*A.

On the basis of the RFLP patterns observed when probed with the *tfd*A gene probe, there were 10 distinct types of *tfd*A - containing strains. Those strains which had identical restriction fragment length polymorphisms, such as TFD6, TFD14, and TFD15 were assigned the same RFLP class designation. Not surprisingly, most strains produced the same restriction pattern when probed with *tfd*A from either pJP4 or from strain RASC.

Four types of *tfd*B genes could be resolved on the basis of hybridization signals: those that hybridized at high, medium, low, and not at all to *tfd*B. However, differences in RFLP patterns indicated 10 unique types of *tfd*B

	*tfd*A	*tfd*B	*tfd*C
JMP134, TFD41	■	■	■
K712, TFD9	■	▨	▒
TFD23	■	▒	▒
TFD31, B6-9	▒	▒	□
TFD6, TFD20	▒	■	■
RASC, TFD2	▒	▒	□
2,4-D1, M1	■	▒	□
B6-5, B6-10	□	□	□

Figure 2. Hybridization patterns with *tfd*A, B and C suggest a mosaic structure of these 2,4-D degrading strains. Solid boxes indicate high similarity to pJP4; striped boxes indicate medium stringency; stippled boxes indicate low stringency; and white boxes indicate that no hybridization was observed.

classes. A *tfd*B-like element was phylogenetically the most widely distributed type of tfd gene in the strains probed.

Three types of *tfd*C genes could be resolved on the basis of hybridization intensity: those that hybridized at high stringency, at low stringency and not at all to *tfd*C. By RFLP pattern differences, seven unique types of *tfd*C classes could be determined.

All but four of the strains used in this study hybridized to at least one of the probes used in this study. Only strains M1, B6-5, and B6-10 hybridized to none of the probes, indicating 2,4-D degradation gene sequences different from those of JMP134.

The hybridization patterns clearly showed that nearly identical *tfd*A genes are paired with different *tfd*B and *tfd*C genes in different strains (Figure 2). This mosaic structure of tfd genes can be clearly seen in strains JMP134, strain TFD6 and strain RASC. In this example, the *tfd*A gene in RASC and TFD6 are identical and different from JMP134. Interestingly, hybridization analysis shows that strains JMP134 and TFD6 share identical *tfd*B and C genes, which are different in strain RASC.

Comparison to 16S Phylogeny: Representatives of unique strains were compared to a phylogeny (chapter 2) of these strains made on the basis of 16S sequence comparisons (Figure 3). This figure illustrates that *tfd*A and C genes similar to those from pJP4 are found only in the beta and gamma *Proteobacteria*. Furthermore, the *tfd*B gene was detected at low stringency in only three of the alpha *Proteobacteria* .

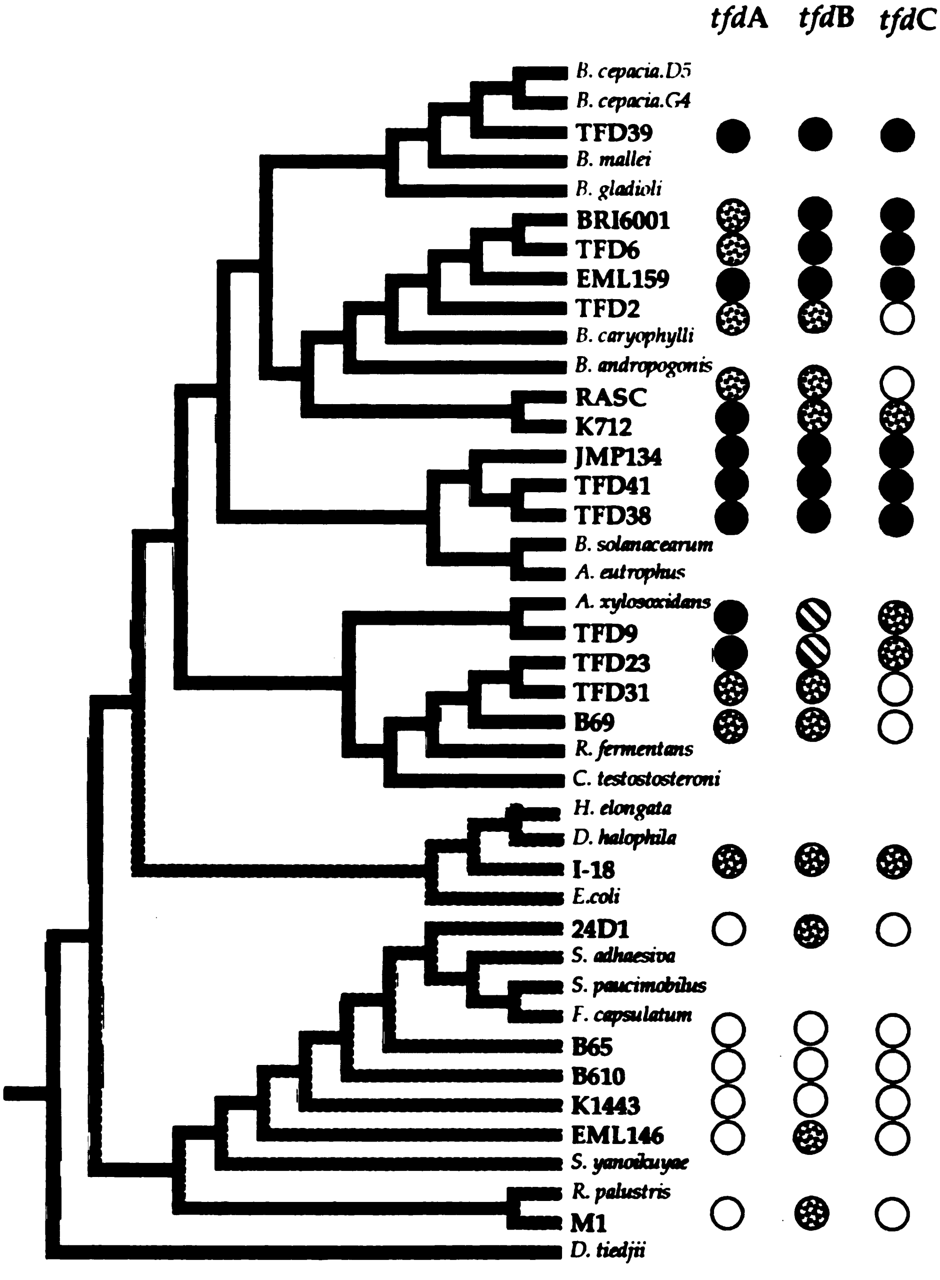


Figure 3. Hybridization patterns of *tfd* A,B and C probes from pJP4 to 2,4-D strains (in bold type) arranged in phylogenetic grouping (branch lengths not to scale). Solid dots indicate high similarity; striped dots indicate low similarity; stippled dots indicate low similarity; and open circles indicate no hybridization. Solid branches are members of the beta *Proteobacteria*, striped are gamma *Proteobacteria*, stippled are alpha *Proteobacteria* and the lone solid branch is delta *Proteobacteria*.

DISCUSSION

This study showed a greater diversity of 2,4-D degradation genotypes than has previously been reported. This diversity is partly due to 2,4-D degrading genes assembled with other 2,4-D genes in different combinations in different strains. This assembly if identical genes in novel arrangements has created a mosaic of genes in the 2,4-D degradation pathway. It appears that *tfd*A and *tfd*C genes similar those of strain JMP134 are phylogenetically limited to the beta and gamma *Proteobacteria*.

Diversity: High, medium, and low hybridization stringencies can detect a greater diversity of 2,4-D degrading genes which may be missed by high stringency hybridization alone. For example, using high stringency conditions alone, *tfd* genes in strains RASC, TFD2 and others would not be detected. Using a strategy of hybridizing at three different stringencies with *tfd*A, *tfd*B and *tfd*C, we found eight hybridization classes of 2,4-D degraders, representing a greater diversity of 2,4-D gene types than the four classes found by Ka et al. 1994.

We also found that the diversity of *tfd* hybridization patterns of these strains correlates fairly well to the diversity of REP classes (Fulthorpe, personal communication; Tonso et a. 1994). In all cases but one, unique REP classes gave unique hybridization classes. The one exception was E2w1, isolated from Puerto Rico, which belongs to REP class 1 along with 10 other strains (including TFD6) used in this study (Fulthorpe, personal communication; Tonso et al. 1994), but which has a hybridization pattern different from the others. The different fragment sizes containing *tfdA* in strains TFD6 and E2w1 may be explained either by gene rearrangement in the host or by a point mutation resulting in the loss of an *EcoRI* site.

Mosaic pathways: The results presented in this study indicate mixing of genes in the 2,4-D pathway between strains, which supports the notion that evolution has occurred by a process of molecular "tinkering" in which bits and pieces of DNA are spliced into new functional units (Jacob, 1977). For example, some strains such as TFD23, K712 and TFD9 contain *tfdA* genes with greater than 99% similarity to JMP134, yet contain genes for the third step in the pathway which are less than 60% similar to the *tfd*C gene from JMP134. Strain B6-9 also appears to have a gene that is > 90% similar to *clcA*, whereas its close relative, TFD31 does not.

Various pathways for 2,4-D degradation may have arisen by the splicing together of different genes in the pathway. For example, some *tfdA* genes highly similar to those in JMP134 are combined with low stringency *tfd*B genes, as is the case with TFD9 and K712. Another example is that *tfd*B and C genes highly similar to those of pJP4 are present in TFD6, but not in strain RASC, which appears to have a *tfdA* gene identical to that of TFD6, which has been shown to be the case (chapter 4). Among the strains that hybridize to *tfdA* at high stringency, strains TFD19, K712, and TFD 41 hybridize to *tfdB* , whereas B6-9 and TFD31 do not. Among the strains with *tfdA* genes nearly identical to strain RASC, only strains with RFLP patterns identical to TFD6 and E2w1, have *tfdB* genes nearly identical to JMP134, whereas strains RASC and TFD2 do not. The apparent rearrangements become even more mixed: strains TFD6 and E2w1 contain *tfd*C genes highly similar to those of pJP4, whereas RASC and TFD2 do not hybridize to this gene probe at all. It is as if

TFD6 and related strains evolved from the recombination of *tfd*A from a RASC-like strain and *tfd*B and C from a JMP134-like strain (Figure 4).

The recombination of *tfdA* , *tfdB,* and *tfdC* suggests that some of these genes may be in separate operons, as has been shown for the *tfdA,* the *tfdB* and the *tfdCDEF* operons of JMP134. This may be the case for those strains which hybridize to plasmid pJP4, including TFD41, K712, and TFD23. In these strains, the *tfdA* and *tfdC* genes are on different *EcoRI* fragments. The *tfdB* gene appears to be more closely situated to either the *tfd*A, in the case of K712, or to *tfd*C, in the case of TFD41 and JMP134. Others strains, in contrast, appear to have genes for the whole pathway in close proximity. For example, in strain RASC, the *tfdA* and *tfdB* genes are located on the same 7.0 kb *EcoRI* fragment, which is indeed the case as confirmed by Suwa et al. (1994) and Kamagata (in preparation) in sequence analysis of the *tfdA* and *tfdB* genes. Also, it appears that the entire pathway is contained within a single *EcoRI* fragments in strains TFD6 and in TFD2.

Gene transfer: The recombination of genes from apparently different sources may be explained by gene transfer. When the hybridization data presented here is compared with the phylogeny based on comparisons of partial 16S rDNA sequence data, it becomes apparent that similar tfd genes are found in phylogenetically diverse strains. One explanation for this is that the tfd genes have transferred between species.

It appears that gene transfer is limited to within deep phylogenetic divisions, since *tfd*A and *tfd*C genes are found only in the beta and gamma *Proteobacteria,* but not in the alpha *Proteobacteria.*

Similar 2,4-D genes found in different strains provide evidence of gene

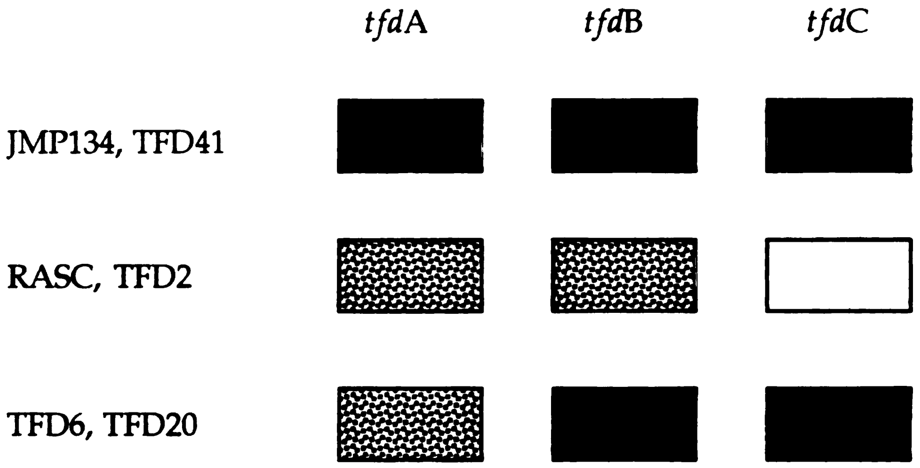


Figure 4. Mosaic pattern of *tfd* genes from three strains indicates recombination. The solid boxes represent genes that are highly similar to those found in JMP134 as determined by hybridization analysis in the case of *tfd*B and *tfd*C. The stippled boxes represent genes identical to those from RASC as determined by sequence analysis in the case of *tfd*A.

exchange. For example, strain JMP134, TFD 19, and TFD39 represent three unique strains based on FAME, Biolog, and REP patterns (Tonso et al. 1994; this paper) and on 16S rDNA sequence data (chapter 2). However, all three of these strains appear to carry identical 2,4-D genes. This is not surprising, since plasmid pJP4 from JMP134 is a broad host range IncP1 plasmid which has been shown to transfer into all Gram negative bacteria except *Bacteroides* (Don and Pemberton, 1981; Harker et al., 1989; Friedrich et al. 1983). These strains differ by more than 9% in 16S rDNA sequences, suggesting that these strains diverged more than 100 million years ago (Moran, 1993). However, identical RFLP patterns in the 2,4-D genes of these strains which represent up to at least 40 kb of conserved DNA sequence (cumulative sizes of hybridized fragments) indicate that the 2,4-D genes have changed very little, probably less than one percent (Matheson et al. 1994; Suwa et al. 1994; and Moran et al. 1993). Because the similarity of the genes is so high and the divergence of the strains is so great, this provides some evidence for interspecies gene transfer *in situ*. This hypothesis is treated more explicitly in the following chapter.

Different *tfd* genes have been found in identical strains, which further suggests that gene exchange has occurred. Strain TFD23 and TFD31 are identical at the level of 16S rDNA (chapter 2), yet they differ from one another in hybridization patterns for 2,4-D genes. Because the host organisms are so closely related, but the genes have different restriction patterns and sequence similarities to pJP4, this is evidence of gene transfer.

Gene flow of the *tfd* genes may be restricted because of some evolutionary constraints since the hybridization classes clustered into two broad categories based on phylogeny: those that hybridize with the *tfdA* probe and those that do not. All those which hybridized to *tfdA* were members of the beta and gamma groups of *Proteobacteria* and those that did not were members of the alpha subgroup of *Proteobacteria.* Furthermore, all but three of these beta *Proteobacteria* that hybridize to *tfdA* are most closely related to the genus *Burkholderia* (Yabuuchi et al. 1992).

All of the strains that did not hybridize to *tfd*A were found to be members of the alpha group of *Proteobacteria,* according to the classification of Woese (1979) and all but one of these were found to be closely related to the genus of *Sphingomonas.* Interestingly, some members of the *Sphingomonas* group of 2,4-D degraders hybridized to the *tfd*B gene probe at low stringency, indicating a gene with a sequence similarity of about 60% to that of *tfd*B.

The one strain that did not hybridize to *tfdA* and was not closely related to the genus *Sphingomonas* was strain M1. This oligotrophic strain was most closely related (97%) to the photosynthetic bacterium *Rhodopseudomonas palustris,* which is a member of the alpha class 2 of *Proteobacteria.* Strain M1 may contain tfd genes which differ significantly from pJP4 and from the *Sphingomonas* strains, though this was not detectable in these hybridization analyses.

This work has shown that the diversity of 2,4-D pathways looks like a mosaic of genes derived from different places. "Evolution does not produce novelties from scratch," wrote Francois Jacob in 1977. "This paramount reshuffling of genetic elements provides tremendous potentialities of adaptation." It appears that much of the reshuffling genes in 2,4-D pathways has been a result of interspecies gene transfer; however, this transfer appears to be limited to within deep, phylogenetic groupings.

ACKNOWLEDGEMENTS

I thank Dr. Roberta Fulthorpe for her collaboration in the hybridization work and for the use of her strains. I also thank Dr. William Holben for 2,4-D strains and *tfdA, tfdB* and *tfdC* gene probes and Dr. Jong-Ok Ka, Dr. Nancy Tonso, Dr. Penny Amy and Dr. Charles Greer for the use of their strains. Thanks to Yuichi Suwa for the use of his gene probe from RASC. I also thank the people in the Research on Microbial Evolution Laboratory for discussion. This work was supported by the National Science Foundation grant number BIR912006 and by Japan Research and Development Corporation grant number 1.

Chapter Four

Interspecies Gene Transfer in the Evolution of 2,4-Dichlorophenoxyacetic Acid-Degrading Bacteria in Nature and in the Laboratory

SUMMARY

Many phylogenetically diverse 2,4-D degrading bacteria exist in nature, yet it is not known how this diversity of organisms evolved. In this study, the role of gene transfer in the evolution of this diversity was examined. To do this, the phylogeny inferred from their 16S ribosomal DNA sequences was compared to a dendrogram of sequences for the *tfdA* gene, which encodes the first enzyme in the 2,4-D degradation pathway. DNA molecules from 2,4-D degrading strains of *Proteobacteria* were amplified by PCR using primers made from conserved regions of the previously sequenced *tfd*A genes from *Alcaligenes eutrophus* JMP134 and strain RASC. Three classes of *tfdA*-like sequences were found in these strains. One class of *tfd*A sequences was identical (or nearly so) to that from JMP134 and the other two classes each differed by 23.0% and 22.4% from JMP134 and 6.4% from each other. The tree derived from these *tfdA* genes differed dramatically from the phylogeny of the 16S DNA from the same organisms, providing evidence of interspecies horizontal gene transfer. Furthermore, identical *tfdA* gene sequences were distributed among several deep branches of the beta and gamma subgroups of *Proteobacteria*. To discover whether these strains could mobilize 2,4-D genes *in vitro*, they were mated with a 2,4-D^- recipient strain, *Burkholderia cepacia* D5. Several strains transferred genes which expressed the ability to degrade 2,4-D. Those that rapidly transferred and expressed the 2,4-D phenotype included strains with *tfd*A sequences greater than 99% similar to *tfdA* from JMP134. Those strains that did not transfer the ability to degrade 2,4-D, or did so only at extremely low frequencies, had *tfdA* gene sequences that differed by up to 23% from JMP134.

INTRODUCTION

How did the diversity of 2,4-dichlorophenoxyacetate-degrading microorganisms evolve? This is more than an esoteric question, for to learn how xenobiotic degraders evolved may assist in better management of xenobiotic pollution. One hypothesis to explain the evolution of diversity is horizontal gene transfer between species (Wilson, 1977).

Many studies have examined interspecies gene transfer (Brisson-Noel et al., 1988; Chatterjee and Chatterjee, 1987; Datta and Hedges, 1972; Fulthorpe et al., 1992; Mazodier et al., 1989; Natarajan and Oriel, 1991; and Trieu-Cout et al., 1987) and many review articles and books have been written on this topic (Coughter and Stewart, 1989; Fry and Day, 1989; Levy and Marshall, 1988; Levy and Miller, 1989; Stotzky,1989; Stotzky and Babich, 1986; and Trevors et al., 1986) yet few studies have examined gene transfer between different phylogenetic groups to gain an understanding of the evolution of organisms in nature (Wilson, 1977).

Horizontal gene transfer of the 2,4-D degrading plasmid pJP4 has been documented in laboratory studies (Don and Pemberton, 1981; Harker et al., 1989; Kukor et al., 1989; Friedrich, 1983), yet it is not known to what extent other 2,4-D genes have been exchanged in nature resulting in the evolution of new strains. The aim of this research was to assess the role of horizontal gene transfer in the evolution of 2,4-D degrading strains.

To demonstrate that gene transfer has occurred in nature involves comparing the phylogenies of two separate genes in a group of organisms, a "phylogenetic congruency test" (Wilson, et al., 1977) or by determining that identical genes are found in widely different strains. Within the same strains, different genes having different phylogenies indicates that horizontal gene transfer has occurred (Dykhuizen and Green, 1991; Wilson et al., 1977).

To test the hypothesis that gene exchange was responsible for generating the diversity of 2,4-D degrading strains, I performed a phylogenetic congruency test comparing 16S rDNA sequences, discussed previously (chapter 2), with the *tfdA* gene sequences discussed here.

The *tfdA* gene was chosen for this because it is the only gene in the 2,4-D pathway known to be used exclusively in 2,4-D degradation, unlike *tfd*B and *tfd*C, which have homologs found in other degradative pathways. The *tfd*A gene encodes an alpha-ketoglutarate-dependent 2,4-D dioxygenase (Fukumori and Hausinger, 1993) and is the first enzyme in the 2,4-D pathway which converts 2,4-D into 2,4-dichlorophenol and glyoxylate. This 861 bp gene, first sequenced from *Alcaligenes eutrophus* JMP134 (Streber et al., 1987) has identical sequences to those of strains EML159 and TFD38, (Fukumori, personal communication) and TFD41 (Nakatsu, personal communication). (TFD38 is identical to strain TFD41 by REP-PCR, by fatty acid methyl ester analysis and substrate utilization profiles (Tonso, et al., 1995) and by hybridization analysis (chapter 3)). These *tfd*A sequences differ by 25% from *Burkholderia* strain RASC (Suwa, 1994) and *Burkholderia* strain TFD6 (Matheson, 1994) which contain identical *tfd*A sequences.

