THESIS
3
2000

This is to certify that the

dissertation entitled

Impact of host population and catabolic plasmid on the fitness of 2,4-dichlorophenoxyacetic acid degrading bacteria

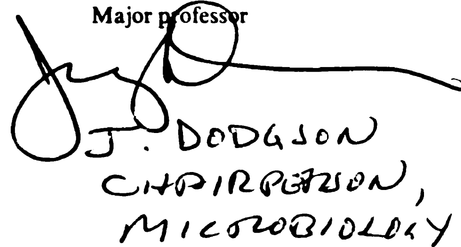
presented by

Kirsti Maria Ritalahti

has been accepted towards fulfillment of the requirements for

Ph.D. degree in Microbiology

Major professor



Date 11 May, 2000

MSU is an Affirmative Action/Equal Opportunity Institution

O-12771

PLACE IN RETURN BOX to remove this checkout from your record.
TO AVOID FINES return on or before date due.
MAY BE RECALLED with earlier due date if requested.

DATE DUE	DATE DUE	DATE DUE

11/00 c:/CIRC/DateDue.p65-p.14

IMPACT OF HOST POPULATION AND CATABOLIC PLASMID ON THE FITNESS OF 2,4-DICHLOROPHENOXYACETIC ACID DEGRADING BACTERIA

By

Kirsti Maria Ritalahti

A DISSERTATION

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

DOCTOR OF PHILOSOPHY

Department of Microbiology
Center for Microbial Ecology

May, 2000

ABSTRACT

IMPACT OF HOST POPULATION AND CATABOLIC PLASMID ON THE FITNESS OF 2,4-DICHLOROPHENOXYACETIC ACID DEGRADING BACTERIA

By

Kirsti Maria Ritalahti

The acquisition of catabolic plasmids and genes carried by these plasmids affect the competitive ability of bacterial populations. Competition experiments between two different 2,4-dichlorophenoxyacetic acid (2,4-D) degrading bacteria (*Burkholderia cepacia* strain DB01 and *Ralstonia eutropha* strain JMP228), carrying either of two different 2,4-D degradative plasmids (pJP4 and pEMT3), were performed. The two plasmids that carried different gene alleles for the 2,4-D degradative pathway endowed their bacterial hosts with different growth rates on 2,4-D. The growth rate and half-saturation constants determined for pure cultures were used to predict the competitively superior strain. Both host-associated and plasmid-associated traits influenced the outcome of competition for 2,4-D under different growth conditions. Host-associated traits had a pronounced effect on competitive outcome. In batch culture competition experiments characterized by a high initial 2,4-D concentration, *Ralstonia* populations became dominant. When hosting the same plasmid, *Ralstonia* exhibited a faster growth rate than *Burkholderia* when 2,4-D was provided as the

limiting resource. In contrast, *Burkholderia* was more successful under continuous culture conditions characterized by very slow growth rates, where the populations were forced to grow at less than half of their maximum growth rates on 2,4-D. Plasmid-associated characteristics also influenced the competitive outcome, particularly in the batch culture environment. Harboring a particular plasmid was of importance when its presence provided the increased benefit of a higher growth rate. Consistently in intra-species competitions, the presence of pJP4 endowed a competitive advantage over strains with pEMT3 under batch culture conditions, and the pJP4 carrying strain became dominant. However, intra-species competition experiments in continuous culture demonstrated that identical strains bearing different plasmids coexisted, suggesting that pJP4 confers considerably less of a selective advantage under low nutrient conditions. Different environments impose different selective pressures. The populations that compete under different environmental conditions adapt to lifestyles that are advantageous in one habitat, and less desirable for another. The ability to compete can be influenced by the presence of different catabolic pathways for the utilization of a specific substrate.

I dedicate this work to the memory of my father, Tapani J. Ritalahti, and to my mother Susan, who encouraged me to "follow my bliss". Their inspiration has instilled in me a drive to never quit. I want to thank them both for believing in my ability to succeed.

ACKNOWLEDGMENT

I would like to thank the Center for Microbial Ecology and Department of Microbiology at Michigan State University and NSF grant #DEB912006 to the Center for Microbial Ecology, the sources which have funded my Ph.D. dissertation research. I want to thank Dr. Terry L. Marsh and Dr. Frank E. Löffler, for scientific discussion, critical proofreading and moral support. I would like to thank the members of my Graduate committee, Dr. Mike Klug, Dr. Pat Oriel, Dr. Rosetta Reusch and Dr. Rich Lenski for their review of the work, and Dr. Larry J. Forney, for opening the door to exploring in the Research on Microbial Evolution (ROME) Lab group. I'd like to extend thanks to Dr. Michael Roberts, who has always lent an ear and answered my innumerable questions. And finally, thanks to all of my colleagues in the ROME lab, who I worked with, over the past years.

TABLE OF CONTENTS

LIST OF TABLES.. viii

LIST OF FIGURES.. x

INTRODUCTION.. 1
Role of Catabolic Genes and Host Genotype in Competition.............. 1

CHAPTER 1:
Competition Theory and 2,4-D Degradation.................................. 9
Competition for Limited Resources...................................... 9
2,4-dichlorophenoxyacetic Acid Degradation as a Model System for Studying Interactions among Bacterial Populations.. 20
References.. 38

CHAPTER 2:
The Impact of Similar Degradative Genes on Different Plasmids to the Fitness of Isogenic *Ralstonia eutropha* Strains Competing for 2,4-Dichlorophenoxyacetic Acid.. 44
Abstract.. 44
Introduction.. 46
Materials and Methods.. 52
Results.. 67
Discussion.. 78
References.. 81

CHAPTER 3:
Ralstonia eutropha and *Burkholderia cepacia* Host Populations as Reservoirs for 2,4-Dichlorophenoyacetic Acid Degradative Plasmids... 83
Summary.. 83
Introduction.. 85
Materials and Methods.. 90
Results.. 108
Discussion.. 127
References.. 136

CHAPTER 4:
Two Hosts and Two Plasmids in Simple Environments: The influence of 2,4-D Degradative Plasmids to the Competitive Fitness of a Host Strain........ 137
Abstract........ 137
Introduction........ 139
Materials and Methods........ 148
Results........ 160
Discussion........ 194
References........ 207

CONCLUDING REMARKS 211

LIST OF TABLES

CHAPTER 1
Table 1.1. Experimental evidence for microbial competition.......... 13
Table 1.2. 2,4-D degrading populations carry mosaics of catabolic genes... 31
Table 1.3. Diversity of 2,4-D degrading isolates. 34

CHAPTER 2
Table 2.1. Genotypic and phenotypic comparison of pJP4, pEMT1 and pEMT3.. 47
Table 2.2. Bacterial strains used to determine the influence of the plasmid backbone on competition......................... 54
Table 2.3. Plasmids used to determine the outcome of competition in isogenic populations.. 55
Table 2.4. Transconjugants obtained with the filter mating technique... 56
Table 2.5. Growth rates of parent strains and transconjugants on succinate and 2,4-D.. 69
Table 2.6. Carrying capacity of *R. eutropha* strains in batch and chemostat conditions... 76

CHAPTER 3
Table 3.1. Bacterial Strains used in the competition experiments..... 93
Table 3.2. Plasmids used in the competition experiments..................... 94
Table 3.3. Transconjugants used in the competition experiments........ 96
Table 3.4. Testing the antibiotic resistance profiles of strains................ 108
Table 3.5. BIOLOG substrate utilization by Ralstonia eutropha JMP228 and Burkholderia cepacia DB01................ 111
Table 3.6. Growth rates of strains supplied with succinate or 2,4-D under batch culture conditions.............................. 115
Table 3.7. One-tailed t-test on growth rates of strains determined under batch culture conditions... 117

Table 3.8. Parameters used to calculate expected growth curves based on the Monod model, using Stella Research Software.. 122

CHAPTER 4
Table 4.1. Pure culture characteristics for strains grown on mineral salts medium with succinate or 2,4-D under batch culture conditions.. 148

Table 4.2. Competition experiments.. 152

Table 4.3. Values for constants in the Stella model........................ 177

LIST OF FIGURES

CHAPTER 1
Figure 1.1. Competitive edge due to a lower resource threshold concentration(R*) 18
Figure 1.2. 2,4-D degradation pathway of plasmid pJP4 of *R. eutropha JMP*.134. 23

CHAPTER 2
Figure 2.1. *Bgl* II restriction analysis of plasmids isolated from transconjugant strains. 57
Figure 2.2. Schematic representation of the chemostat used in the competition experiments under low nutrient conditions.............................. 66
Figure 2.3. Growth of pure cultures carrying plasmid pJP4 or pEMT1 on 2,4-D.............................. 70
Figure 2.4. Competition among *R. eutropha* strains JMP228/pJP4, and JMP228/pEMT1 on 200 ppm 2,4-D.............................. 74
Figure 2.5. *Ralstonia eutropha* hosting pJP4 or pEMT1 are equal competitors against *Burkholderia cepacia* hosting pJP4.............................. 75
Figure 2.6. *R. eutropha* with pJP4 or pEMT1 in pure culture or in competition. 77

CHAPTER 3
Figure 3.1. Restriction of plasmids isolated from transconjugants and cut with enzyme EcoR1.............................. 97
Figure 3.2. 2,4-D depletion curves used to calculate rates of 2,4-D degradation for use in calculating K_S for strain DB01/pJP4.............................. 103
Figure 3.3. Lineweaver-Burke plot to determine the KS for each of the four host/plasmid combinations.............................. 104
Figure 3.4. Growth model developed on the Stella Research Program v 5.0 to generate expected population growth curves and 2,4-D depletion curves for pure cultures.............................. 107
Figure 3.5. Average population sizes for selected strains grown in batch culture under 24 and 72 hours transfer conditions.............................. 119

Figure 3.6. Allocation of ring-labeled ^{14}C-2,4-D in growing cultures of JMP228/pJP4, JMP228/pEMT3, DB01/pJP4 and DB01/pEMT3.. 122
Figure 3.7. Chemostat growth of JMP228R/pJP4, JMP228S/pJP4, JMP228R/pEMT3 and JMP228S/pEMT3.................. 124
Figure 3.8. Chemostat Growth of DB01R/pJP4, DB01T/pJP4, DB01R/pEMT3 and DB01T/pEMT3....................... 125

CHAPTER4
Figure 4.1. An overview of the genetic contribution of different genes in the competition experiments.................................... 142
Figure 4.2. Growth of JMP228/pEMT3 and JMP228/pJP4 competing for 5 mM succinate as a limiting resource.................. 162
Figure 4.3. Plasmids pEMT3 and pJP4 do not govern fitness of *R. eutropha* competing for succinate.................................... 162
Figure 4.4. JMP228 is a superior competitor to DB01 when succinate is supplied as the limiting resource.................................... 164
Figure 4.5. ln of the population sizes of JMP228/DB01 when succinate is supplied as the limiting resource.................................... 164
Figure 4.6. Effect of antibiotic resistance markers to fitness of JMP228/pJP4 and JMP228/pEMT3.................................... 165
Figure 4.7. Competition between JMP228/pJP4 and JMP228/pEMT3 for 2,4-D in batch culture.................................... 167
Figure 4.8. Effect of antibiotic resistance markers on fitness of DB01/pJP2 and DB01/pEMT3.................................... 168
Figure 4.9. Competition between DB01/pJP4 and DB01/pEMT3 for 2,4-D in batch culture.................................... 169
Figure 4.10. Competition between JMP228/pJP4 and DB01/pJP4 for 2,4-D in batch culture.................................... 171
Figure 4.11. Competition between JMP228/pEMT3 and DB01/ pEMT3 for 2,4-D in batch culture.................................... 172
Figure 4.12. Competition between JMP228/pJP4 and DB01/pEMT3 for 2,4-D in batch culture.................................... 173
Figure 4.13. Competition between JMP228/pEMT3 and DB01/pJP4 for 2,4-D in batch culture.................................... 174

Figure 4.14. The model that was used by the Stella Research Program to generate expected population growth curves for two competing populations.. 176

Figure 4.15. Chemostat competition between JMP228S/pJP4 and JMP228R/pEMT3.. 180

Figure 4.16. Chemostat competition between JMP228R/pJP4 and JMP228S/pEMT3.. 181

Figure 4.17. Chemostat competition between DB01T/pJP4 and DB01R/pEMT3.. 182

Figure 4.18. Chemostat competition between JMP228S/pJP4 and DB01R/pJP4.. 184

Figure 4.19. Different K_S values for DB01 influence competition between JMP228S/pJP4 and DB01R/pJP4 in chemostats.. 186

Figure 4.20. Chemostat competition between JMP228S/pJP4 and DB01R/pJP4.. 188

Figure 4.21. Chemostat competition between JMP228R/pEMT3 and DB01T/pEMT3.. 189

Figure 4.22. Chemostat competition between JMP228R/pEMT3 and DB01T/pJP4.. 191

Figure 4.23. Chemostat competition between JMP228R/pEMT3 and DB01T/pEMT3.. 192

INTRODUCTION

ROLE OF CATABOLIC GENES AND HOST GENOTYPE IN COMPETITION

Competition for limiting resources in the environment has captured the attention of ecologists and microbiologists. In both disciplines competition has been implicated as a force driving speciation, promoting extinction, and influencing the distribution of populations that rely on the same set of resources. The introduction of man-made compounds to the environment potentially changes the balance of functional ecological systems at the microbial and macro community level. Microbial community changes may be due to negative (e.g. toxicity) or positive (e.g. enrichment) effects of the introduced chemical. When a toxic compound is applied, susceptible populations decline in numbers relative to resistant ones. The increase in the relative abundance of resistant populations produces a shift in the community composition. In most environments, microbial communities are responsible for the mineralization of many natural and anthropogenic compounds, and many compunds are used as a source of carbon and/or energy. Populations that are capable of utilizing anthropogenic compounds gain a distinct growth advantage over populations that cannot use the resource. The result may be observed as an

enrichment of populations that are capable of benefiting from the compound. Some of the populations that grow with the introduced compound may become involved in a competitive interaction with other populations that can degrade the same compound. Even among very simple communities comprised of only a few populations, our interpretation of changes in population dynamics is still rudimentary. When changes in community composition occur, an comprehensive view of the factors that influence the dominant population, include the host population, its genetics and the surrounding environment.

The rate at which a population grows and the efficiency at which it utilizes a resource are phenotypic features that influence competitive fitness. Among sexually reproducing organisms it has been suggested that some components of competitive fitness relate to the manner in which populations reproduce. The mode of reproduction, including growth rate, age of reproductive maturity and the amount of time during which a female is reproductive, as well as the allocation of energy to producing and raising offspring, contribute to the "life history" of a population. Asexually reproducing populations, like bacteria, face a similar set of criteria when the relationship of life history to competitive fitness is considered. The rate of reproduction, (growth rate), the time at which division begins (lag time) and the efficiency of resource use (allocation to reproduction) can be used to define competitive properties of microbial populations.

Bacteria are prime candidates for use as model organisms to test competition theory among asexual populations. Bacterial populations have advantageous properties because (i) they attain high cell densities relative to sexually reproducing organisms, (ii) they exhibit very short generation times, and (iii) they are distinguished by easily selectable phenotypic markers, which may be naturally present or genetically introduced.

The many phenotypic differences in the growth characteristics of bacterial populations are due to differences in their genetic makeup. Genes residing on the chromosome contribute to general metabolism, substrate range and reproduction. While bacteria are considered asexual, novel genetic arrangements can be created by lateral gene transfer. Plasmids are mobile, extrachromosomal genetic elements that can disburse or maintain a variety of different traits in bacteria. They can supply catabolic pathways, such as for the degradation of herbicides, pesticides and aromatic solvents, or can convey antibiotic resistance. The acquisition of broad host range plasmids and the catabolic genes carried by these plasmids (such as for 2,4-dichlorophenoxyacetic acid, toluene and 3-chlorobenzoate degradation) affects the competitive ability of different bacterial populations when the novel substrate is provided as a limiting resource. Since there exists a correlation between a population's genotype and its fitness, it is reasonable to assert that when new catabolic genes are acquired, the fitness of its host may become altered. Recruiting different catabolic plasmids among

members of the same host species results in genetically distinct populations. Subpopulations with different plasmids, or different genes may be competitively equal in the absence of selection, but may result in a dominant population when selection for a specific plasmid-encoded catabolic pathway occurs.

Bacteria are ideal model systems for examining aspects of inter-specific and intra-specific competition. This work focuses on the well-characterized degradation pathway for the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) and two different 2,4-D-degrading bacterial species. *Ralstonia eutropha* strain JMP228 and *Burkholderia cepacia* strain DB01 were used as host populations for different catabolic plasmids carrying alternative gene alleles for 2,4-D degradation. Previous research indicated that the application of 2,4-D to agricultural sites resulted in the enrichment of specific 2,4-D degrading bacterial populations. These findings suggested that different 2,4-D genotypes contributed to the development of the dominant population in soils where 2,4-D had been applied. Since it is likely that alternative degradative genotypes for a particular substrate impact different bacterial populations in different ways, we can determine the fitness advantage of carrying different catabolic plasmids to both inter-specific and intra-specific competition, when the ability to utilize the limiting substrate resides on a catabolic plasmid.

Chapter 1 reviews information about population growth, competition, and the characteristics of the 2,4-D degradative pathway.

The first hypothesis to be tested (Chapter 2) is that in host strain, *R. eutropha*, different plasmid backbones with similar alleles for 2,4-D degradation, result in competitively equivalent populations. The two plasmids, pJP4 and pEMT1, carry homologous alleles for 2,4-D degradative genes in a different plasmid background. Within the *R. eutropha* host, similar 2,4-D degradative alleles on different plasmid backbones resulted in similar growth and 2,4-D degradation rates. Competition studies with the two strains under batch culture conditions demonstrated that the strains coexisted, which suggested that the influence of the plasmid backbone was minimal under non-limiting resource conditions (2,4-D was in excess).

The second hypothesis tested whether possessing different plasmids and degradative alleles affected competition within the same host population under batch and chemostat conditions. Both *R. eutropha* strain JMP228 and *B. cepacia* strain DB01, each of which carried the two different 2,4-D degradative plasmids (pJP4 or pEMT3) were examined. Plasmids pJP4 and pEMT3 encode for different alleles of the 2,4-D degradation pathway, expressed from similar plasmid backbones. Prior to carrying out competition experiments, pure culture growth data were obtained, and the differences between *R. eutropha* and *B. cepacia* were explored (Chapter 3).

While the growth rates on 2,4-D were different for all four strains, there was no measured difference in the half-saturation constants.

In batch competition experiments of Chapter 4, the population with the fastest growth rate became numerically dominant. Among members of the same host species, plasmid pJP4 conferred dominance to its host, and the pEMT3 carrying strain was out competed. In controlled, low growth rate conditions of a chemostat, members of the same host population with either set of alleles coexisted and the selective advantage of the fast growth rate conferred by pJP4 was not as significant.

The third hypothesis was to determine if carrying the same plasmid and allele combination affected competition among members of different host species under two different growth conditions. Based on the pure culture growth parameters determined in Chapter 3, *R.. eutropha* strains were predicted to dominate in batch culture due to having the faster growth rate. Experimental results in Chapter 4, indicate that when *R.. eutropha and B. cepacia* both carried the same plasmid, be it pJP4 or pEMT3, *R.. eutropha* dominated under batch conditions. Conversely, *B. cepacia* became dominant under chemostat growth conditions. Collectively, these results suggested that the host population played a significant role in controlling population dynamics under different growth conditions.

The fourth hypothesis was to test if the presence of either plasmid or allele combination would change the results of competition so that *B.*

cepacia could out compete *R.. eutropha* in batch culture, or if *R.. eutropha* could out compete *B. cepacia* in the chemostats (Chapter 4). In batch culture, the order of dominance correlated with the expected pattern predicted by the growth rates: *R. eutropha* (pJP4) > *B. cepacia* (pEMT3) and *B. cepacia* (pJP4) > R. *eutropha* (pEMT3). As in the previous example, faster growth rates lead to dominance in batch culture competitions. In contrast, in the chemostats with a low dilution rate (0.03 h^{-1}), and low nutrient concentrations, the *B. cepacia* strains became dominant, supporting evidence that the *B. cepacia* population is more competitive under the low growth rate selected growth regime.

The data presented in the following chapters support the hypothesis that phenotypic characteristics of a population influence success under different environmental conditions. Different genotypes conferring catabolic pathways with similar function, such as 2,4-D utilization, do influence fitness when competition occurs for that limiting substrate. Under the conditions that were tested, dominance due to plasmid-borne alleles was observed in the conditions where a faster growth rate was advantageous. Experimental results described herein indicated that the host genotype in concert with the appropriate environment played a significant role in determining dominance. The outcome of carrying different alleles for 2,4-D degradation on a catabolic plasmid was important to intra-specific

competition. In highly similar host backgrounds, the strain carrying pJP4 became dominant.

Based on physiological differences, *R.. eutropha* and *B. cepacia* species have slightly different life history strategies. While both species are widely distributed opportunistic organisms, *R.. eutropha* uses a rapid response to environmental stimuli and a fast growth rate to gain an advantage under high nutrient conditions (e.g. batch transfers). On the other hand, *B. cepacia* can use a lower resource threshold and hence, has an ability to maintain high population densities at low resource concentrations, (e.g. chemostat conditions).

CHAPTER 1:

COMPETITION THEORY AND 2,4-D DEGRADATION

COMPETITION FOR LIMITED RESOURCES

Populations and Community Diversity

Who wins and why has been a key question in ecological research for decades. Some populations gain a prominent position within a community, while others follow a road to extinction. Competition for limiting resources has been considered a driving force in determining the distribution and abundance of populations within a community. It has been implicated as a force of evolution, and for driving the divergence of one species into many. (Darwin, 1859; Arthur, 1982; Connell, 1978; Vadas, 1990). Much of the foundation for research on competitive interactions was initiated in eukaryotic systems. Darwin set the stage by suggesting that competition was central to the struggle for existence (Darwin, 1859). His work on finches was an example of evolution, where one ancestor gave rise to many different lines of finches; each finch suited a particular habitat. In the years that followed, experimental studies comparing fluctuations of competing populations were observed directly, and spread to include microbial

competitions as well, (Table 1.1). Microbial systems are ideally suited to study both interspecific and intraspecific competition. Several studies demonstrated that limiting nutrient conditions are important factors in determining dominance.