Although the *tfdA* gene of *Alcaligenes eutrophus* JMP134 is on a plasmid, other *tfdA* genes appear to be chromosomally located, including RASC (chapter 5; Suwa, et al, in prep); TFD6 (Matheson, et al., 1994); and BRI6001 (Greer, et al., 1990) which appears to be identical to TFD6 (chapter 3).

Furthermore, Ka et al. (1994) found that 25% of the *tfd*A hybridizing strains in his collection appeared to have this gene located on the chromosome.

The 2,4-D degraders used in this study belong to the alpha, beta and gamma subgroups of *Proteobacteria,* a diverse group of Gram negative bacteria (Murray et al., 1990). The beta subgroup of 2,4-D degraders includes strains related to *Alcaligenes eutrophus, Burkholderia cepacia, Alcaligenes xylosoxidans,* and *Rhodoferax fermentans.* A single member of the gamma *Proteobacteria* most closely related to the genus *Halomonas* was also examined. An additional strain used in this study clustered with the genus *Sphingomonas,* a member of the alpha *Proteobacteria.* It was found that all strains belonging to the beta and gamma subgroup of *Proteobacteria* hybridized to *tfdA,* whereas none of the strains clustering with the alpha *Proteobacteria* hybridized to *tfd*A (chapter 3; Fulthorpe, et al., 1995).

In this chapter, I describe experiments designed to test the hypothesis that the genetic diversity of 2,4-D degrading organisms is due, in part, to interspecies gene transfer. I examined the phylogenies of the *tfd*A and 16S rDNA genes from a group of 2,4-D degrading strains, and found that their phylogenies were different, indicating that gene exchange had occurred in nature. To further test the hypothesis that gene transfer occurs among this group of 2,4-D degrading strains, *in vitro* mating experiments were conducted with these strains. These experiments showed that 2,4-D genes were transferred at relatively high frequencies from strains which hybridized to plasmid pJP4 and at very low to undetectable frequencies in those strains which did not hybridize to plasmid pJP4.

MATERIALS AND METHODS

Media and growth conditions: 2,4-D degrading strains were grown in 2,4-D MMO containing 500 ppm 2,4-D (Sigma) in a mineral salts medium (Stanier, et al., 1966). When appropriate, antibiotics including kanamycin, carbenicillin, and bacitracin, were added at concentrations of 50 µg/ml. PTYG contained 0.25 g peptone, 0.25 g tryptone, 0.5 g yeast extract and 0.5 g glucose, 0.03 g magnesium sulfate, and 0.003 g calcium chloride per liter. Trypticase soy broth (TSB) was obtained from Difco (Detroit, MI). All cultures were grown at 30°C. Broth cultures were aerated by shaking on Innova 4300 shaker (New Brunswick Scientific Co., New Brunswick, NJ) at 200 rpm.

Bacterial strains: The strains used in the phylogenetic congruency tests are described in chapter 2 and listed in Table 1. Additional 2,4-D degrading strains used in mating experiments are listed in Table 2. All of the 2,4-D degrading strains are sensitive to 50 µg/ml each of the antibiotics kanamycin, carbenicillin, and bacitracin. *Burkholderia cepacia* D5, which contains neomycin phosphotransferase genes carried on Tn5, was supplied by Ron Olsen (Walch and Ballou, 1983) and is resistant to 50 µg/ml each of kanamycin, bacitracin and carbenicillin.

Sequencing of *tfdA*-like genes: Conserved primers internal to *tfdA*, designated TVL and TVR, were prepared by Tatiana Vallaeys and Alice Wright (Wright et al., in prep). These primers were used to amplify sequences by PCR from the following organisms: TFD2, B6-9, K712, TFD9, TFD39, TFD31, TFD23, I-18, and K1443. Negative controls (water) were used to insure no contamination of PCR products. Amplified DNA fragments were size fractionated on a 1.5% electrophoresis gel. Primers were removed from the PCR amplified DNA using the GeneClean Kit (Bio101, La Jolla, California). Approximately 20 pmol of primer TVL was added to 150 ng amplified DNA in 20µl total reaction volume. DNA was sequenced using the Applied Biosystems Model 373A automated sequencer (Perkin Elmer Cetus) at Michigan State University Sequencing Facility.

Mating studies: A ten-fold excess of recipient strains *B. cepacia* D5 in broth (~ 10^9 cells) was mixed with donor cells and centrifuged briefly to concentrate. Mixed cells were then pipetted either onto a 0.2 µm filter, which was placed onto nutrient agar plates, or were pipetted directly onto selective agar media. Cells were allowed to incubate together overnight and portions were then resuspended in H_20 and serially diluted before plating, and other portions were streaked directly onto selective plates of 2,4-D kanamycin, carbenicillin and bacitracin. JMP134 was mated with *B. cepacia* D5 as a positive control. The recipient alone and the 2,4-D degrading strains alone were carried through the mating procedures as negative controls.

2,4-D biodegradation: Disappearance of 2,4-D was monitored by HPLC. Supernatants were centrifuged at 14,000 rpm and filtered through 0.2 µ filters and were then analyzed by HPLC on Lichrosorb Rp-18 column (Anspec Co., Ann Arbor) using 60% methanol and 40% percent 0.1% H_3PO_4 as eluant. 2,4-D was detected at 230 nm.

Hybridization: DNA from the entire pJP4 plasmid and the *npt*II gene of Tn5 were labeled using random hexanucleotides incorporating (∞-32P)dCTP (3,000 Ci/mmol; New England Nuclear, Boston, MA). Hybridizations were done under low stringency conditions using 50% formamide and Denhardts solution (Maniatis, et al., 1985) at 42°C.

RESULTS

Strains: The strains chosen for comparison of 16S and *tfd*A DNA sequences are listed in Table 1. With the exception of strain K1443, which contains a 16S rRNA sequence belonging to the alpha *Proteobacteria* and which did not hybridize to *tfdA*, all strains are members of the beta and gamma *Proteobacteria* (chapter 2) and all hybridize to *tfd*A from pJP4 at either high, medium or low stringency (chapter 3). All strains were shown to be unique either by 16S rDNA sequences, by unique repetitive extragenetic palindromic (REP) PCR patterns (Fulthorpe et al., 1995), and/or by unique hybridization patterns with probes from internal regions of the *tfd*A, *tfd*B, or *tfd*C genes encoding the first three reactions in the 2,4-D pathway (chapter 3). These strains were isolated from diverse locations in Australia, Oregon, Saskatchewan, Ontario, and Michigan.

Sequencing of *tfdA*-like genes: Conserved primers internal to the *tfdA* gene were used to amplify DNA from strains TFD2, B6-9, K712, TFD9, TFD39, TFD23, TFD31, I-18, and K1443. These amplified DNA fragments were approximately 320 nucleotides in length as determined by size fractionation on a 1.5% electrophoresis gel. This is the same size as the *tfdA* gene fragment from pJP4 and strain RASC.

Sequences of *tfd*A DNA were initially aligned in SeqEd (ABI) and were imported into MacClade for further analysis. Additional *tfdA* sequences from JMP134 (GenBank accession number M16730), from strain RASC (courtesy

Table 1. Strains used for comparison of *tfd*A and 16S rDNA sequences.

Strain	Location	Class*	*tfdA* Reference	pJP4**	Most Similar To:	*Proteobacteria* Subgroup
JMP134	Australia	I	Don and Pemberton, 1981	+	*Alcaligenes eutrophus*	Beta 2
EML159	Oregon	I	Amy et al, 1985	+	*Burkholderia* sp.	Beta 2
TFD39	Saskatchewan	I	Tonso et al., 1995	+	*Burkholderia* sp.	Beta 2
K712	Michigan	I	Ka et al., 1994	+	*Burkholderia* sp.	Beta 2
TFD9	Saskatchewan	I	Tonso et al., 1995	+	*Alcaligenes xylosoxidans*	Beta 2
TFD41	Michigan	I	Tonso et al., 1995	+	*Alcaligenes eutrophus*	Beta 2
TFD23	Michigan	I	Tonso et al., 1995	+	*Rhodoferax fermentans*	Beta 2
RASC	Oregon	II	Amy et al, 1985	-	*Burkholderia* sp.	Beta 2
TFD6	Michigan	II	Tonso et al., 1995	-	*Burkholderia* sp.	Beta 2
TFD2	Michigan	II	Tonso et al., 1995	-	*Burkholderia* sp.	Beta 2
TFD31	Saskatchewan	III	Tonso et al., 1995	-	*Rhodoferax fermentans*	Beta 1
B6-9	Ontario	III	Fulthorpe et al., 1995	nd	*Rhodoferax fermentans*	Beta 1
I-18	Oregon	III	Malteseva et al., 1995	nd	*Halomonas* genus	Gamma
K1443	Michigan	ø	Ka et al., 1994	-	*Sphingomonas* sp.	Alpha 4

* Classification of *tfd*A genes is given in text.

** A "+" indicates this strain hybridized to plasmid pJP4 at high stringency. A "-" indicates no hybridization occurred, and "nd" indicates that hybridization was not determined.

of Alice Wright and Yuichi Suwa), from EML159 (courtesy of Fumiyasu Fukumori) and from TFD41 (courtesy of Cindy Nakatsu) were added to the alignment (Figure 1). Three classes of *tfdA* gene sequences were found: class I included JMP134, EML159, TFD41 and strain TFD39 which differed by only one nucleotide from JMP134, and strains TFD23, K712, and TFD9, which differed from JMP134 by 1 base pair. The class II *tfd*A sequence was identical to that found in RASC and TFD6 and included TFD2. The *tfd*A sequence in this class differed by 23% from class I. (Note that this percentage is less than the 25% difference when the entire *tfd*A sequence is taken into account.) Class III *tfd*A sequences, which included strains TFD31 and B6-9, differed by 22.4% from class I and 6.4% from class II. The alignment in Figure 1 includes the *tfd*A sequences from JMP134, TFD39, and TFD23 as representatives of class I, RASC as representative of class II and B6-9 as representative of class III.

It is interesting that both class II and III *tfd*A genes differed from each other and from class I in the same nine sites, indicating probable hypervariable hot spots in the wobble position.

Strain K1443, an alpha *Proteobacterium* which did not hybridize to *tfd*A from JMP134 nor from strain RASC, (Ka, et al., 1994; and chapter 3) yielded a 320 bp PCR band, although its intensity was only half that of the other strains. However, it is not known whether this amplified DNA from K1443 was from a functional *tfd*A analog, since it could not be aligned to the other *tfd*A genes using SeqEd or other sequence alignment software, including Sequencher from Genecodes and BLASTN and BLASTX (Altschul, et al., 1990) from NCBI. Because the amplified fragment from K1443 did not hybridize to *tfdA* from pJP4 even at low stringency, the *tfdA* nucleotide

```
[                   10        20        30        40        50]
[                    .         .         .         .         .]
JMP134      CATCACCAGATCTCCCACGTTCCAGCGATGCCGGTACACGAATTCCCGCT   [50]
TFD39       ..................................................   [50]
TFD23       ..................................................   [50]
B69         ......G........G..T.......T......A.....A..C.T...T.   [50]
RASC        ......A.....C..G..T.......T......A..G..A..C.T.G.T.   [50]

[                   60        70        80        90       100]
[                    .         .         .         .         .]
JMP134      GTGTCGCGTGCTCGAGAAGCTCCGCAAGCAGCATCCGGCCTTCGGCCACC   [100]
TFD39       ..................................................   [100]
TFD23       ..................................................   [100]
B69         .C....................A..C........G..A........G..G   [100]
RASC        .C.....A..............T...........G..A........G..G   [100]

[                  110       120       130       140       150]
[                    .         .         .         .         .]
JMP134      GGAAGGCCTTCGACGTGGCTCGCGTGCGCGCCGATGAAGAGAAACTTGCG   [150]
TFD39       ..................................................   [150]
TFD23       ...................C..............................   [150]
B69         ...C....C....T...A.C...A..A..C.....A..A...........   [150]
RASC        ...C.........T...A..A..A..A..C.....A..A...........   [150]

[                  160       170       180       190       200]
[                    .         .         .         .         .]
JMP134      CCCGGAGCCGGCGTGGGTTCGAACCAGCGGCCAGTTGACCGGCGGCATGG   [200]
TFD39       ..............................A...................   [200]
TFD23       ..............................A...................   [200]
B69         T..A..T..C........G..G.....T.....AC.C.....A.....C.   [200]
RASC        T..A..T..C........G..G.T..........C.C.....A.....C.   [200]

[                  210       220       230       240       250]
[                    .         .         .         .         .]
JMP134      CATTGCGTTGCGCTTCCGAATAGTCGGTGTCGCCGAGCAGGAAGCGGGAG   [250]
TFD39       ..................................................   [250]
TFD23       ..................................................   [250]
B69         ..........ACT...A........C..A.....A..A.T.........A   [250]
RASC        ..........ACT...A.....C..T........A..A.TA........A   [250]

[                  260       270       280       290       300]
[                    .         .         .         .         .]
JMP134      TTCAGTGCGTAGTGCTCGGCACGCAGCCCTTCCAACTCGGATTGGAGGTC   [300]
TFD39       ..................................................   [300]
TFD23       ...................................T..............   [300]
B69         .....C...........T..G....AT..C.G..GT..CTTC.T..AA..   [300]
RASC        .G...C...........T.......AT..C.G..GT..CTTC.T..AA..   [300]

[                  310 ]
[                    . ]
JMP134      CCGAGGCAGCGCG   [313]
TFD39       .............   [313]
TFD23       .............   [313]
B69         .TCG..G..AT..   [313]
RASC        .TCG..A..AT..   [313]
```

Figure 1. Alignment of 313 nucleotides of internal fragments of *tfd*A genes from representative strains. Nucleotides identical to *tfdA* from pJP4 are represented by periods.

sequence in K1443, if present, is probably less than 55% similar to that of pJP4.

A distance matrix was prepared from this alignment using 313 aligned nucleotides. Only JMP134, TFD39, TFD23, RASC and TFD31 were used to represent the other identical strains (Table 2).

A dendrogram of the *tfdA* genes was prepared from the aligned sequences using maximum parsimony with MacClade (Figure 2). This shows that the *tfd*A genes of JMP134, TFD39, TFD23, TFD9, K712, EML159 and TFD41 are all nearly identical. The class II and class III *tfdA* genes are more closely related to each other than either is to the class I *tfd*A gene. Because there is no outgroup to the *tfd*A sequences, a rooted phylogeny could not be made, since it not known whether the ancestral *tfd*A gene more closely resembles that from JMP134, RASC or TFD31.

The different classes of *tfd*A gene were plotted on a map of North America to show the geographic distribution of these genes. It is interesting to note that identical *tfd*A sequences were found in organisms geographically remote from each other (Figure 3).

Phylogenies are incongruent: The 16S sequence data described in chapter 2 were condensed to construct a phylogenetic tree using only those strains for which *tfd*A sequence data is presented above. The most parsimonious tree constructed using PAUP contained strain K1443 as an outgroup. This tree has 13 strains with 10 unique 16S rDNA sequences, each differing by more than 2 - 5%, indicating a high diversity of 16S rDNA for these strains. This tree was then compared to the tree derived from *tfd*A sequence data (Figure 4).

A cursory observation of the most parsimonious phylogenetic trees for

Table 2. Distance matrix of *tfd*A-like genes from strains JMP134, TFD39, TFD23, RASC and TFD31. Numbers above diagonal represent percent similarities; numbers below diagonal represent number of base pair differences in 313 nucleotides.

Strain	**JMP134**	**TFD39**	**TFD23**	**RASC**	**B6-9**
JMP134	–	99.7%	99.0%	77.0%	77.6%
TFD39	1	–	99.4%	76.7%	77.3%
TFD23	3	2	–	76.7%	80.0%
RASC	72	73	73	–	93.6%
B6-9	70	71	69	20	–

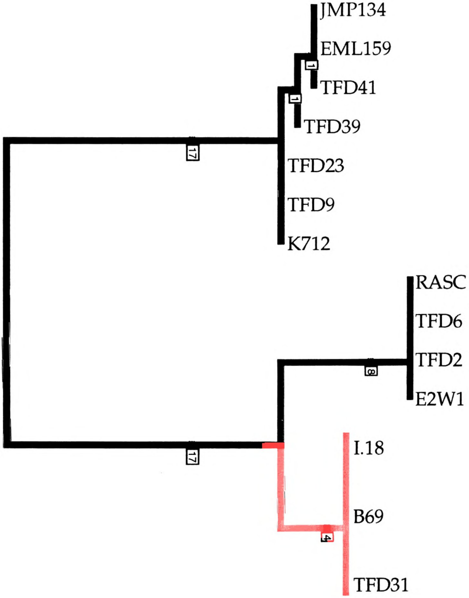


Figure 2. Dendrogram of 2,4-D degrading strains based on *tfd*A sequences. Numbers on branches indicate numbers of unambiguous base pair changes.

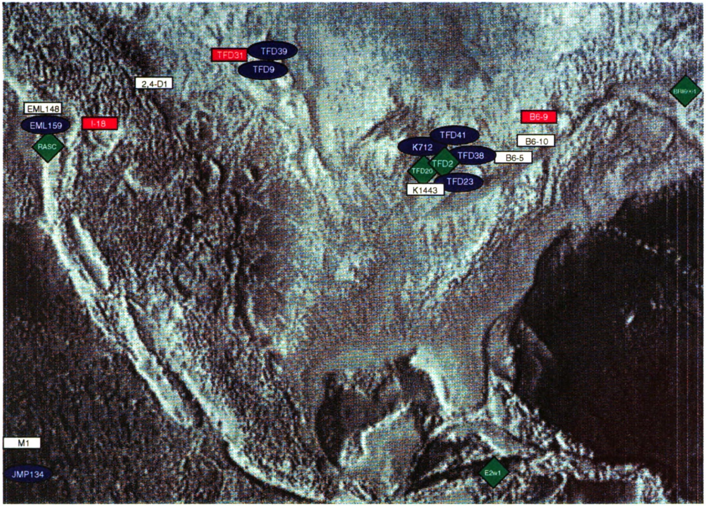


Figure 3. Geographic distribution of *tfd*A genes. White rectangles indicate strains with *tfd*A sequences less than 50% similar to *tfd*A from JMP134. JMP134 is from Australia and M1 is from French Polynesia. Red rectangles indicate strains with *tfd*A like B6-9; green diamonds indicate *tfd*A like RASC, and blue ovals indicate genes like that of JMP134.

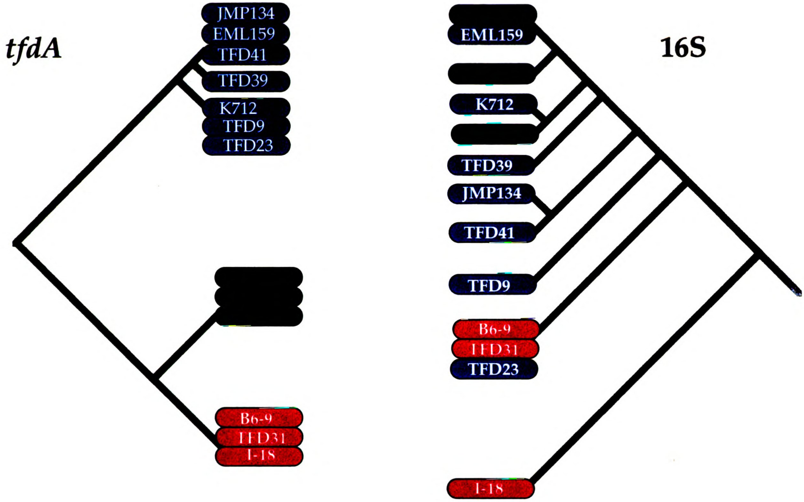


Figure 4. Phylogenetic incongruency for *tfd*A genes and 16S rDNA from diverse 2,4-D degrading bacteria. A dendrogram for *tfd*A is on the left and one for 16S is on the right. Colors indicate type of *tfd*A sequence.

16S rDNA and for *tfdA* shows that the two trees are clearly different. For one thing, the unrooted tree for *tfd*A shows only three, deeply divided branches, representing three *tfd*A classes and five unique sequences, whereas the phylogeny for 16S for the same strains show 10 unique sequences with fairly deep branches, representing sequence differences between 2 and 18 percent.

Another way to show the relationship of *tfd*A genes to 16S rDNA was to observe the distribution of *tfd*A classes among the branches of the 16S rDNA phylogenetic tree. Figure 5 clearly shows that identical *tfd*A genes are distributed throughout the beta and gamma *Proteobacteria*, and that adjacent branches don't necessarily contain identical genes, as would be expected if no interspecies gene transfer had occurred. Figure 5 shows several distantly related strains (as determined by 16S rDNA comparisons) contain identical *tfd*A genes. For example, strain RASC and TFD2 contain identical *tfd*A sequences, yet their 16S sequences differ by 7%. A more dramatic incongruency is seen in the case of TFD9 and TFD23. Although their *tfdA* genes are identical, these strains differ in 45 of 283 16S rDNA nucleotides, or 16%. The degree of divergence in 16S genes is even more striking when one considers that the highly conserved 16S genes probably evolve at a much slower rate (Moran et al., 1993; Wilson et al., 1977) than the *tfd*A genes.

Sometimes gene duplication within strains is a problem in determining phylogenies (Doolittle and Brown, 1994) though this was not a concern in this study. For example, it could be argued that two or more types of *tfdA* genes could be found in individual strains, thereby confounding the argument for gene transfer. However, this was not the case as supported by two lines of evidence. First, PCR amplifications were made on total genomic

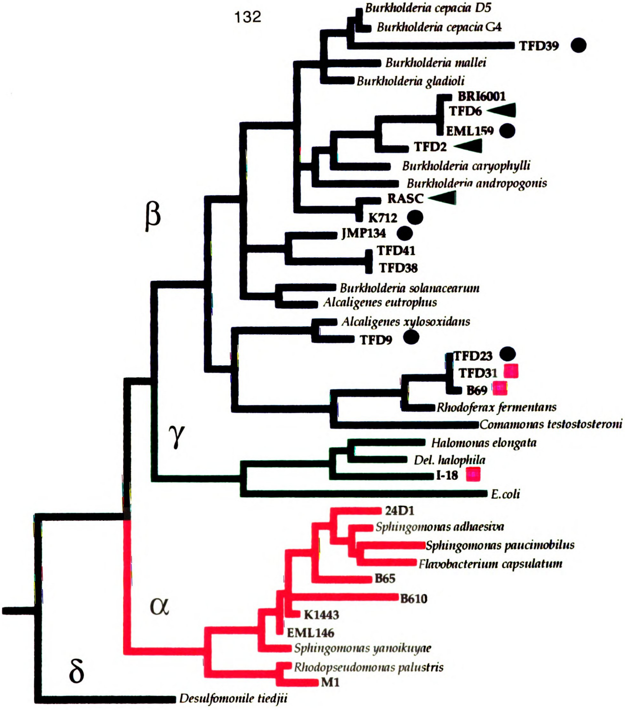


Figure 5. Comparison of *tfd*A genes to phylogeny of 16S rDNA from 2,4-D degrading bacteria, indicated in bold letters, and reference strains, indicated in italics. Colored lines indicate phylogenetic groups of *Proteobacteria*: blue are beta, green are gamma, red are alpha, and black is delta *Proteobacteria*. Types of *tfd*A genes are indicated as either blue dots, green arrows, or red squares.

DNA, and if there were duplicate, differing *tfd*A genes, sequencing would have revealed ambiguous data, which was not the case. Secondly, only one type of *tfd*A gene was observed for each strain in hybridizations studies (discussed in chapter 3).

***In vitro* transfer of 2,4-D genes:** To determine whether these 2,4-D degrading strains could transfer the ability to degrade 2,4-D in the lab, mating experiments were performed using *B. cepacia* D5 as a recipient strain. In addition to the strains used for the sequence comparisons, other related strains were added to test for their ability to transfer 2,4-D genes. These included strains that hybridized to plasmid pJP4 and those that did not (Matheson, 1994; Tonso, et al., 1995). To determine whether putative transgenic strains could completely mineralize 2,4-D, they were grown in 500 ppm 2,4-D medium and 2,4-D disappearance was tested by HPLC. All transgenic strains completely degraded 2,4-D as indicated by its disappearance.

Only some of the strains transferred and expressed the 2,4-D phenotype. Interestingly, only those strains which had class I *tfd*A genes and which also hybridized to plasmid pJP4 could transfer the 2,4-D phenotype to *Burkholderia cepacia* D5. Those strains that belonged to class II or class III or did not hybridize to pJP4 neither transferred nor expressed the 2,4-D phenotype to *B. cepacia* D5 (Table 3), with the exception of strain RASC, discussed below. The inability of the class II and class III *tfd*A strains to transfer and express the 2,4-D genes may have been a result of the particular conditions used here, such as an unsuitable recipient strain. However, extensive mating experiments were conducted with one of these class II strains, RASC (described in next chapter), which failed to transfer the 2,4-D

Table 3. Strains used for mating studies and results of in vitro 2,4-D phenotype transfer.

Strain	Location	Reference	pJP4 Hyb*	Transfer@
JMP134	Australia	Don and Pemberton, 1981	+	+
EML159 (19)	Oregon	Amy et al, 1985	+	+
TFD39	Saskatchewan	Tonso et al., 1995	+	+
K712	Michigan	Ka, et al., 1994	+	+
TFD9	Saskatchewan	Tonso et al., 1995	+	+
TFD41	Michigan	Tonso et al., 1995	+	+
TFD23	Michigan	Tonso et al., 1995	+	+
TFD31	Saskatchewan	Tonso et al., 1995	-	-
B6-9	Ontario	Fulthorpe et al., 1995	nd	nd
RASC	Oregon	Penny Amy	-	+
TFD6	Michigan	Tonso et al., 1995	-	-
TFD2	Michigan	Tonso et al., 1995	-	-
K1443	Michigan	Ka et al., 1994	-	-
EML146	Oregon	Amy et al, 1985	-	-
2,4-D1	Washington	Rob Sanford	-	-
TFD38	Michigan	Tonso et al., 1995	+	+
TFD20	Michigan	Tonso et al., 1995	-	-
TFD33	Michigan	Tonso et al., 1995	+	+
24L	Michigan	Tonso et al., 1995	-	-
TFD34	Michigan	Tonso et al., 1995	-	-
TFD27	Michigan	Tonso et al., 1995	-	-
TFD21	Michigan	Tonso et al., 1995	-	-
TFD13	Michigan	Tonso et al., 1995	-	-
TFD5	Michigan	Tonso et al., 1995	-	-
TFD8	Michigan	Tonso et al., 1995	-	-
TFD4	Michigan	Tonso et al., 1995	-	-
TFD22	Michigan	Tonso et al., 1995	-	-
TFD14	Michigan	Tonso et al., 1995	-	-
TFD15	Michigan	Tonso et al., 1995	-	-
TFD12	Michigan	Tonso et al., 1995	-	-
26D	Saskatchewan	Tonso et al., 1995	+	+
TFD18	Saskatchewan	Tonso et al., 1995	-	-
TFD29	Saskatchewan	Tonso et al., 1995	+	+
TFD42	Saskatchewan	Tonso et al., 1995	+	+
EML148	Oregon	Amy et al, 1985	-	-

* Strains which hybridized to pJP4.

@ Strains which could transfer and express the 2,4-D phenotype in *B. cepacia* D5.

phenotype to a 2,4-D- sibling, suggesting that host incompatibility was not necessarily responsible for limiting gene exchange.

Transfer of DNA from the 2,4-D degraders to *P. cepacia* D5 was confirmed by hybridizing colony blots of donor, recipients, and at least three different transgenic strains with the entire pJP4 plasmid labeled with ^{32}P. Plasmid pJP4 was used as a probe for tracking gene transfer because it was previously shown that these particular strains hybridized to pJP4 (Tonso, et al., 1994). In all cases of transfer except one, the transgenic strains also hybridized to plasmid pJP4 (Table 3). Figure 6 shows colony blots for a select group of donor, recipient, and transgenic colonies probed with plasmid pJP4. To confirm that the transgenic strains retained the recipient's chromosome, colonies were also hybridized with the *nptII* internal gene fragment from transposon Tn5 (data not shown).

One strain showed an unusual pattern of transfer. The only strain that did not hybridize to pJP4 yet transferred its 2,4-D genes *in vitro* was strain RASC. This strain was unusual because it transferred the 2,4-D genes only very infrequently and at a very slow rate. Because it was so unusual, this gene transfer event was studied in more detail as discussed in the next chapter.

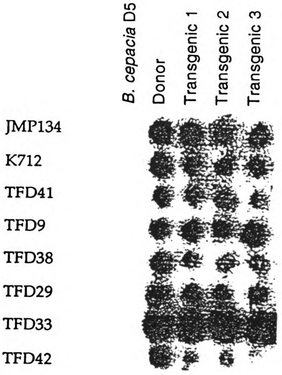


Figure 6. Colonies of *B. cepacia D5*, donors, and three transconjugants were hybridized with total plasmid pJP4. This demonstrates that a plasmid like pJP4 transferred in these strains.

DISCUSSION

This work has shown that gene exchange is responsible for generating much of the diversity of 2,4-D degrading bacteria. The evidence for this is that the *tfd*A genes have been transferred to and expressed in different species of bacteria in nature, as indicated by the incongruent phylogenies of the *tfd*A and 16S rDNA sequences for these strains. Evidence of interspecies gene transfer can also be observed in that identical *tfd*A genes are found in strains with 16S rDNA sequences differing by up to 16%, even though 16S rDNA is regarded as a highly conserved gene and *tfd*A is unlikely to be conserved.

Further evidence for interspecies gene transfer is given by the results that different *tfdA* genes were found in strains with the same 16S genes. For example, strains TFD6 and EML159 contained identical 16S partial sequences, yet their *tfdA* genes differed by 23%. Strains TFD23 and TFD31 also contained identical 16S partial sequences, yet they also carried *tfdA* sequences differing by 20%. Perhaps certain strains contain metabolic machinery, such as a maleylacetate reductase genes (Kukor et al., 1989; Kaschabek and Reineke, 1992) which make them receptive hosts to a variety of different *tfd*A sequences.

Barrier to gene flow or expression

The results of this study suggest a possible barrier to gene flow or expression between members of the major subgroups of *Proteobacteria*. Strains with *tfdA* sequences 55% or more similar to *tfdA* from pJP4 did not appear to transfer from the beta group to the alpha subgroup, as indicated by lack of gene transfer in laboratory experiments and the lack of hybridization of any of the alpha group to *tfdA*(pJP4) (chapter 3).

This apparent barrier of gene flow between the beta and gamma and alpha Proteobacteria is also found in plasmid pJP4, since no pJP4-like *tfd*A genes were found among the alpha or gamma *Proteobacteria.* This is curious since plasmid pJP4 is a broad host range plasmid and has been known to transfer to alpha *Proteobacteria,* such as *Rhizobium* and *Agrobacterium,* and to gamma bacteria such as *Pseudomonas putida, P. fluorescens,* and *P. aeruginosa* (Don and Pemberton, 1981). However, in those laboratory experiments, plasmids were transferred, but the 2,4-D pathways were not expressed. This inability to express the phenotype may be due to lack of accessory genes such as a maleylacetate reductase gene, required for complete degradation of 2,4-D molecule (Kukor, et al., 1989) or to possible lack of 2,4-D permease genes (Tim Sassanella, personal communication). The data from nature, however, suggests that the transfer and retention of 2,4-D genes between members of the alpha and beta subgroups of *Proteobacteria* probably occurs rarely, since hybridization studies failed to show evidence of these genes in the alpha *Proteobacteria* and since none of the members of the alpha *Proteobacteria* hybridized to pJP4 (chapter 3 and Matheson, 1994,).

Limited diversity of *tfdA* sequences

This study has shown only three basic types of *tfdA* sequences found in strains belonging to 10 distinct 16S rDNA classes. Furthermore, Wright, et al. (in prep) have sequenced two additional unique strains with *tfdA* sequences identical to those of B6-9 reported here, including one from a French agricultural isolate and another from an unculturable 2,4-D degrading soil community. With the exceptions of the minor differences in various pJP4-like *tfd*A sequences, why were no intermediate forms of these *tfdA* sequences detected in these many unique strains? Presumably, these three genes diverged long ago and only recently (perhaps only after massive application of 2,4-D in the past half century) have these surviving *tfd*A genes been particularly successful and have propagated throughout many populations via gene exchange.

Broad host range transfer via plasmid and chromosome

In addition to demonstrating that gene exchange occurs in nature, many 2,4-D degrading strains demonstrated gene transfer in the laboratory, and the information from these *in vitro* mating experiments gave some clues as to the mechanism of gene transfer. This study showed that in many cases gene exchange was probably due to transfer of conjugal 2,4-D plasmids, although another mechanism of gene transfer is suspected to have occurred as well.

This study indicates that the class I *tfdA* genes (like those from pJP4) are probably plasmid encoded. The evidence for this is threefold. First, all donor and transconjugant strains hybridized to plasmid pJP1, whereas the recipient strain alone did not. Second, all strains with a class I *tfd*A gene examined so far contain broad host range, self transmissible plasmids containing 2,4-D genes. For example *tfd*A genes are located on plasmids found in JMP134 (Don and Pemberton, 1981), in TFD41 (Nakatsu, personal communication), in EML159 (Amy, et al., 1985) and K712 (Ka and Tiedje, 1994). Third, all of the strains with a class I *tfd*A gene transferred the 2,4-D phenotype at relatively high frequencies, a behavior more typical of conjugal transfer rather than transformation of transduction.

The data in this study show that class I *tfd*A genes have a broad host range among the beta *Proteobacteria*. These nearly identical *tfd*A genes have been found in strains with 16S rDNA sequences differing from 7.6% up to 19% indicating a broad range of interspecies and intergeneric gene transfer. Further evidence of the broad host range of these strains is provided by the fact that plasmid pJP4 and K712 are both members of the IncP plasmid incompatibility group (data not shown, and Top, personal communication) which includes highly self-mobilizable, broad host range plasmids (Thomas and Smith, 1987).

Another type of *tfdA* gene which could be transferred in nature and to a limited extent in laboratory experiments is that of strains RASC, TFD2, and TFD6. The *tfd*A genes of these strains are probably chromosomally located as appears to be true for RASC (Suwa et. al., 1994), TFD6 (Matheson et al., 1994) and strain BRI6001 (Greer et al., 1991). The identical *tfdA* sequence is found in strains RASC, TFD2, and TFD6 which differ in 16S rDNA nucleotide sequences by 5.2 to 6.9 percent. Interestingly, this particular *tfd*A sequence was found only in strains clustering into the genus *Burkholderia,* determined by 16S rDNA sequences, but were not found outside this group, as were the class I *tfd*A genes.

The limited host range, among genus *Burkholderia,* of the class II *tfdA* gene may be due to its limited ability to transfer and express 2,4-D genes, as observed in laboratory experiments. The experiments performed here did not eliminate the possibility that gene transfer was not observed due to the host used. However, an unsuitable recipient may not have limited transfer since strain RASC failed to transfer its 2,4-D phenotype to its sibling that was 2,4-D$^-$ (lacking a functional *tfd*B gene). None of the strains with a class II or class III *tfdA* genes transferred and expressed the 2,4-D phenotype in lab experiments, with the exception of strain RASC which did so at extremely low frequency. Perhaps there is a barrier to gene transfer or expression for this type of *tfdA* gene or its operon. This barrier may simply be due to the fact that this type of *tfd*A gene is confined to the chromosome, or to an extremely large, relatively immobile or integrated plasmid.

The third class of *tfdA* genes, which includes B6-9, I-18 and TFD31, may have a very broad host range. Strain, I-18, a halophile isolated from a salt flat, contains a *tfdA* gene identical to TFD31 and B6-9. Strain I-18 is a member of the gamma *Proteobacteria,* whereas TFD31 and B6-9 are members of the beta *Proteobacteria.* Despite the broad host range observed in natural isolates, transfer was not duplicated in laboratory experiments Again, the lack of observed transfer may have been due to incompatibility of a particular recipient. It is not known whether TFD31 carries its 2,4-D genes on a plasmid, however it is known that TFD31 carries no plasmid related to pJP4, as indicated by its inability to hybridize to this plasmid (Tonso, et al., 1994).

Anastomosis

Although this study includes a dendrogram based on partial sequence from *tfdA* genes, this does not necessarily reflect the true phylogeny, that is historical evolution, of these strains; rather, it may only reflect the phylogeny of the *tfdA* molecules contained within these strains. Similarly, the phylogeny of the 16S genes may not necessarily be construed as the true phylogeny of these organisms, since organisms are amalgams of different genes. Instead, the *tfd*A and 16S rDNA sequence data indicate that the evolution of these strains has been by a process of "anastomosis", (or reticulate evolution) in which branches of the phylogenetic tree merge, forming a network (Margulis, 1992).

The 16S rRNA molecule has revolutionized our understanding of prokaryotic phylogeny, but until at least some portion of the remaining 99.99 percent of genes in any strain are also analyzed for comparable phylogenies, we should be cautious in relying solely on 16S, which Olsen et al., have called "a key to prokaryotic phylogeny" (1994), for determining the phylogeny of the entire organism. Many microbiologists, including myself, seem to be swept up by the "Winds of (Evolutionary) Change," (Olsen, et al., 1994). However, in the enthusiasm for having discovered a possible Rosetta stone for deciphering the evolution of all life, we should not overlook the fact that 16S rRNA sequences account for only a small portion of the entire genome. In the future, comparing the phylogenies of other genes to the phylogenies derived from 16S rRNA should address this concern.

So far, phylogenies based on ribosomal sequence data have only been corroborated by a handful of other molecules, including elongation factors EF-1 alpha (Tu) and EF-2(G), and the alpha and beta subunit of ATP-synthase (Iwabe, et al., 1989) and cytochromes (Woese, et al, 1980), and in a handful of others in a limited number of strains (Doolittle and Brown, 1994). Even with these data there still exists the problem of gene duplication and horizontal gene transfer (Doolittle and Brown, 1994) and inability of current methods to discern a true phylogeny (Doolittle and Brown, 1994, Creti, et al., 1994).

In addition to corroborating phylogenies created with ribosomal data, researchers have performed phylogenetic congruency tests on other molecules. Dykhuizen and Green found that the phylogenies of the *phoA*, *gnd*, and *trp* genes were incongruent (Dykhuizen, 1991). However, their study was confined to one species, where (by definition in the eukaryotic/sexual sense) horizontal gene transfer can occur.

Some may object to using "accessory genes," such as genes for 2,4-D degradation, for comparing phylogenies of organisms, because similar genes are not necessary under all circumstances. Although accessory genes are not essential to bacteria under all conditions, these genes are absolutely essential for survival under some conditions and may comprise a significant portion of the bacterial genome. For these reasons it is only reasonable to include the ancestry of these accessory genes in the phylogeny of an organism. Though this task is orders of magnitude more complex and less convenient for systematists who have enough of a challenging task to determine the phylogeny of a single gene, it is a more accurate representation of reality.

Phylogeny of the gene is not necessarily the phylogeny of the organism

Prior to systematics based on 16S sequencing, the primary means of categorizing bacteria was phenotypic. In our enthusiasm for the simplicity of a system based on 16S, we should not forget that phenotypes are also based on genetic data, which are as amenable to phylogenetic analysis as is ribosomal DNA. To infer that strain A is more like strain B than like strain C based on similarities of their 16S genes is like concluding that a Mitsubishi pickup is more related to a Mitsubishi toaster than it is to a Ford pickup, just because the former two share a Mitsubishi microchip for heater control.

A true phylogeny of an organism can only be inferred when the phylogenies of several genes per organism have been compared. Comparing ribosomal RNAs has created a revolution in understanding evolution and comparing the phylogenies of more molecules will lead to an even bigger revolution in understanding microbial evolution.

This study has shown that interspecies gene transfer is important in the evolution of 2,4-D degrading bacteria. The evolution of these organisms cannot be inferred by examining 16S rDNA alone or *tfd*A genes alone, but when examined together, these two genes present a clearer picture of the evolution of these bacteria in nature.

ACKNOWLEDGEMENTS

I thank Dr. William Holben for 2,4-D strains and *tfdA, tfdB* and *tfdC* gene probes and Dr. Jong-Ok Ka, Dr. Nancy Tonso, Dr. Penny Amy and Dr. Charles Greer for the use of their strains. I would like to thank Dr. Richard Lenski for valuable discussions in comparing phylogenies of *tfd*A and 16S rDNA and for encouraging me to partially sequence the *tfd*A genes. I especially thank Dr. Alice Wright and Dr. Roberta Fulthorpe for their assistance in sequencing the *tfd*A genes. This work was supported by the National Science Foundation grant number BIR912006 and by the Japan Research and Development Corporation.

Chapter Five

Interspecies Transfer of Chromosomally Encoded 2,4-Dichlorophenoxyacetic Acid-Degrading Genes

Interspecies Transfer of Chromosomally Encoded 2,4-Dichlorophenoxyactic Acid Degrading Genes

SUMMARY

Identical chromosomally located *tfd*A genes, which encode the first enzyme for the degradation of 2,4-dichlorophenoxyacetate, have been found in phylogenetically diverse strains, yet it is not known by what mechanism these genes have transferred. Here it is shown that the *tfd*A and B genes have were transferred from the chromosome of strain RASC to the chromosome of *Burkholderia cepacia* strain D5 in the laboratory. Transfer of the 2,4-D genes was not via the only detectable plasmid (11 kb) harbored by RASC. Results from independent mating experiments indicated that the genes inserted into different sites in the recipient *B. cepacia* D5 chromosome. The transferred 2,4-D phenotype appeared stable indicated by the fact that serial transfers of RASC and transgenic strain *B. cepacia* D5(RASC) in nonselective medium over 300 generations showed no loss of the 2,4-D phenotype. Integration of the 2,4-D genes into the new host's chromosome may have involved the concomitant transfer of a Repetitive Extragenomic Palindromic (REP) fragment from RASC into the new host.

INTRODUCTION

It is thought that the broad diversity of chloroaromatic degrading organisms has evolved via transfer of degradation genes (Fox,1983; Kellogg et al., 1981; Yano, 1980). Generally, this transfer is thought to occurred via conjugation (Sayler et al., 1990). Chakrabarty and Gunsalas first found that camphor degradative traits were on plasmids, and since then, genes for degrading many chlorinated aromatic compounds have been found on plasmids. Until now, the only reported means for transfer of such genes to other organisms is via conjugation of plasmids (for review see Sayler, 1990), or via transposons residing at least temporarily on plasmids (Wyndham, et al, 1988; Fulthorpe, et al., 1991; Nakatsu, 1992; Burlage et al., 1989; Jeenes et al., 1982). So far there has been no report of transfer of catabolic genes from the chromosome of one strain into the chromosome of another.