The influence of interspecific competition among animals and plants was demonstrated in many field studies, as summarized in reviews by Schoener (1983) and Connell (1983). Both supported the notion that competition was widespread, at least among the species under investigation. Connell suggested that in many of the studied cases where interspecific competition was demonstrated, a high possibility of finding the interaction could have influenced the choice of the research subjects. In other words, where the influence of competition was expected, it was often affirmed. Finally, the absence of present-day competition does not mean that competition did not take place in the past. The composition of current communities may be a reflection of the winners of prior competition, with the losing populations driven to extinction, and the existing populations coexisting due to minimal or balanced competitive interactions.

The "ghost of competition past" (Connell, 1983) is evidenced in our current evaluation of microbial populations that degrade the herbicide 2,4-dichlorophenoxyacetate (2,4-D). A common set of catabolic genes for utilizing this herbicide exists in many of the dominant 2,4-D degrading

populations. The presence of these alleles correlated with the prior application history of the herbicide at a particular site (Ka et al., 1994a, Dunbar et al., 1997). Further addition of the herbicide tended to enrich for greater numbers of the populations carrying the favorable genotypes.The catabolism of 2,4-D, and the populations that compete for this resource, will be addressed later in this chapter.

Competitive Exclusion or Coexistence?

The research on competitive interactions in *Paramecium* lead to the development of two key concepts in community interactions (Gause, 1934). The first principle was called "Competitive Exclusion". Gause observed that if a single resource is supplied to two populations of *Paramecium*, they will compete for it. The one that uses the resource most rapidly will displace the population that is less efficient. Not every competitive interaction inevitably leads to a complete extinction of a population, but may lead instead to a significant reduction in numbers. Simply stated, a competitive interaction is one that decreases the growth and reproductive rate, or increases the death rate, of each population due to a shared demand on a common resource base. Competition may be due to exploitation or interference. Competition by exploitation is due to consumption of a resource, so that it is not available to a competitor population. Interference

competition involves a direct interaction of populations and may include the use of toxins or force to eliminate an antagonist.

One principle of classical ecological theory states that two different species can not coexist while competing for the same set of limiting resources in a similar manner, (Gause, 1934). In order to avoid competition, habitat differentiation can occur leading to coexistence and increased diversity. Research on eukaryotic populations noted that instances of cohabitation by two species of the same genera in the same habitat was rare (Elton, 1946). In cases where cohabitation does occur, the populations usually coexisted by utilizing different portions of the environment. Gause (1934), noted that two different populations of *Paramecium* could coexist, as long as one fed on yeast cells in the bottom of the culture vessel, and the other fed on bacteria growing in the aerobic, upper portion of the vessel. If both populations of *Paramecium* fed on both food types, competitive exclusion would occur, assuming the resources were limiting.

Table 1.1. Experimental evidence for microbial competition.

Populations	Limiting resource	details	Reference
Various species of *Paramecium.*	Yeast, *E. coli.*	Competitive exclusion among some competitive pairs, Niche differentiation among others	Gause, 1934
Chromatium weissei and *Chromatium vinosum*	Light in batch and chemostat	Continuous light selects for *C. vinosum*, while intermittent light allows for coexistence, since *C. weissei* has a faster rate of sulfide oxidation in the dark	Van Gemerden, 1974
Heterotrophic and autotrophic nitrifiers	Ammonia and oxygen in chemostats	Most cases showed no interaction. Under high C/N, or low oxygen, the heterotrophic populations dominated.	VanNiel, 1993
High mutating *E.coli* vs low mutating *E. coli*	Glucose in chemostats	High mutating strain wins in chemostat if present at greater than 7 x 10-5 starting ratio, due to development and replacement by faster growing mutants	Chao and Cox, 1983
E. coli vs *P. aeruginosa*	Tryptophan in chemostats	Measurements of μ, K_S and calculated J, predicted the outcome of competition.	Hansen and Hubbell 1980
Methylosinus trichosporium OB3b and *Methylomonas albus* BG8	Methane copper nitrate in chemostats	*M. albus* dominant under methane limitation, *M. Trichosporium* under nitrate limitation.	Graham et al, 1993
Ancestral vs glucose evolved lines of *E. coli*	Glucose	Derived strains had less lag, and faster growth rates on glucose	Lenski et al. 1998

Microbial population dynamics

Microbial populations, especially the well studied bacterium *Escherichia coli* (Levin, 1972; Lenski et al., 1994; Lenski et al., 1998. Dykhuizen and Hartl, 1983), have served as useful tools for testing specific cases associated with the fitness of populations and competition theory. Frequently the behavior of populations can be modeled using some fairly simple assumptions. For example, the logistic growth equation describes population growth without the influence of a competitor. In early stages of growth, growth proceeds exponentially, with the population increasing at a maximum rate. As resources become scarce, there is an increase in competitive pressure from individuals within the same population. Population growth slows, and as a result, the population size plateaus at a level that has been described as the carrying capacity of the environment. Natural populations are often maintained well below their carrying capacities due to predators or environmental perturbations.

The growth of a microbial population can be based on measurable physiological traits. The two traits of particular relevance are the maximum growth rate (μ_{max}), and the half saturation constant (K_s) for the compound supplied as a sole resource (Van Niel et al., 1993; Gottschal, 1992; Duetz et al. 1994). The half saturation constant represents the resource concentration that allows a population to grow at half the maximum growth rate. Often, the outcome of competition between two competing

populations was correlated with the relationship of these parameters (Duetz et al., 1994). Using substrate-limited growth kinetics derived by Monod (1949), it became increasingly common to perform single-resource microbial competitions with outcomes in agreement with theory. The Monod equation and resulting models are presented in Chapter 4.

As a life history strategy, some populations take advantage of a fast maximum growth rate (a high μ_{max}), or a high reproductive output, when resource concentrations are plentiful. Other populations maximize the efficiency at which they utilize low concentrations of a substrate, and therefore can grow and maintain viable population sizes even when resources are limited. In the laboratory, the typical test tube or shake flask simulates a high growth-rate favored habitat. The flask has a high initial concentration of resource that is readily utilized. The population that grows faster utilizes a greater proportion of the resource and increases its population size accordingly. In contrast, a population with a very high affinity but a low growth rate on a substrate would maintain a viable population size when nutrients are meager. A constant and low substrate concentration, which leads to a low growth rate, can be simulated in the laboratory with the use of a chemostat. Generally, chemostats are used to maintain a continuously growing culture, where the flow rate of the substrate into the culture vessel is constant.

In a continuous flow environment, waste products are removed as new resources are provided. The rate at which the populations divide is controlled by the rate at which the substrate is provided (Gottchal, 1992; Dykhuizen and Hartl, 1983). A population will divide at the maximum rate allowed by the substrate provided, as long as the dilution rate (the rate at which the culture volume is replaced) does not exceed the culture's growth rate. When the dilution rate exceeds the growth rate, a population will become diluted out over time. Under chemostat conditions suited to a low growth rate, the substrate concentration also tends to be extremely low, because incoming nutrients are consumed immediately by the culture. Consequently, K_S becomes more significant than μ_{max}. For two populations with the same maximal growth rate, the one with a lower K_S value may have a faster growth rate at a lower substrate concentration. The population with a lower K_S value will reduce the concentration of substrate in the environment to a level that will not allow its competitor to grow. For example, if population A reduces the concentration of substrate below that capable of supporting pouplation B, A will eventually become dominant, as its competitor B starves, cannot reproduce, and is diluted out of the chemostat. Thus, at low substrate concentrations, affinity (K_S) for the resource is crucial. Additionally, with low substrate concentrations, the energy and carbon provided would first have to be used for maintenance of cell integrity, and less would be allocated to growth and cell division.

Expanding on the work of Monod, growth rate and K_S were cleverly combined into an equation to describe the J parameter, and experiments were carried out in mixed continuous cultures with multiple bacterial strains, (Hansen and Hubbell, 1980). J represents the minimum threshold concentration of a growth limiting substrate that will support a viable population size. Similar experiments were carried out with diatoms, but the parameter was termed R*, (Tilman, 1981; Smith, 1990; Smith, 1993). Among competitors, the population that can grow at resource concentrations lower than those which can support a competitor will have an advantage, and therefore should dominate. The minimum threshold concentration (J or R*) for the substrate can be calculated. Figure 1.1 describes how R* can be used to predict the winner of a single resource competition. The dilution rate for a chemostat determines the population growth rate. In this example, population A has an R* of 0.2 g L^{-1} at a dilution rate of 0.1 h^{-1}, and will outcompete population B which has an R* of 1 g L^{-1}. Population A will dominate (win), because the substrate in the chemostat will reach a steady state concentration of 0.2 g L^{-1}. At this substrate concentration, B will have a growth rate of 0.025 h^{-1}, which is less than the dilution rate of the chemostat. Consequently, population B will be washed out.

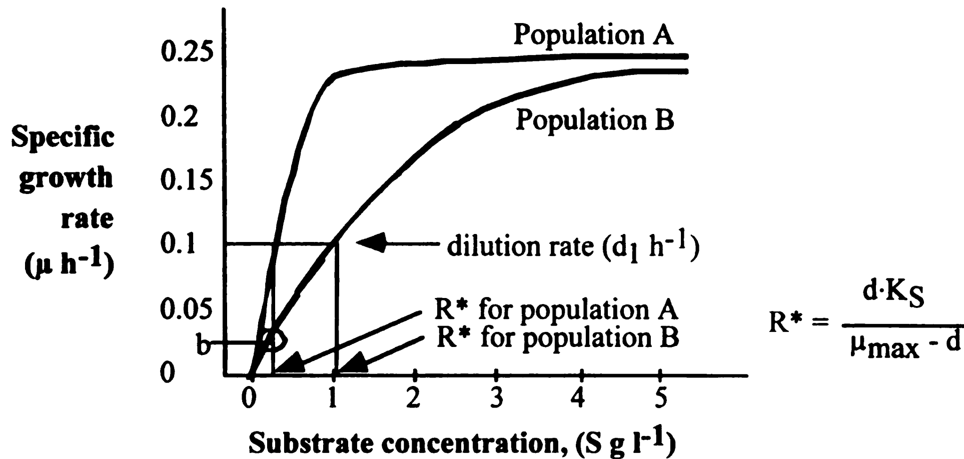


Figure 1.1. Competitive edge due to a lower resource threshold concentration(R*). Graphical representation and equation of the concept of resource threshold (R*). Calculation of R* uses the values for μ_{max}, (maximum growth rate), K_S (half saturation constant) and d (dilution rate of the chemostat). "d" is equal to the specific growth rate, μ. Population A will outcompete B because $R^*_A < R^*_B$. Growth rate b in the diagram represents the growth rate of population B when the substrate concentration is reduced by the presence of population A, at a dilution rate of 0.1 h^{-1}.

In an experiment to determine the validity of the theory, Tilman demonstrated the value of R* in predicting the outcome of competition among two species of freshwater algae competing for two limiting substrates. In a case of interspecific competition, coexistence was obtained between the two algal species (*Asterionella formosa* and *Cyclotella meneghiniana*), when silicate and phosphate were limiting (Tilman 1977). In the results of a long term study of 76 competition experiments, the species-specific characteristics involved in the utilization of silicate and

phosphorous as resources influenced the competitive outcome of two populations. When both populations were phosphate limited, *Asterionella* predominated; when both were silicate limited, *Cyclotella* dominated, and when each was growth-rate-limited by a different resource, both could coexist. These results matched predictions based on the Monod model, which, as mentioned previously, describes the dependence of the growth rate on the concentration of limiting resource.

An organism's capacity to utilize resources present in the environment has a profound impact on its competitive ability. When a bacterial population detects the presence of a compound that may be used as a source of carbon and energy, first the compound must be transported into the cell. Following this, the organism must transform the substrate into useful metabolic intermediates, which can be utilized for energy and biomass production. Differences in the detection limit for a compound, the rates of uptake and metabolism are all important to the reproductive potential of a population. In addition to properties directly involved with resource utilization, other biological attributes, such as motility, attachment, substrate range and resilience to environmental perturbations may also play a significant role.

2,4-D Degradation as a Model System for Studying Interactions among Bacterial Populations

2,4-dichlorophenoxyacetic acid degradation

The bacterial utilization of the broad-leaf herbicide, 2,4-D was chosen as the model system for testing competition theory. This herbicide has been widely studied since its initial use during the 1940's in both agricultural and domestic capacity. Shortly after application, it was found that 2,4-D could be readily degraded both by the combined efforts of microbial communities as well as by numerous autocthenous bacterial populations in the soil (Ka et al., 1994; Fulthorpe et al., 1994; Dunbar et al., 1996). To date, many microorganisms capable of growth on 2,4-D as their sole source of carbon and energy have been isolated, most often from sites with prior application histories. Research has been focused on the enzymes involved in the degradation of 2,4-D and the evolution of these pathways.

Competition experiments, described in chapters 2 and 4, take advantage of a specific genotype which used 2,4-D as a sole source of carbon and energy. Within the 2,4-D degrading bacterial community, there is diversity at the allelic, vector (plasmid), and host levels that can be exploited to address questions of inter- and intra-species competition among 2,4-D degrading populations. In addition to comparing fitness differences among different microbial species, the effect of different gene alleles to the

fitness' of a host species can also be tested within the same genetic background.

2,4-D catabolic pathway and types of tfd genes

The 2,4-D degradation pathway contains allelic variation. Initially this variation was described relative to the genes characterized for the most thoroughly studied 2,4-D catabolic plasmid pJP4 from *Ralstonia eutropha* JMP134 (formerly *Alcaligenes eutrophus* JMP134) (Don and Pemberton, 1985; Don and Pemberton, 1981; Don et al., 1985). The genes carried on pJP4 have been sequenced and characterized, and the operon structure has been determined (Streber et al., 1987; Ghosal et al., 1988, Perkins et al., 1990). This plasmid carries five of the genes encoding for enzymes responsible for the degradation of 2,4-D to 2-chloromaleylacetate (CMA) (Fig.1.2) (Don et al., 1985; Perkins et al., 1990; Fukumori and Hausinger, 1993). The enzymes responsible for the subsequent transformation of CMA to intermediates of the Krebs cycle, were found to be chromosomally encoded (Don et al. 1985; Kukor et al., 1989; Bollmer et al., 1993). The *tfd*A gene is an α-ketoglutarate dependent dioxygenase, which cleaves the acetate moiety from the 2,4-D molecule, resulting in 2,4-dichlorophenol and glyoxylate. Glyoxylate is transformed to succinate, which is shunted into the bacteria's metabolic pathways (Fukumori and Hausinger, 1993). The transcriptional units of the genes *tfd*BCDEF encode for enzymes involved in

the catabolic transformation of 2,4-dichlorophenol to CMA, which is then degraded by host encoded enzymes. The *tfd*R and *tfd*S genes code for regulatory proteins of *tfd*A, *tfd*B and *tfd*CFEF gene cassettes. (Don and Pemberton, 1985; Don et al., 1985; Fukumori and Hausinger, 1993; Streber et al., 1987; Harker et al., 1989; Kaphammer et al., 1990; Kaphammer and Olsen, 1990; Perkins et al., 1990; Matrubuthan and Harker, 1994). The gene *tfd*K has been implicated in the transport of 2,4-D into the cell (Leveau et al. 1998). The genes of pJP4 have been extensively analyzed, and are an important tool for analyzing and comparing the 2,4-D degradative genes among bacterial isolates.

Different 2,4-D alleles have been identified among 2,4-D degrading isolates. Most of the differences have been found using the *tfd* genes of pJP4 as a probe at different hybridization stringencies against the catabolic plasmids, and chromosomes of strains that grow on this compound. One of the plasmids isolated using exogenous plasmid-trapping experiments, is pEMT3 (Top et al., 1994). Plasmid pEMT3 was the chosen representative of a set of plasmids, pEMT2-pEMT7, all of which exhibited similar restriction patterns and hybridization profiles to each other, yet differed from those of pJP4. Only the gene for *tfd*A was detected under high stringency conditions, whereas *tfd*B could be seen under medium, and *tfd*C under low stringency conditions. (Top et. al. 1994). The *tfd*A, -B and -C genes of pEMT3 are still related, but different from those of pJP4.

Furthermore, when the sequence and enzyme differences of the *tfd*B and -C genes of pEMT3 were determined, it was found that differences in the two sets of alleles, pJP4 and pEMT3, are in their specificity toward chlorinated and non-chlorinated substrates. These findings were confirmed by studying the substrate specificities of the individual enzymes (Maltseva, 1996). Enzymes encoded by the pJP4-like genes have higher activity toward chlorinated substrates while those of pEMT3 are better suited to the non-chlorinated counterpart.

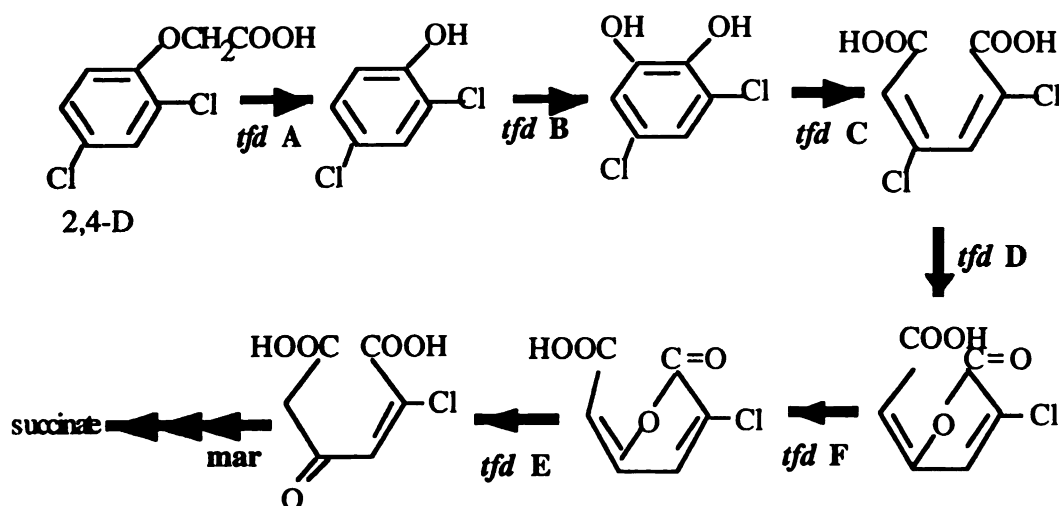


Figure 1.2. 2,4-D degradation pathway of plasmid pJP4 of *R. eutropha JMP*.134. *Tfd*A: α–ketoglutarate dependent dioxygenase, *tfd*B: 2,4-DCP hydroxylase, *tfd*C: chlorocatechol-1,2-dioxygenase, *tfd*D, chloromuconate cycloisomerase, *tfd*F, trans-chlorodienelactone isomerase, *tfd*E, chlorodienlactone hydrolase. The *mar* gene, chloromaleylacetate reductase, is chromosomally encoded.

Another plasmid, pEMT8, was isolated and carried only a *tfd*A gene that could complement a pathway in *which tfdA* had been knocked out. The *tfd*A gene of pEMT8 shares high sequence similarity to that of pJP4. Another 2,4-D degrading isolate, *Burkholderia cepacia* strain RASC carried another *tfd*A gene that did not hybridize to pJP4, while the *tfd*B and *tfd*C genes did. Sequencing *tfd*A(RASC) indicated the gene was only 77% similar to that of pJP4 (Suwa et al., 1994). Although the *tfd*A(pJP4) and *tfd*A(RASC) have a 23% difference in their DNA sequence, they still encode for proteins with 81.9% amino acid identity and 96.5% similarity. Curiously, the *tfd*A, *tfd*B and *tfd*CDEF genes are located sequentially, in the same type of grouped cluster as those on plasmid pEMT3 (Top et al., 1994). The hybridization patterns indicated sequence differences, but with a familiar spatial organization. Hence, it is likely that the *tfd*A gene has evolved for some period of time. Some strains have genes much like pJP4, others like that of *Burkholderia cepacia* strain RASC, and yet others without clear homology to either (Table 1.2).

A distinct pattern of non-homology is seen in many of the *Sphingomonas* strains that are phylogenetically distant from other isolated 2,4-D degraders (Ka et al. 1994a). *tfd*A, -B and -C from pJP4 do not generally hybridize to DNA isolated from these strains. Despite not having sequence similarity to *tfd*C, this strain exhibits chlorocatechol dioxygenase

activity when grown on 2,4-D (Fulthorpe et al., 1994). Although no *tfd*A-like activity has yet been detected, there may be another oxygenase that may fulfill this function.

Since many of the 2,4-D degrading isolates do not carry sequences with recognizable homology to the previously identified 2,4-D degradative genes, there is reason to believe that other pathways for the catabolism of this herbicide exist (Matheson et al., 1996; Ka et al.,1994; Fulthorpe et al., 1995). At this time, preliminary evidence for alternative pathways for the catabolism of 2,4-D have been demonstrated. There is evidence for degradation through a hydroxyquinone pathway in *Nocardia* species (Haugland et al., 1990). Some isolates have been shown to lack *tfd*A activity while other strains were shown to use an alternative ether-cleavage enzyme that degrades 4-NPA to nitrophenol (Sassanella 1994). Isolates from low nutrient, and pristine environments, Kamagata et al. (1997) detected a novel ether-cleavage activity which did not exhibit the α-keto acid dependence as does *tfd*A of pJP4. Since many of these novel isolates have not been examined, other distinctive mechanisms and pathways for 2,4-D degradation may exist.

2,4-D degradative plasmids and mosaics of catabolic genes

The 2,4-D degradation genes from various bacterial isolates are located on plasmids (Don and Pemberton 1981, Mae et al. 1993, Ka and Tiedje 1994, Top et al. 1994) or on the chromosome (Mae et al. 1993, Fulthorpe et al. 1994, Ka and Tiedje, 1994, Matheson et al. 1994, Suwa et al. 1994). Among isolates capable of utilizing 2,4-D, by way of catabolic plasmids, often the degradative and regulatory genes occur in different spatial arrangements. An analysis of the catabolic genes from plasmids of 2,4-D degrading bacteria indicated that homologous *tfd* genes are located on different plasmids, or in a different spatial order from those of pJP4. In Australia, Don and Pemberton (1981) obtained identical plasmids from both *Ralstonia eutropha* JMP134 (pJP4) and from an *Alcaligenes paradoxus* strain (pJP3). Several years later, *Alcaligenes* species EML159 was isolated from Oregon soil, and this isolate contained a plasmid similar to pJP4 based on restriction analysis and cross-hybridization experiments. Despite the similarities in their encoding functions, the two plasmids did exhibit different host ranges for the transfer of 2,4-D degradative ability (Amy et al., 1985). The latter result suggests that the degradative plasmid may have a different transfer or replication function, but the features leading to incompatibility and host range were not included in the analysis of the 2,4-D degrading phenotype.