Plasmid mediated transfer of 2,4-D degradation has been well documented, beginning with the best characterized 2,4-D plasmid, pJP4 from *Alcaligenes eutrophus* JMP134 (Fisher, et al., 1978; Don and Pemberton, 1981; Friedrich, et al., 1983). Since the discovery of plasmid pJP1, several other researchers have reported 2,4-D genes carried on plasmids in a broad range of organisms (Don and Pemberton, 1981; Chaudry and Huang, 1988; Ausmees, et al., 1990, Amy, et al., 1985; Bhat, et al., 1994; chapter 4) whereas there have been only suggestions of chromosomally encoded 2,4-D genes (Amy, 1985; Greer, 1990; Ka, 1993; Suwa, 1994). Genes for 2,4-D degradation have been shown to transfer only via conjugation of plasmids (Don and Pemberton, 1981; Amy, et al., 1985; Bhat, et al., 1994; Chaudry and Huang, 1988).

However, a broad range of 2,4-D degraders with apparently chromosomally encoded 2,4-D genes may be more widely distributed than strains harboring plasmid-borne, 2,4-D-degrading strains (Matheson, et al., 1994; Chapter 2). The genes in these strains are 60-80 percent similar to those of pJP4, (Fulthorpe, et al, submitted; Matheson, et al., submitted; Suwa, et al. 1994) yet they appear to be on the chromosome (Suwa, et al., 1994.)

In this study we investigated the ability of these apparently chromosome-borne 2,4-D genes to be transferred. Here we present evidence for dissemination of the 2,4-D phenotype from the chromosome of strain RASC to the chromosome of *B. cepacia* which suggests a paradigm for transfer of 2,4-D genes in nature other than by conjugation.

MATERIALS AND METHODS

Media and growth conditions: 2,4-D MMO containing 500 ppm 2,4-D (Sigma) in a mineral salts medium (Stanier, 1966) was used to grow 2,4-D degrading strains. Antibiotics including kanamycin, carbenicillin, and bacitracin, were added at concentrations of 50 µg/ml. Peptone, tryptone, yeast extract, glucose (PTYG) medium is a dilute nutrient broth containing 0.25 g peptone, 0.25 g tryptone, 0.5 g yeast extract and 0.5 g glucose, 0.03 g magnesium sulfate, and 0.003 g calcium chloride per liter. TSB contains 15 g Trypticase soy broth (Difco, Detroit, MI) per liter. All cultures were grown at 30°C. Broth cultures were aerated by shaking on an Innova 4300 shaker (New Brunswick Scientific Co., New Brunswick, NJ) at 200 rpm.

Bacterial strains. Strain RASC was isolated by Dr. Penny Amy from return activated sludge from the Corvalis, Oregon waste water treatment plant (Dr. Penny Amy, personal communication). It is sensitive to the antibiotics kanamycin, carbenicillin, and bacitracin. *B. cepacia* strain D5 was supplied by Dr. Ron Olsen (Walch and Ballou, 1983). It was made kanamycin resistant by the introduction of neomycin phosphotransferase genes carried on Tn5 (Holben, personal communication) into a nonessential region of *B. cepacia* strain DBO1. This strain is resistant to 50 µg/ml each of kanamycin, bacitracin and carbenicillin. *B. cepacia* DBO113R1 is a spontaneous rifampicin resistant, methionine auxotroph of *B. cepacia* D5. RASCd2 is a 2,4-D$^-$ mutant made by Tn5 interruption of the *tfdB*-analogous gene of RASC (Suwa, 1994). *Escherichia coli* (pR388) contains the Inc W plasmid pR388 which is resistant to streptomycin. *Alcaligenes eutrophus* JMP134(pJP4) contains 2,4-D genes located on its 80 kb plasmid (Don and Pemberton, 1981) and was provided by Dr. Ron Olson.

Mating Studies. A ten-fold excess of recipient *B. cepacia* D5 in broth (~ 10^9 cells) was mixed with RASC donor cells and centrifuged briefly to concentrate. These cells were then pipetted either onto 0.2 µm filter paper, (Whatman), which was placed onto agar plates, or onto agar media directly. Strain RASC and *B. cepacia* D5 were mated under diverse conditions of varying surfaces and substrates (agar, broth, or filters), duration, and media. The donor strain was grown either in TWIG, TSB, or 2,4-D. The recipient strains were grown either with or without kanamycin in TSB or PTYG. Agar plates contained 2,4-D with or without antibiotics, PTYG or TSB. Cells were allowed to incubate together for various times ranging from 2 hours to 16 days. Mating mixtures were then either resuspended in H_20 and serial dilutions made before plating, or were streaked directly onto selective media of 2,4-D kanamycin, carbenicillin and bacitracin. JMP134 was mated with *B. cepacia* D5 as a positive control.

2,4-D Biodegradation. Disappearance of 2,4-D was monitored by HPLC. Supernatants were centrifuged at 14,000 rpm and filtered through 0.2 μ filters. Samples were then analyzed by HPLC on a Lichrosorb Rp-18 column (Anspec Co., Ann Arbor) using 60% methanol and 40% 0.1% H_3PO_4 as eluant. 2,4-D was detected at 230 nm.

Transformation studies. An excess (5 μg) of DNA from strain RASC was added to 1 ml (~ 10^8 cells) of *B. cepacia* D5 concentrated to 100 μl by brief centrifugation and incubated either in PTYG broth, half strength TSB plates or on 2,4-D MMO with 50 μg/ml each kanamycin, carbenicillin, and bacitracin. After one week incubation, a loopful from each medium was streaked onto agar plates containing 2,4-D MMO with and without 50μg/ml each of kanamycin, carbenicillin, and bacitracin. Putative transformant colonies were tested purified by streaking onto fresh 2,4-D plates.

DNA preparation and restriction analyses. DNA was isolated using the procedure of Ausubel, et al., 1992. DNA was digested according to manufacturers directions using restriction enzymes from Boehringer Mannheim. These included *Eco*RI, *Not*I, *Hind*III, *Sal*I, *Pst*I, *Pvu*II, *Bgl*II, *Sau*II, *Sma*I, *Sfi*I, and *Swa*I. DNA molecular weight markers included bacteriophage lambda digested with *Hind*III (Boehringer Mannheim), MegaBase II marker (Biorad, Hercules, CA), and 100 bp marker (Bethesda Research Laboratories, Gaithersburg, MD). DNA was transferred to nylon membrane (Amersham Hybond-N^{TM}) and covalently linked to the membrane with ultraviolet radiation using a Stratalinker^{TM} 1800 (Stratagene, La Jolla, CA).

Hybridization. The 11 kb plasmid, pRASC and the *nptII* gene were labeled using random hexanucleotides incorporating (^{32}P)dCTP (3,000 Ci/mmol; New England Nuclear, Boston, MA). DNA probes were made from internal fragments of the *tfdA, B, C, and D* genes from plasmid pJP4 (Holben, et al, 1993) and from the 3.0 kb and 2.2 kb fragments from strain RASC which included the *tfdA*-like gene from this strain (Suwa, in press). Plasmids containing DNA fragments were digested with appropriate restriction enzymes, purified by gel electrophoresis, excised from agarose, and labeled with digoxygenin-11-dUTP using the Random Primers Labeling Kit (Boehringer Mannheim) according to the manufacturer's instructions. Hybridizations using probes from JMP134 were carried out under low stringency conditions using no formamide and a temperature of 62°C. Hybridization with the probes from RASC were carried out under high stringency conditions using 50% formamide and 62°C as described in chapter 3.

Phenotype stability. Stability of the 2,4-D phenotype was examined by growing RASC and D5(RASC) for 300 generations by transferring 25 µl (approximately 1.7 x 10^6 cells as determined by plate counts) into 25 ml non-selective broth (PTYG) each day for 30 days. Strains JMP134 and *B. cepacia* D5(pJP4) were treated in the same manner as a control. After each 24 hour period cells were counted by plating, and found to number approximately 1.7 x 10^9 cells per ml.

The number of generations per day was calculated as :

$(\log \text{Population}_{\text{final}} - \log \text{Population}_{\text{initial}}) / \log 2$

The number of generations per day, found to be 10, was multiplied by 30 days resulting in 300 generations total. Colonies were counted on non-selective PTYG medium and on 2,4-D medium on days 1, 2, 7, 8, 9 and 30.

Large Plasmid Detection. Protocols of Kado and Liu, (1981) of Wheatcroft et al., (1990) and of Eckhardt (1978) were used and modified for trying to detect large plasmids from strain RASC using JMP134 as a positive control. The largest plasmid yield for pJP4 was obtained using the modified method of Kado and Liu. Overnight culture grown in TSB was pipetted into a 2 ml microfuge tube and pelleted by centrifugation at 14,000 rpm. Cells were resuspended in 200 µl of Buffer E (Kado and Liu, 1981) and mixed with 400 µl of lysis buffer. The lysate solution was incubated at 68°C for 1 hour. A volume of 600 µl of phenol:chloroform (1:1) was added and the lysate was inverted to mix. The lysate was then centrifuged at 14,000 in 4°C and the supernatant was transferred to a clean microfuge tube. Plasmids were precipitated by addition of 100 µl of 3 M NaAcetate, pH = 5.3, and 700 µl isopropanol and then stored overnight at -20. DNA was pelleted by centrifugation and pellets were resuspended in 40 µl $T_{10}E_1$ and then separated by gel electrophoresis on a 0.7% agarose gel.

REP-PCR - DNA from strains was subjected to PCR amplification using reverse extragenomic palindromic (REP) primers (Right = 5'GCCIGATGICGICGI-3' and Left = 5'-ICGICTTATCIGGCCTAC-3') synthesized by the Macromolecular Sequencing Facility at Michigan State University (deBruijn, 1993). Reagents for PCR were added according to the protocol of deBruijn, 1993. Taq polymerase was purchased from Perkin Elmer. Thermal cycling was done in a Perkin Elmer 9700 Thermal Cycler using the following conditions: Melting at 95°C 1 min; cycling 35 times at 94°C for 1 min, 40°C for 1 min, and 65°C for 2 min followed by an elongation time of 8 min at 65°C. PCR products were separated by electrophoresis on a 1.5% agarose gel (Boehringer Mannheim) at 38V for 12 hours using a 100 base pair ladder as a size marker. The gel was stained with ethidium bromide and photographed with Polaroid positive negative film 55.

RESULTS

Mating experiments. Strain RASC and *B. cepacia* were initially mated on a cellulose filter overlying nonselective TSB agar, incubated 25 hours and then serially diluted and spread plated onto selective media containing 2,4-D as the sole carbon and energy source and 50 ppm each of carbenicillin, bacitracin, and kanamycin to select against the donor. Negative controls with donor cells or recipient cells alone yielded no colonies on the selective medium. Colonies of donor cells from mating mixture were counted on 2,4-D medium and numbered 2.6×10^8 donors per ml. Colonies of recipient cells in mating mixture, counted on PTYG 50 ppm each of carbenicillin, bacitracin, and kanamycin, numbered 2.1×10^9. Transgenic strains were observed only after four weeks incubation on selective plates. The frequency of transfer was very low and could not be reliably quantitated since several plates with dilutions of 10^{-1}, 10^{-1} and 10^{-2} contained approximately the same number of colonies (1, 66 and 2 colonies, respectively). This is a frequency of between 2.5×10^{-7} and 3.8×10^{-7} transgenic strains per donor. In contrast, a positive control mating with EML159 and *B. cepacia D5* yielded 2×10^{-4} transconjugants per donor which appeared after only three days. In another control experiment, JMP134 mated with *B. cepacia* D5 yielded 50% transconjugants per donor.

Transgenic colonies were transferred onto fresh selective agar three times to isolate pure colonies. The colonies grew more quickly with each successive transfer.

To determine whether the putative transgenic strains could completely mineralize 2,4-D, they were grown in 500 ppm 2,4-D medium and 2,4-D disappearance was tested by HPLC. All transgenic strains showed 100% disappearance of 2,4-D and no appearance of any other U.V. absorbing peaks. **Transfer of DNA.** Transfer of DNA from strain RASC to *B. cepacia* D5 was confirmed by hybridizing Southern blots of *EcoRI* digested donor, recipient and transgenic strains with the *tfdA* probe from pJP4 at low stringency. A 7 kb band appeared on lanes with both RASC and D5(RASC), but not on the lane of the recipient strain *B. cepacia* D5. Hybridization with *tfdB* yielded the same pattern with the appearance of an additional 3.5 kb band in both donor and transgenic strains (Figure 1). Interestingly, the *tfdC* and *tfdD* gene probes showed no hybridization signals, even at very low stringency. A probe constructed of a 3 kb fragment from RASC containing the *tfdA*-like gene from this strain also hybridized to the 7 kb *Eco*RI fragment (Figure 2).

To determine whether transfer of the 2,4-D phenotype was concomitant with the transfer of the resident 11 kb plasmid in RASC, the 11 kb plasmid from RASC was labeled with ^{32}P and used to probe donor, recipient and transgenic strains (Figure 3). Since the plasmid did not hybridize to the transgenic strain, this indicated that the genes were not located on the 11 kb plasmid. The conclusion was subsequently confirmed by Yuichi Suwa in this lab by plasmid curing experiments.

To see if the transferred genes were inserted in the chromosome, total genomic DNA from donor and transgenic strains was digested with a seven different restriction enzymes and probed with a 3.0 kb fragment of DNA from RASC that contained both *tfdA*- and *tfdB*-like genes. Two of the restriction enzymes, *Bgl*II and *Pvu*II yielded different restriction patterns, indicating that

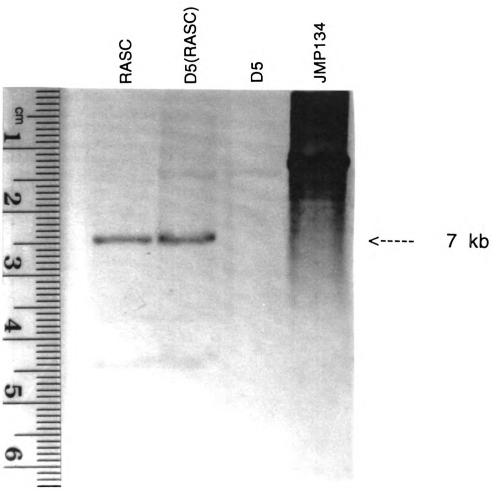


Figure 1. Southern blot of *EcoRI*-digested total genomic DNA from RASC, *B. cepacia* D5, and *B. cepacia* D5(RASC) was hybridized at low stringency with *tfdB* from pJP4. This probe hybridized to the same 7.0 kb and 3.5 kb fragments in RASC and D5(RASC), indicating transfer of genetic material from one strain to the other. This hybridized with an intense signal to the positive control JMP134, but not to the negative control *B. cepacia* D5.

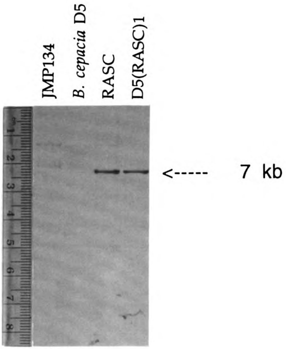


Figure 2. DNA from JMP134, RASC, D5, and D5(RASC) were digested with *EcoRI* and hybridized with the 3.0 kb fragment from RASC which contains the *tfdA*-like gene.

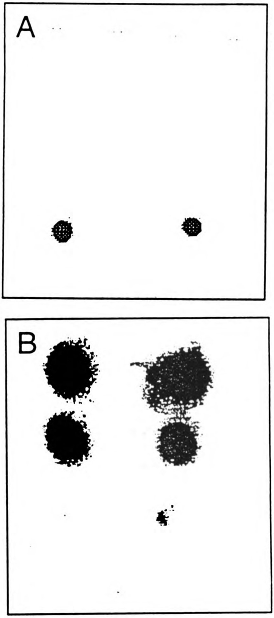


Figure 3A. Duplicate colonies of RASC, *B. cepacia* D5 and transgeneic strains *B. cepacia* D5(RASC) were hybridized to the 11 kb plasmid isolated from strain RASC. The blot in figure B was hybridized to *npt*II from Tn5, which is contained on *B. cepacia* D5. The labeled plasmid from RASC did not hybridize to the transgenic strain indicating that the genes for 2,4-D degradation were not located on this plasmid.

the 2,4-D genes had either inserted into a site in the recipient's chromosome or rearranged after entering the new host (Figure 4).

Stability of 2,4-D phenotype. After more than 300 generations in nonselective broth medium 100% of the strains of RASC, D5(RASC), and JMP134 or D5(pJP4) (used as controls) retained the 2,4-D degradation phenotype. This stability indicates that the genes are not on an unstable transposon or plasmid, but are located on the chromosome, or on a very stable transposon or plasmid.

Reproducibility. To reproduce and optimize this gene transfer event, 29 different mating conditions were designed to optimize the frequency of DNA transfer from strain RASC to *B. cepacia.* These different experiments employed different media, different incubation times and different surfaces to optimize conditions for transfer (Table 1). Of these 29 independent mating experiments, transgenic strains were observed in only six, and these only after a period of incubation on plates for at least three weeks. Media type and mating support (i.e. filter or agar) appeared to be less important to gene transfer than sufficient time.

To determine whether the 2,4-D genes could be transferred back into their original host, transgenic strain D5(RASC) was mated with a 2,4-D Tn5 mutant of RASC unable to grow on 2,4-D. This produced no transgenic strains capable of growth on 2,4-D.

To see whether the genes could be transferred from *B. cepacia* D5(RASC) to a sibling of *B. cepacia* D5 , transgenic strain D5(RASC) was mated with *B. cepacia* DBO113R1, a spontaneous rifampicin resistant, methionine auxotrophic mutant of DBO1. This produced no transgenic colonies.

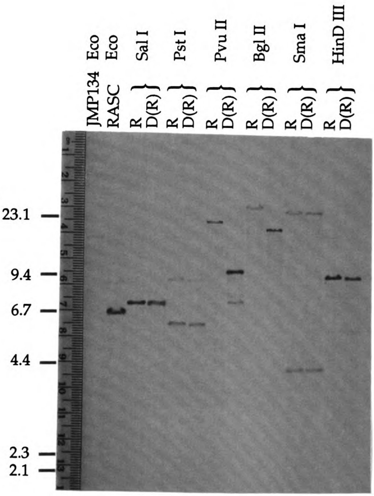


Figure 4. Total genomic DNA from RASC and *B. cepacia* D5(RASC) was digested with seven restriction enzymes and then probed with *tfd*A from RASC. The restriction enzymes *PvuII* and *BglII* yielded different restriction fragments, indicating that the DNA had a different genomic arrangement in *B. cepacia* than in strain RASC.

Table 1. Conditions used in matings

Broth Media

RASC	D5	Support	Agar	Time*
PTYG	PTYG	Filter/24 h	PTYG	3.5 weeks
PTYG	PTYG	Plate/24 h	PTYG	-
PTYG/2,4D	PTYG	Plate	2,4-D KCB	-
PTYG/2,4D	PTYG	Replica Spot	2,4-D KCB	3 weeks
PTYG	PTYG	Spot	2,4-D KCB	3 weeks
PTYG	PTYG	Filter/24	PTYG	-
TSB	TSB	Filter/ 2 weeks	PTYG	-
TSB	TSB	Filter/ 2 weeks	PTYG	-
TSB	TSB	Spot/16 days	2,4-D,HMK	2 weeks
2,4-D	PTYG	Filter/ 24 h	2,4-D	-
2,4-D	PTYG	Filter/ 24 h	PTYG	-
2,4D	PTYG K[1]	Filter/48	2,4-D KCB	-
2,4D	PTYG K	Filter/48	PTYG	-
2,4-D	PTYG K	Filter/48	2,4-D KCB	-
2,4-D	PTYG K	Filter/48	TSB/2,4D	5 weeks
2,4-D	PTYG K	Filter/2	2,4-D	-
TSB	TSB K	Filter/2	TSB/2,4D	-
TSB	TSB K	Filter/24	TSB/2,4D	-
TSB	TSB K	Filter/48	TSB/2,4D	-
TSB	TSB K	Filter/3	2,4D KCB	-
TSB	TSB K	Filter/24	2,4D KCB	5 weeks
TSB	TSB K	Filter/48	2,4D KCB	-
TSB	TSB K	Filter/4	2,4D	-
TSB	TSB K	Filter/24	2,4D	-
TSB	TSB K	Filter/48	2,4D	-
2,4-D	TSB K	Filter/3	TSB/2,4D	-
2,4-D	TSB K	Filter/24	TSB/2,4D	-
2,4-D	TSB K	Filter/3	2,4D	-
2,4-D	TSB K	Filter/24	2,4D	-

* Time at which transgenic colonies were first observed. A "-" indicates no transgenic colonies were seen.

1) "K" indicates addition of kanamycin

To see whether the same restriction patterns were found in independent transgenic strains, DNA from two mating experiments was isolated and digested with *Bgl*II (Figure 5). The different restriction fragment lengths of the two transgenic strains indicate that unique transfer events yielded unique insertion sites.

Transposon Trapping. To investigate the possibility that the transferred genes might be on a transposon, transposon trapping experiments were performed using plasmid p388, a broad host range Inc W plasmid as the "trap" (Valentine and Kado, 1989; Bennett and Grinsted, 1988). This plasmid, carrying streptomycin resistance, was mated from a proline deficient auxotrophic strain of *E. coli* into RASC. The mating mixture was plated on MMO agar containing 2,4-D and streptomycin to recover transconjugants. This transfer occurred at a frequency of 61% transconjugants per donor. These resulting transconjugant RASC(pR388) strains were then mated with *B. cepacia* D5. No 2,4-D degrading transconjugants were observed. The experiment was repeated with the RASC(pR388) strains stressed by starvation and transconjugants were still not observed.

Transformation studies. To determine whether DNA from strain RASC was transferred via transformation, DNA isolated from strain RASC was added to cultures of *B. cepacia* D5 and allowed to incubate for one week. In one experiment the cells and DNA were left to incubate on selective agar plates; the second incubation was on nonselective agar (one half strength TSB) and in the third experiment cells and DNA were incubated in PTYG broth without mixing. In three experiments no transformants were found.

Transfer of REP fragment. Strains of donors RASC, recipient D5, and seven

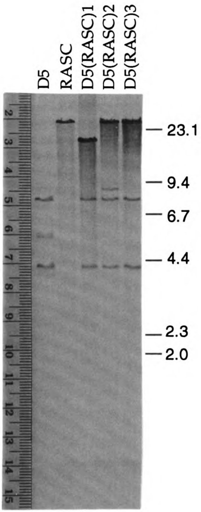


Figure 5. Total genomic DNA from *P. cepacia* D5, RASC and 3 separate transgenic strains was digested with BglII and hybridized with a 3 kb fragment from RASC containing its *tfdA* like gene as well as a portion of Tn5. DNA from RASC hybridized to different size fragments in the transgenic strains, indicating different insertion sites in each as a result of different gene transfer events.

transgenic strains of D5(RASC) were PCR amplified using repetitive extragenomic palindromic (REP) sequences as primers. Banding patterns for *B. cepacia* D5 and D5(RASC) differed from that of strain RASC, as expected. However, *B. cepacia* D5 and D5(RASC) shared identical banding patterns except that all the transgenic D5(RASC) strains shared an additional 1.2 kb fragment, not found in *B. cepacia*, not found in strain RASC (Figure 6). Hence, it appeared that a 1.2 kb fragment from RASC amplified by REP PCR had transferred to *B. cepacia* D5.

To confirm that this apparent new band was from RASC, and not an artifact of PCR, the 1.2 kb band was excised from the gel, labeled with digoxygenin, and used to probe a Southern blot of the PCR amplified REP fragments (Figure 7). This 1.2 kb probe hybridized only with RASC and the transgenic strains of D5(RASC), and not to *B. cepacia* D5.

To investigate whether this PCR amplified REP fragment was directly adjacent to the *tfdA* or *tfdB* genes in RASC, DNA from RASC, *B. cepacia* D5, D5(RASC) and JMP134 digested with *EcoRI*, separated by gel electrophoresis, blotted onto nylon filters and probed with the 1.2 kb fragment from RASC (Figure 8). The resulting fragment size which hybridized to this probe was 4.7 kb, much smaller than the 7.2 and 3.5 kb *Eco*RI fragments containing the *tfdA* and *tfdB*-like genes.

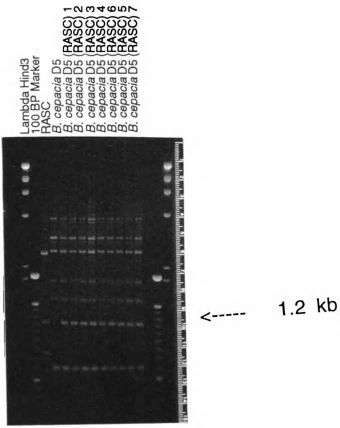


Figure 6. Strains RASC, *B.cepacia* D5 and seven transgenic strains of *B. cepacia* D5(RASC) were PCR amplified using REP primers. RASC contains amplified bands differing from other strains. The transgenic strains all had the same size fragments as those of *B. cepacia* D5 with the addition of a 1.2 kb fragment shared with strain RASC.

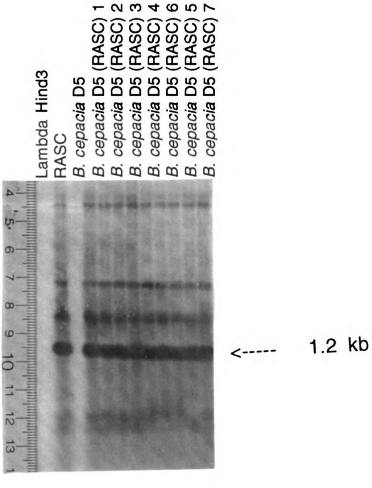


Figure 7. REP-PCR amplified DNA from strains RASC, *B. cepacia* D5 and 7 transgenic strains of *B. cepacia* D5(RASC) was blotted and probed with a 1.2 kb fragment from strain RASC. Only strain RASC and the transgenic strains hybridized to this fragment.

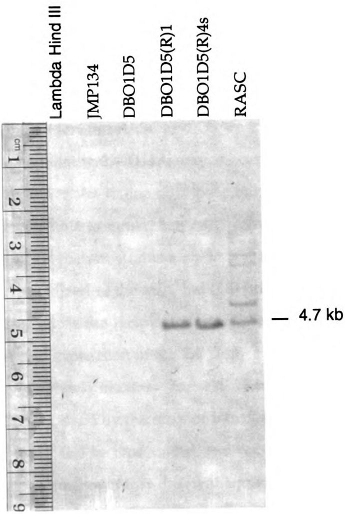


Figure 8. Hybridization of *Eco*RI digested total genomic DNA with a 1.2 kb REP amplified fragment isolated from RASC which appears to have transferred into separate transgenic strains of *B. cepacia* D5(RASC).

DISCUSSION

The *tfd*A and *tfd*B genes in the 2,4-D degradation pathway transferred from the chromosome of strain RASC into the chromosome of *B. cepacia* D5. This was shown by the fact after RASC and D5 were mated, colonies appeared on selective media containing 2,4-D, kanamycin, carbenicillin, and bacitracin, whereas controls of either strain alone did not. Furthermore, these transgenic strains were able to mineralize 2,4-D completely when cultured in broth (unlike each of the parent strains alone). It was also shown that the transgenic strains hybridized to the *tfd*A and *tfd*B probes from JMP134 and from strain RASC, whereas the recipient strain did not.

The transfer and expression of the 2,4-D phenotype occurred infrequently and very slowly *in vitro*. At least 15 kb of DNA transferred from RASC into D5 as judged by the sizes of transferred restriction fragments. The gene transfer could also be reproduced, and was in six different experiments. It was also shown that different transgenic strains had tfd genes inserted into different sites in the chromosome, as indicated by different RFLP patterns.

It is not surprising that DNA from strain RASC transferred to and was expressed in a strain of *B. cepacia*, since strain RASC is member of the genus *Burkholderia* (Yabuuchi, et al., 1992) as determined by 16S rRNA (chapter 2). Strain RASC has been shown to be very similar to another 2,4-D degrader (isolated from a distant part of the U.S.) strain K712, differing by only 2% in 16S genes (Ka, et al., 1994; chapter 2). Interestingly, a close relative of strain

K712 has 2,4-D genes located on a plasmid which has been shown to integrate into the chromosome, (Ka, et al., 1994d).

It is perhaps not surprising that 2,4-D genes could be expressed in *B. cepacia* since other strains of *B. cepacia* have previously been shown to be involved in 2,4-D degradation (Bhat, et al., 1994; Greer, et al., 1990) . *B. cepacia* CSV90 has been discovered which contains a 2,4-D plasmid with a *tfdC* gene identical with that of pJP4 (Bhat, 1994). Another *B. cepacia* strain, BRI6001, which was isolated from peat in Quebec, Canada, reportedly has 2,4-D genes located on the chromosome. Interestingly, strain BRI6001 contains a *tfdA* gene which is identical to that of the *tfdA* gene of strain RASC (chapter 4).

Mechanism of transfer: This story would be more satisfying if we knew the mechanism of transfer or of integration of the 2,4-D genes. However, since the gene exchange and expression required such a long incubation time, there is only little data on gene exchange mechanisms. Still, some observations were made and I would like to present them here.

The slow rate at which the 2,4-D genes transferred to *B. cepacia* may be a clue to the mechanism of gene transfer or of integration into the chromosome. The long incubation time required before the transfer and expression of the 2,4-D genes could be the result gene rearrangement before expression of the 2,4-D degradation phenotype. Perhaps the long incubation time was required for the lysis of RASC and uptake of DNA into *B. cepacia* D5. Another possibility is that the transfer event occurred quickly, but the expression required time for appropriate gene rearrangement in the new host. Many researchers have observed a long period of adaptation of microbial communities before expression of a particular biodegradative phenotype (reviewed by van der Meer, et al., 1992), which can be attributed to genetic recombination. Because the restriction patterns of donor RASC and transgenic *B. cepacia* D5 (RASC) differed, this suggested genetic recombination in the new host. In addition, the slow time for expression of the 2,4-D pathway in all six transfer events suggested the possibility that the genes required time to rearrange themselves into a proper orientation before properly expressing the 2,4-D phenotype.

Several experiments were designed to investigate the possibility of conjugation as a mechanism for transfer, and all were negative. First, the only readily detectable plasmid in strain RASC, an 11 kb plasmid, did not transfer, although the 2,4-D phenotype did. Second, several different protocols were used to detect large plasmids in strain RASC and the transconjugant. Although plasmids from the positive control, JMP134, could be detected, no large plasmids were detected in strain RASC. Another line of evidence that may rule out the possibility of conjugation is the fact that the 2,4-D phenotype did not transfer from *B. cepacia* D5 back into strain RASC nor into another strain of B. cepacia. Conjugation also seems unlikely due to the long incubation times required (discussed above).

Another possible mechanism of gene exchange is transformation. It is possible that the genes transferred via natural transformation, although at an extremely low frequency below the levels of detection used in the experiments reported here. Other bacteria closely related to *B. cepacia* have been shown to transfer their genes via transformation. *P. stutzeri* and *A. calcoaceticus* and *Neisseria* are all members of the beta *Proteobacteria*, (like *B.*

cepacia) and are naturally competent. Transformation has been observed in *B. cepacia* (Mark Frischer, personal communication). Transformation is important in nature in the evolution of new pathways (Stewart and Carlson, 1986). Although this event occurred slowly *in vitro* on a geological time scale this could represent an important source of gene transfer in nature. Infrequent events in small areas become frequent events when extrapolated over large areas. For example, Frischer et al. (1994) have calculated that a rate of 10^{-15} transformants per liter per ng DNA per recipient per day reported for marine bacteria becomes 10^{11} transformation events per year when extrapolated to all of Tampa Bay Estuary in Florida.

Transduction is another possible explanation for the transfer event. Almost 50% of *Burkholderia cepacia* strains examined are susceptible to lysogenic phage, and approximately 10% of *B. cepacia* strains carry transducing phage (Matsumoto, 1986). Although two experiments were done to test for transduction (data not shown) neither yielded positive results. This should not be weighed too heavily since a positive control was not done.

Mechanism of integration: How the genes from RASC integrated into their new host chromosome is unknown. Integration of 2,4-D genes into the chromosome has been reported by Ka who observed loss of a 41 kb 2,4-D plasmid in from *Alcaligenes paradoxus* strain 2118P with concomitant insertion of a fragment that hybridized to the *tfdA* gene probe into the chromosome of this organism that could still degrade 2,4-D (Ka, et al., 1994). A plasmid nearly identical to this, pKA4, which was isolated from a strain isolated from the same plot as 2118C at the Kellogg Biological Station, is similar to pJP4, in that it is also an Inc P1ß plasmid, and also hybridizes at medium stringency to *tfdA* and *tfdB* from pJP4. Because of the similarity to the pJP4 plasmid, this type of integration is probably different from that of strain RASC, which contains no plasmids similar to pJP4.

Perhaps these genes integrated into a site in *B. cepacia* D5 which is homologous to that of RASC. One potential candidate for homologous DNA fragments are the repetitive extragenomic palindromic (REP) sequences. REP sequences are highly conserved, inverted repeats of about 18 bp which appear to be ubiquitous in gram negative bacteria. Although the presence of REP sequences has been known since 1981, (Higgins, et al., 1982) the function of these sequences is uncertain (Lupski and Weinstock, 1992; deBruijn, et al., 1993; Higgins, et al., 1988). There has been speculation that REP sequences may be involved in gene conversions and gene rearrangements (Higgins, et al., 1988; Stern, et al., 1984; Gilson, 1987), or that they may have the potential for transposition (Stern,1984) and are probably important for the evolution of bacterial chromosomes (Lupski, 1992). Since the REP fragments from RASC transferred to D5 along with the 2,4-D genes, it is tempting to speculate that the REP sequences may have played some role in the integration of RASC DNA into the D5 chromosome. There have been unpublished reports that recombination occurred between the REP sequences on a plasmid and on the chromosome (Stern, et al., 1984.) Other reports have indicated that chromosomal duplications of the histidine operon in *E. coli* are located between REP sequences. These REP fragments are not found within the coding region of genes (Stern, 1984) but directly adjacent to them. *E. coli* contains between 500-1000 copies of REP sequences which occupy up to one % of the total genome (Higgins, 1988; Stern, 1984). Although REP sequences, like *Alu* sequences of humans, may be shown to have other primary functions, they still may be sites of recombination.

Another possibility mechanism for integration into the chromosome may come from transposons, although the few experiments performed here do not support this possibility. Since the transferred DNA was stably integrated into the genome of strain RASC and the transgenic strain D5(RASC) this indicates that the genes are not on an unstable transposon, as is the case for the 3-chlorobenzoate transposon Tn 5271 which resides on plasmid pBRC60 (Nakatsu, et al., 1991; Fulthorpe and Wyndham, 1991) or the toluene transposon on plasmid pWWO Chakrabarty, et al., 1978; Tsuda and Ieno, 1987; Burlage, et al., 1989).

There is evidence that the 2,4-D genes in this strain are not on located on a highly mobile transposon, since self mobilizing plasmids introduced into RASC failed to carry the 2,4-D genes from RASC. This, however, still does not rule out the possibility that these genes are flanked by low frequency mobilizing insertion sequences.

Additional observations: Strain RASC did not hybridize to the *tfdC* or *tfdD* gene probes from pJP4 indicating that it has some type of gene which is less than 55% similar in nucleotide homology. RASC and the transgenic strains also failed to hybridize to the *clcABC* gene cluster of pAC27 which encodes the degradative genes for 3-chlorobenzoate degradation. RASC probably contains a dichlorocatechol dioxygenase, since the 2,4-D pathway in strain RASC proceeds by the *ortho* pathway (Maltseva, personal communication). This would be a unique dichlorocatechol dioxygenase gene since the three known chlorocatechol dioxygenase genes (from *tfdC*, *clcA*, and *tcbC*) all share at least 53% amino acid similarity (van der Meer, et al., 1992). Perhaps the ring cleavage enzyme in RASC is similar to 3,4-dihydroxybenzoate (protocatechuate) dioxygenase enzyme, which has been described in *B. cepacia* (Walsh, 1983). This enzyme has been shown to degrade 6-chloro protocatechuate (Walsh, 1983) and may conceivably be the enzyme responsible for further catabolism of 3,5-dichlorocatechol.

Since there were no probes available to determine whether genes from the lower pathway of 2,4-D degradation transferred from RASC, it is not known whether the entire pathway transferred or only the first two genes transferred. Either case is possible. Since at least 15 kb of DNA from RASC transferred, it is feasible for the lower pathway, including genes encoding ring cleavage enzymes, to have been transferred as well. However, it is equally likely that the remaining genes could have already been present in *B. cepacia* since this organism also contains the ß-keto adipate (*ortho*) cleavage pathway, which is required for the lower pathway of 2,4-D degradation in pJP4. The recipient strain, *B. cepacia* D5, is derived from an ancestral strain which contains the 3,4-dihydroxybenzoate (protocatechuate) dioxygenase enzyme (Walsh and Ballou, 1983).

Perhaps one of the reasons for the catholic appetite of *B. cepacia* is the many IS elements it contains, which could conceivably be rearranged in new ways to accommodate new compounds (Lessie, 1990). It is widely accepted that the ß-ketoadipate pathway has evolved to accept many different aromatic compounds (Ornston, et al., 1990). *B. cepacia,* formerly more aptly named *Pseudomonas multivorans* , has the reputation of degrading more types of compounds than any other known species (Stanier, et al., 1966; Lessie, et al., 1990). The genetic plasticity and nutritional versatility of this strain may explain why it can accommodate genes for 2,4-D degradation.

In summary, the *tfd*A and *tfd*B genes from strain RASC were shown to be transferred to and expressed in B. cepacia D5. Although gene exchange occurred only very slowly, it was reproducible, and transgenic strains with very stable 2,4-D phenotypes were obtained. This transfer of 2,4-D genes *in vitro* supports the observation seen in the phylogenetic congruency test (chapter 4) in which identical *tfd*A genes are found in phylogenetically diverse strains.

ACKNOWLEDGEMENTS

I thank Dr. Yuichi Suwa for his probes from strain RASC and for useful comments. Special thanks are given to Dr. Roberta Fulthorpe for helpful discussions and to Dr. Larry Forney for encouragement. I also thank the people in the Research on Microbial Evolution Laboratory for discussions and ideas. This work was supported by the National Science Foundation grant number BIR912006.

BIBLIOGRAPHY

BIBLIOGRAPHY

Allewell, N. (1989). "Evolving to dissimilate hydrocarbons." TIBS 14: 473-474.

Altschul, S. F., W. Gish, W. Miller, E. W. Myers and D. J. Lipman (1990). "Basic Local Alignment Search Tool." J. Mol. Biol. 215: 403-410.

Amann, R., W. Ludwig and K.-H. Schleifer (1994). "Identification of uncultured bacteria: a challenging task for molecular taxonomists." ASM News 60(7): 360-365.

Amy, P. S., J. W. Schulke, L. M. Frazier and R. J. Seidler (1985). "Characterization of aquatic bacteria and cloning of genes specifying partial degradation of 2,4-dichlorophenoxyacetic acid." Appl. Env. Microbiol. 49(5): 1237-1245.

Ausmees, N. R. and A. L. Heinaru (1990). "New plasmids of herbicide 2,4-D degradation." Genetica 26(4): 770-772.

Bagdasarian, M., R.Lurz, B. Ruckert, F. C. H. Franklin, M. M. Bagdasarian, J.Frey and K. N. Timmis (1981). "Specific-purpose plasmid cloning vectors. II. broad host range, high copy number, RSF1010-derived vectors, and a host-vector system for gene cloning in Pseudomonas." Gene 16: 237-247.

Bakkeren, G., Z. Koukolikova-Nicola, N. Grimsley and B. Hohn (1989). "Recovery of *Agrobacterium tumefaciens* T-DNA molecules from whole plants early after transfer." Cell 57: 847-857.

Bale, M. J., M. J. Day and J. F. Fry (1988). "Novel method for studying plasmid transfer in undisturbed river epilithon." Appl. Environ. Microbiol. 54(11): 2756-2758.

Bale, M. J., J. C. Fry and M. J. Day (1987). "Plasmid transfer between strains of *Pseudomonas aeruginosa* on membrane filters attached to river stones." J. Gen. Microbiol. 133: 3099-3107.

Bale, M. J., J. C. Fry and M. J. Day (1988). "Transfer and occurence of large mercury resistance plasmids in river epilithon." Applied and Env. Microbiol. 54(4): 972-978.

Barns, S. M. R. E. Fundyga, M. W. Jeffries, and N. R. Pace. (1994). "Remarkable archael diversity detected in a Yellowstone National Park hot spring environment." Proc. Natl. Acad. Sci. 91:1609-1613.

Baron, M., D. G. Norman and I. D. Campbell (1991). "Protein modules." TIBS 16: 13-17.

Baum, D. (1992). "Phylogenetic species concepts." TREE 7(1).

Begley, S. and T. Waldrop (1989). "Microbes to the rescue." Newsweek (June 19): 56-57.

Bell, G. R. (1957). "Some morphological and biochemical characteristics of a soil bacterium which decomposes 2,4-dichlorophenoxyacetic acid." Can. J. Microbiol. 3: 821-840.

Bennett, P. M. and J. Grinsted, Eds. (1988). Detection and use of transposons. Methods in Microbiology. London, Academic Press.

Bentjen, S. A., J. K. Fredrickson, P.VanVoris and S. W. Li (1989). "Intact soil-core microcosms for evaluating the fate and ecological impact of the release of genetically engineered microorganisms." Appl. and Env. Microbiol. 55(1): 198-202.

Berg, D. E. and M. M. Howe, Eds. (1989). Mobile DNA. Washington,D.C., American Society for Microbiology.

Bertrand, J. C., M. Almallah, M. Acquaviva and G. Mille (1990). "Biodegradation of hydrocarbons by an extremely halophilic archaebacterium." Let. Appl. Microbiol. 11: 260-263.

Bhat, M. A., M. Tsuda, K. Horiike, M. Nozaki, C. S. Vaidyanathan and T. Nakazawa (1994). "Identification and characterization of a new plasmid carrying genes for degradation of 2,4-dichlorophenoxyacetate from *Pseudomonas cepacia* CSV90." AEM 60(1): 307-312.

Bleakley, B. H. and D. L. Crawford (1988). "The effects of varying moisture and nutrient levels on the transfer of a conjugative plasmid between *Streptomyces* species in soil." Can. J. Microbiol. 35: 544-549.

Bogosian, G. and J. F. Kane (1991). Fate of recombinant *Escherichia coli* K-12 strains in the environment. *in* Advances in Applied Microbiology. Academic Press. pp 87-131.

Bollag, J. M., C. S. Helling and M. Alexander (1968). "2,4-D metabolism." J. Agr. Food Chem. 16(5): 826-833.

Bounds, H. C. and A. R. Colmer (1964). "Detoxification of some herbicides by streptomyces." Weeds : 249-252.

Bradley, D. E. and P. A. Williams (1982). "The TOL plasmid is naturally derepressed for transfer." J. Gen. Microbiol. 128: 3019-3024.

Brisson-Noel, A., M. Arthur and P. Courvalin (1988). "Evidence for natural gene transfer from Gram-positive cocci to *Escherichia coli.*" Jour. Bact. 170: 1739-1745.