In addition to pJP4-like plasmids, other plasmids carry 2,4-D degrading genes. 2,4-D catabolic plasmids can range from moderately sized (45 kb) to large (160 kb) and are represented among different incompatibility groups. Using incompatibility tests and restriction profiles with hybridization, some plasmids such as pJP4 and pEMT3 were shown to belong to the broad host range IncP1 plasmid group (Don and Pemberton, 1981; Top et al., 1994). Other plasmids, such as pTFD41, pRC10 and pEMT1 belong to a different, yet unknown, incompatibility group (Ka and Tiedje, 1994; Top et al., 1994). The plasmid, pRC10, isolated from *Flavobacterium* sp. strain 50001 by Chaudry and Huang (1988) encodes for many of the same functions as pJP4 and hybridization of pJP4 *tfd* genes indicates high sequence similarity. However, no homology was noted for pRC10 and pJP4 for those regions that encode for functions of maintenance, replication, conjugation or incompatibility (Don et al., 1985). Similarly, the large conjugative plasmid (160kb) of *Ralstonia* sp. strain TFD41 possesses *tfd* with nearly 100% nucleotide sequence identity to those of pJP4, yet the plasmid backbone and gene order is different. Plasmid pTFD41 maintains a duplicate copy of the *tfd*B and a partial duplication of the *tfd*CDEF operons. The *tfd*A and *tfd*R genes are located in close proximity on pTFD41, and these are grouped farther away from the rest of the operon as in the pathway carried on pJP4 (Nakatsu et al., 1994).

Different types of *tfd* genes have been described, and the mosaic structure of the 2,4-D degradative pathway of several populations are depicted in Table 1.2 (data adapted from Fulthorpe et al., 1995 sources listed as references). As further evidence for the assortment of 2,4-D degradative genes, an experiment was carried out to characterize diverse 2,4-D degradative plasmids occurring in soil. Top used *R. eutropha* JMP228 (JMP134, rif^R, cured of pJP4) as a genetic sink to trap catabolic plasmids. Examination of the recovered 2,4-D degrading JMP228 transconjugants showed that seven different plasmids, designated pEMT1-pEMT7, had been captured (Top et al., 1994). All plasmids were digested with restriction enzymes, and the resulting fragments were hybridized with the pJP4 *tfd*A, -B, -C, -D, -E, -F and -R probes. Plasmids pEMT2- pEMT7 were similar to pEMT3, described above. An EcoR1 digest showed pEMT1 to be very different from pJP4 and the other six captured plasmids. As with pTFD41, all the *tfd* genes of pJP4 hybridized strongly to fragments of pEMT1 under high stringency conditions. Although the individual fragment sizes carrying these *tfd* genes did not match those of pJP4 and the distances between pEMT1 genes varied, the spatial arrangement of the *tfd* genes remained the same as for the well studied pJP4. Therefore very similar gene sequences are found on different plasmids.

The mosaic gene pattern for the *tfd* genes that arose among the analyzed 2,4-D degrading isolates, is probably due to plasmid transfer

followed by recombination. It is known that catabolic plasmids can move between soil populations (Mergeay et al., 1994; Top et al., 1994; Kinkle et al., 1993; Ka and Tiedje, 1994). Due to the conjugative nature of these plasmids, they serve as vectors for shuttling catabolic pathways between microbial populations. Plasmids carrying homologous gene sequences and different spatial arrangements of *tfd* genes could recombine with homologous sequences of other plasmids or chromosomes, exchanging *tfd* gene cassettes. In this way, genes carried on different replicons could become arranged in new combinations. This rearrangement could explain why genes with nearly identical hybridization profiles are found in different sites, at different times and within different organisms (Don and Pemberton, 1981; Top et al., 1994).

Although variations in gene sequences, arrangement and location have been found, the implications of these differences to the fitness of the 2,4-D degrading populations have not been determined. Many isolates have genes with varying degrees of similarity to those of pJP4, yet most seem to utilize similar biochemical steps as the canonical pathway. It is not known how the fitness of the host population is affected by the efficiencies of these enzymes with observed differences in gene sequence. Slight differences in amino acid sequence can have profound repercussions as to the function and structure of a protein. Unfortunately, there is very little data to indicate if a particular combination of *tfd* genes confers a competitive advantage to a

bacterial strain when compared to the same strain carrying a different permutation of *tfd* genes. It is not yet understood what the fitness effect of different *tfd* gene sequences or spatial arrangements are among competitors utilizing 2,4-D as a substrate.

2,4-D organism diversity

Although a few pure cultures of microorganisms with the ability to grow on 2,4-D as the sole source of carbon have been obtained from pristine soils without previous exposure to 2,4-D, most of the bacteria capable of catabolizing the compound have been isolated from sites with prior exposure to the herbicide (Fulthorpe et al., 1994; Kamagata et al., 1995). Multiple bacterial populations with this ability can be isolated from a single soil sample (Fulthorpe et al., 1995; Tonso et al., 1994; Don and Pemberton; 1981, Top et al., 1994, Dunbar et al., 1994) (Table 1.3). Most frequently, 2,4-D degrading bacterial isolates are members of the gram negative Proteobacteria, however, some gram positive bacteria and fungi can also degrade 2,4-D (Häggblom ,1992).

Table 1.2. 2,4-D degrading populations carry mosaics of catabolic genes. Southern hybridization analysis of 2,4-D degrading strains with the tfdA, tfdB and tfdC gene probes from plasmid pJP4, are shown with asterixes, *** is high stringency hybridization (>90% sequence similarity), ** is medium stringency (75-90%), and * is low stringency (60-75% sequence similarity). Other notations are: -(hs) no hybridization under high stringency, (rasc) low stringency hybridization to tfdA of pJP4, high sequence similarity to tfdA from strain RASC. Fulthorpe et al, 1995

				similarity to pJP4			
genus	**strain**	**plasmid**	**2,4-D genes**	**tfdA**	**tfdB**	**tfdC**	**Reference**
Ralstonia eutropha	JMP134	pJP4	P[a]	***	***	***	Don 1981
Ralstonia eutropha	JMP228	pEMT1	P	***	***	***	Top 1995
Ralstonia eutropha	JMP228	pEMT2-7	P	***	**	*	Top 1995
Alcaligenes paradoxus	2811P	pKA2	P	***	-(hs)	-(hs)	Ka 1994
Alcaligenes xylosoxidans	TFD9			***	**	*	Fulthorpe 1995
Burkholderia sp.	TFD2			*	*	-	Fulthorpe 1995
Burkholderia sp.	RASC		C[b]	*	***	***	Suwa 1996
Pseudomonas mallei	BRI6001		C	(rasc)	***	***	Greer 1990
Pseudomonas andropogonis	K712	pKA4	P	***	**	*	Fulthorpe 1995
Variovorax sp.				*			Kamagata 1997
Rhodoferax fermentans	TFD23			***	*	*	Fulthorpe 1995
Rhodoferax fermentans	TFD31			-	-	-	Fulthorpe 1995
Rhodoferax fermentans	B6-9			**	-	*	Fulthorpe 1995
Sphingomonas paucimobilis	1443		C?	-	-	-	Ka 1994
Sphingomonas sp.	K1443		C	-	*	-	Fulthorpe 1995
Sphingomonas sp.	EML146		C	-	*	-	Fulthorpe 1995
Sphingomonas sp.	TFD44		C	-	*	-	Fulthorpe 1995
Sphingomonas sp.	B6-10		C	-	-	-	Fulthorpe 1995
Halomonaddaceae sp.	I-18			***	***	***	Maltseva 1996

[a] P indicates plasmid- borne 2,4-D genes.

[b] indicates chromosomally located 2,4-D genes.

In addition to genus level bacterial diversity, genetic diversity has also been demonstrated to occur within a single species (Dunbar et al., 1994; Dunbar et al., 1995). Consistent with the results of prior hybridization experiments, Dunbar isolated 136 different bacterial isolates belonging to the same species, but that could be distinguished into 32 different taxonomic groups based on 16S rDNA restriction profiles (ARDRA). Groups separated by ARDRA share a similar phylogeny. The restriction groups were further subdivided by REP-PCR (DeBruijn et al., 1992). In another similar set of experiments, using a variety of phenotypic and genotypic methods for evaluating the diversity found among the 2,4-D utilizing groups of microorganisms, Tonso et al. (1994) also characterized 46 isolates of 2,4-D degrading bacteria. The isolates were obtained from seven geographically distinct sites and were divided into five taxonomic groups based on carbon source utilization patterns (BIOLOG) and fatty acid profiles (FAME). Genetic fingerprints by REP-PCR indicated that these groups could be further sub-divided based on identical, similar or unique banding patterns (Tonso et al., 1994). Additionally, when the 16S rDNA phylogenies of 21 different 2,4-D degrading isolates were compared with known reference strains, the degraders were largely represented among α-, β- and γ- proteobacteria (Fulthorpe et al., 1994). Since so many 2,4-D degrading populations occur in natural environments, inter-specific and

intra-specific competition may play a relevant role in determining the microbial community structure.

Consequence of the high diversity in 2,4-D degradative genes

As mentioned above, several different gene clusters and arrangements for the known 2,4-D degradative alleles have been described. A single species of bacteria may have different sets of alleles, depending on the selective pressures of its environment. Consequently, similar sets of alleles may be found in different species of bacteria, especially when the pathway is found on a catabolic plasmid. When a plasmid is beneficial, it propagates, both by assisting in the growth and reproduction of its host, as well as by promoting its own lateral transfer. The result is a skewed distribution of the 2,4-D degradative alleles toward the fittest genotype when selection pressure is applied.

Table 1.3. Diversity of 2,4-D degrading isolates.

group	genus	strain	source	Reference
β-Proteobacteria				
	Alcaligenes eutrophus	JMP134	Australia	Don 1981
	Alcaligenes paradoxus	2811P	KBS, Michigan	Ka 1994
	Alcaligenes xylosoxidans	TFD9	Saskatchewan	Fulthorpe 1995
	Burkholderia sp.	TFD2	KBS, Michigan	Fulthorpe 1995
	Burkholderia sp.	RASC	activated sludge	Suwa 1996
	Burkholderia mallei	TFD6	KBS, Michigan	Matheson et al. (1996)
	Burkholderia mallei	BRI6001	Peat, Nova Scotia	Greer 1990
	Pseudomonas picketii	712	KBS, Michigan	Ka 1994)
	Pseudomonas andropogonis	K712	KBS, Michigan	Fulthorpe 1995
	Variovorax sp.		Hawaiian soil	Kamagata 1997
	Rhodoferax fermentans	TFD23	Michigan	Fulthorpe 1995
	Rhodoferax fermentans	TFD31	Saskatchewan	Fulthorpe 1995
	Rhodoferax fermentans	B6-9	Ontario	Fulthorpe 1995
α-Proteobacteria				
	Bradyrhizobium sp.		Hawaiian soil	Kamagata 1997
	Sphingomonas paucimobilis	1443	KBS, Michigan	Ka (1994
	Sphingomonas sp.	EML146	Oregon	Fulthorpe 1995
	Sphingomonas sp.	TFD44	Montana	Fulthorpe 1995
	Sphingomonas sp.	B6-10	Ontario	Fulthorpe 1995
Halomonadaceae	*Halomonaddaceae sp.*	I-18	Oregon, Alkali Lake	Maltseva 1996
Flavobacterium	*Flavobacterium sp.*	MH	2-(2,4-DCP)PA	Chaudry 1988
Gram Positive	*Nocardioides simplex*	3E	USSR, 2,4,5-T	Kozyreva 1993
	Arthrobacter sp.		soil	Loos 1967
	Arthrobacter sp.		soil	Tiedje 1969
	Corynebacterium sp.		soil	Rogoff 1956
Fungi	*Aspergillus niger*		soil	Shailubhai, 1984

The maintenance of a high diversity of degradative genes and vectors within populations is ecologically advantageous. A more diverse gene pool will allow for the formation of more host-plasmid combinations, that may offer advantages under variable environmental conditions. From an evolutionary standpoint, an understanding of the true evolutionary origin of the 2,4-D phenotype would be useful. Since the herbicide 2,4-D is structurally similar to the plant hormone auxin, the pathway may have developed as a means of degrading this type of natural compound. Auxins however, are not degraded by the 2,4-D pathway. The majority of the 2,4-D degradation pathway utilizes *tfd*B, (hydroxylase) and *tfd*CDEF (ring cleavage) genes, specific for chloroaromatic compounds.

Different degradation mechanisms may have evolved to degrade the assortment of phenolic products that result from the breakdown of lignin. The fact that the 2,4-D genes of plasmid pJP2 have undergone a duplication event, is indirect evidence that different pathways may arise by subsequent duplication and modification. Acts of further evolution of the duplicated genes may lead to divergent evolution of the pathway. In addition to the genes for hydroxylation and ring cleavage, 2,4-D must first be converted to a chlorophenol, which frequently occurs as a result of the enzyme encoded by the gene *tfd*A. The function of the *tfd*A gene is the initial step in 2,4-D degradation, and several different homology groups of the gene have been identified, (pJP4, RASC and pEMT8). While the fundamental function of

the ancestral *tfd*A gene is not known, Hogan et al. (1998) found the presence of genes highly similar to *tfd*A in 70% of heterotrophic soil isolates from Michigan Agricultural soil. None of the populations were capable of utilizing 2,4-D as a sole source of carbon and energy, and the function of *tfd*A in these isolates is unknown.

It is unclear whether the gene sequences encoding for catabolic enzymes, the characteristics of the plasmids carrying these genes, or the organism's species-specific properties determine competitive superiority among populations utilizing the same limiting resource. The natural diversity of 2,4-D degradative populations, vectors and alleles provide a series of variables that can be used to investigate intra- and inter-species competition. Because the canonical pathway for 2,4-D utilization is well characterized, it makes a good system for determining whether competitive outcome among organisms is due to the influence of the organism, or the *tfd* gene sequences. The variables of allele and plasmid to host strain fitness can be determined for a single species, which facilitates intra-specific competition experiments comparing different 2,4-D genotypes. The variables of allele and plasmid to the fitness' of two competing host species allow for inter-specific comparisons. Previously, Ka et al. (1994) had noted a correlation between the plasmid carried by *B. cepacia* DB01 and its growth rate on 2,4-D, although the two plasmid-bearing strains were not subjected to conditions of direct competition. Thus, there is reason to

believe that the catabolic genes encoding for enzymes of 2,4-D degradation pathways can influence the outcome of competition in bacteria.

2,4-D degradative genes may influence competitive fitness

To determine how various *tfd* gene alleles affect competition, isogenic hosts (spontaneous antibiotic resistant mutants of one natural isolate) differing in their plasmid-encoded genes for the utilization of an aromatic substrate (2,4-D) will be competed under different growth conditions. Two of the bacterial species that can degrade 2,4-D when they contain a 2,4-D plasmid, are *Burkholderia* and *Ralstonia*. By comparing the results of competition experiments among the same species carrying two different types of plasmids with different sets of alleles, it can be determined if there is a significant benefit to carrying one of the allelic groups. By forcing competition among different host species with the same sets of degradative alleles, the contribution of host phenotype to fitness can be compared.

References:

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. Environ. Microbiol. 49:1237-1245.

Arthur, W. 1982. The evolutionary consequences of inter-specific competition. Adv. Ecol. res. 12:127-187

Bollmer, M.D., K. Stadler-Fritzche, and M. Schlömann. 1993. Conversion of 2-cloromaleylacetate in *Alcaligenes eutrophus* JMP134. Arch. Microbiol. 159:182-188

Chaudhry, 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. 170:3897-3902.

Chao, L. and E. C. Cox, 1983. Competition between high and low mutating strains of *E. coli*. Evolution 37:125-134.

Connell, J. H. 1978. Diversity and the coevolution of competitors, or the ghost of competition past.. Oikos 35: 131-138

Connell, J.H. 1983 On the prevalence and relative importance of interspecific competition: evidence from field experiments. American Naturalist, 122:661-696

Darwin, C. 1859 The Origin of Species by Means of Natural Selection. Penguin Books, Harmondsworth (reprint 1968), p116

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

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

Don, R. H., A.J. Weightman, H.J. Knackmuss and K. N. Timmis. 1985b. Transposon mutagenesis and cloning analysis of the pathways for degradation of 2,-4dichlorphenoxyacetic acid and 3-chlorobenzpate in Alcaligenes eutrophus JMP134 (pJP4). J. Bacteriol. 161:85-90.

Duetz W. A., C. DeJong, P.A. Williams, and J. G. Van Andel. 1994. Competition in chemostat culture between *Pseudomonas* strains that use different

pathways for the degradation of toluene. Appl. Environ. Microbiol. 60:2858-2863.

Dunbar, J., D.C. L. Wong, M.J. Yarus, and L. J. Forney. 1996. Autoradiographic mothod for isolation of diverse microbial species with unique catabolic traits. Appl. Envrion. Microbiol. 62:4180-4185.

Dunbar, J., S. White, and L. Forney. 1997. Genetic diversity through the looking Glass: effect of enrichment bias. Appl. Envrion. Microbiol. 63:1326-1331.

Dykhuizen, D. E. and D. L. Hartl, 1983. Selection in chemostats Microbiol. Rev. 47:150-168.

Elton, C. 1946. Competition and the structure of ecological communities. Journal of Animal Ecology, 15:54-68

Fukumori F. and R.P. Hausinger. 1993. Alcaligenes eutrophus JMP134 "2,4-D dichlorophenoxyacetate monooxygenase" is an a-ketoglutarate-dependent dioxygenase. J. Bacteriol. 175:2083-2086.

Fukumori F. and R.P. Hausinger. 1993. Purification and characterization of 2,4-D dichlorophenoxyacetate/a-ketoglutarate dioxygenase. J. Biol. Chem. 268:24311-24317.

Fulthorpe, R.R., C. Mcgowan, O.V. Maltseva, W.E. Holben, and J.M. Tiedje. 1995. 2,4-Dichlorophenoxyacetic acid-degrading bacteria contain mosaics of catabolic genes. Appl. Environ. Microbiol. 61:3274-3282

Gause, G.F. 1934. The struggle for existence. Hafner, New York. 1964. pp 163-.

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. 1988. Genes specifying degradation of 3-chlorobenzoic acid in plasmids pAC27 and pJP4. Proc. Nat. Acad. Sci. 82:1638-1642.

Graham, D. W. , J. A. Chaudhary, R. S. Hanson and R. G. Arnold. 1993. Factors affecting competition between Type I and Type II methanotrophs in two-organisms, continuous-flow reactors. Microbial Ecology, 25:1-17

Gottschal, J. C. 1992. Continuous Culture. *In* "Encyclopedia of Microbiology", Vol.1. Academic Press Inc. London. pp559-572.

Hansen S. R. and S. P. Hubbell. 1980. Single-nutrient microbial competition: qualitative agreement between experimental and theoretically forecast outcomes. Science. 207:1491-1493.

Harker A.R., R.H. Olsen and R.J. Seidler. 1989. Phenoxyacetic acid degradation by the 2,4-dichlrophenoxyacetic acid (TFD) pathway of plamid pJP4: mapping and characteriztion of the TFD regulatory gene tfdR. J. Bactriol. 171:314-320.

Haugland, R. A., D. J. Schlemm, R. P. Lyons III, P. R Sferra and A. M. Chakrabarty. 1990. Degradation of the chlorinated phenoxyacetate herbicides 2,4-dichlorophenocyacetic acid and 2,4,5-trichlorophenoxyacetic acid by pure and mixed bacterial cultures. Appl, Environ. Microbiol. 56:1357-1362.

Häggblom, M. M. 1992. Microbial breakdown of halogenated aromatic pesticides and related compounds. FEMS microbiology rev. 103: 29-72.

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. 60:1121-1128

Ka, J.O. and J.M. Tiedje 1994b. Integration and Excision of a 2,4-dichlorophenoxyacetioc acid-degradative plasmid in Alcaligenes paradoxus and Evidence of its natural intergeneric transfer. J. BActeriol. 176:5284-5289,

Kamagata, Y., R.R. Fulthorpe, K. Tamura, H. Takami, L.J. Forney and J.M. Tiedje (1997). Pristine environments harbor a ne wgroup of oligotrophic 2,4-Dichlorophenoxyacetic acid-degrading bacteria. Appl. Environ. Microbiol. 63:2266-2272

Kaphammer, B., J.J. Kukor and R.H. Olsen. 1990a. Regulation of tfdCDEF by tfdR of the 2,4-dichlorophenoxyacetic acid degradation plasmid pJP4. J. Bacteriol. 172:2280-2286.

Kaphammer, B., and R.H. Olsen. 1990b. Cloning and characterization of tfdS, the repressor-activator gene of tfdB, from the 2,4-dichlorophenoxyacetic acid degradation plasmid pJP4. J. Bacteriol. 172:5856-5862.

Kinkle, B.K., M.J. Sadowsky, E.L. Schmidt and W. C. Koskinen. 1993. Plasmids pJP4 and r68.45 can be transferred between populations of Bradyrhizobia in non-sterile soil. Appl. Environ. Microbiol. 59:1762-1766.

Kozyreva L. P., Y. V. Shurukhin, Z. I. Finkel'shtein, B. P. Bashunov, and L. A. Golovleva. 1993. Metabolism of the Herbicide 2,4-D by a Nocardioides simplex strain. Microbiology. 62:78-85.

Kukor, J.J, R.H. Olsen and J.S. Siak. 1989. Recruitment of a chromosomally encoded maleylacetate reductase for degradation of 2,4-dichlorophenoxyacetic acid by plasmid pJP4. J. Bacteriol. 171:3385-3390.

Lenski, R.E., S.C. Simpson, and T.T. Nguyen. 1994. Genetic analysis of a plasmid-encoded host genotype-specific enhancement of bacterial fitness. Journal of Bacteriology.