Brokamp, A. and F. R. J. Schmidt (1991). "Survival of *Alcaligenes xylosoxidans* degrading 2,2-dichloropropionate and horizontal transfer of its halidohydrolase gene in a soil microocosm." Curr. Microbiol. 22: 299-306.

Brosius, J., M. L. Palmer, P. J. Kennedy and H. F. Noller (1978). "Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli.*" Proc. Natl. Acad. Sci. 75(10): 4801-4805.

Bruggen, A. H. C. v., K. N. Jochimsen, E. M. Steinberger, P. Segers and M. Gillis (1993). "Classification of *Rhizomonas suberifaciens,* an unnamed *Rhizomonas* species, and *Sphingomonas* spp. in rRNA superfamily IV." International Journal of Systematic Bacteriology 43(1): 1-7.

Burlage, R. S., L. A. Bemis, A. C. Layton, G. S. Sayler and F. Larimer (1990). "Comparative genetic organization of incompatibility group P degradative plasmids." J. Bacteriol. 172(12): 6818-6825.

Burlage, R.S. , S. W. Hooper, and G. S. Sayler. (1989). "The TOL (pWWO) catabolic plasmid." Appl. Environ. Microbiol. 55:1323-1328.

Busse, H.-J., T. el-Banna, H. Oyaizu and G. Auling (1992). "Identification of xenobiotic-degrading isolates from the beta subclass of the proteobacteria by a polyphasic approach including 16S rRNA partial sequensing." Internatioanl Journal of Systematic Bacteriology 42(1): 19-26.

Calabrese, V. G. M., W. E. Holben, B. M. Schroeter, N. L. Tonso and A. J. Sextone (1991). Assessement of diversity of 2,4-D-catabolizing plasmids in bacteria isolated from different soils. Agronomy Society of America Annual Meeting, Denver, Colorado,

Cano, R. J. (1994). "Bacillus DNA in amber: a window to ancient symbiotic relationships?" ASM News 60(3): 129-134.
Chakrabarty, A. M. and I. C. Gunsalas (1971). "Degradative pathways specified by extrachromosomal gene clusters in Pseudomonads." Genetics 68(1): s10.

Chapman, P. J. (1976). "Microbail degradation of halogenated compounds." Biochem. Soc. Trans. 4: 463-466.

Chatterjee, D. K. and P. Chatterjee (1987). "Expression of degradative genes of *Pseudomonas putida* in *Caulobacter crescentus.*" Jour. Bacteriol. 169(7): 2962-2966.

Chatterjee, D. K., S. T. Kellogg, D. R. Watkins and A. M. Chakrabarty (1988). Plasmids in the biodegradation of chlorinated aromatic compounds. in *Plasmids* Ed. Levy.

Chatterjee, D. K., J. J. Kilbane and A. M. Chakrabarty (1982). "Biodegradation of 2,4,5-trichlorophenoxyacetic acid in soil by a pure culture of *Pseudomonas cepacia.*" Appl. Env. Micriobiol. 44(2): 514-516.

Chaudry, G. R. and G. H. Huang (1988). "Isolation and characterization of a new plasmid from a *Flavobacterium* sp. which carries the genes for degradation of 2,4-dichlorophenoxyacetate." J. Bacteriol. 170(9): 3897-3902.

Chaudry, G. R., G. A. Toranzos and A. R. Bhatti (1989). "Novel method for monitoring genetically engineered microorganisms in the environment." Appl. Env. Microbiol. 55(5): 1301-1304.

Chen, S. and M. Alexander (1989). "Reasons for the acclimation for 2,4-D biodegradation in lake water." J. Environ. Qual. 18: 153-156.

Cheng, H.-P. and T. G. Lessie (1994). "Multiple replicons constituting the genome of Pseudomonas cepacia 17616." J. Bact. 176(13): 4034-4042.

Christie, P. J. and R. Z. Korman (1987). "Two conjugation systems associated with *Streptococcus faecilis* plasmid pCF10. Identification of a conjugative transposon that transfers between *S. faecilis* and *B. subtilis.*" J. Bact. 169: 2529-2536.

Clewell, D., Ed. (1993). Bacterial Conjugation. New Yorks, Plenum Press.

Colwell, R. K. (1985). "Viable but non-culturable Vibrio cholerae and related pathogens in the environment: implication for the release of genetically engineered microorganisms." Bio/technology 3: 817-820.

Corbett, J. R., K. Wright and A. C. Baille (1984). The Biochemical Mode of Action of Pesticides. London, Academic Press.

Cork, D. J. and J. P. Krueger (1991). Microbial transformation of herbicides and pesticides. Advances in Applied Microbiology Eds. S. E. Neidleman and A. I. Laskin. San Diego, Academic Press. 364.

Coughter, J. P. and G. J. Stewart (1980). "Genetic exchange in the environment." Antonie van Leeuwenhoek 55: 15-22.

Creti, R., E. Ceccarelli, M. Bocchetta, A. M. Sanangelantoni, O. Tiboni and P. Palm (1994). "Evolution of translational elongation factor (EF) sequences: reliability of global phylogenies inferred from EF-1alpha(Tu) and EF-2(G) proteins." Proc. Natl. Acad. Sci., USA 91: 3255-3259.

Czelusniak, J., M. Goodman, N. D. Moncrief and S. M. Kehoe (1990). Maximum parsimony approach to construction of evolutionary trees from aligned homologous sequences. Molecular Evolution: Computer analysis of protein and nucleic acid sequences Ed. R. F. Doolittle. San Diego, Academic Press. 736.
Dagley, S. (1976). "Microbial catabolism and the carbon cycle." Biochem. Soc. Trans. 4: 455-458.

Datta, N. and R. W. Hedges (1972). "Host ranges of R factors." Jour. Gen. Microbio. 70: 453-460.

Davidson, M. W., M. J. Page and M. F. French (1991). "Drugs for Bugs." Am. Lab. 23(12): 34-338.

De Vos, P., M. Goor and J. D. Ley (1985). "Ribosomal ribonucleic acid cistron similarities of phytopathogenic *Pseudomonas species*." International Journal of Systematic Bacteriology 1985(2): 169-184.

deBruijn, F. J. (1992). "Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergeneric consensus)sequences and the polymerase chain reaction to fingerprint the genomes of Rhizobium meliloti isolates and other soil bacteria." Applied and Environmental Microbiology 58(7): 2180-2187.

Dewhirst, F. E., B. J. Paster and P. L. Bright (1989). "*Chromobacterium, Eikenella, Kingella, Neisseria, Simonsiella,* and *Virteoscilla* species comprise a major branch of the beta group of *Proteobacteria* by 16S ribosomal ribonucleic acid sequence comparison: tranfer of *Eikenella* and *Simsonsiella* to the family *Neisseriaceae* (emend.)." International Journal of Systematic Bacteriology 39(3): 258-266.

Dewhirst, F. E., B. J. Paster, I. Olsen and G. J. Fraser (1992). "Phylogeny of 54 representative strains of species in the family *Pasteurellaceae* as determinedby comparison of 16S rRNA." J. Bacteriol. 174(6): 2002-2013.

Dickerson, R. E. (1980). "Evolution and gene transfer in purple photosynthetic bacteria." Nature 283: 210-212.

Dobzhansky, T. (1973). "Nothing in biology makes sense except in the light of evolution." American Biology teacher 25: 125-129.

Don, R. H. and J. M. Pemberton (1981). "Properties of six pesticide degradation plasmids isolated from *Alcaligenes paradoxus* and *Alcaligenes eutrophus.*" Jour. Bacteriol. 145(2): 681-686.

Don, R. H. and J. M. Pemberton (1985). "Genetic and physical map of the 2,4-dichlorophenoxyacetic acid-degrading plasmid pJP4." J. Bacteriol. 161(1): 466-468.

Don, R. H., A. J. Weightman and H. J. K. a. K. N. Timmis (1985). "Transposon mutagenesis and cloning of the pathways for degradation of 2,4-dichlorophenoxyacetic acid and 3-chlorobenzoate in *Alcaligenes eutrophus* JMP134(pJP4)." :

Doolittle, R. F., Ed. (1990). Molecular Evolution: Computer Analysis of Protein and nucleic Acid Sequences. Methods in Enzymology. San Diego, Academic Press.
Doolittle, R. F., D. F. Feng, K. L. Anderson and M. R. Alberro (1990). "A naturally occuring horizontal gene transfer from a eukaryote to a prokaryote." J. Mol. Evol. 31: 383-388.

Doolittle, W. F. (1988). "Bacterial evolution." Can. J. Microbiol. 34: 547-551.

Doolittle, W. F. and J. R. Brown (1994). "Tempo, mode, the progenote, and the universal root." Proc. Natl. Acad. Sci. USA 91: 6721-6728.

Doolittle, W. F. and C. Sapienza (1980). "Selfish genes, the phenotype paradigm and genome evolution." Nature 284: 601-603.

Drake, J. W. (1991). "A constant rate of spontaneous mutation in DNA-based microbes." Proc. Natl. Acad. Sci. USA 88: 7160-7164.

DuBose, R. F., D. E. Dykhuizen and D. Hartl (1988). "Genetic exchange among natural isolates of bacteria: recombination within the *pho*A gene of *Escherichia coli*." Proc. Natl. Acad. Sci. 85: 7036-7040.

Duxbury, J. M., J.Tiedje, M. Alexander and J. E. Dawson (1970). "2,4-D metabolism: enzymatic conversion of chloromalylacetic acid to succinic acid." J Agr Food Chem 18(2): 199-201.

Dykhuizen, D. E. and L. Green (1991). "Recombination in *Escherichia coli* and the definition of biological species." J. Bact. 173(22): 7257-7268.

Eberhard, W. G. (1990). "Evolution in bacterial plasmids and levels of selection." Quarterly Rev. Biol. 61(1): 3-22.

Eckhardt, T. (1978). "A rapic method for the identification of plasmid desoxyribonucleic acid in bacteria." Plasmid 1: 584-588.

Eden, P. A., T. M. Schmidt, R. P. Blakemore and N. R. Pace (1991). "Phylogenetic analysis of *Aquaspirillum magnetotactium* using polymerase chain reaction-amplified 16S rRNA-specific DNA." International Jouranl of Systematic Bacteriology 41(2): 324-325.

Elsas, J. D. V., J. T. Trevors, M. E. Starodub and L. S. V. Overbeek (1990). "Transfer of plasmid RP4 between pseudomonads after introduction into soil; influence of spacial and temporal aspects of inoculation." FEMS Microbiol. Ecol. 73: 1-12.

Fallenleaf (1990). Proceedings of the Fallen Leaf LAke Conference on Promiscuous Plasmids in Gram-Negative and -Positive Bacteria, Fallen Leaf Lake, South Lake Tahoe, California, Plasmid.

Felsenstein, J. (1981). "Evolutionary trees from DNA sequences: a maximum likelihood approach." Journal of Molecular Evolution 17: 368-376.

Felsenstein, J. (1985). "Confidence limits on phylogenies: an approach using the bootstrap." Evolution 39(4): 783-791.

Felsenstein, J. (1988). "Phylogenies from molecular sequences: Inference and reliability." Annu. Rev. Genet. 22: 521-565.

Felsenstein, J. (1992). "Phylogenies from restriction sites: a maximum-likelihood approach." Evolution 46(1): 159-173.

Fisher, P. R., J. Appleton and J. M. P{emberton (1978). "Isolation and characterization of the pesticide-degrading plasmid pJP1 from *Alcaligenes paradoxus*." J. Bacteriol. 135(3): 798-804.

Flores, E. and C. P. Wolk (1985). "Identification of facultatively heterotrophic, N2-fixing cyanobacteria able to receive plasmid vectors from *Escherichia coli* by conjugation." J. Bacteriol. 162(3): 1339-1341.

Fox, G. E., E. Stackebrandt, R. B. Hespell, J. Gibson, J. Maniloff, T. A. Dyer, R. S. Wolfe, et al. (1980). "The phylogeny of prokaryotes." Science 209: 457-463.

Fox, G. E., J. D. Wisotzkey and P. Jurtshuk (1992). "How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity." :

Fox, J. L. (1983). "Soil microbes pose problems for pesticides." Science 221: 1029-1031.

Fox, J. L. (1989). "Native microbes'role in Alaskan clean-up." Bio/Technology 7: 852.

Frey, J. and M. Bagdasarian (1989). The molecular biology of IncQ plasmids. Promiscuous Plasmids of Gram-Negative Bacteria . Academic Press Limited. 79-94.

Friedrich, B., M. Meyer and H. Schlegel (1983). "Transfer and expression of the herbicide-degrading plasmid pJP4 in aerobic autotrophic bacteria." Arch. Microbiol. 134: 92-97.

Fries, M. R., J. Zhou, J. Chee-Sanford and J. M. Tiedje (1994). "Isolation, characterization, and distribution of denitrifying toluene degraders from a variety of habitats." Appl. Environ. Microbiol. 60(8): 2802-2810.

Frischer, M. E., G. J. Stewart and J. H. Paul (submitted). "Plasmid transfer to indigenous marine bacterial populations by natural transforamtion."

Frisher, M. E., J. M. Thurmond and J. H. Paul (1990). "Natural plasmid transformation in a high-frequency-of-transformation marine *Vibrio* strain." Applied and Environmental Microbiology 56(11): 3439-3444.

Fry, J. C. and M. J. Day (1990). Bacterial Genetics in Natural Environments. Cambridge, Chapman and Hall.

Fukuda, M. (1993). "Diverstiy of chloroaromatic oxygenases." Current Biology 4: 339-343.

Fukumori, F. and R. P. Hausinger (1993). "*Alcaligenes eutrophus* JMP134 "2,4-Dichlorophenoxyacetate monooxygenase" is an alpha-ketoglutarate-dependent dioxygenase." J. Bacteriol. 175(7): 2083-2086.

Fulthorpe, R. and R. C. Wyndham (1992). "Involvement of a chlorobenzoate-catabolic transposon, Tn 5271, in community adaptation to chlorobiphenyl, chloroaniline, and 2,4-dichlorophenoxyacetic acid in a freshwater ecosystem." Appl. Environ. Microbiol. 58(1): 314-325.

Fulthorpe, R. R., N. A. Straus and R. C. Wyndham (1989). Bacterial adaptation to chlorobenzoate contamination in the Niagara region investigated by DNA:DNA colony hybridization. *In* Aquatic Toxicology and Environmental Fate. Eds. G. W. S. II and M. A. Lewis. Philadelphia, American Society for Testing and Materials. 59-71.

Fulthorpe, R. R. and R. C. Wyndham (1991). "Transfer and expression of the catabolic plasmid pBR60 in wild bacterial recipients in a freshwater ecosystem." Appl. Environ. Microbiol. 57(5): 1546-1553.

Fulthorpe, R. R., C. McGowan, O. V. Maltseva, W. H. Holben, and J. M. Tiedje. (1995) 2,4-Dichlrophenoxyacetic acid degrading bacteria are mosaics of catabolic genes. (submitted.)

Ghisalba, O. (1983). "Chemical wastes and their biodegradation - an overview." Experientia : 1247-1257.

Ghosal, D. and I.-S. You (1988). "Nucleotide homology and organization of chlorocatechol oxidation genes of plasmids pJP4 and pAC27." Mol. Gen. Genet. 211: 113-120.

Ghosal, D., I.-S. You, D. K. Chatterjee and A. M. Chakrabarty (1985). "Genes specifying degradation of 3-chlorobenzoic acid in plasmids pAC27 and pJP4." Proc. Nat. Aca. Sci. USA 82: 1638-1642.

Ghosal, D., I.-S. You, D.K.Chatterjee and A. M. Chakrabarty (1985). "Microbial degradation of halogenated compounds." Science 228(4696): 135-142.

Gibson, D. T., Ed. (1984). Microbial Degradation of Organic Compounds. Microbiology Series. New York, Marcel Dekker.

Gilson, E., J.-M. Clement, D. Brutlag and M. Hofnung (1984). "A family of dispersed repetitive extragenic DNA sequences in *E. coli.*" EMBO 3(6): 1417-1421.

Gilson, E., J. M. Clément, D. Perrin and M. Hofnung (1987). "Palindromic units: a case of highly repetitive DNA sequences in bacteria." Trends in Genetics 3(8): 226-230.

Giovannoni, S. J., S. Turner, G. J. Olsen, S. Barns, D. J. Lane and N. Pace (1988). "Evolutionary relationships among cyanobacteria and green chloroplasts." J. Bacteriol. 170: 3584-3592.

Gish, W. and D. J. States (1993). "Identification of protein coding regions by database similarity search." Nature Genetics 3: 266-272.

Goodfellow, M., D. Jones and F. G. Priest, Eds. (1985). Computer-assisted Bacterial Systematics. Special Publication of the Society for Gneral Microbiology. London, Academic Press.

Graham, J. B. and C. A. Istock (1978). "Genetic exchange in *Bacillus subtilis* in soil." Mol. Gen. Gen. 166: 287-290.

Graham, J. B. and C. A. Istock (1979). "Gene exchange and natural selection cause *Bacillus subtilis* to evolve in soil culture." Science 204: 637-639.

Greer, C. W., J. Hawari and R. Samson (1990). "Influence of envrionmental factors on 2,4-dichlorophenoxyacetic acid degradation by *Pseudomonas cepacia* isolated from peat." Arch. Micriobiol 154: 317-322.

Greer, L. E., J. A. Robinson and D. R. Shelton (1992). "Kinetic comparison of seven strains of 2,4-dichlorophenoxyacetic acid-degrading bacteria." Appl. Environ. Microbiol. :

Guerry, P., D. J. LeBlanc and S. Faldow (1973). "General method for the isolation of plasmid deoxyribonucleic acid." J. Bact. 116(2): 1064-1066.

Harayama, S. and R. H. Don (1985). Catabolic plasmids: their anaysis and utilization in the manipulation of bacterial metabolic activities. Genetic Engineering Principles and Methods Eds. J. K. Setlow and A. Hollander. New York, Plenum Press. 283-307.

Harayama, S. and M. Kok (1992). "Functional and evolutionary relationships among diverse oxygenases." Annu. Rev. Microbiol. 46: 565-601.

Hardy, K. G., Ed. (1987). Plasmids: A Pracitical Approach. The Practical Approach Series. Oxford, IRL Press.

Hardy, K. G. (1987). Purification of plamids. Plasmids: A Practical Approach . Oxford, IRL Press. 180.

Harker, A. R. and Y. Kim (1990). "Trichloroethylene degradation by two independent aromatic degrading pathways in *Alcaligenes eutrophus* JMP134." Appl. Env. Microbiol. 56(4): 1179-1181.

Harker, A. R., R. H. Olsen and R. J. Seidler (1989). "Phenoxyacetic acid degradation by the 2,4-Dichlorophenoxyacetic acid (TFD) pathway of plasmid pJP4: mapping and characterization of the TFD regulatory gnen, *tfd*R." J. Bacteriol. 171(1): 314-320.

Harrigan, W. F. (1970). Probability tables for the estimation of microbial numbers by the multiple tube technique. Laboratory Methods in Food and Dairy Microbiology . London, Harcourt, Brace Jovanovich.

Hartnett, C., E. L. Neidle, K.-L. Ngai and L. N. Ornston (1990). "DNA sequences of genes encoding *Acinetobacter calcoaceticus* protocatechuate 3,4-dioxygenase: evidence indicating shuffling of genes and of DNA sequences within genes during their evolutionary divergence." Journal of Bacteriology 172(2): 956-966.

Hazen, T. C. and L. Jimenez (1988). "Enumeration and identification of bacteria from environmental samples using nucleic acid probes." Microbiol. Sciences 5(11): 340-343.

Heinemann, J. A. (1991). "Genetics of gene transfer between species." TIG 7(6): 181-185.

Heinemann, J. A. and G. F. Sprague (1989). "Bacterial conjugative plasmids mobilize DNA transfer between bacteria and yeast." Nature 340: 205-209.

Helling, C. S., J.-M. Bollag and J. E. Dawson (1968). "Cleavage of ether-oxygen bond in phenoxyacetic acid by an *Arthrobacter* species." J. Agric. Food Chem. 16(3): 538-539.

Hensche, R. B. and F. R. J. Schmidt, Eds. (1990). Use of wide hst range promoters to monitor the fate of recombinant DNA in soil.

Henschke, R. B. and F. R. J. Schmidt (1990). "Plasmid mobilization from genetically engineered bacteria to members of the indigenous soil microflora *in situ.*" Curr. Microbiol. 20: 105-110.

Higgins, C. F., R. S. McLaren and S. F. Newbury (1988). "Repetitive extragenomic palindromic sequences, mRNA stability and gene expression: evolution by gene conversion? - a review." Gene 72: 3-14.

Hiraishi, A. (1992). "Direct automated sequencing of 16S rDNA amplified by polymerase chain reaction from bacterial cultures without DNA purification." Letters in Applied Microbiology 15: 210-213.

Hiraishi, A., Y. Hoshino and T. Satoh (1991). "*Rhodoferax fermentans* gen. nov., sp. nov., a phototrophic purple nonsulfur bacterium previously referred to as the "*Rhodocyclus gelatinonus*-like" group." Arch. Microbiol. 155: 330-336.

Holben, W. E., V. G. M. Calabrese, D. Harris, J. O. Ka and J. M. Tiedje (1993). Analysis of structure and selection in microbial communities by molecular methods. Trends in Microbial Ecology Eds. R. Guerrero and C. Pedrós-Alió. Spanish Society for Microbiology. 367-370.

Holben, W. E., V. G. M. Calabrese, D. Harris, J. O. Ka and J. M. Tiedje (1993). "Analysis of structure and selection in microbial communities by molecular methods." Trends in Microbial Ecology : 367-370.

Holben, W. E., J. K. Jansson, B. K. Chelm and J. M. Tiedje (1988). "DNA probe method for the detection of specific microorganisms in the soil bacterial community." Appl. Environ. Microbiol. 54: 703-711.

Holben, W. E., B. M. Schroeter, V. G. M. Calabrese, R. H. Olsen, J. K. Kukor, V. O. Biederbeck, A. E. Smith, et al. (1992). "Gene probe analysis of soil microbial population selected by amendment with 2,4-dichlorophenoxyacetic acid." Appl. Env. Microbiol. 58(12): 3941-3948.

Istock, C. A., K. E. Duncan, N. Ferguson and X. Zhou (1992). "Sexuality in a natural population of bacteria -- Bacillus subtilus challenges the clonal paradigm." Molecular Ecology 1: 95-103.

Jacob, F. (1977). "Evolution and tinkering." Science 196: 1161-1166.

Jacobsen, C. S. and J. C. Pedersen (1992). "Growth and survival of *Pseudomonas cepacia* DB01(pRO101) in soil amended with 2,4-dichlrophenoxyacetic acid." Biodegradation 2: 245-252.

Jacobson, C. S. and J. C. Pedersen (1992). "Mineralization of 2,4-dichlorophenoxyacetic acid (2,4-D) in soil inoculated with *Pseudomonas cepacia* DBO1(pRO101) and *Alcaligenes eutrophus* JMP134(pJP4): effects of inoculation level and substrate concentration." Biodegradation 2: 253-263.

Jain, R. K., G. S. Sayler, J. T. Wilson, L. Houston and D. Pacia (1987). "Maintenance and stability of introduced genotypes in groundwater aquifer material." Appl. Env. Microbiol. 53(5): 996-1002.

Jansson, J. (1988). Development of gene probe methodology for detection of specific bacteri in environmental samples. PhD Dissertastion. Michigan State University.

Jeenes, D. J. and P. A. Williams (1982). "Excision and integration of degradative pathway genes from TOL plasmid pWWO." J. Bact. 150(1): 188-194.

Johnson, J. L. and N. J. Palleroni (1989). "Deoxyribonucleic acid similarities among *Pseudomonas* species." International Journal of Systematic Bacteriology 39(3): 230-235.

Ka, J. O., W. E. Holben and J. M. Tiedje (1994a). "Analysis of competition in soil among 2,4-dichlorophenoxyacetic acid-degrading bacteria." Appl. Environ. Microbiol. 60(4):

Ka, J. O., W. E. Holben and J. M. Tiedje (1994b). "Genetic and phenotypic diversity of 2,4-dichlorophenoxyacetic acid (2,4-D)-degrading bacteria isolated from 2,4-D-treated field soils." Appl. Environ. Microbiol. 60(4):

Ka, J. O., W. E. Holben and J. M. Tiedje (1994c). "Use of gene probes to aid in recovery and identification of functionally dominant 2,4-Dichlorophenoxyacetic acid-degrading populations in soil." Appl. Environ. Microbiol. 60(4):

Ka, J. O. and J. M. Tiedje (1994d). "Integration and excision of a 2,4-dichlorophenoxyacetic acid-degradative plasmid in *Alcaligenes paradoxus* and evidence of its natural intergeneric transfer." J. Bact. 176(17): 000-000.

Kado, C. I. and S.-T. Liu (1981). "Rapid procedure for detection and isolation of large and small plasmids." J. Bacteriol. 145(3): 1365-1373.

Kamagata, Y. (1994). personal communication.

Kaphammer, B., J. J. Kukor and R. H. Atlas (1990). "Regulation of *tfd*CDEF by *tfd*R of the 2,4-dichlorophenoxyactic acid degradation plasmid pJP4." J. Bacteriol. 172(5): 2280-2286.

Kellogg, S. T., D. K. Chatterjee and A. M. Chakrabarty (1981). "Plasmid-assisted molecular breeding: New technique for enhanced biodegradation of persistent toxic chemicals." Science 214: 1133-1135.

Kilbane, J. J., D. K. Chatterjee and A. M. Chakrabarty (1983). "Detoxification of 2,4,5-trichlorophenoxyacetic acid from contaminated soil by *Pseudomonas cepacia.*" Appl. Env. Microbiol. 45(5): 1697-1700.

Kimura, M. (1983). The neutral theory of molecular evolution. *In:* Evolution of genes and proteins Eds. M. Nei and R. K. Koehn. Sunderland, MA, Sinauer Associates.

Klingmüller, W. (1991). "Plasmid transfer in natural soil: a case by case study with nitrogen-fixing *Enterobacter.*" FEMS Microbial Ecology 85: 106-116.

Kobayashi, H. and B. E. Rittmann (1982). "Microbial removal of hazardous organic compounds." Environ. Sci. Techno. 16(3): 170-181.

Kozyreva, L. P., Y. V. Shurukhin, Z. I. Finkel'shtein, B. P. Baskunov and L. A. Golovleva (1993). "Metabolism of the herbicide 2,4-D by a *Nocardioides simplex* strain." Microbiology 62(1): 78-84.

Krishnapillai, V. (1988). "Molecular genetic analysis of bacterial plasmid promiscuity." FEMS Microbiol. Rev. 54: 223-238.

Kukor, J. J., R. H. Olsen and J.-S. Siak (1989). "Recruitment of a chromosomally encoded malylacetate reductase fro degradation of 2,4-dichlorophenoxyatecit acid by plasmid pJP4." J. Bacteriol. 171(6): 3385-3390.

Kumar, S., K. Tamura and M. Nei (1993). MEGA: Molecular Evolutionary Genetics Analysis, version 1.01. University Park, PA, The Pennsylvania State University.

Kuramitzu, H. K. and M. J. Casadaban (1986). "Transposition of the gram positive transposon TN917 in *E. coli.*" J.Bact. 167: 711-712.

Laguerre, G., M.-R. Allard, F. Revoy and N. Amarger (1994). "Rapid identification of Rhizobia by restriction fragment length polymorphis analysis of PCR-amplified 16S rRNA genes." AEM 60(1): 56-63.

Lake, J. A. (1991). "Tracing origins with molecular sequences: metazoan and eukaryotic beginnings." TIBS 16: 46-50.

Lane, D. J., B. Pace, G. J. Olsen, D. A. Stahl, M. Sogin and N. R. Pace (1985). "Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses." P.N.A.S. 82: 6955-6959.

Lang, B. F. (1984). "The mitochondrial genome of the fission yeast *S. pombe*: highly homologous introns are inserted in the same positions of the otherwise less conserved Cox 1 genes in S. pombe and Apergillus nidulans." EMBO 3: 2129-2136.

Latorre, J., W. Reineke and H.-J. Knackmuss (1984). "Microbial metabolism of chloroanilines: enhanced evolution by natural genetic exchange." Arch. Microbiol. 140: 159-165.

Lenski, R. E. and M. Travisano (1994). "Dynamics of adaptation and diversification: a 10,000-generation experiment with bacterial populations." Proc. Natl. Acad. Sci. USA 91: 6808-6814.

Lerner, S. A., T. T. Wu and E. C. C. Lin (1964). "Evolution of a catabolic pathway in bacteria." Science 146: 1313-1315.

Lessie, T. G., M. S. Wood, A. Byrne and A. Ferrante (1990). Transposable gene-activating elements in *Pseudomonas cepacia*. In: *Pseudomonas: Biotransformations, Pathogenesis, and Evolving Biotechnology* Ed. Silver. Washington, D.C., American Society for Microbiology.

Levy, S. B. and B. M. Marshall (1988). Genetic transfer in the natural environment. In: *The Release of Genetically-Engineered Microorganisms*. New York, Academic Press. 61-76.

Levy, S. B. and R. V. Miller (1989). Gene transfer in the environment. New York, McGraw Hill Publishing Comapany.

Lewin, R. (1989(?)). "Fish to bacterium gene transfer." Science 227: 1020.

Ley, J. d., P. Segers, K. Kersters, W. Mannheim and A. Lievens (1986). "Intra- and intergeneric similarities of the *Bordatella* ribosomal ribonucleic acid cistrons: proposal for a new family, *Alcaligenaceae*." Interantional Journal of Systematic Bacteriology 36(3): 405-414.

Li, W.-H. (1986). "Evolutionary change of restriction cleavage sites and phylogenetic inference." Genetics 113: 187-213.

Li, X., M. Dorsch, T. D. Dot, L. I. Sly, E. Stackebrandt and A. C. Haywood (1993). "Phylogenetic studies of the rRNA group II pseudomonads based on 16S rRNA gene sequences." Journal of Applied Bacteriology 74: 324-329.

Loos, M. A., R. N. Roberts and M. Alexander (1967). "Formation of 2,4-dichlorophenol and 2,4-dichloroanisole from 2,4-dichlorophenoxyacetate by *Arthrobacter* sp." Can. J. Microbiol. 13: 691-699.

Loos, M. A., R. N. Roberts and M. Alexander (1967). "Phenols as intermediates in the decomposition of phenoxyacetates by an *Arthrobacter* species." Can. J. Microbiol. 13: 679-690.

Lorenz, M. G., B. W. Aardema and W. Wackernagel (1988). "Highly efficient genetic transformation of *Bacillus subtilis* attached to sand grains." J. Gen. Microbiol. 134: 107-112.

Lorenz, M. G. and W. Wackernagel (1991). "High frequency of natural genetic transformation of *Pseudomonas stutzeri* in soil extract supplemented with a carbon/energy and phosphorus source." Appl. Env. Microbiol. 57(4): 1246-1251.

Lupski, J. R. and G. M. Weinstock (1992). "Short, interspersed repetitive DNA sequences in prokaryotic genomes." J. Bacteriology 174(14): 4525-4529.

Macrina, F. L., D. J. Kopecko, K. R. Jones, D. J. Ayers and S. M. McCowen (1978). "A multiple plasmid containing *Escherichia coli* strain: Convenient source of size reference plasmid mlecules." Plasmid 1: 417-420.

Maddison, W. P. and D. R. Maddison (1992). MacClade: Analysis of phylogeny and character evolution. Version 3.0. Sunderland, MA, Sinauer Associates.

Madsen, E. L. (1991). "Determining *in situ* biodegradation: facts and challenges." Environ. Sci. Technol. 25(10): 1663-1672.

Mäe, A. A., R. O. Maritis, N. R. Ausmees, V. M. Koiv and A. L. Heinaru (1993). "Characterization of a new 2,4-dichlorophenoxyacetic acid degrading plasmid pEST4011: physical map and localization of catabolic genes." Journal of General Microbiology 139: 3165-3170.

Mancini, P., S. Fertels, D. Nave and M. Gealt (1987). "Mobilization of plasmid pHSV106 from *Escherichia coli* HB101 in a laboratory-scale waste treatment facility." Appl. Environ. Microbiol. 53(4): 665-671.

Maniatis, T., E. F. Fritsch, J. Sambrook (1982). *Molecular Cloning: a Laboratory Manual.* Cold Spring Harbor Laboratory Press. New York.

Margulis, L. (1991). Symbiosis in Evolution: Origins of cell motility. In: *Evolution of Life: Fossils, Molcules and Culture* ,Eds. S. Osawa and T. Honjo. Tokyo, Springer-Verlag. 305-324.

Margulis, L. (1992). "Biodiversity: molecular biological domains, symbiosis and kingdom origins." BioSystems 27: 39-51.

Marshall, E. (1991). "A is for apple, Alar and ... alarmist?" Science 254: 20-22.

Martin, J. P. and I. Fridovitch (1981). "Evidence for a natural gene transfer from the ponyfish to its bioluminescent symbiont Photobacter leignathi." Jour. Biol. Chem. 256(12): 6080-6089.

Massol-Deya, A. A., D. A. Odelson, R. F. Hickey and J. M. Tiedje (in press). Bacterial community fingerprinting of amplified 16S and 16-23S ribosomal DNA gene sequences and restriction endonuclease analysis (ARDRA). In: *Molecular Microbial Ecology Manual,* Ed. F. deBruijn. Kluwgr pub.

Matsumoto, H., Y. Itoh, S. Ohta and Y. Terawaki (1986). "A generalized transducing phage of *Pseudomonas cepacia.*" J. Gen. Microbiol. 132: 2583-2586.

Mazodier, P., R. Petter and C. Thompson (1989). "Intergenic conjugation between *Escherichia coli* and *Streptomyces* species." Jour. Bacteriology 171(6): 3583-3585.

McCall, P. J., S. A. Vrona and S. S. Kelley (1981). "Fate of uniformly carbon-14 ring labeled 2,4,5-trichlorophenoxyacetic acid and 2,4,-dichlorophenoxyacetic acid." J. Agric. Food. Chem. 29(1):

McClure, N. C., J. C. Fry and A.J.Weightman (1991). "Survival and catabolic activity of natural and genetically engineered bacteria in a labolratory-scale activated sludge unit." Appl. Env. Microbiol. 57(2): 366-373.

McClure, N. C., A. J. Weightman and J. C. Fry (1989). "Survival of *Pseudomonas putida* UWC1 containing cloned catabolic genes in a model activated-sludge unit." Appl. Environ. Microbiol. 55(10): 2627-2634.

Mergeay, M., D. Springael and E. Top (1990). Gene transfer in polluted soils. In: *Bacterial Genetics in Natural Environments* , Eds. J. C. Fry and M. J. Day. London, Chapman and Hall. 259.

Meulien, P. and P. Broda (1982). "Identification of chromosomally integrated TOL DNA in cured derivatives of *Pseudomonas putida* PAW1." J. Bacteriol. 152(2): 911-914.

Michel, F. and B.Dujon (1986). "Genetic exchanges between bacteriophage T4 and filamentous fungi?" Cell 46: 3231.

Milkman, R. and M. M. Bridges (1990). "Molecular evolution of the *Escherichia coli* chromosome. III Clonal fragments." Genetics 126: 505-517.

Milkman, R. and A. Stoltzfus (1988). "Molecular evolution of the *Escherichia coli* chromosome. II Clonal segments." Genetics 120: 359-366.

Miller, J. H. (1972). Experiments in Molecular Genetics. Cold Spring Harbor, Cold Spring Harbor Laboratory.

Miller, R. V., T. A. Kokjohn and G. S. Sayler (1990). Environmental and molecular characterization of systems which affect genenome alteration in *Pseudomonas aeruginosa*. In: *Pseudomonas: Biotransformations, Pathodenesis, and Evolving Biotechnology* Ed. Silver. Washington, D.C., American Society for Microbiology. 252-268.

Moore, E. R. B., R. Wittich, P. Fortnagel and K. N. Timmis (1993). "16S ribosomal RNA gene sequence characterization and phylogenetic analysis of a dibenzo-p-dioxin-degrading isolate within the new genus *Sphingomonas*." Letters in Applied Microbiology 17: 115-118.

Moran, N. A., M. A. Munson, P. Bauman and H. Ishikawa (1993). "A molecular clock in endosymbiotic bacteria calibrated using the insect hosts." Proc. R. Soc. Lond. B. 253: 167-171.

Morgan, P. and R. J. Watkinson (1989). "Microbiological methods for the cleanup of soil and ground water contaminated with halogenated organic compounds." FEMS Microbiol. Rev. 63: 277-300.

Mortlock, R. P., Ed. (1992). *The Evolution of Metabolic Function*. Boca Raton, CRC Press.

Mortlock, R. P. and M. A. Gallo (1992). "Experiments in the evolution of catabolic pathways usig modern bacteria." In: *The Evolution of Metabolic Function* Ed. R. P. Mortlock. Boca Raton, CRC Press. 1-13.

Moyer, C. L., F. C. Dobbs and D. M. Karl (1994). "Estimation of diversity and community structure through restriction fragment length polymorphism distribution analysis of bacterial 16S rRNA genes from a microbial mat at an active, hydrothermal vent system, Loihi Seamount, Hawaii." Applied and Environmental Microbiology 60(3): 871-879.

Murray, R. G. E., D. J. Brenner, R. R. Colwell, P. D. Vos, M. Goodfellow, P. A. D. Grimont, N. Pfennig, et al. (1990). "Report of the Ad Hoc Committee on approaches to taxonomy within the *Proteobacteria*." International Journal of Systematic Bacteriology 40(2): 213-215.

Nakatsu, C., J. Ng, R. Singh, N. Staius and C. Wyndham (1991). "Chlorobenzoate catabolic transposon Tn5271 is a composite class I element with flanking class II insertion sequences." Proc. Natl. Acad. Sci. In Press,:

Nakatsu, C. H. (1992). The biochemical pathway and catabolic genes for chlorobenzoate degradation encoded on the transopson Tn 5271 of *Alcaligenes* sp. strain BR60. PhD Thesis, Carleton University.

Natarajan, M. R. and P. Oriel (1991). "Conjugal transfer of recombinant transposon Tn916 from *Escherichia coli* to *Bacillus stearothermophilus*." Plasmid 26: 67-73.

Nei, M. and W.-H. Li (1979). "Mathematical model for studying genetic variation in terms of restriction endonucleases." Proc. Natl. Acad. Sci. 76(10): 5269-5273.

Neilson, J. W., K. L. Josephson, S. D. Pillai and I. L. Pepper (1992). "Polymerase chain reaction and gene probe detection of the 2,4-dichlorophenoxyacetic acid degradation plasmid, pJP4." Applied and Environmental Microbiology 58(4): 1271-1275.

Nüßlein, K., D. F. Dwyer, D. Maris and K. Timmis (In press). "Expression and transfer of catabolic pathways harbored by *Pseudomonas* spp. introduced into activated sludge microcosms." :

Oh, K.-H. and O. H. Tuovinen (1990). "Degradation of 2,4-dichlorophenoxyacetic acid by mixed cultures of bacteria." J. Indust. Microbiol. 6: 275-278.

Olsen, G. J., R. Overbeek, N. Larsen, T. L. Marsh, M. J. McCaughey, M. A. Maciukenas, W.-M. Kuan, et al. (1992). "The Ribosomal Database Project." Nucleic Acids Research 20: 2199-2200.

Olsen, G. J., C. R. Woese and R. Overbeek (1994). "The winds of (Evolutionary) change: breathing new life into microbiology." J. Bact. 176(1): 1-6.

Orgel, L. E. and F. H. C. Crick (1980). "Selfish DNA: the ultimate parasite." Nature 284: 604-607.

Ornston, L. N., J. Houghton, E. L. Neidle and L. A. Gregg (19). Subtle selection and novel mutation during evolutionary divergence of the ß-ketoadipate pathway.

Ornston, L. N. and D. Parke (1976). "Evolution of catabolic Pathways." Biochem. Soc. Trans. 4: 468-473.

Ornston, L. N. and W. K. Yeh (1979). "Origins of metabolic diversity: evolutionary divergence by sequence repitition." Proc. Natl. Acad. Sci. 76(8): 3996-4000.

Pallernoni, N. J., R. W. Ballard, E. Ralston and M. Doudoroff (1972). "Deoxyribonucleic acid homologies among some *Pseudomonas* species." J. Bacteriol. 110(1): 1-11.

Palleroni, N. J., R. Kunisawa, R. Contopoulou and M. Doudoroff (1973). "Nucleic Acid Homologies in the genus *Pseudomonas*." Int. J. Sys. Bacteriol. 24(4): 333-339.

Paster, B. J., F. E. Dewhirst, I. Olsen and G. J. Fraser (1994). "Phylogeny of *Bacteroides, Prevotella,* and *Porphyromonas* spp. and related bacteria." J. Bact. 176(3): 725-732.

Pemberton, J. M. (1981). "Genetic engineering and biological detoxification of environmental pollutants." Residue Reviews 78: 1-11.

Pemberton, J. M., B. Corney and R. H. Don (1979). Evolution and spread of pesticide degrading ability among soil micro-organisms. Plasmids of Medical, Environmental and Commercial Importance, Spitzingsee, F.D.G., Elsevier.

Perkins, E. J., M. P. Gordon, O. Caceres and P. F. Lurquin (1990). "Organization and sequence analysis of the 2,4-dichlorophenol hydroxylase and dichlorocatechol oxidative operonsa of plasmid pJP4." J.Bacteriol. 172(5): 2351-2359.

Pertsova, R. N., F.Kunc and L. A. Golovleva (1984). "Degradation of 3-chlorobenzoate in soil by pseudomonads carrying biodegradative plasmids." Folia Microbiol. 29: 242-247.

Pfennig, N. (1984). Microbial behavior in natural environments. In: *The Microbes* Eds. D. P. Kelly and N. G. Carr. Cambridge, Cambridge University Press.

Pinkney, M. and C. M. Thomas (1987). " Replication and maintenace of promiscuous plasmids of Gram-negative bacteria." Microbiol. Sciences 4(6): 186-191.

Piruzyan, L. A., A. G. Malenkov and G. M. Barrenboym (1985)." Chemistry and the biosphere." In: *Global Ecology Ed. C. H. Southwick.* Sunderland, Massachusetts, Sinauer Associates. 323.

Postel, S. (1988). "Controlling toxic chemicals." In: IEd. L. R. Brown. New York, W. W. Norton. 237.

Reineke, W., D. Jeenes, P. A. Williams and H.-J. Knackmuss (1982). "TOL plasmid pWWO in constructed halobenzoate-degrading Pseudomonas strains: prevention of meta pathway." J. Bact. 150(1): 195-210.

Reineke, W. and H.-J. Knackmuss (1988). "Microbial degradation of haloaromatics." Ann. Rev. Microbiol. 42: 263-287.

Reineke, W. and H. J. Knackmuss (1979). "Construction of haloaromatics utilising bacteria." Nature 277(1): 385-386.

Reineke, W. and H. J. Knackmuss (1980). "Hybrid pathway for chlorobenzoate metabolism in *Pseudomonas* sp. B13 derivatives." J. Bacteriol. 142: 467-473.

Roberts, L. (1987). "Discovering microbes with a taste for PCBs." Science 237: 975-977.