Lenski R. E., J. A. Mongold, P. D. Sniegowski, M. Travisano, F. Vasi, P.J. Gerrish and T. M. Schmidt. 1998 Evolution of competitive fitness in experimental populations of *E. coli*: What makes one genotype a better competitor than another. Antonie von Leeuwenhoek. 73:35-47

Leveau, J.H.J., A.J.B. Zehndera nd J. R. van der Meer. 1998. The *tfdK* gene product facilitates uptake of 2,4-dichlorophenoxyacetate by *Ralstonia eutropha* JMP134. J. Bacteriol. 176:2348-2353

Levin, B. 1972. Coexistence of two asexual strains on a single resource. Science. 175:1272-1274

Mäe A.A., O. M. Reet, N.R., Ausmees, V.M.Koiv and A.L. Heinaru. 1993. Characterization of a new 2,4-dichlrophenoxyacetic acid degrading plasmid pEMT4011: physical map and localization of catabolic genes. J. Gen. Microbiol. 139:3165-3170.

Maltseva, O. 1996. Personal Communication.

Matheson, V.G., L. J. Forney, Y. Suwa, Ch. H. Nakatsu, A.J. Sextone and W. E. Holben. 1996. Evidence for acquisition in nature of a chromosomal 2,4-dichlrophenoxyacetic acid/a-ketoglutarate dioxygenase gene by different Burkholderia spp. Appl. Environ. Microbiol. 62:2457-2463.

Matrubuthan, U. and A.R. Harker. 1994. Analysis of duplicated gene sequences associated with tfdR and tfdS in Alcaligenes eutrophus JMP134. J. Bacteriol. 176:2348-2353.

Mergeay, M., H. De Rore, E. Top, D. Srinagael, M. Höfte, D. Van der Lelei, R. Dijkmans, and W. Verstraete. 1994 Plasmid biology and risk assessment: Elements for modelling approach of plasmid-mediated gene release in soil environments. *In* Environmental Gene Release: Models, experiments and risk assessment. Eds. J. J. Bazin, and J. M. Lynch. Chapman & Hall, London. pp.67-75.

Monod, J. 1949. The growth of bacterial cultures. Annu. Rev. Microbiol. 3:371-394.

Nakatsu, C. 1994. Genetic organization and molecular evolution of the 2,4-D catabolic pathway. *In* JRDC-MSU Joint Workshop on Microbial Evoultion and Biodegradation. Organizing Committee: L. Forney, M. Fukuda, T. Kudo and Y. Tanaka.. JRDC-MSU. pp.51-59.

Perkins E.J., G.P. Milton, O. Caceres and P.R. Lurquin. 1990. Organization and sequence anlaysis of the 2,4-dichlorophenol hydroxylase and dichlorocatechol oxidative operons of plasmid pJP4. J. Bacteriol. 2351-2359.

Rogoff, M. H and J. J. Reid. 1956. Bacterial decomposition of 2,4-dichlorophenoxyacetic acid. J. Bacteriol. 71:303-307

Sassanella 1994, personal communication

Schoener, T.W. 1983. Field experiments on interspecific competition. American Naturalist, 122:240-285

Shailubhai, K., S. R. Sahasrabudhe, K. A. Vora and V. V. Modi. 1984. Degradation of chlorobenzoates by *Aspergillus niger.* Experentia, 40: 406-407.

Smith, V. 1993, Implications of Resource-Ratio Theory for microbial ecology. Advances in Microbial Ecolgy. 13:1-37.

Smith, V. 1993, Applicability of Resource-Ratio Theory to microbial ecology. Limnol. Oceanogr. 38:239-249.

Streber W. R., K. N. Timmis and M.H. Zenk. 1987. Analysis, cloning and high-level expression of 2,4-Diclrophenoxyacetate monnoxygenase gene tfdA of *Alcaligenes eutrophus* JMP134. J. Bacteriol. 169:2950-2955.

Suwa, Y. A.D. Wright, F. Fukumori, K.A. Nummy, R.P. Hausinger, W.E. Holben and L.J. Forney. 1996. Characterization of a Chromosoamlly encoded 2,4-Dichlorphenoxyacetic acid/alpha-Ketoglutarate dioxygenase from *Burkholderia* sp. Strain RASC. Appl. Environ. Microbiol. 62:2464-9.

Top, E., I. De Smet, W. Verstraete, R. Dijkmans, and M. Mergeay. 1994. Exogenous isolation of mobilizing plasmids from polluted soils and sludges. Apple. Environ. Microbiol. 60:831-839.

Top E.M., W.E. Holben and L.J. Forney (1995) Characterization of diverse 2,4-Dichlorophenoxyacetic acid-degrading plasmids isolated from soil by complementation. Appl. Environ. Microbiol. 61:3274-3281

Tilman, D. 1977. Resource competition between planktonic algae: an experimental and theoretical approach. Ecology: 58:338-348.

Tilman, D. 1981. Tests of resource competition theory using four species of Lake Michigan algae. Ecology. 62:802-815.

Van Gemerden, H. 1974. Coexistence of organisms competing for the same substrate: an example among the purple sulfur bacteria. Microbial Ecology. 1:104-119.

Vadas R. L. 1990. Competitive exclusion, character convergence, or optimal foraging: which should we expect? Oikos 00: 123:228

Van Niel, E.W.J. , L. A. Robertson, and J. G. Kuenen. 1993. A mathematical description of the behaviour of mixed chemostat cultures of an autotrophic nitrifier and a heterotrophic nitrifier/arerobic denitrifier: a comparison with experimental data

CHAPTER 2

THE IMPACT OF SIMILAR DEGRADATIVE GENES ON DIFFERENT PLASMIDS TO THE FITNESS OF ISOGENIC *RALSTONIA EUTROPHA* STRAINS COMPETING FOR 2,4-DICHLOROPHENOXYACETIC ACID.

ABSTRACT

Genes encoding the 2,4-D degradative pathway have been identified on different plasmid backbones. *Ralstonia eutropha* strains acquire the ability to degrade 2,4-D when they possess the appropriate catabolic genes. This research examines the hypothesis that for *R. eutropha* strain JMP228, 2,4-D degradative alleles with high sequence similarity that reside on different plasmid backbones, will result in populations of equal competitiveness when 2,4-D is supplied as the limiting resource. Both strains carrying pJP4 and pEMT1, exhibit very similar growth characteristics in terms of growth rate on 2,4-D, and identity based on colony morphology and time of colony appearance. Previously it was shown that the two plasmids, pJP4 and pEMT1, carry homologous alleles for the genes encoding the 2,4-D degradative pathway on different replicons. The genes of pEMT1 hybridize to probes generated from the degradative genes of pJP4 under high stringency conditions. To test the hypothesis, pure strains of *R. eutropha*, carrying either pJP4 or pEMT1,

were grown with 2,4-D as their sole limiting substrate. Similar growth rates (μ=0.19) and rates of 2,4-D degradation by the host, were observed in *R. eutropha* containing either pJP4 or pEMT1. Growth parameters derived from pure culture studies indicated that plasmids pJP4 and pEMT1 conferred very similar growth rates and degradation rates in this host. Pair-wise combinations of *R. eutropha* carrying pJP4 and pEMT1 were competed in batch culture conditions with 10 % of the culture transferred daily for 5 days. During this period, the strains coexisted at nearly equal ratios, which suggested that the influence of the plasmid backbone was minimal. The slope of the line generated from natural logarithm of population sizes did not vary significantly from zero ($P>0.1$). In chemostats, pure cultures of *R. eutropha* carrying either pJP4 or pEMT1 reached similar population densities of 3-6 x 10^8 CFU/ml at a dilution rate of 0.033 h^{-1}. When competed in the chemostats, the two populations coexisted, and the total cell numbers were similar to those attained by either pure culture.

INTRODUCTION

Among bacteria, plasmid transfer is a common mechanism for acquiring new catabolic functions. Some of the prevalent broad-host range plasmids of the proteobacteria can carry genes encoding for the degradation of various aromatic compounds. One of the best studied catabolic traits to be distributed by conjugative plasmids is the ability to degrade 2,4-D. Self-mediated horizontal transfer of plasmid pJP4 has been shown to occur in soils, as well as in the laboratory. (DiGiovanni et al., 1996,; Neilson et al., 1994; Friedrich et al., 1983; Kinkle et al., 1993; Top et al., 1998).

The propensity of these plasmids to propagate among the proteobacteria allowed Top et al. (1995) to use *Ralstonia eutropha* JMP228 as a recipient strain for capturing several naturally occurring 2,4-D degradative plasmids from the environment. Transconjugants that gained the ability to degrade 2,4-D were isolated, and their plasmids were characterized using gene probes targeting internal fragments derived from the well-studied plasmid pJP4 (Holben et al., 1992; Top et al., 1995). All of the 2,4-D genes of plasmid pEMT1 hybridized under high stringency conditions to those of pJP4. Upon examination of the plasmid backbone with restriction analysis and a gene probe generated from the 750 bp Hae II fragment of vector pULB2420, carrying the inc-P/rep function of the Inc-

Inc-P1 plasmid RK2 it was concluded that pEMT1 was not an Inc-P plasmid like pJP4 (Top et al., 1995). The gene cassettes of pJP4 and pEMT1 were found to be in a different arrangement, with different distances between the cassettes (Table 2.1). Similar results were seen in a bacterial isolate identified as *Pseudomonas putida*, where the catabolic genes were found in a different arrangement, on a plasmid backbone that did not hybridize to the one of pJP4 (Mäe et al., 1993). Consequently, cassette shuffling and lateral transfer could lead to the persistence of highly homologous alleles residing in slightly different genetic backgrounds even within the same species of bacteria.

Table 2.1. Genotypic and phenotypic comparison of pJP4, pEMT1 and pEMT3. Characteristics of plasmids pJP4 and pEMT1 used in experiments of this chapter, and characteristics of plasmid pEMT3 used in Chapters 3 and 4 (Top et al., 1995)

	pJP4	pEMT1	pEMT3
Size (Kb)	88	96	63
tfd gene homology		High to *tfd*A (pJP4) High to *tfd*BCDEF (pJP4)	High to *tfd*A (pJP4) Moderate to *tfd*B (pJP4) Low to *tfd*CDEF (pJP4)
Tfd gene order	*tfd*A, *tfd*CDFE *tfd*B	*tfd*A, *tfd*CDFE *tfd*B	*tfd*A, *tfdB*, *tfdF*, *tfd*D, *tfd*C
Distance from *tfd*A to *tfd*B	Ca. 16 Kb	Ca. 7 Kb	Ca. 2 Kb
Inc group	Inc-Pβ	Non-Inc-P	Inc-Pβ
Host range		α, β, γ -proteobacteria (all)	

Since the same 2,4-D degradative genes appear to be located in several different plasmid backgrounds, it raises the question of whether a host strain population carrying these different plasmids would behave in a competitively equivalent manner. Previous evidence supports the idea that genetically similar bacterial populations do coexist in natural environments (Dunbar et al., 1996). Analysis of a set of phenotypically identical, but genetically distinct subspecies of 2,4-D degrading *Variovorax paradaxus* populations were isolated from a single small soil sample. The great diversity, indicated the coexistence of intra-specific variants (Dunbar et al., 1996). In soil competition experiments among denitrifying bacteria added to soils, or among epiphytic bacteria, generally both populations would coexist. When one population dominated, inoculum density or prior colonization served as the primary determinants of the interaction (Murray et al., 1992; Wilson and Lindow, 1994).

While several broad host range plasmids have been found to carry homologous cassettes for the degradation of 2,4-D, the magnitude of sequence differences is not precisely known. Plasmid pEMT1 carries a set of 2,4-D degradative alleles that hybridize to those of pJP4 under high stringency conditions, indicating that they are >90 % similar in sequence, but are the genes identical? (Top et al., 1995; Fulthorpe et al., 1995). Based on this result it is both possible that the genes are identical, or that

they may contain some slight differences. Very small differences in nucleotide composition, perhaps due to 3rd codon nucleotide variation in the DNA sequence, may not significantly affect the enzyme activities of the proteins carrying out the consecutive degradative steps of the pathway. Conversely, there may be differences that are not accounted for, if they occur in the portion of the gene that was not used as the probe.

When a wild type bacterial population obtains a single genetic mutation to generate different strains with a phenotypic marker that can readily be identified in the laboratory, the resulting mutants are often termed isogenic. While the populations are of the same species as the wild type strain, and have nearly identical genomes, they now have a slight genetic difference. The small differences in phenotype that arise as a consequence of mutations utilized for genetic markers may or may not impact the competitive fitness of the bacterial host under different environmental conditions. When utilized as strains for competition experiments, it is desirable to obtain the mutation in a gene that does not influence the fitness of the host under the competition conditions. Evidence from previous research has established the possibility that among isogenic strains of bacteria, slight differences in genotype or the presence of a plasmid may or may not affect the relative competitive abilities of bacterial populations under particular laboratory or environmental conditions (Levin, 1972; Bouma and Lenski, 1988; Rutter and Nedwell,

1994) . Dominance is not only affected by growth kinetics (Chapter1) but also by a large number of factors, ranging from inoculum density to the environment or culture conditions used for the competition experiments. Therefore, it is possible that the same genes carried on different conjugative plasmids could result in strains with very similar phenotypes, and with very little observed differences in fitness under limiting resource conditions. On the other hand, the converse could prove to be true. There may be factors carried on the plasmids that do not allow for the evolution of compatible host-plasmid combinations, and thus strains which are expected to display similar fitness may prove to be significantly different.

The research presented in this Chapter examined whether there was a measurable difference in the phenotype and fitness of two isogenic *Ralstonia. eutropha* JMP228 strains, when they served as hosts to plasmids pJP4 and pEMT1 and utilized 2,4-D as the limiting resource. Antibiotic resistant mutants of *R. eutropha* JMP228, bearing either streptomycin resistance or rifampicin resistance were used as the isogenic host populations for competition experiments. Presumably, the rifampicin and streptomycin resistance markers are neutral with regard the 2,4-D degrading phenotype, but to control for the influence of the genetic markers, both plasmids pJP4 and pEMT1 were introduced to both isogenic strains. Pure culture growth characteristics in both batch cultures and chemostats, were determined for the strains before competition experiments. To determine

experiments. To determine how the two isogenic populations compared to a member of a different species (non-isogenic), the pJP4 and pEMT1 carrying *R. eutropha* JMP228 strains were competed with the pJP4 carrying *Burkholderia cepacia* DB01 strain in batch culture. If the two plasmids conferred equivalent fitness upon *R. eutropha*, then they should also boast similar population dynamics in competition with the *B. cepacia* strain. Fitness differences due to the presence of the different plasmid backbones of pJP4 and pEMT1 were addressed with the isogenic host populations bearing the different plasmids. If the plasmids are neutral with respect to succinate metabolism, isogenic populations with the different plasmids should exhibit very similar growth rates on a non-selective carbon source, such as succinate. If the fitness differences are influenced by the plasmid backbone, then the phenotypes and/or competitive fitness of the pJP4 and pEMT1 carrying strains may differ from each other. If, on the other hand, the different plasmid backbones have no effect on the 2,4-D degradative phenotype, no differences in competitive fitness should be detected.

MATERIALS AND METHODS

Bacterial strains. *Ralstonia eutropha* JMP228, is the pJP4-cured strain of *R. eutropha* JMP134 (Don 1981). Spontaneous mutants of *R. eutropha* JMP228, resistant to rifampicin (250 μgml^{-1}) (Sigma, St. Louis, MO) and streptomycin (250 $\mu g\ ml^{-1}$) (Sigma, St. Louis, MO) were used to distinguish between plasmid bearing populations (JMP228R and JMP228S). The rifampicin resistant isolate was obtained by Top (1995) and the streptomycin resistant strain was generated for these competition experiments as follows. Strain JMP228S was a spontaneous mutant prepared by growing a culture of *R. eutropha* JMP228) to late log phase in minimal medium containing sodium succinate (5 mM) (Sigma, St. Louis, MO. Cells (1 ml of culture fluid) were collected by centrifugation at 6000 rpm in a microcentrifuge (Beckman, Westbury, NY), washed once with sterile phosphate buffered saline (PBS) and resuspended in 100 µl PBS. The suspension was serially diluted in ten-fold increments and 100 µl was spread over the surface of a minimal medium agar plate containing 2,4-D (200 $\mu g\ ml^{-1}$) (Sigma, St. Louis, MO) and streptomycin (100, 150, 200 and 250 $\mu g\ ml^{-1}$). Three of the colonies which grew on the 10^{-2} dilution plate with 250 $\mu g\ ml^{-1}$ streptomycin were streaked out to the same medium three times. All spontaneous mutants were equivalent to one another and the parental strain, in terms of growth rates on succinate and colony

morphology on LB and R2A (Difco, Detroit, MI) agar plates (Sambrook et al., 1989). In the absence of selection for streptomycin resistance, the marker was not readily lost. When JMP228S was grown in the presence or absence of streptomycin, then diluted and plated to R2A agar with or without the antibiotic, equal numbers of colonies were obtained for both the non-selective and selective plates. *B. cepacia* strain DB01, naturally moderately resistant to streptomycin (100 $\mu g\ ml^{-1}$), served as the non-isogenic host strain. A mutant resistant to rifampicin (100, 150, 200, 250 $\mu g\ ml^{-1}$) was obtained in the same manner as for *R. eutropha* JMP228. Three colonies from the highest antibiotic concentration were chosen and purified by three consecutive transfers to R2A agar containing rifampicin. The mutants did not readily lose the rifampicin marker under non-selective conditions, and the resistant *Burkholderia* strain was designated DB01R. *Ralstonia* and *Burkholderia* strains can also be distinguished based on their different colony morphologies on R2A agar plates. Bacterial strains used in this experiment are listed in table 2.2.

Table 2.2. Bacterial strains used to determine the influence of the plasmid backbone on competition

Species	Strain	Antibiotic resistance	Reference
Ralstonia eutropha	JMP228	rif^S str^S	Don and Pemberton 1981
Ralstonia eutropha	JMP228R	rif^R str^S	Top et al. 1995
Ralstonia eutropha	JMP228S	rif^S str^R	This study
Burkholderia cepacia	DB01	rif^S tri^S	Holben et al. 1993
Burkholderia cepacia	DB01R	rif^R tri^S	This study

Plasmids and transconjugant construction. Plasmid pJP4, was isolated from *R. eutropha* JMP134, and carries genes encoding the best characterized 2,4-D degradative pathway (Don 1995). Plasmid pEMT1 was obtained in a previous study as a result of an exogenous plasmid trapping experiment using strain JMP228R as a recipient (Top 1995). Plasmid pEMT1 carries homologous *tfd*A *tfd*B and *tfd*C genes to those of pJP4, but on a different replicon backbone, as determined by hybridization experiments using the replication origin for Inc-P plasmids as a probe (Top 1995). The plasmids used in this study are described in Table 2.3.

Table 2.3. Plasmids used to determine the outcome of competition in isogenic populations

		Hybridization with[a]				
		tfd genes of pJP4			IncP replicon	
Plasmid	Size (kb)	tfdA	tfdB	tfdC	rep R	Reference
pJP4	88	***	***	***	***	Don and Pemberton 1981
pEMT1	96	***	***	***	none	Top et al. 1995

[a] *** indicates hybridization under high stringency conditions and correlates to >90% sequence similarity.

Plasmids pJP4 and pEMT1 were transferred from JMP228/pJP4 or JMP228R/pEMT1 into the *R. eutropha* JMP228R and JMP228S strains using a filter mating technique. Donor cells ($1x10^7$) were combined with recipient cells ($2x10^6$) on a 13 mm, 0.02 μm Poretics filter (Fisher, Pittsburgh, PA) which had been placed on LB agar plates for 8 hours. Cell material from the filters was suspended by placing the filter in 1 ml A+N minimal medium broth (composition below), and vortexing the filter for 30 seconds. The suspended cells were serially diluted in 10-fold increments, and plated to A+N minimal media plates containing antibiotics to counter-select the donor strain, and with 2,4-D to assure the presence of the degradative plasmid in resulting transconjugants. The resulting transconjugants are listed in Table 2.4.

Table 2.4. Transconjugants obtained with the filter mating technique

Transconjugant	Donor	Recipient	2,4-D utilization [a]	Antibiotic resistance [b]
	JMP228/pJP4		++	rif^S str^S
	JMP228R pEMT3		+	rif^R str^S
	JMP228R pEMT1		++	rif^R str^S
		JMP228R	-	rif^R str^S
		JMP228S	-	rif^S str^R
		DB01R	-	rif^R
JMP228R/pJP4	JMP228/pJP4	JMP228R	++	rif^R str^S
JMP228S/pJP4	JMP228/pJP4	JMP228S	++	rif^S str^R
JMP228R/pEMT1	indigenous	JMP228R	++	rif^R str^S
JMP228S/pEMT1	JMP228R pEMT1	JMP228S	++	rif^S str^R
JMP228R/pEMT3	indigenous	JMP228R	+	rif^R str^S
JMP228S/pEMT3	JMP228R pEMT3	JMP228S	+	rif^S str^R
DB01R/pJP4	JMP228/pJP4	DB01R	++	rif^R

[a] A minus indicates that no growth with 2,4-D occurred, a plus indicates slow growth, two plusses indicate fast growth..

[b] The antibiotics rifampicin (rif) and streptomycin (str) were used at a final concentration of 250 μg ml^{-1}

Plasmid pJP4 was introduced into *B. cepacia* DB01R as described for *R. eutropha* JMP228. The presence of the plasmid in the transconjugants was verified by isolation of plasmid DNA using the QIAGEN midi-prep column procedure as described in the manual (QIAGEN, Chatsworth, CA). The plasmid was restricted by combining 0.5 μg of plasmid with 3 μl buffer REact 3, 1μl of enzyme Bgl II and water to a total volume of 25 μl (Gibco, Grand Island, NY), and incubated at 37 °C for 3 hours. Digestion products were separated and viewed on a

3% agarose gel in 1X TAE buffer stained with ethidium bromide. (fig. 2.1). For storage, the transconjugant and recipient cultures were mixed with sterile 60% glycerol to a final glycerol concentration of 15%, and frozen in Corning cryovials (Fisher, Pittsburgh, PA).

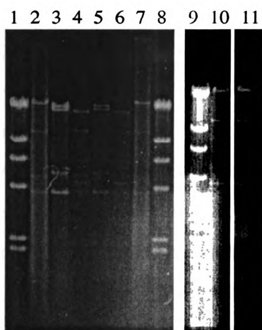


Figure 2.1. ***Bgl*** **II restriction analysis of plasmids isolated from transconjugant strains.** Lane 1: *Hind* III lambda marker, Lane 2: JMP228R/pJP4, Lane 3: JMP228R/pEMT3 (not used in this study), Lane 4: JMP228R/pEMT1, Lane 5: JMP228S/pEMT3 (not used in this study), Lane 6: JMP228S/pEMT1, Lane 7: JMP228S/pJP4, Lanes 8 and 9: Hind III lambda marker, Lane 10: JMP228R/pJP4, Lane 11: DB01R/pJP4.

Cultivation of cells. Cells were grown in a mineral salts medium supplemented with carbon sources and antibiotics. Per liter, the A+N minimal medium consisted of 1.71 g $K_2HPO_4 \cdot 3H_2O$, 0.34 g $NaH_2PO_4 \cdot H_2O$, 0.33 g $(NH_4)_2SO_4$, 0.25 g $MgSO_4 \cdot 7H_2O$, and 10 ml of a trace metals solution. The trace metal solution contained, per liter: 12.0 g $Na_2EDTA_4 \cdot 2H_2O$, 2.0 g NaOH, 0.4 g $ZnSO_4 \cdot 7H_2O$, 0.4 g $MnSO_4 \cdot 4H_2O$, 0.1 g $CuSO_4 \cdot 5H_2O$, 3.0 g $FeSO_4 \cdot 7H_2O$, 5.2 g Na_2SO_4,0.1 g $NaMoO_4 \cdot 2H_2O$. (Wyndham et al. 1986). The pH was adjusted with 10 N H_2SO_4 to 7.2 prior to autoclaving. Agar (1.7%, difco, Detroit, MI) or sodium succinate (5 mM) was added prior to autoclaving. The substrate 2,4-D and the selective antibiotics, rifampicin and streptomycin were added after autoclaving and cooling the medium to 55 °C from filter-sterilized (0.22 μm) stock solutions. The 2,4-D stock solution consisted of 20 mg ml^{-1} 2,4-D dissolved in 0.1 M NaH_2PO_4 (pH 7.2), the rifampicin stock was 25 mg ml^{-1} in 100% methanol, and the streptomycin stock was 25 mg ml^{-1} in water. Stock solutions were added to final concentrations of 200 μg ml^{-1} (2,4-D) and 250 μg ml^{-1} rifampicin or streptomycin. LB agar solid media consisted (per liter) of 10 g yeast extract (Difco, Detroit, MI), 5 g tryptone (Difco), 5 g NaCl (Baker, VWR, Plainsfield, NJ) and 17 g agar. For each independent experiment, cultures were started from a loopful of frozen culture stock. The cell cultures were grown to late log phase in 16 ml test

grown to late log phase in 16 ml test tubes with 3 ml A+N minimal media with 2,4-D (200 μg ml^{-1}) and the appropriate antibiotic. A 2.5 % inoculum to A+N medium supplied with succinate (5mM) or 2,4-D (200 μg ml^{-1}) as the sole source of carbon and energy, was used to start the cultures for growth and competition experiments.

Growth rate with 2,4-D. The growth rates of the four strains were determined with 2,4-D (200 μg ml^{-1}) as the carbon source using two methods. In a more traditional method, three replicate cultures containing 100 ml A+N minimal medium in 500 ml Erlenmeyer flasks were inoculated with a 1% inoculum, and grown at 30 °C on a rotary shaking incubator (200 rpm). During growth with 2,4-D, samples were periodically removed to determine optical density, and the concentration of 2,4-D. In addition, the viable population size was determined by plating 100 μl of culture fluid onto A+N minimal medium agar plates. The optical density of the growing cultures were monitored spectrophotometrically with a Hewlett Packard 8452A diode array spectrophotometer (Hewlett Packard , Waldbronn, Germany) at 600 nm. The maximum growth rates were determined by regression analysis of the linear portion of the logarithmic growth phase. A one-tailed t-test was conducted with the calculated growth rates of replicate samples using Excel 5.0 (Microsoft, Seattle, WA).

A second method for measuring growth was used. The cultures were grown in Falcon 96-well microtiter plates (Fisher, Pittsburgh, PA), and

optical density measurements were made with a BIO-TEC Microtiter EL312e microplate reader (BIO-TEK instruments Inc., Wenooski, VT). A 1 % inoculum from cultures grown in A+N minimal medium with succinate (5 mM) was transferred into 25 ml of A+N minimal medium with succinate (5 mM) in a 125 ml Erlenmeyer flask. The cells were grown to stationary phase (24 h, 30 °C, 200 rpm in a shaking rotary incubator) in order to exhaust the resource, and to become nutrient limited. . A 1 ml aliquot of the cell culture was spun down gently (4,000 rpm, 20 min in a microcentrifuge), and suspended in sterile A+N medium without a carbon source to an OD of 0.5 at 600 nm. Aliquots (20 µl) of this cell suspension were then added to wells of a 96-well microtiter plate, that contained 230 µl A+N medium per well. The medium was supplemented with either 2,4-D (100 or 200 µg ml^{-1}) or succinate (5 mM). The Kcalc program (BIO-TEK instruments Inc., Wenooski, VT) was used to program the microtiter reader. Microtiter plates were incubated at the ambient temperature inside the reader (~25°C). Every hour for 60 hours the microtiter plate was shaken at high speed for 45 seconds, and the optical density was determined spectrophotometrically at 590 nm. Microtiter plates were covered and parafilm sealed during the course of the growth experiment. No evaporation of media was observed for the inoculated wells. The data was saved to a computer disk and analyzed using Microsoft Excel 5.0. Comparison of the growth rates was carried out using a one-tailed t-Test.

High Performance Liquid Chromatography (HPLC). To measure the 2,4-D concentration by HPLC, 1 ml samples from growing cultures were centrifuged at 4 °C for 15 minutes at 13,000 rpm in an eppendorf microcentrifuge (Brinkmann, Westbury, NY) to remove bacterial cells. The supernatant was filtered through a 13 mm minispike 0.45 μm Acrodisc® filter (Fisher, Pittsburgh, PA), and the first 0.5 ml were discarded. One filter was used for 5 consecutive samples so that by discarding the first 0.5 ml, 2,4-D remaining from the previous sample was washed out of the filter unit. The remaining 0.5 ml was transferred an HPLC vial, crimp sealed and frozen until all the samples could be run consecutively using an autosampler. Prior to analysis, all samples were acidified by adding 10 μl of 10% phosphoric acid . The protonated and deprotonated forms of 2,4-D have slightly different retention times, and acidification to below the pKa of 2,4-D ($pKa_{2,4\text{-}D}$ = 2.34) resulted in a single peak. Samples were analyzed with a Hewlett Packard Series 1050 HPLC equipped with a 250 mm x 4.6 mm Lichrosorb RP-18, 10 μm particle size, chromatography column (Alltech, Bannockburn, IL), and a UV detector which was set to 260 nm. A mixture of 70% methanol/30% phosphoric acid (0.1%) (vol/vol) was used as the eluent at a flow rate of 1.5 ml per minute. In order to quantify the concentrations of 2,4-D in the samples, the resulting peak areas were compared with those of a standard curve generated in triplicate, with 5, 10, 25, 50, 100 and 250 μg ml^{-1} 2,4-D. A linear relationship was observed

through the entire concentration range that was examined, and concentrations as low as 2.5 μg ml^{-1} could be detected.

Batch Culture Competitions. All cultures were grown in succinate (5mM) media as described above, rinsed with sterile media without a carbon source, then resuspended to OD_{600} = 0.1 in minimal media without 2,4-D. Competition experiments were carried out using equal numbers of competitor cells (4-6 x 10^7 CFU ml^{-1}). Bacterial growth was monitored by measuring the optical density at 600 nm. Viable cell counts on A+N agar medium with 2,4-D (250 μg ml^{-1}) were performed in quadruplicate. Equal volumes (500 μl each) of the rinsed and resuspended JMP228S/pJP4 and JMP228R/pEMT1 cells were combined in two Eppendorf tubes and mixed to yield similar starting population densities. As reciprocal pairs, two tubes of JMP228R/pJP4 and JMP228S/pEMT1 mixtures were also prepared. In six replicate wells, 20 μl of each competition mixture was added to 1080 μl of A+N amended with 2,4-D (200 μg ml^{-1}), in the alternating wells of Falcon, flat-bottom, 24-well tissue culture plates (Becton Dickinson, Lincoln Park, NJ) to avoid cross contamination between adjacent wells. Plates were sealed with parafilm and incubated on a rotary shaker at 30 °C. For 5 days, the CFU ml^{-1} was determined daily by dilution and plating to A+N minimal media agar with 2,4-D (250 μg ml^{-1}) and the selective antibiotic to distinguish between competing populations. 10% of the mixed culture volume was transferred to fresh medium in a new, sterile microtiter

culture volume was transferred to fresh medium in a new, sterile microtiter plate to continue the competition. Analysis of the relative fitness of the competing populations was determined by plotting the ratios of the natural logarithms of the population sizes as described by Lenski et al. (1992). A deviation in the slope of this line away from zero, indicated that one strain is competitively dominant to another. Competitive pairs consisted of combinations of strains with different antibiotic susceptibilities, and carrying different plasmids. Intra-species competitions relied on the antibiotic resistance markers to identify the competing populations. In the inter-specific competition experiments between *R. eutropha* and *B. cepacia*, microtiter plates were inoculated in the same manner as the inter-species competitions described above. Four replicate competitions were carried out for each of the two pairs: JMP228R/pJP4 with DB01R/pJP4 and JMP228R/pEMT1 with DB01R/pJP4. Differences in the colony morphologies were used to distinguish between the *R. eutropha* and *B. cepacia* populations.

Chemostat growth. Chemostats modified from the design of Chao et al. (1977) were constructed and used to compare the growth characteristics of strains JMP228S/pJP4, JMP228R/pEMT1 and JMP228S/pEMT1, at low dilution rates. A schematic representation of the chemostat is shown in Figure 2.2. The starter cultures were initiated in the same manner as for the

attained mid to late log phase, which correlated with an optical density of ~ 0.1 at 600 nm (2-3 x 10^7 CFU ml^{-1}), the cells were harvested (4000 rpm, 20 minutes in a Sorvall SS34 rotor). After rinsing with A+N minimal media, the pellets were suspended to a final OD_{600} of 0.2 in A+N with 200 μg ml^{-1} 2,4-D. Either 75 ml of the pure culture or 37.5 ml of each competitor was placed in a chemostat and 2,4-D was added from a 20 mg/ml stock solution to a final concentration of 200 μg ml^{-1}. Samples (3 ml) were taken from each chemostat at the onset of the experiment. The chemostats were incubated for 8 hours at 30° C, before constant flow conditions were initiated. The dilution rate was set to 1.25 volume exchanges per day (2.5 ml h^{-1}), with a reservoir 2,4-D concentration of 200 μg ml^{-1}. The chemostats were operated for 120 h. Since the dilution rate (d) of a chemostat determines the growth rate (μ) of the populations, if d= 2.5 ml h^{-1} for a 75 ml volume then μ = 0.033 h^{-1}, which correlates to a 17.5 h doubling time. This growth rate was well below the maximum growth rate for the strains under batch culture conditions. To sample the chemostats, the port was swabbed with 70% ethanol. Using a 3 ml Hamilton syringe fitted with a sterile spinal tap needle (4 mm) (Fisher, Pittsburgh, PA), a 3 ml sample was removed. The sample was used for obtaining measurements of the 2,4-D concentration, optical density and the CFU ml^{-1} of each competing strain on A+N agar with 200 μg ml-1 2,4-D and the appropriate selective antibiotics. Triplicate plates were established for each time point to

determine the average population size. As with the Batch culture competitions, the change in the ln ratio for competitive fitness were determined. The slopes for the lines are reported from the time following nutrient depletion in the chemostats.

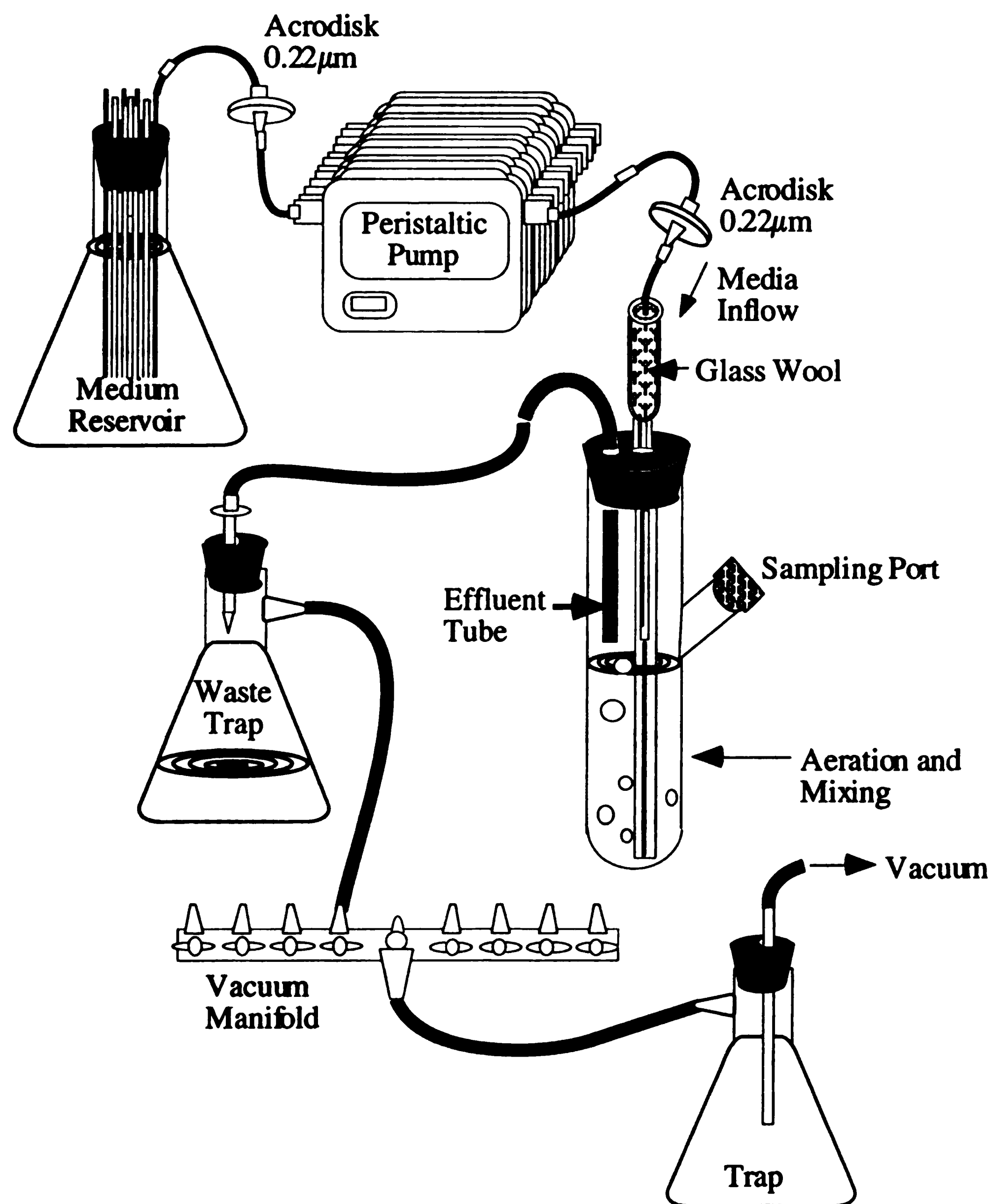


Figure 2.2. Schematic representation of the chemostat used in the competition experiments under low nutrient conditions. All connections were made with luer-lock fittings. A glass wool plug surrounding the media inflow tube filtered the air, which was drawn into the chemostat using vacuum. Vacuum was also used for drawing media into the culture vessel, and for aeration and mixing.

RESULTS

Growth of pure cultures under batch conditions. Plasmids pJP4 and pEMT1 benefit their host *Ralstonia eutropha* by supplying a catabolic pathway that can convert the herbicide 2,4-D to metabolic intermediates which then enter the TCA cycle. Both plasmids, pJP4 and pEMT1, were conjugally transferred into two isogenic host strains, *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) JMP228R and strain JMP228S as described in Table 2.4. The growth curves determined by optical density measurements and 2,4-D depletion curves of the constructed strains are shown in figure 2.3. The inset panel indicates the log-linear (exponential) portion of the growth curve used to calculate the growth rate.

The growth rates for *R. eutropha* strains carrying pJP4 and pEMT1 did not differ significantly when succinate served as the source of carbon and energy. The data for the batch cultures grown in Erlenmeryer flask and/or the microtiter plate grown cells were comparable, and the growth rates for the strains are compared in Table 2.5. When grown with succinate (5 mM) as a limiting resource, both strains had μ_{max} of 0.54 to 0.60 h^{-1} (n=6, P =0.08). Neither of the *R. eutropha strains* appeared to be significantly different. Furthermore, for *R. eutropha* growing on 5 mM succinate, chromosomal antibiotic resistance had no effect on the growth

rate (n =6, P = 0.96.) (Table 2.5). Growth rates of the strains were also determined on 200 μg ml^{-1} 2,4-D as the carbon source to determine if there was a difference between the plasmids (table 2.5). Plasmids pJP4 and pEMT1 both conferred a relatively fast growth rate of 0.23 (+/- 0.04) h^{-1} upon strains JMP228R/pJP4, JMP228S/pJP4, JMP228R/pEMT1 and JMP228S/pEMT1 when 2,4-D was supplied as a limiting substrate. Under batch culture conditions, both pJP4 and pEMT1 harboring *R. eutropha* strains maintained equivalent population densities of approximately 4 X 10^8 CFU ml^{-1} (Table 2.6) .

The Microtiter plate experiments showed that the growth curves obtained on succinate and on 2,4-D (100 or 200 μg ml^{-1}) were alike for JMP228S/pJP4, JMP228R/pJP4, JMP228S/pEMT1 and JMP228R/pEMT1. Replicate microtiter plate results were comparable, and while initial rates were the same for both 100 and 200 ppm 2,4-D, the growth rates decreased more rapidly in the 100 ppm 2,4-D samples as the substrate was consumed. The comparison with flask experiments use rates obtained with 200-250 μg ml^{-1} 2,4-D to calculate the values in Table 2.5, since a longer growth phase was observed. There was no significant difference in the growth rates of JMP228R/pJP4 and JMP228S/pEMT1 (n=12, p=0.96) as indicated by a one-tailed t-test.

Table 2.5. Growth rates of parent strains and transconjugants on succinate and 2,4-D. Growth rates (h^{-1}) ± standard deviation. Number of replicates is shown in parentheses.

Strain	Carbon and energy source [a]			
	Succinate		2,4-D	
	Flask (h^{-1})	Microtiter (h^{-1})	Flask (h^{-1})	Microtiter (h^{-1})
JMP228R	0.47 ± 0.012 (3)	0.57 ± 0.067 (6)	No growth	No growth
JMP228S	0.42 ± 0.003 (3)	0.54 ± 0.018 (6)	No growth	No growth
JMP228R/pJP4	0.51 ± 0.048 (4)	0.58 ± 0.030 (6)	0.20 ± 0.009 (3)	0.24 ± 0.048 (6)
JMP228S/pJP4	0.48 ± 0.007 (3)	0.56 ± 0.037 (6)	0.24 ± 0.004 (3)	0.22 ± 0.026 (6)
JMP228R/pEMT1	0.50 ± 0.034 (4)	0.60 ± 0.037 (6)	0.23 ± 0.011 (3)	0.24 ± 0.059 (6)
JMP228S/pEMT1	0.50 ± 0.007 (3)	0.55 ± 0.052 (6)	0.21 ± 0.004 (3)	0.23 ± 0.043 (6)
DB01R/pJP4	0.47 ± 0.036 (4)	0.41 ± 0.048 (6)	0.15 ± 0.012 (3)	0.20± 0.052 (6)

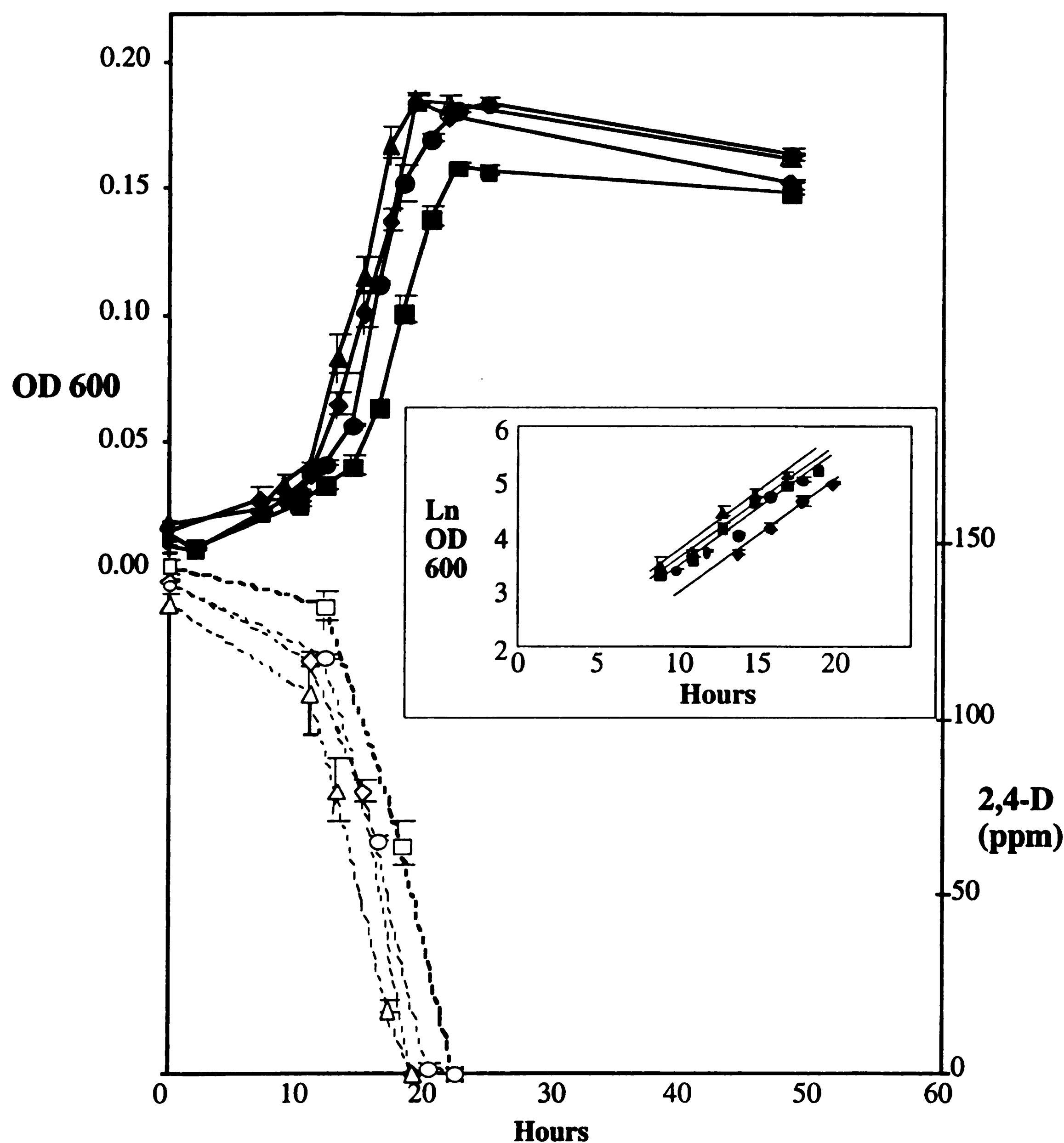


Figure 2.3. Growth of pure cultures carrying plasmid pJP4 or pEMT1 on 2,4-D. JMP228R/pJP4 (squares), JMP228S/pJP4 (diamonds), JMP228R/pEMT1 (triangles) and JMP228S/pEMT1 (circles) on 2,4-D minimal media (solid symbols). 2,4-D depletion is shown in open symbols. Each point is the average of measurements of three replicate cultures. Error bars represent one standard deviation around the average. The inset panel shows the natural log of the population size over time. From top to bottom the lines are JMP228R/pEMT1, JMP228S/pJP4, JMP228S/pEMT1 and JMP228R/pJP4.