Roberts, M. S. and F. M. Cohan (1993). "The effect of DNA sequence divergence on sexual isolation in *Bacillus.*" Genetics 134: 401-408.

Roberts, R. C., R. Burioni and D. K. Helsinki (1990). "Genetic characterization of the stabilizing functions of a region of broad-host-range plasmid RK2." J. Bacteriol. 172(11): 6204-6216.

Rochkind, M. L., J. W. Blackburn and G. Sayler, Eds. (1986). Microbial Decomposition of Chlorinated Aromatic Compounds. Cincinnati, U.S. E.P.A.

Rosenshine, I., R. Tchelet and M. Mevarech (1987). "The mechanism of DNA transfer in the mating system of an *Archaebacterium.*" Science (245): 1387-1389.

Roszak, D. B. and R. R. Colwell (1987). "Survival strategies of bacteria in the natural environment." Microbiol. Rev. 51: 365-379.

Saunders, J. R., A. Docherty and G. O. Humphreys (1984). "Transformation of bacteria by plasmid DNA." Meth. Microbiol 17: 61-95.

Saye, D. J., O. Ogunseitan, G. S. Sayler and R. V. Miller (1987). "Potential for transduction of plasmids in a natural freshwater environment: effect of plasmid donor concentration and a natural microbial community on transduction in Speudomonas aeruginosa." Appl. Environ. Microbiol. 53(5): 987-995.

Saye, D. J., O. A. Ogunseitan, G. S. Sayler and R. V. Miller (1990). "Transduction of linked chromosomal genes between *Pseudomonas aeruginaosa* strains during incubation *in situ* in a freshwater habitat." Appl. Environ. Microbiol. 56(1): 140-145.

Sayler, G. S., S. W. Hooper, A. C. Layton and J. M. H. King (1990). "Catabolic plasmids of environmental and ecological significance." Microb. Ecol 19: 1-20.

Schilf, W. and W. Klingmüller (1983). "Experiments with *Escherichia coli* on the dispersal of plasmids in environmental samples." Recombinant DNA Technical Bulletin 6(3): 101-102.

Schlömann, M. (1991). Enzyme und gene des Abbaus mono- und dichlorsubstituierter Brenzkatechine: Woher kommt ein neuer Abbauweg? Biologischer Abbau von Chlorkohlenwasserstoffen Ed. B. Weigert. Berlin, Technishe Universität Berlin. 87-107.

Schlömann, M., D. H. Pieper and H. J. Knackmuss (1990). Enzymes of haloaromatic degradation: variation of *Alcaligenes* on a theme by *Pseudomonas*. In: *Psuedomonas: Biotransformations, pathogenesis, and evolving biotechnology*. Eds, Silver, S., A. M. Chakrabarty, B. Iglewski and S. Kaplan, Washington, D.C., American Society of Microbiologists.

Schlömann, M., E. Schmidt and H. J. Knackmuss (1984). "Different types of a key enzyme of haloaromatic degradation in benzoate utilizing bacteria." Appl. Sys. Microbiol. 5: 259.

Schmidt, T. M., E. F. DeLong and N. R. Pace (1991). "Analysis of a marine picoplankton community by 16S rRNA gene cloning and sequencing." J. Bacteriol. 173(14): 4371-4378.

Schofield, P. R., A. H. Gibson, W. F. Dudman and J. M. Watson (1987). "Evidence for genetic exchange and recombination of *Rhizobium* symbiotic plasmids in a soil population." Appl. Env. Microbiol. 53(12): 2942-2947.
Short, K. A., J. D. Doyle and R. J. King (1991). "Effects of 2,4-Dichlorophenol, a metabolite of a genetically engineeered bacterium, and 2,4-dichlorophenoxyacetate on some microorganism-mediated ecological processed in soil." Appl. Env. Microbiol. 57(2): 412-418.

Sikorski, R. S., W. Michaud, H. L. Levin, J. D. Boeke and P. Heiter (1990). "Trans-kingdom promiscuity." Nature 345: 581-582.

Silver, S., A. M. Chakrabarty, B. Iglewski and S. Kaplan, Eds. (1990). *Psuedomonas: Biotransformations, pathogenesis, and evolving biotechnology.* Washington, D.C., American Society of Microbiologists.

Simon, R., U. Priefer and A. Puehler (1983). "A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram negative bacteria." Bio/Tech November: 784-791.

Simon, R., U. Priefer and A. Pühler (1983). Vector plasmids for in-vivo and in-vitro manipulation of gram-negative bacteria. Molecular Genetics of the Bacteria-Plant Interaction Ed. A. Pühler. Berlin, Springer-Verlag.

Sinclair, M. I., P. C. Maxwell, B. R. Lyon and B. W. Holloway (1986). "Chromosomal location of TOL plasmid DNA in *Pseudomonas putida.*" J. Bact. 168:

Siuda, J. F. and J. F. DeBernardis (1973). "Naturally occurring halogenated organic compounds." :

Slater, J. H. (1985). "Gene transfer in microbial communities." Microbiol Ecol. : 89-98.

Smith, C. A. and C. M. Thomas (1989). Relationships and evolution of IncP Plasmids. In: *Promiscuous plasmids of Gram-Negative Bacteria.* Academic Press Limited.

Smith, G. R. (1991). "Conjugal recombination in *E. coli*: myth and mechanisms." Cell 64: 19-27.

Smith, J. M. (1989). "Generating novelty by symbiosis." Nature 341: 248-285.

Smith, J. M., C. G. Dowson and B. G. Spratt (1991). "Localized sex in bacteria." Nature 349: 29-31.

Smith, J. M., N. H. Smith, M. O'Rourke and B. G. Spratt (1993). "How clonal are bacteria?" Proc. Natl. Acad. Sci., USA 90: 4384-4388.
Sogin, M. L. (1991). The phylogenetic significance of sequence diversity and length variations in eukaryotic small subunit ribosomal RNA coding regions. In: *New Perspectives on Evolution.* Wiley-Liss. 175-188.

Sonea, S. (1989). "A new look at bacteria." ASM News 55(11): 584-585.

Souza, V., T. T. Nguyen, R. R. hudson, D. Piñero and R. E. Lenski (1992). "Hierachical analysis of linkage disequilibrium in *Rhizobium* populations: Evidence for sex?" Proc. Natl. Acad. Sci., USA 89: 8389-8393.

Spratt, B. G., L. D. Bowler, Quian-Yun Zhang, J. Zhou and J. M. Smith (1992). "Role of interspecies transfer of chromosomal genes in the evolution of penicillin resistance in pathogenic and commensal *Neisseria* species." J. Mol. Evol. 34: 115-125.

Stachel, S. E. and P. C. Zambryski (1989). "Generic trans-kingdom sex?" Nature 340: 190-191.

Stackebrandt, E., R. G. E. Murray and H. G. Trüper (1988). "*Proteobacteria* classis nov., a name for the phylogenetic taxon that includes the "purple bacteria and their relatives"." International Journal of Systematic Bacteriology 38(3): 321-325.

Stanier, R. Y., N. J. Palleroni and M. Douderoff (1966). "The aerobic *Pseudomonads*: a taxonomic study." J. Gen. Microbiol. 43: 159-279.

Slatkin, M. (1985). "Gene flow in natural populations." Ann. Rev. Ecol. Syst. 16: 393-430.

Slatkin, M. and W. P. Maddison (1989). "A cladistic measure of gene flow inferred from the phylogenies of alleles." Genetics 123: 603-613.

Steffan, R. J., A. Breen, R. M. Atlas and G. S. Sayler (1989). "Monitoring geneically engineered microorganisms in freshwater microcosms." J. Indust. Microbiol. 4: 441-446.

Stern, M. J., G. F.-L. Ames, N. H. Smith, E. C. Robinson and C. F. Higgins (1984). "Pepetitive extragenic palindromic sequences: a major component of the bacteria genome." Cell 37: 1015-1026.

Stewart, C.-B. (1993). "The powers and pitfalls of parsimony." Nature 361: 603-607.

Stewart, G. J. (1986). "The biology of natural transformation." Ann. Rev. Microbiol. 40: 211-235.

Stewart, G. J. and C. D. Sinigalliano (1990). "Detection of horizontal gene transfer by natural transformation in native and introduced species of bacteria in marine and synthetic sediments." Appl. Environ. Microbiol. 56(6): 1818-1824.

Stotzky, G. (1989). "Gene transfer among bacteria in soil." In: *Gene transfer in the Environment* Eds. S. B. Levy and R. V. Miller. New York, McGraw-Hill Publishing Company. 434.

Stotzky, G. and H. Babich (1986). "Survival of, and genetic transfer by, genetically engineered bacteria in natural environments." Adv. Appl. Microbiol. 31: 93-138.

Streber, W. R., K. N. Timmis and M. H. Zenk (1987). "Analysis, cloning, and high-level expression of 2,4-dichlorophenoxyacetate monooxygenase gene *tfd*A of *Alcaligenes eutrophus* JMP134." J. Bacteriol. 169(7): 2950-2955.

Suwa, Y., A. D. Wright, F. Fukumori, K. A. Nummy, R. P. Hausinger, L. J. Forney, and W. E. Holben (1995). Gene cloning and sequence analysis of a chromosomally encoded 2,4-D dichlorophenoxyacetate monooxygenase gene *tfd*A of *Alcaligenes eutrophus* JMP134. J. Bacteriol. (submitted).

Swofford, D. L. (1991). PAUP: Phylogenetic Analysis Using Parsimony, version 3.0s. Champaign, Illinois, Illinois Natural History Survey.

Thomas, C. M., Ed. (1989). *Promiscuous Plasmids of Gram-Negative Bacteria.* San Diego, Academic Press Limited.

Thomas, C. M. and C. A. Smith (1987). "Incompatibility group P plasmids: genetics, evolution, and use in genetic manipulation." Ann. Rev. Microbiol. 41: 77-101.

Thomashow, M. F., R.Nutter, K. Postle, M. D. Chilton, F. R. Blattner, A.Powell, M. P. Gordon, et al. (1980). "Recombination between higher plant DNA and the Ti plasmid of *Agrobacterium tumefaciens*." Proc. Natl. Acad. Sci. USA 77(11): 6448-6452.

Tiedje, J. M., J. M. Duxbury, M. Alexander and J. E. Dawson (1969). "2,4-D metabolism: Pathway of degradation of chlorocatechols by *Arthrobacter* sp." J. Agr. Food Chem. 17(5): 1021-1025.

Timmis, K. N. and A. Pühler (1979). Plasmids of Medical, Environmental and Commercial Importance. In: *Plasmids of Medical Environmental and Commercial Importance,* Spitzingsee, Elsevier.

Tonso, N., N. Iwashita and M. Fukuda (1994). Phylogenetic analysis and aromatic degradative capability of several Rhodococci isolated from contaminated soils. American Society of Microbiology Meeting, Las Vegas. Poster Q 171.

Tonso, N. L., V. G. M. Calabrese and W. E. Holben (submitted). "Polyphasic characterization of a suite of bacterial isolates capable of degrading 2,4-D."

Top, E., M. Mergeay, D. Springael and W. Verstraete (1990). " Gene escape model: transfer of heavy metal resistance genes from Escherichia coli to *Alcaligenes eutrophus* on agar plates and in soil samples." Appl. Environ. Microbiol. 56: 2471-2479.

Top, E., I. d. Smet, W. Verstraete, R. Dijkmans and M. Mergeay (1994). "Exogenous isolation of mobilizing plasmids from polluted soils and sludges." Appl. Environ. Microbiol. 60(3): 831-839.

Torsvik, V., J. Goksøyr and F. L. Daae (1990). "High diversity in DNA of soil bacteria." Appl. Environ. Microbiol. 56(3): 782-787.

Torsvik, V., K. Salte, R. Sørheim and J. Goksøyr (1990). "Comparison of phenotypic diversity and DNA heterogeneity in a population of soil bacteria." Appl. Environ. Microbiol. 56(3): 776-781.

Tourova, T. P. and A. S. Antonov (1987). "Identification of microorganisms by rapid DNA-DNA hybridization." Methods in Microbiology 19: 333-355.

Trevors, J. T. (1987). "Survival of Escherichia coli donor, recipient, and transconjugant cells in soil." Water Air Soil Pollut. 34: 409-414.

Trevors, J. T., T. Barkay and W. Bourquin (1986). "Gene transfer among bacteria in osil and aquatic environments: a review." Can. J. Microbiol. 33: 191-198.

Trevors, J. T. and G. Berg (1989). "Conjugal RP4 transfer between pseudomonads in soil and recovery of RP4 plasmid DNA from soil." Syst. Appl. Microbiol. 11: 223-227.

Trevors, J. T. and K. M. Oddie (1986). "R-plasmid transfer in soil and water." Can. J. Microbiol 32: 610-613.

Trevors, J. T. and M. E. Starodub (1987). "R-plasmid transfer in non-sterile agricultural soil." Syst. Appl. Microbiol. 9: 312-315.

Trieu-Cuot, P., C. Carlier, P. Martin and P. Courvalin (1987). "Plasmid transfer by conjugation from *Escherichia coli* to Gram-positive bacteria." FEMS Microbiological Letters 48: 289-294.

US.EPA Pesticide Fact Handbook. Park Ridge, N.J., Noyes Data Corporation.

Valdés, A. M. and D. Piñero (1992). "Phylogenetic estimation of plasmid exchange in bacteria." Evolution 46(3): 641-656.

Van Bruggen, A. H. C., K. N. Jochimsen, E. M. Steinberger, A. Segers and M. Gillis (1993). "Classification of *Rhizomonas suberifaciens*, and unnamed *Rhizomonas* species, and *Sphingomonas* spp. in rRNA Superfamily IV." Internatioanl Journal of Systematic Bacteriology 43(1): 1-7.

Van der Meer, J. R., W. M. de Vos, S. Harayama and A. J. B. Zehnder (1992). "Molecular mechanisms of genetic adaptation to xenobiotic compounds." Microbiol. Rev. 56(4): 677-694.

Van der Meer, J. R., R. I. L. Eggen, A. J. B. Zehnder and W. M. d. Vos (1991). "Sequence analysis of the *Pseudomonas* sp. strain P51 *tcb* gene cluster, which encode metabolism of chlorinated catechols: evidence for specialization of catechol 1,2-dioxygenases for chlorinated substrates." J. Bact. 173(8): 2425-2434.

Van Elsas, J. D. and J. T. Trevors (1990). "Plasmid transfer to indigenous bacteria in soil and rhizosphere: problems and perspectives." In: *Bacterial Genetics in Natrual Environments* Eds. J. C. Fry and M. J. Day. Cambridge, Chapman and Hall. 188-199.

Verstraete, W. and E. Top (1992). Holistic Environmental Biotechnology. .

Vollmer, M. D., K. Stadler-Fritzsche and M. Schlömann (1993). "Conversion of 2-chloromaleylacetate in *Alcaligenes eutrophus* JMP134." Arch. Microbiol. 159: 1-7.

Walsh, T. A. and D. P. Ballou (1983). "Halogeneated protocatechuates as substrates for protocatechuate dioxygenase from *Pseudomonas cepacia.*" The J. Biol. Chem. 258(23): 14413-14421.

Ward, D. M., M. M. Bateson, R. Weller and A. L. Ruff-Roberts (preprint). "Ribosomal RNA analysis of microorganisms as they occur in nature." Advances in Microbial Ecology :

Wayne, L. G., D. J. Brenner, R. R. Colwell, P. A. D. Grimont, O. Kandler, M. I. Krichevsky, L. H. Moore, et al. (1987). "Report of the Ad Hoc Committee on reconciliation of approaches to bacterial systematics." International Journal of Systematic Bacteriology 37(4): 463-464.

Weinberg, S. R. and G. Stotzky (1972). "Conjugation and genetic recombination of *Escherichia coli* in soil." Soil Biol. Biochem. 4: 191-180.

Weisburg, W. G., S. M. Barns, D. A. Pelletier and D. J. Lane (1991). "16S ribosomal DNA amplification for phylogenetic study." J. Bact. 173(2): 697-703.

Wellington, E. M. H., N. Cresswell and V. A. Saunders (1990). "Growth and survival of streptomycete inoculants and extent of plasmid transfer in sterile and nonsterile soil." Appl. Environ. Microbiol. 46(4): 1413-1416.

Wheatcroft, R., D. G. McRae and R. W. Miller (1990). "Changes in the *Rhizobium meliloti* genome and the ability to detect supercoiled plasmids during bacteroid development." Molecular Plant-Microbe Interactions 3(1): 9-17.

Wheelis, M. L., O. Kandler and C. R. Woese (1992). "On the nature of global classification." Proc. Natl. Acad. Sci. 89: 2930-2934.

Wickham, G. S. and R. M. Atlas (1988). "Plasmid frequency fluctuation in bacterial populations from chemically stressed soil communities." Appl. Environ. Microbiol. 54(9): 2192-2196.

Willems, A., J. d. Ley, M. Gillis and K. Kersters (1991). "*Comamodaceae,* a new family encompassing the acidovorans rRNA complex, including *Variovorax paradoxus* gen. nov., comb. nov., for *Alcaligenes paradoxus* (Davis 1969)." International Journal of Systematic Bacteriology 41(3): 445-450.

Willets, N. (1985). Plasmids. Genetics of Bacteria Eds. J. Scaife, D. Leach and A. Galizzi. London, Academic Press. 165-195.

Willets, N. and C. Crowther (1981). "Mobilization of the non-conjugative IncQ plasmid RSF1010." Genet. Res. Camb 37: 311-316.

Williams, P. A. and M. J. Worsey (1976). "Plasmids and catabolism." Biochem. Soc. Trans. 4: 466-468.

Wilson, A. C., S. S. Carlson and T. J. White (1977). "Biochemical Evolution." Ann. Rev. Biochem. 46: 573-639.

Woese, C. (1987). "Bacterial evolution." Microbiol. Rev. 51: 221-271.

Woese, C. R. (1994). "There must be a prokaryote somewhere: microbiology's search for itself." Microbiol. Rev. 58(1): 1-9.

Woese, C. R., P. Blanz and C. M. Hahn (1984). "What isn't a *Pseudomonad*: the importance of nomenclature in bacterial classification." System. Appl. Microbiol. 5: 179-195.

Woese, C. R., J. Gibson and G. E. Fox (1980). "Do genealogical patterns in purple photosynthetic bacteria reflect interspecific gene transfer?" Nature 283: 212-216.

Wöhrman, K. and J. Tomiuk, Eds. (1993). *Transgenic Organisms: Risk Assessment of Deliberate Release*. Advances in Life Sciences. Basel, Birkhäuser Verlag.

Wolfe, C. J. and M. G. Haywood (1991). "Restriction fragment length polymorphism analysis reveals high levels of genetic divergence among the light organ symbionts of flashlight fish." Biol. Bull. 181: 135-143.

Wong, F. Y. K., E. Stackebrandt, J. K. Ladha, D. E. Fleischman, R. A. Date and J. A. Fuerst (1994). "Phylogenetic analysis of *Bradyrhizobium japonicum* and photosynthetic stem-nodulating bacteria from *Aeschynomene* species grown in separated geographical regions." Appl. Environ. Microbiol. 60(3): 940-946.

Wyndham, C., R. K. Singh and N. A. Straus (1988). "Catabolic instability, plasmid gene deletion and recombination in *Alcaligenes* sp. BR60." Arch. Microbiol. 150: 237-243.

Wyndham, R. C., A. E. Cashore, C. H. Nakatsu and M. C. Peel (1994). "Catabolic transposons." Biodegradation in press.

Wyndham, R. C. and N. A. Straus (1988). "Chlorobenzoate catabolism and interactions between *Alcaligenes* and *Pseudomonas* species from Bloody Run Creek." Arch. Microbiol. 150: 230-236.

Yabuuchi, E., Y. Kosako, H. Oyaizu, I. Yano, H. Hotta, Y. Hashimoto, T. Ezaki, et al. (1992). "Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus, with the type species *Burkholderia cepacia* (Palleroni and Holmes 1981) comb. nov." Microbiol. Immunol. 36(12): 1251-1275.

Yadav, J. S., R. E. Wallace and C. A. Reddy (submitted). "Degradation of chlorobenzenes by the white-rot fungus *Phanerochaete chrysosporium.*"

Yano, K. (1980). "Degradative plasmids: aspects of microbial evolution." In: *Molecular Breeding and Genetics of Applied Microorganisms* Eds. K. Sakaguchi and M. Okanishi. Tokyo, Kodansha Ltd.

Zilberman, D., A. Schmitz, G. Casterline, E. Lichtenberg and J. B. Siebert (1991). "The economics of pesticide use and regulation." Science 253: 518-522.

Zuckerkandl, E. and L. Pauling (1965). "Molecules as documents of evolutionary history." J. Theor. Biol. 8: 357-366.

Zylstra, G. J. and D. T. Gibson (1991). "Aromatic hydrocarbon degradation: a molecular approach." In: *Genetic Engineering: Principles and Methods* Ed. J. K. Setlow. New York, Plenum Press. 183-203.

Zylstra, G. J., R. H. Olsen and D. P. Ballou (1989). "Cloning, expression, and regulation of the *Pseudomonas cepacia* protocatechuate 3,4-dioxygenase genes." J. Bact. 171(11): 5907-5914.

Zylstra, G. J., R. H. Olsen and D. P. Ballou (1989). "Genetic organization and sequence of the *Pseudomonas cepacia* genes for the alpha and beta subunits of protocatechuate 3,4-dioxygenase." J. Bact. 171(11): 5915-5921.

(1990) "Validation of the publication of new names and new combinations previously effectively published outside the IJSB." International Journal of Systematic Bacteriology 40(3): 320-321.

(1992). "Validation of the publication of new names and new combinations previously effectively published outside the IJSB." International Journal of Systematic Bacteriology 42(1): 191-192.