Colony morphology on agar plates. *R. eutropha* JMP228 carrying plasmids pJP4 or pEMT1 had the same colony morphology on both R2A agar plates, and on 2,4-D minimal media plates. On R2A agar, colonies were round, of uniform size and tan in color. The translucent colonies had smooth edges and appeared after an incubation period of 48 hours at 20 °C. On 2,4-D minimal media plates, the colonies maintained the round, smooth, morphology, and translucent appearance. Colonies could be observed after an incubation period of 72 hours, but were much easier to count after 96 hours.. The *R. eutropha* strains could readily be distinguished from the *B. cepacia* strain on R2A agar plates. *B. cepacia* DB01r/pJP4 was characterized by opaque cream colored colonies, with irregular size and rough edges. Furthermore, the *B. cepacia* strain did not form colonies before 60 hours of incubation. The *B. cepacia* strains also formed colonies on 2,4-D agar plates after incubation for 72 hours, however, visual distinction from *R. eutropha* was difficult. Based on these results, plate counts were carried out after 72 hours (3 days) with the R2A agar plates, and after 120 hours (5 days) with the 2,4-D minimal medium plates.

Batch competition experiments. *R. eutropha* JMP228 carrying plasmids pJP4 and pEMT1 (JMP228R/pJP4 and JMP228S/pEMT1, JMP228S/pJP4 and JMP228R/pEMT1) coexisted at a constant ratio in intra-specific batch culture competitions. Panel A in Figure 2.4 indicates the

average population sizes for the competing strains. No effect was observed for the strains carrying different antibiotic markers ($n = 12$, $P = 0.21$). In 24 independent, replicated batch culture competition experiments between JMP228R/pJP4 and JMP228S/pEMT1 or the reciprocal set, both strains coexisted at a relatively constant ratio. The slope of the line generated by the ratios of the natural logarithms of the population sizes did not differ significantly from zero, as indicated by the very low r^2 values (average slope = -0.1025, $r^2 = 0.1794$) (Figure 2.4 B). When the natural log of the rifampicin resistant to streptomycin resistant strains was compared, the slope of the line also did not deviate significantly from zero (slope = 0.0108, $r^2 = 0.11942$). Similar results were obtained in competition experiments with JMP228S/pJP4 versus JMP228R/pEMT1 (data not shown).

In another set of experiments, equal numbers of JMP228R/pJP4 orJMP228R/pEMT1 were competed against DB01R/pJP4. In four replicate competition flasks, the *R. eutropha* strains became dominant over the *B. cepacia* strain when 200 μg ml^{-1} 2,4-D was supplied as the limiting substrate. Furthermore, both JMP228R/pJP4 and JMP228R/pEMT1 followed the same pattern of dominance over DB01R (Fig. 2.5 A). The average slope of the line positively deviated from zero, indicating dominance by *R. eutropha.* JMP228R/pEMT1 had an average slope of 2.809 and expressed an r^2=0.9680, whereas JMP228R/pJP4 had an average slope of 2.9637 and an r^2=0.9842). Comparing the slopes of the lines generated by

the two *R. eutropha* strains indicated no significant differences in the ability of both strains to compete with *B. cepacia* (n=8, P=0.27). The plateau of the ratio of competing populations represents the point at which no more *B. cepacia* colonies could be detected on plates otherwise solely consisting of *R. eutropha* colony morphotypes (Fig. 2.5, panel B).

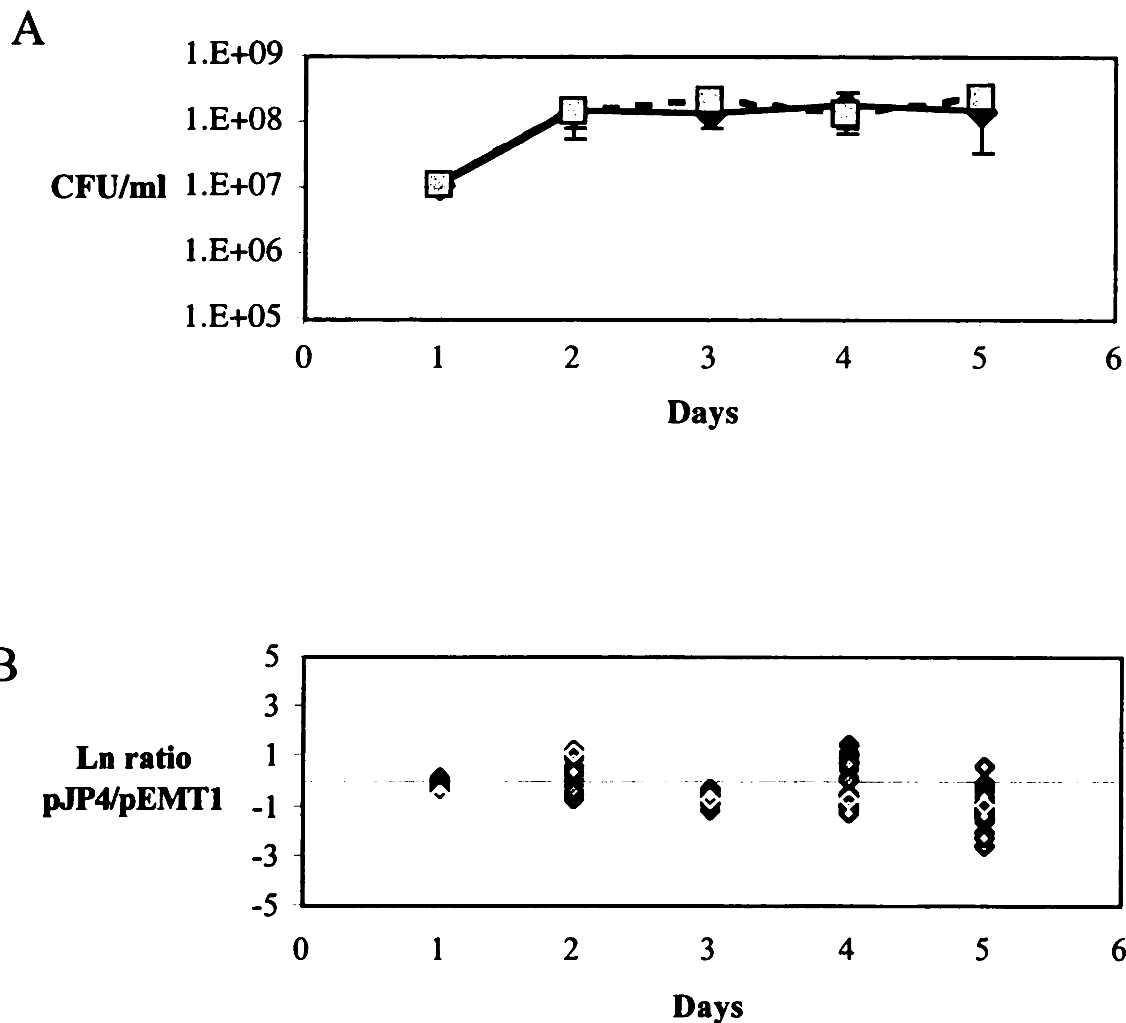


Figure 2.4. Competition among *R. eutropha* strains JMP228/pJP4, and JMP228/pEMT1 on 200 ppm 2,4-D. Panel A: Averages of the colony forming units of 24 replicate competition flasks. Diamonds are JMP228/pJP4 and squares are JMP228/pEMT1. Error bars are the standard deviation around the mean value for the population sizes of all competition flasks. Panel B: Natural logarithm of ratio of the pJP4 carrying JMMP228 to the pEMT1 carrying strains. Each of the 24 replicates is shown by diamonds. Diamonds of different shades of gray represent different experimental pairs.

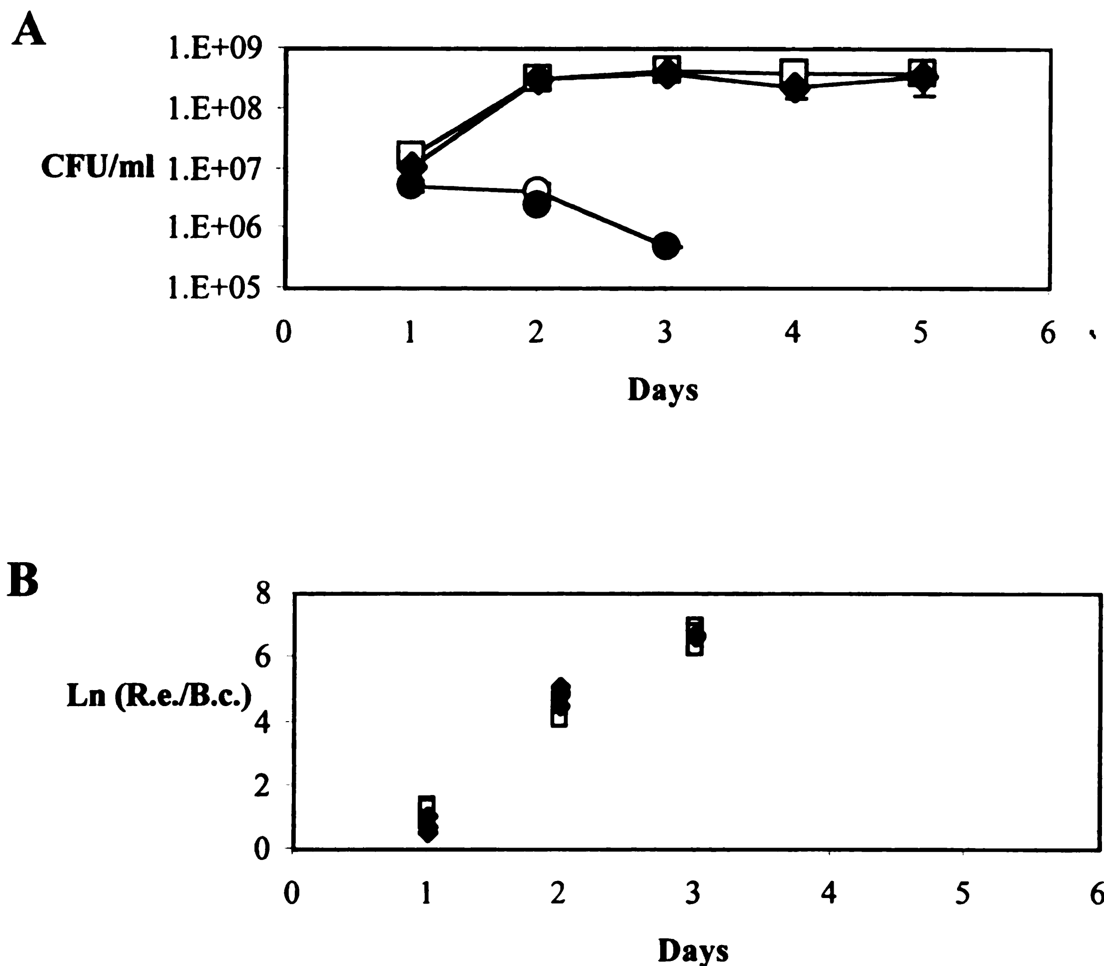


Figure 2.5. ***Ralstonia eutropha*** **hosting pJP4 or pEMT1 are equal competitors against** ***Burkholderia cepacia*** **hosting pJP4.** Batch culture competitions in minimal medium amended with 250 ppm 2,4-D were transferred to fresh media every 24 hours for five consecutive days.
A: Each point represents the average of four replicates. Error bars are the standard deviation. Filled symbols represent competition between JMP228R/pJP4 (diamonds) and DB01R/pJP4 (circles). Open symbols represent competition between JMP228R/pEMT1 (squares) and DB01R/pJP4 (circles). Panel B: The natural logarithm of the ratio of *R. eutropha* with pJP4 or pEMT1 to *B. cepacia* hosting plasmid pJP4. Filled diamonds are four separate competitions of JMP228R/pJP4 versus DB01R/pJP4. Open squares are JMP228R/pEMT1 versus DB01R/pJP4. DB01 populations could not be detected below 1% of the total population.

Chemostat growth and competition experiments. Both pure culture and mixed culture competition experiments were performed concurrently in chemostats. Strains JMP228S/pJP4 and JMP228R/pEMT1 attained cell densities of approximately 4-6 x 10^8 CFU ml^{-1}. Strain JMP228S/pEMT1 grew to 2-4 x 10^8 CFU ml^{-1} (Table 2.5). In all culture vessels, the concentration of 2,4-D was utilized to undetectable levels within the first 24 hours. These results suggested that the pJP4 and pEMT1 carrying *R. eutropha* populations appeared to behave similarly under mixed and pure culture growth conditions, and coexist in a chemostat operated at low dilution rates. In a single chemostat experiment, strains JMP228R/pEMT1 and JMP228S/pJP4 were competed. The two strains coexisted, and neither appeared to have a definitive fitness advantage during the course of 6 days (Figure 2.6).

Table 2.6. Carrying capacity of *R. eutropha* strains in batch and chemostat conditions

Strain	Carrying Capacity	
	Batch [a]	Chemostat [b]
JMP228R/pJP4	3.8 x 10^8 ± 2.3 x 10^7	nd
JMP228S/pJP4	3.7 x 10^8 ± 5.3 x 10^7	6 x 10^8 ± 1 x 10^8
JMP228R/pEMT1	4.6 x 10^8 ± 6.1 x 10^7	6 x 10^8 ± 1 x 10^8
JMP228S/pEMT1	4.3 x 10^8 ± 1.2 x 10^8	3 x 10^8 ± 3 x 10^8

[a] Pure culture growth on 200 ppm 2,4-D

[b] Pure culture growth with 200 ppm 2,4-D in the reservoir, and a 0.33 h^{-1} dilution rate. 2,4-D concentration in the chemostats was at undetectable concentrations. Each experiment was run in triplicate..

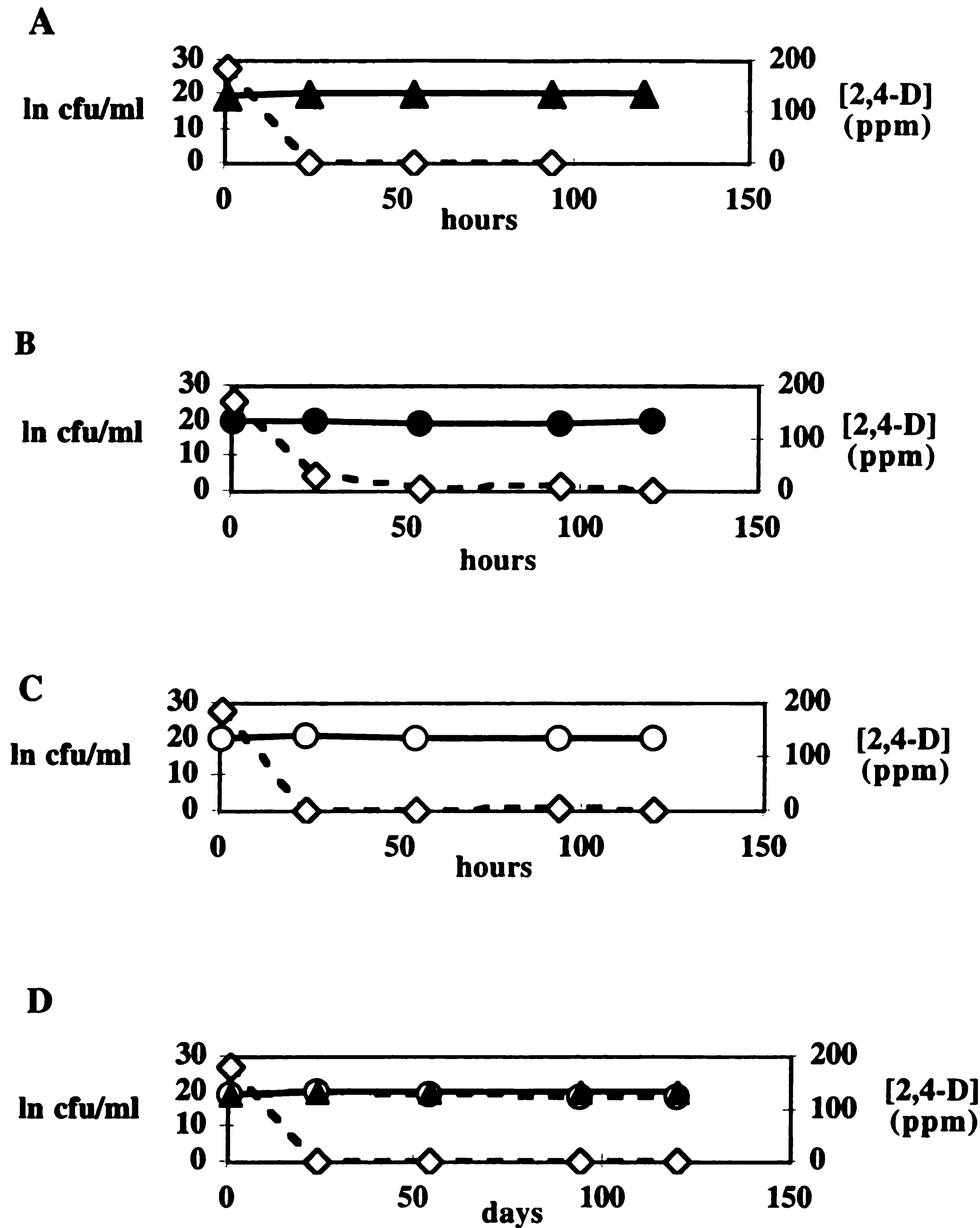


Figure 2.6. ***R. eutropha*** **with pJP4 or pEMT1 in pure culture or in competition in chemostats.** Panels A–C: pure culture experiments. (A) JMP228S/pJP4, (B) JMP228R/pEMT1, (C) JMP228S/pEMT1. Panel D: competition experiment. JMP228S/pJP4 (diamond) and JMP228R/pEMT1 (squares). 2,4-D concentration in chemostats is shown by the open diamonds. Chemostats were run at a dilution rate of 0.033 h-1, and each time point represents the mean of three replicate samples. Standard deviations fall within the presented symbols.

DISCUSSION

Many bacteria isolated from soils degrade 2,4-D, often by means of catabolic genes carried on plasmids. The effect of different plasmids carrying homologous degradative pathways with very similar genes for 2,4-D utilization on competitive fitness was examined. The plasmids were readily maintained within sub-populations of a single host strain. The goal was to determine if two isogenic populations behaved in a similar manner when 2,4-D was supplied as the sole source of carbon and energy. To determine if the growth rates of an *R. eutrophus* host carrying different 2,4-D degradative plasmids (pJP4 and pEMT1) influenced competitive outcome, strains JMP228R/pJP4, JMP228S/pJP4, JMP228R/pEMT1 and JMP228S/pEMT1 were competed in batch competition. When the relative fitness of the strains were determined in head to head competition experiments, coexistence was observed. The ratio of the two competing populations remained constant. In this instance, it appears that the two populations are nearly equally fit to compete for 2,4-D, and therefore the different plasmid backbones of pEMT1 and pJP4 did not significantly influence the individual strains fitness under the experimental conditions chosen.

Both pJP4 and pEMT1 containing cultures exhibited similar growth rates on succinate, a chromosomally encoded pathway. The similarity of growth rates on succinate indicated that the downstream portion of the 2,4-D degradative pathway was comparable in both strains. Plasmids pJP4 and pEMT1 benefited their host strain by supplying a catabolic pathway which converts the herbicide 2,4-D to metabolic intermediates, including succinate, which then enter the TCA cycle. Therefore, following degradation to 2-chloromaleylacetate, the genes needed to further metabolize the intermediates are encoded by the host (Kukor et al., 1989). Since the chromosomally-encoded genes for the degradation of 2-chloromaleylacetate are the same for the two *R. eutropha* strains, any differences in fitness when grown on 2,4-D, could be presumed to be due to the plasmid itself, or the genes carried on the plasmid. Therefore, the similarities in both the growth characteristics and competitive ability of strains JMP228R/pJP4, JMP228S/pJP4, JMP228R/pEMT1 and JMP228S/pEMT1, were due to physiologically comparable genes for degrading 2,4-D, carried in relatively neutral plasmid backbones.

Although plasmids pJP4 and pEMT1 belong to different incompatibility groups, they have a similar impact on the growth of their host when competing for 2,4-D. Under the described experimental conditions, no selective advantage was observed when the two populations were grown on 2,4-D. The genes for 2,4-D utilization can be transferred

horizontally on two very different plasmid backbones, and confer comparable physiological capabilities (2,4-D degradation) to the same host species. In nature, however, there may be differences in the survival in these strains due to more complex environmental conditions and different host responses. The survival of the plasmids themselves in the environment and the probability that they transfer to other populations in soil or water ecosystems may be due to the host range and promiscuity of the plasmid.

REFERENCES

Bollmer, M.D., K. Stadler-Fritzche, and M. Schlömann. 1993. Conversion of 2-cloromaleylacetate in *Alcaligenes eutrophus* JMP134. Arch. Microbiol. 159:182-188

Bouma, J.E. and R.E. Lenski. 1988. Evolution of a bacteria/plasmid association. Nature. 335:351-352.

Chao, L., B.R. Levin and F.M. Stewart (1977). Complex community in a simple habitat: Experimental study with bacteria and phage. Ecology. 58:369-378.

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

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

Don, R. H., A.J. Weightman, H.J. Knackmuss and K. N. Timmis. 1985. Transposon mutagenesis and cloning analysis of the pathways for degradation of 2,-4dichlorphenoxyacetic acid and 3-chlorobenzpate in Alcaligenes eutrophus JMP134 (pJP4). J. Bacteriol. 161:85-90.

DiGiovanni G.D., J.W. Neilson, I.L. Pepper and N.A. Sinclair. 1996. Gene transfer of Alcaligenes eutrophus JMP134 plasmid pJP4 to indigenous soil recipients. Appl. Enviorn. Microbiol. 62:2521-2526

Dunbar, J.M. 1996. Effect of selection on the structure of a 2,4-dichlorophenoxyacetic acid (2,4-D) degrading guild in soil. Ph.D dissertation. Michigan State University, Department of Microbiology.

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

Fulthorpe, R.R., C. Mcgowan, O.V. Maltseva, W.E. Holben, and J.M. Tiedje. 1995 2,4-Dichlorophenoxyacetic acid-degrading bacteria contain mosaics of catabolic genes. Appl. Environ. Microbiol. 61:3274-3281

Holben, W.E., B. M. Schroeter, B.G. Calabrese, R.H. Olsen, J.K. Kukor, B.O. Biederbeck, A.E. Smith and J.M. Tiedje. 1992. Gene probe analysis of soil microbial populations selected by amendment with 2,4-dichlorophenoxyacetic acid. . Appl. Eviron. Microbiol. 58:3941-3948.

Kukor, J.J, R.H. Olsen and J.S. Siak. 1989. Recruitment of a chromosomally encoded maleylacetate reductase for degradation of 2,4-dichlorophenoxyacetic acid by plasmid pJP4. J. Bacteriol. 171:3385-3390.

Lenski, 1992, Relative Fitness: its estimation and its significance for environmental applications of microorganisms in Microbial Ecology (eds. M. A. Levin, Seideler R.J. and Rogul, M.) McGraw-Hill, Inc. pp183-198

Levin, B. 1972. Coexistence of two asexual strains on a single resource. Science. 175:1272-1274

Mäe A.A., O. M. Reet, N.R., Ausmees, V.M.Koiv and A.L. Heinaru. 1993. Characterization of a new 2,4-dichlrophenoxyacetic acid degrading plasmid pEMT4011: physical map and localization of catabolic genes. J. Gen. Microbiol. 139:3165-3170.

Murray, R.E., L.L. Parsons and M. S. Smith. 1992, Competition between two isolates of denitrifying bacteria added to soil. Applied and Environmental Microbiology. 58:3890-3895.

Neilson, J.W., K.L. Josephson, I.L. Pepper, R.B. Arlnold G.D. DiGiovanni and N.A. Sinclair. 1994. Frequency of horizontal gene tranfer of a large catabolic plasmid (pJP4) in soil. Appl. Environ. Microbiol. 60;4053-4058.

Rutter, M. and D.B. Nedwell, 1994. Influence of changing temperature on growth rate and competition between two psychrotolerant antarctic bacteria: competition and survival in non-steady state temperature environments

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Second Edition. Cold Spring Harbor Laboratory Press. USA.

Top, E.M., P. Van Daele, N. De Saeyer, and L.J. Forney. 1998. Enhancement of 2,4-dichlorophenoxyacetic acid (2,4-D) degradation in soil by dissemination of catabolic plasmids. Antonie Van Leeuwenhoek. 73:87-94.

Top E.M., W.E. Holben and L.J. Forney. 1995. Characterization of diverse 2,4-Dichlorophenoxyacetic acid-degrading plasmids isolated from soil by complementation. Appl. Environ. Microbiol. 61:3274-3281

Wilson, M. and S.E. Lindow. 1994. Ecological similarity and coexistence of epiphytic ice-nucleating (Ice+) *Pseudomonas syringe* strains and a non-ice nucleating (Ice-) biological control agent. Appl. Environ. Microbiol. 60:3128-3137.

CHAPTER 3

RALSTONIA EUTROPHA AND *BURKHOLDERIA CEPACIA* HOST POPULATIONS AS RESERVOIRS FOR 2,4-DICHLOROPHENOYACETIC ACID DEGRADATIVE PLASMIDS

SUMMARY

Ralstonia eutropha and *Burkholderia cepacia* are two of the many naturally occurring bacterial populations that utilize 2,4-dichlorophenoxyacetic acid (2,4-D) when they possess catabolic genes for the upper degradation pathway for this aromatic compound. The work presented in this chapter explores characteristics that were obtained from pure culture experiments with *R. eutropha* JMP228 and *B. cepacia* DB01 as well as some of the previously documented characteristics of the two strains. The ultimate goal of these experiments was to determine which host-plasmid combinations offer the fittest phenotype when competing for 2,4-D as a limiting resource. Before performing the competition experiments, growth rates and K_S values were determined for pure cultures of each host-plasmid combination. Experimental results were compared with models generated for substrate limited bacterial growth, and will be used to predict the outcome of competition experiments performed in chapter 4.

R. eutropha and *B. cepacia* are representative of their genera based upon BIOLOG profiles. Different substrate utilization profiles were obtained for the two species on BIOLOG GN plates, with *B. cepacia* utilizing a broader range of substrates than *R. eutropha*. Both strains were able to reduce Tween 40 and Tween 80, fructose, two esters (methyl pyruvate and mono-methyl succinate), 15 aliphatic and aromatic carboxylic acids, 12 amino acids, bromosuccinic acid, urocanic acid, inosine and phenyl ethylamine. Only two substrates were uniquely utilized by *R. eutropha:* succinamic acid and L-leucine. *B. cepacia* utilized 30 substrates that *R. eutropha* was unable to oxidize. These included 12 carbohydrates, three carboxylic acids, four amino acids, two amines, two alcohols and three phosphorylated sugars.

When two different plasmids (pJP4 and pEMT3) containing different cassettes for the degradation of 2,4-D were introduced into these two different hosts, the growth rates of the host-plasmid combinations differed. When 2,4-D served as the sole source of carbon and energy, carrying pJP4 was advantageous relative to hosting pEMT3. Pure culture growth rate experiments, using 2,4-D as the limiting resource, indicated that *R. eutropha* JMP228/pJP4 grew fastest (μ= 0.23 h^{-1}). *B. cepacia* DB01/pJP4 also had a relatively fast growth rate (μ= 0.15 h^{-1}), while *R. eutropha* JMP228/pEMT3 (μ= 0.09 h^{-1}), and *B. cepacia* DB01/pEMT3 (μ=0.08 h^{-1}) displayed the slowest growth. Within a host population,

neither plasmid impacted growth rate when the populations utilized the "neutral" carbon source, succinate. This compound was readily metabolized by either host strain, independent of the presence of the catabolic plasmid. *R. eutropha* grew slightly faster (μ= 0.49 h^{-1}) than *B. cepacia* (μ= 0.47 h^{-1}) when succinate served as the sole source of carbon and energy.

INTRODUCTION

The manner in which a population utilizes the resources and grows in its environment may predict which population will become dominant under different habitat conditions. This chapter is dedicated to the physiological and genetic features of *R. eutropha* and *B. cepacia,* the two host strains that will be used in upcoming competition experiments.

Ralstonia eutropha (formerly *Hydrogenomonas eutropha, Alcaligenes eutrophus*) (Davis et al., 1969; Yabuuchi et al. 1996) and *Burkholderia cepacia* (formerly *Pseudomonas cepacia*) (Palleroni, 1981; Yabuuci et al. 1992) were been identified as two naturally occurring bacterial populations that utilize 2,4-D when they possess genes encoding for the degradative pathway. Both bacterial species are members of the β-proteobacteria (Palleroni, 1992a). Some features are shared among the two strains. For instance, both genera are gram negative, non-spore forming rods and the cells tend to occur singly or in pairs, rather than forming long

chains or clumps. Cell aggregates are easily suspended in liquid media, and cultures grow without forming flocks when grown on a rotary shaker. Neither *Ralstonia* nor *Burkholderia* possess sheaths or prosthecae. Both populations are aerobic chemo-organotrophs, and are capable of growth on a wide variety of organic substrates. Neither grows fermentatively. Both are non-phototrophic and do not fix nitrogen. In times of substrate excess *B. cepacia* and *R. eutropha* are both capable of producing poly-β-hydroxybutyrate (PHB) as a storage compound (Palleroli, 1992a; Palleroni, 1992b).

Burkholderia species are metabolically very versatile. Consequently, these organisms can be found in many habitats. *B. cepacia* species are nutritionally diverse, and at least 102 of 136 substrates were utilized by 14 of 18 strains tested by Ballard et al. (1970). *B. cepacia* maintains a wide range of antibiotic resistances to penicillin-type antibiotics, chloramphenicol, aminoglycosides, and polymyxin, largely due to the impermeability of the cell wall and capsule (Burns et al. 1989). All *B. cepacia* strains are sensitive to trimethoprim. For marking the genome of *B. cepacia* for molecular and population studies, rifampicin has proved to be most successful, as the strains can readily be isolated, and resistance is stable. However, there appears to be a slight affect of rifampicin resistance on the competitive ability of bacterial strains bearing this marker (Lewis et al., 1987).

Genetic diversity is tremendous among B*urkholderia cepacia* strains, whose genomic complexity and plasticity are rivaled by few other genera. With the advent of Pulsed Field Gel Electrophoresis, the visualization of the large DNA molecules of the bacterial genome became possible, and sparked the discovery that the *B. cepacia* genome contains multiple chromosomes (Cheng et al., 1994; Hendrickson et al., 1996). On average, *B. cepacia* isolates contain two to four replicons with total genome sizes spanning the range of 5 to 9 Mb. *B. cepacia* strain DB01 (Keyser et al., 1976), exhibits three large replicons of 3.4, 2.4 and 1.3 Mb, with a total genome size of 7.1 MB. Each large replicon has at least one set of rrn operons. In strain DB01, a total of 6 rrn operons were found among its 3 chromosomes.

Mobile genetic elements are commonplace in *Burkholderia* strains, and together with the different chromosome numbers, genome sizes and arrangements, support the idea that lateral gene transfer and genetic rearrangement is a prominent feature of this genus. IS elements are common, and have been implicated as a force driving diversification of the genome and the ability to acquire novel catabolic functions, such as the ability to degrade the herbicide 2,4,5-T (Haugland et al., 1990; Daubaras et al., 1995) The presence of plasmids is also common among populations of *B. cepacia*. Some confer resistance to antibiotics and antibacterial agents. (Chakrabarty et al., 1976; Jacoby, 1986). Some may provide resistance to metals such as mercury, borate and terllurite. Other plasmids have been

shown to increase the catabolic range by providing pathways for the utilization of aromatic compounds such as toluene, benzene, napthalene and salicylate.

Based on rRNA similarity groups, Palleroni (1992b) categorized *B. cepacia* as a Group II Pseudomonad. As such, *B. cepacia* clusters with the β–subgroup of the Proteobacteria, and shares a common ancestry with *Alcaligenes* species. Both these genera are genetically distinct from the members of the γ-subclass, that includes the type species of the Pseudomonads, *Pseudomonas aeruginosa*. A comparison of the 16S rRNA of the *Ralstonia* and *Burkholderia* groups indicates that members of *Burkholderia* share 94% sequence similarity with each other, the genus *Ralstonia* shares from 93-95% similarity and the two groups are joined at a level of 88% sequence similarity (Yabuuchi et al., 1992). The members of the *B. cepacia* group exhibit greater than a 98% similarity in the 16S rRNA sequence (Tabacchioni et al., 1995).

Ralstonia eutropha, is a β-proteobacteria and shares many physiological characteristics with *B. cepacia*. *R. eutropha* is also ubiquitous in soil and freshwater environments, and has frequently been isolated from habitats with high levels of aromatic or heavy metal contaminants. These peritrichously flagelated bacteria function as facultative chemolithotrophs, capable of oxidizing hydrogen. They are oxidase positive, catalase positive, and are capable of anaerobic growth with nitrate as the electron acceptor.

R. eutropha utilizes more carbohydrates than the type strain of *Alcaligenes* from which it was reclassified in 1992. Among its distinguishing catabolic traits are that hydroxybenzoate is metabolized via the gentisate pathway, and protochatechuate via an ortho cleavage pathway (Davis et al., 1969). A variety of aromatic compounds can be utilized, and the regulation of the β-ketoadipate pathway was described by the classic work of Johnson and Stanier (1971). *R. eutropha* makes frequent use of large catabolic plasmids for increasing its metabolic potential. Those isolates obtained from sites contaminated with chlorinated aromatic compounds, such as herbicides, frequently possess large broad-host-range plasmids like pJP4 and pAC27, which carry the genes for degradation of 2,4-dichlorophenol and 4-chlorophenol.

While many of the typical characteristics of *R.eutropha* and *B. cepacia* are known from literature, this research focused on a few of the phenotypic traits that could be used to distinguish between the two population when they are used as hosts for two different catabolic plasmids, pJP4 and pEMT3 (Ka et al., 1992; Top et al. 1995). The 2,4-D degradative pathway encoded by plasmid pJP4 was discussed in Chapter 1. Another plasmid, pEMT3, also encoding the 2,4-D degradative pathway, was obtained by Top et al. (1994). The genetic and physiological characterization of this plasmid is shown in Table 2.1. Plasmid pEMT3 belongs to the Inc-P incompatibility group, like pJP4. Consequently,

pEMT3 carries a similar set of genes for its own transfer and replication. Although the gene encoding for the first enzyme of the 2,4-D degradative pathway (*tfd*A) is homologous among these two plasmids, pEMT3 carries a different set of 2,4-D gene alleles for the *tfd*CDFE operon and for the *tfd*B gene. This plasmid's phenotype is reflected in slower growth when 2,4-D is supplied as a sole source of carbon and energy as compared to pJP4.

MATERIALS AND METHODS

Antibiotic Resistance profiles. Before plasmids were introduced to the host strains to construct the different host-plasmid combinations, antibiotics were evaluated for their ability to serve as suitable selective agents for the plasmid-free populations *Ralstonia eutropha* JMP228 and *Burkholderia cepacia* DB01. Rifampicin (50, 100, and 150 μg ml^{-1}), streptomycin (100, 250, and 500 μg ml^{-1}), trimethoprim (20, 40, and 80 μg/ml), tetracycline (15, 20, and 25 μg ml^{-1}), chloramphenicol (25, 50, and 75 μg ml^{-1}), kanamycin (25, 50, and 75 μg ml^{-1}), naladixic acid (10, 15, and 20 μg ml^{-1}) and ampicillin (50 and 100 μg ml^{-1}) were tested at 22°C. *R. eutropha* and *B. cepacia* were inoculated from freezer stock into LB media (Sambrook et. al, 1990), and allowed to grow for 12 hours. A 5% (vol/vol) inoculum was then transferred to 50 ml fresh LB medium, and grown to late log phase as determined by absorption readings at 600 nm. Aliquots (100 μl) were directly plated onto LB plates containing each of the

antibiotics to determine the susceptibility and the frequency of appearance of resistant mutants. A sample was diluted and plated to LB media to determine the concentration of viable cells in the culture. The frequency of appearance of the resistant phenotype was calculated as the number of resistant colonies/ml divided by the total CFU ml^{-1}.

Bacterial strains. *Ralstonia eutropha* JMP228 (referred to as JMP228), the pJP4 cured strain of *R. eutropha* JMP134 (Don and Pemberton, 1981) and *Burkholderia cepacia* DB01 (referred to as DB01) (Ka and Holben 1994) were used as the recipient host strains for either of two 2,4-D degradative plasmids. Spontaneous mutants of JMP228, resistant to rifampicin or streptomycin were used to distinguish between plasmid bearing populations, and these strains were called JMP228R and JMP228S. The JMP228R was obtained from Top et. al.(1995) and the streptomycin resistant strain was a spontaneous resistant mutant generated for these competition experiments. JMP228S was obtained by growing a culture of JMP228 in minimal medium containing succinate (5 mM) to late log phase. A one ml aliquot of the cell suspension was rinsed with sterile phosphate buffered saline (PBS) and resuspended in 100 µl PBS. This suspension was serially diluted in ten-fold increments and 100 µl was spread over the surface of a minimal medium agar plate containing 2,4-D (200 $\mu g ml^{-1}$) and streptomycin (100, 150, 200 and 250 $\mu g ml^{-1}$). Three colonies which grew on the 10^{-2} dilution plate with 150 µg ml^{-1}

streptomycin were clonally purified on the same medium three times. All spontaneous mutants were equivalent in terms of growth rates and colony morphology. Loss of the streptomycin resistance marker in the absence of selection pressure was not observed. No difference was observed in the growth rates of cultures of JMP288S grown in the presence or the absence of 150 $\mu g\ ml^{-1}$ streptomycin. The number of colonies isolated on R2A agar containing or lacking streptomycin was equal for JMP228S with or without selective pressure. *B. cepacia* strain DB01, served as the other host strain Two antibiotic resistant strains were generated using rifampicin (100, 150, 200, 250 μgml^{-1}) and trimethoprim (2.5, 5 and 7.5 μgml^{-1}). Three colonies from the highest concentration were repeatedly transferred for purification. After verifying that there were no revertants, the resistant *Burkholderia* strains were designated DB01R and DB01T. In addition to the antibiotic resistance markers, *Ralstonia* and *Burkholderia* strains could be distinguished based on the colony morphology on R2A medium. The bacterial strains used in the experiments are listed in Table 3.1.

Table 3.1. Bacterial Strains used in the competition experiments

Species	Strain	Antibiotic resistance	Reference
Ralstonia eutropha	JMP228	rif^S str^S	Don and Pemberton 1981
Ralstonia eutropha	JMP228R	rif^R str^S	Top et al. 1994
Ralstonia eutropha	JMP228S	rif^S str^R	This study
Burkholderia cepacia	DB01	rif^S tri^S	Holben et al. 1993
Burkholderia cepacia	DB01R	rif^R tri^S	This study
Burkholderia cepacia	DB01T	rif^S tri^R	This study

Plasmids and transconjugant construction. Plasmid pJP4, isolated from *R. eutropha* JMP134, belongs to the Inc-P incompatibility group, and carries the most thoroughly characterized 2,4-D degradative pathway (Don 1985). Plasmid pEMT3 was isolated in a previous study as a result of an exogenous plasmid trapping experiment using strain JMP228R as a recipient (Top 1994). Plasmid pEMT3 is an Inc-P plasmid, and carries a *tfd*A gene which hybridizes under high stringency conditions to a gene probe generated from the internal fragment of the *tfd*A gene of plasmid pJP4. In contrast, using internal fragments of *tfd*B and *tfd*C genes of plasmid pJP4 as probes, successful hybridization to pEMT3 only occurs under intermediate and low stringency conditions, indicating differences in the sequences of these genes (Top 1995) (Table 3.2).

Table 3.2: Plasmids used in the competition experiments

	Hybridization [a]				
	tfd genes of pJP4			IncP replicon	
Plasmid	tfdA	tfdB	tfdC	rep R	Reference
pJP4	***	***	***	***	Don and Pemberton 1981
pEMT3	***	**	*	***	Top et al. 1994

[a] *** indicates hybridization under high stringency conditions and correlates to >90% sequence similarity. ** hybridizes under medium stringency and 75-90 % sequence similarity. * hybridizes under low stringency conditions and represents 60 - 75 % sequence similarity

Plasmids pJP4 and pEMT3 were transferred from JMP228R/pEMT3 or JMP228/pJP4 into strains *R. eutropha* JMP228R and JMP228S using filter mating. Donor cells ($1x10^7$) were combined with recipient cells ($2x10^6$) for 8 hours on a polycarbonate filter (Poretics 13 mm, 0.2 μm pore size) placed atop LB agar. The cells from the filter were resuspended in A+N minimal medium broth (composition below), without a carbon source. A dilution series was plated to medium containing antibiotics to prevent donor strain growth, and 2,4-D (200 ppm) as a sole source of carbon to select for transconjugants.

Plasmid pJP4 was introduced to *B. cepacia* DB01R and DB01T as described for the JMP228 populations. Plasmid pEMT3, however, had to be introduced to *B. cepacia* DB01R and DB01T by combining donor cells (1×10^7) and recipient cells (2×10^6) onto a single spot on R2A agar (Difco). After 12 hours of incubation, colony material was transferred with an inoculating loop to A+N minimal medium containing 2,4-D and antibiotics to counterselect the donor strain. The presence of the plasmid was verified by isolation of plasmid DNA using the QIAGEN midi-prep column (QIAGEN). Plasmids from donor strains and transconjugants were compared by RFLP analysis *Eco*-RI (Gibco) digest (Fig 3.1). Transconjugants are listed in table 3.3. All cultures were archived in selective media containing 15% glycerol, final concentration, at –80 °C.

Table 3.3: Transconjugants used in the competition experiments

Transconjugant	Donor	Recipient	2,4-D utilization[a]	Antibiotic resistance[b]
	JMP228/pJP4		++	rif^S str^S tri^S
	JMP228R pEMT3		+	rif^R str^S tri^S
		JMP228R	-	rif^R str^S tri^S
		JMP228S	-	rif^S str^R tri^S
		DB01R	-	rif^R tri^S
		DB01T	-	rif^S tri^R
JMP228R/pJP4	JMP228/pJP4	JMP228R	++	rif^R str^S tri^S
JMP228S/pJP4	JMP228/pJP4	JMP228S	++	rif^S str^R tri^S
JMP228R/pEMT3	indigenous	JMP228R	+	rif^R str^S tri^S
JMP228S/pEMT3	JMP228R pEMT3	JMP228S	+	rif^S str^R tri^S
DB01R/pJP4	JMP228/pJP4	DB01R	++	rif^R tri^S
DB01T/pJP4	JMP228/pJP4	DB01T	++	rif^S tri^R
DB01R/pEMT3	DB01/ pEMT3	DB01R	+	rif^R tri^S
DB01T/pEMT3	JMP228R/pEMT3	DB01T	+	rif^S tri^R

[a] A minus indicates that no growth with 2,4-D occurred, a plus indicates slow growth, two plusses indicate fast growth..

[b] The antibiotics rifampicin (rif) and streptomycin (str) were used at a final concentration of 250 µg ml^{-1} and trimethoprim (tri) resistance was used at a final concentration of 75 µg ml^{-1}.

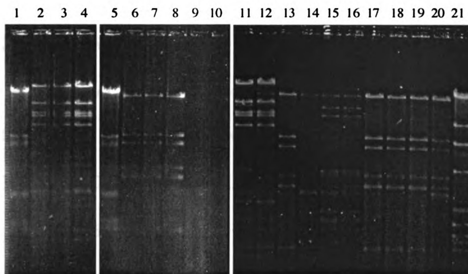


Figure 3.1: Restriction of plasmids isolated from transconjugants and cut with enzyme EcoR1. Lanes 1, 5, 21 *Hind*III digested lambda DNA.; Lane 2, plasmid pJP4 from JMP134 (Don and Pemberton, 1981); Lane 3, JMP228r/pJP4; Lane 4, JMP228s/pJP4; Lane 6 plasmid pEMT3 from K19 (JMP228/pEMT3) (Top et al. 1995); Lane 7 JMP228r/pEMT3; Lane 8, JMP228s/pEMT3; Lane 9, JMP228r; Lane 10, *B. cepacia* DB01; Lane 11, DB01r/pJP4; Lane 12, DB01t/pJP4; Lane 13, DB01t/pEMT3; Lane 14, DB01t/pEMT3-212a; Lane 15, DB01t/pEMT3-212b1; Lane 16, DB01t/pEMT3-212b2; Lane 17, DB01t/pEMT3-213; Lane 18, DB01r/pEMT3 Lane 19, DB01r/pEMT3-236; Lane 20, DB01t/pEMT3-241. The DB01 transconjugants in Lanes 14, 15, 16, 17, 19 and 20 were not used in this study. Lanes 14, 15, 16 and 20 suggest changes in the plasmid backbone of pEMT3 could occur in strain DB01.

Cultivation of cells. Cells were grown in A+N mineral salts medium to which carbon sources and antibiotics were added (Wyndham, et al., 1986). A+N medium consisted of 1.71 g $K_2HPO_4 \cdot 3H_2O$, 0.34 g N $aH_2PO_4 \cdot H_2O$, 0.33 g $(NH_4)_2SO_4$, 0.25 g $MgS0_4 \cdot 7H_2O$, and 10 ml of a trace metals solution. The trace metal solution contained, per liter: 12.0 g $Na_2EDTA_4 \cdot 2H_2O$, 2.0 g NaOH, 0.4 g $ZnSO_4 \cdot 7H_2O$, 0.4 g $MnSO_4 \cdot 4H_2O$, 0.1 g $CuSO_4 \cdot 5H_2O$, 3.0 g $FeSO_4 \cdot 7H_2O$, 5.2 g Na_2SO_4, 0.1 g $NaMoO_4 \cdot 2H_2O$. (Wyndham 1986). The pH was adjusted with 10 N H_2SO_4 to 7.2 prior to autoclaving (45 min, 121 psi). Prior to autoclaving, amendments of 5 mM sodium succinate (Sigma), or 1.7 % agar (Difco, Detroit, MI) were added if needed. Stock solutions of 2,4-D (20 mg ml^{-1} in 0.1 M NaH_2 PO_4, pH7.2) (Sigma), rifampicin (25 mg ml^{-1} in 100 % methanol), streptomycin (25 mg ml^{-1} in water) and Trimethoprim (7.5 mg ml^{-1} in 100% methanol) were filter sterilized prior to supplementing autoclaved media. Solid LB agar medium consisted, per liter, of 10 g yeast extract (Difco), 5 g tryptone (Difco), 5 g NaCl (Sigma) and 17g agar (Difco). For each independent experiment, cultures were started from a frozen culture stock. The cell cultures were grown to late log phase in 16 ml test tubes with 3 ml A+N minimal medium amended with 2,4-D (200 $\mu g ml^{-1}$) and the appropriate antibiotic (rifampicin, streptomycin or trimethoprim). A 2.5% (vol/vol) inoculum to A+N medium with succinate

(5mM) or 2,4-D (200 ppm) was used to start any other culture used in the growth and competition experiments. Population identification in the competition experiments was carried out using 2,4-D minimal medium plates supplemented with the appropriate antibiotics (Table 3.3). Differences in colony morphology among *Ralstonia* and *Burkholderia* were determined on R2A agar plates.

Substrate utilization. Substrate utilization profiles were determined using BIOLOG GN plates with 95 different carbon sources. JMP228r, JMP228s, DB01r and DB01t were grown in A+N media supplemented with 5 mM succinate to an absorbance of 0.3 at 600 nm. The bacteria were harvested by centrifugation at 5000 rpm (Sorvall SS34 rotor), rinsed twice in sterile saline (0.85% NaCl), and suspended to an absorbance reading of 0.1 at 600 nm in sterile saline. Triplicate BIOLOG plates were inoculated with the different strains by adding 300 μl of culture fluid to each well. Development of purple color in the wells due to reduction of the reazurin dye was used as the criterion for substrate use. Color formation was determined visually after 8, 24 and 48 hour intervals. The 48 hour readings were used as the endpoint, as no additional wells developed color when incubated longer.

Growth Parameters. *Culture Growth Rates.* Growth rates of the different strains were determined with succinate and 2,4-D, using two two methods described in chapter 2. In the traditional method, three replicate

cultures were initiated in 100 ml of medium in a 500 ml Erlenmeyer flask with a 1 % (vol/vol) inoculum. The Flasks were incubated at 30 °C in a rotary shaker (200 rpm, 30 °C). During growth with 2,4-D, 3 ml samples were periodically removed to determine the viable population size, optical density, and concentration of 2,4-D. The optical density of a growing culture was monitored spectrophotometrically with an HP 8452A diode array spectrophotometer (Hewlett Packard, Waldbronn, Germany) at 600 nm, and the growth rates were determined by regression analysis. Bacterial growth rates were calculated using the equation $N_1 = N_0e^{\mu t}$ where N is the population size, and N_0 is the population size at time 0. μ is the growth rate and t is the time elapsed from N_0 to N_1. The variable to solve for is μ, and it is the slope of the line from N_0 to N_1 during time t. During exponential growth, an increase in population size is reflected by a linear increase in absorbance. The growth rate μ can be determined from the slope of the straight line obtained on a log-linear plot by regression analysis during exponential growth. The maximum growth rate for a substrate is the intrinsic rate of increase in cell number above which additional substrate will not allow for more rapid growth.

The second method determined growth rates in 96-well microtiter plates. Optical densities at 590 nm were measured using a BIO-TEC Microtiter EL312e microplate reader (BIO-TEK instruments Inc., Wenooski, VT) as described in Chapter 2. The data was saved to a

computer disk and analyzed using Microsoft Excel 5.0. Growth rates were determined by regression analysis.

Disappearance of Substrate. Concentrations of 2,4-D were determined with High Performance Liquid Chromatography (HPLC). The samples were prepared as described previously in chapter 2. To calculate the concentrations of 2,4-D in the samples, the peak areas were compared with a 6-point calibration curve ranging from 5 to 250 µg ml^{-1} 2,4-D standards. The samples were analyzed with a Hewlett Packard Series 1050 HPLC with a 250 mm x 4.6 mm Lichrosorb RP-18, 10 µm particle size, chromatography column (Alltech, Bannockburn, IL) at a flow rate of 1.5 ml per minute and with 70% methanol and 30% phosphoric acid (0.1%) (vol/vol) as the solvent.

The Half-Saturation Constant. The half saturation constants (Ks) were determined for strains JMP228R/pJP4, JMP228R/pEMT3, DB01R/pJP4 and DB01R/pEMT3, by measuring the rate of uptake and incorporation of ring-labeled ^{14}C- 2,4-D. 100 ml-cultures of each strain were grown to late log phase with 200 µg ml^{-1} 2,4-D in A+N medium. The optical densities of the cultures were measured and an appropriate volume of culture fluid that provided 1 x 10^{10} CFU ml^{-1} , were harvested by centrifugation at 8000 rpm (Sorvall SS34 rotor) for 10 minutes. The cell pellets were rinsed twice with phosphate buffered saline (PBS) solution (Löffler, 1997), and resuspended in 1 ml PBS. CFU ml^{-1}, were determined

for each sample. Actual cell counts were approximately 2 x 10^9 CFU ml^{-1}. At time zero, a sample was removed from each vial before inoculation with the test culture. A 10 µl aliquot of the dense cell suspension was added at 1 minute intervals to 100 ml serum bottles each containing 10 ml of a solution containing ^{14}C ring-labeled 2,4-D at concentrations of 10, 1, 0.2, 0.1, 0.67 and 0.05 µg ml^{-1} 2,4-D. The bottles were capped with Teflon-lined rubber stoppers, crimp-sealed, placed on a rocking platform after inoculation, and incubated at room temperature. At each of the designated intervals of 0, 6, 13, 20, 27, 34 and 41 minutes, 0.4 ml samples were removed from each bottle using a syringe. The samples were transferred to Centricon micro-concentrator filter units (Amicon) containing 40 µl 2N HCl in the bottom of each unit. After centrifugation at 12,000 rpm in a microcentrifuge for 1 minute, the filters containing the cells were placed into scintillation vials containing 8 ml of scintillation fluid. The micro-concentration tubes containing the flow through, without the caps, were placed upside down into another scintillation vial. The DPMs for all samples were counted with a scintillation counter. The resulting counts were transformed into µg ml^{-1} 2,4-D using a standard curve generated using samples with known 2,4-D concentrations and the DPMs obtained for the different dilutions. 2,4-D uptake curves for each strain were fitted to first order degradation kinetics using the Kaleidagraph software. An example of the degradation curves is shown in Figure 3.2, for strain DB01R/pJP4.

Similar depletion curves were prepared for the four strains tested. The equations generated by the graphs were used to calculate the initial rates of 2,4-D uptake at each of the substrate concentrations mentioned above. The inverse of the substrate concentrations and rates, were used to create Lineweaver-Burke graphs for each of the strains by plotting 1/S over 1/V (Figure 3.3). The point at which the line intercepted the X-axis represented $-1/K_S$, which was then used to find the half saturation constant for JMP228R/pJP4, JMP228R/pEMT3, DB01R/pJP4 and DB01R/pEMT3.

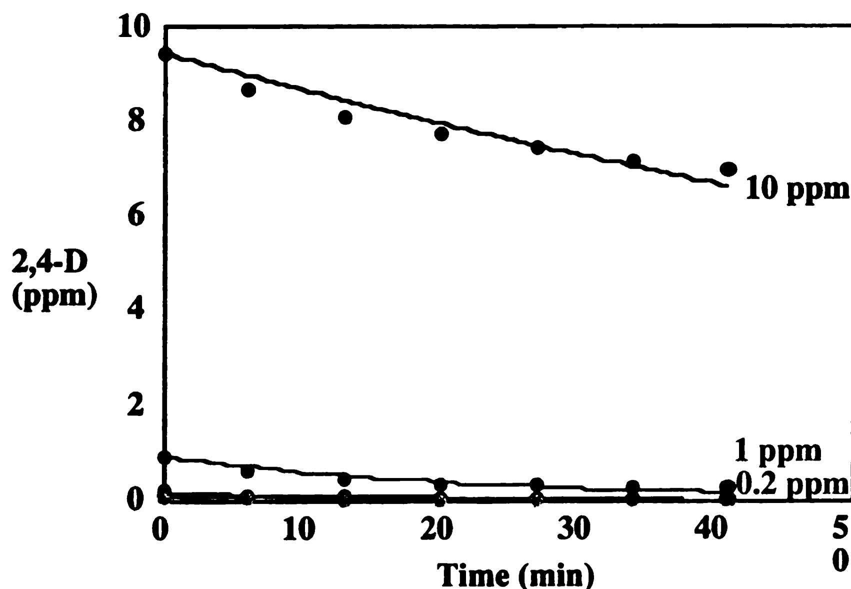


Figure 3.2. 2,4-D depletion curves used to calculate rates of 2,4-D degradation for use in calculating Ks for strain DB01R/pJP4. This graph illustrates curves for 10, 1 and 0.2 ppm. Curves for 0.067 and 0.05 ppm cannot be resolved on this scale. Kaleidagraph software was used to determine the initial degradation rate for each curve, which was then used to generate a lineweaver-burke plot.

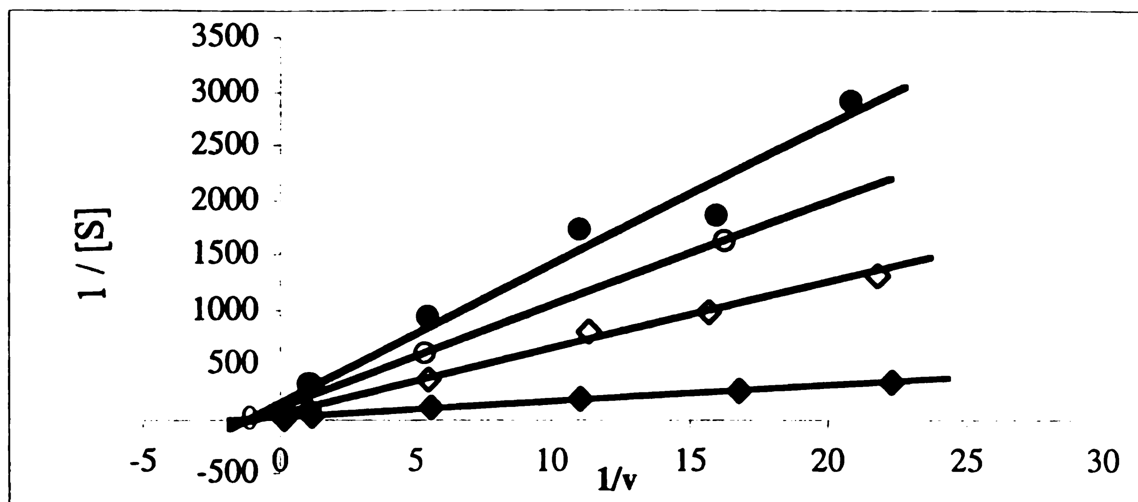


Figure 3.3. Lineweaver-Burke plot to determine the Ks for each of the four host/plasmid combinations. Open diamonds are DB01/pEMT3, filled diamonds are DB01/pJP4, light grey circles are JMP228/pEMT3, dark filled circles are JMP228/pJP4. The Ks values calculated for each of the strains based on the interception of the line with the X axis are 1.2 ppm, 0.9 ppm, 0.8 ppm and 1.2 ppm respectively.

Chemostat Conditions: Chemostats of each pure culture were operated as described in Chapter 2 for a duration of 12 days. Operating parameters were as follows. A. Succinate-grown culture in mid-late log phase (optical density of ~ 0.1 at 600 nm, about 2-3 x 10^7 CFU ml^{-1}), was gently harvested (4,000 rpm for 20 minutes in a Sorvall SS34 rotor), rinsed with A+N minimal medium, and suspended to a final OD_{600} of 0.2 in the same medium. This cell suspension (75 ml) was placed in a chemostat and 2,4-D was added to a final concentration of 200 μg ml^{-1}. Each chemostat was incubated for 8 hours at 30° C, before a constant flow conditions were

initiated. The dilution rate (D) of a chemostat determines the growth rate (μ), and for the 75 ml volume chemostats, D= μ = 0.033 h^{-1} which is well below the maximum growth rate for the strains under investigation. To sample the chemostats, the port was swabbed with 70% ethanol and 3 ml of culture was removed with a 3 ml syringe. The withdrawn sample was used to obtain measurements of the 2,4-D concentration (HPLC), optical density and the CFU ml^{-1}. Culture fluids aliquots (100 μl) were plated onto A+N agar plates containing 200 μg ml^{-1} 2,4-D and R2A agar plates, both amended with the appropriate selective antibiotics to obtain viable cell numbers. Three measurements of each chemostat were used for each time point to determine the average population size.

Equation (a)

$$\frac{dN}{dt} = N \mu_{max} \frac{S}{K_S+S} - DN$$

In order to evaluate the growth of the different strains in chemostats, the values for μ_{max}, K_S and yield were inserted into the Monod equation (a) (Dykhuizen and Hartl, 1983). Then models were run using the Stella Research Software 5.0 (High Performance Systems Inc. Hanover, NH). The critical equation in this model is the Monod equation: dN/dt is the rate of change in population size, N is the population size, μ_{max} is the maximum growth rate on the resource and S is the substrate concentration. The model

automatically calculates the population sizes over time and the remaining concentration of 2,4-D, based on the substrate utilization equation by Monod. Equation (b) represents the utilization of the substrate in the chemostats for a single population. S_0 and S are substrate concentration in the reservoir and chemostat respectively. Y represents the growth yield, (CFU/mg substrate). An illustration of the model is presented in Figure 3.4. The values to be tracked are population size and 2,4-D concentration, shown by the black boxes in Figure 3.4. Arrows connected to clouds indicate the direction of energy flow, and the equation associated with the arrow will calculate the rate at which the population size or resource concentration changes depending on the various inputs represented by open circles (in this case using the Monod equation. The efficiency of utilization (mg/CFU) is the inverse of the growth yield, CFU/mg 2,4-D, as calculated for strains grown on 2,4-D medium. The dilution rate (D) determines the growth rates that the populations experience.

Equation (b)

$$\frac{dS}{dt} = D(S_0 - S) - YN \frac{S}{Ks + S}$$

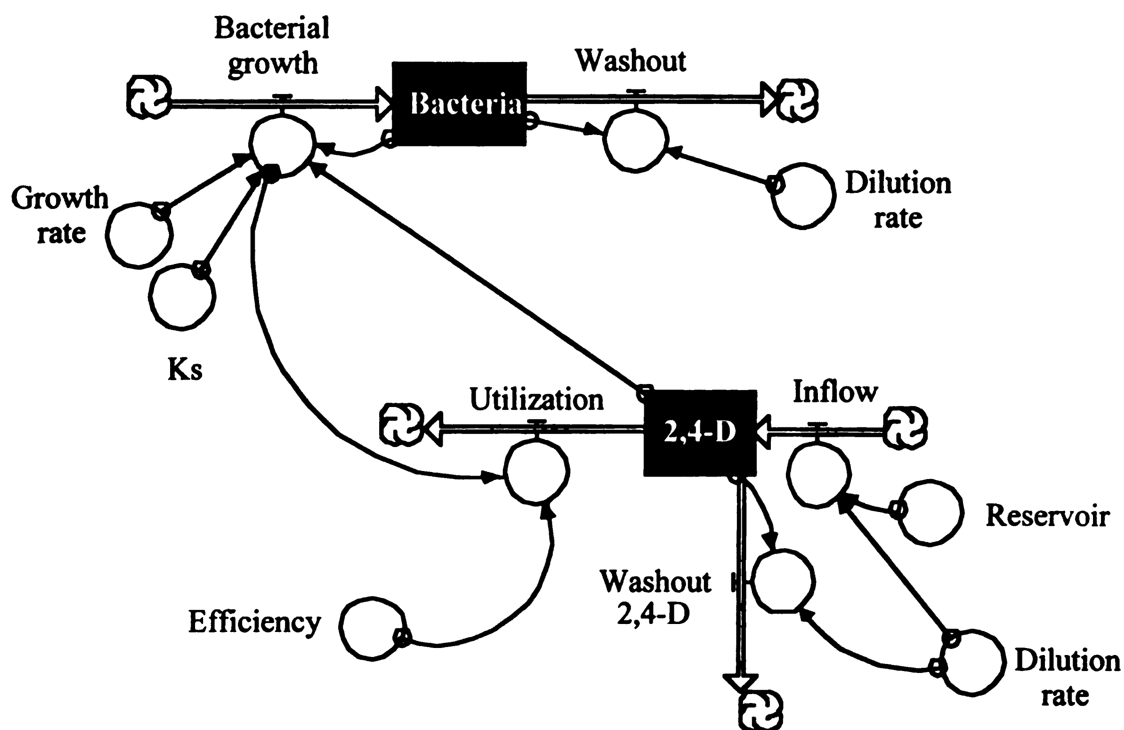


Figure 3.4. Growth model developed on the Stella Research Program to generate expected population growth curves and 2,4-D depletion curves for pure cultures. The constants for K_S, growth rate, and efficiency were chosen using an average value determined for each host/plasmid combination. The initial population size (N) and 2,4-D concentrations (200 ppm) that were used for the model, were obtained using values from the experimental chemostats.

RESULTS

Antibiotic resistance profiles. To determine which antibiotics to use as selective markers, 1.7×10^8 to 2.7×10^8 cells of each bacterial strain were plated to LB agar plates containing the different antibiotics.

Table 3.4: Testing the antibiotic resistance profiles of strains

		Number of resistant colonies / total number of colonies	
Antibiotic	μg/ml	*R. eutropha* JMP228	*B. cepacia* DB01
Rifampicin	50	6.1×10^{-09}	8.8×10^{-09}
	100	$9. \times 10^{-09}$	3.7×10^{-09}
	150	6.2×10^{-09}	4.0×10^{-09}
Streptomycin	100	3.9×10^{-09}	TNTC[a]
	250	5.8×10^{-09}	7.4×10^{-07}
	500	3.9×10^{-09}	9.8×10^{-09}
Trimethoprim	20	TNTC	2.3×10^{-08}
	40	TNTC	5.8×10^{-08}
	80	7.5×10^{-07}	0
Tetracycline	15	0[b]	TNTC
	20	0	TNTC
	25	0	3.7×10^{-06}
Chloramphenicol	25	TNTC	TNTC
	50	5.1×10^{-07}	TNTC
	75	6.4×10^{-08}	1.6×10^{-06}
Kanamycin	25	0	0
	50	0	0
	75	0	0
Naladixic acid	10	3.4×10^{-07}	TNTC
	15	1.5×10^{-07}	TNTC
	20	6.1×10^{-08}	1.4×10^{-07}
Ampicillin	50	TNTC	TNTC
	100	TNTC	TNTC

[a] TNTC indicates a lawn of bacteria, or colonies were too numerous to be counted on the plate.

[b] 0 indicates that no visible colonies were formed on the plate.

Table 3.4 displays the antibiotics that were tested. Chloramphenicol, naladixic acid and ampicillin were not suitable, since the *B. cepacia* strains were resistant, and the *R. eutropha* strains readily generated spontaneously resistant mutants. Kanamycin produced no spontaneous mutants for all of the populations tested. Results of the selection experiments and literature review (Lewis 1987) indicated that rifampicin would be a good choice for both *R. eutropha* and *B. cepacia.* Only a few spontaneous mutants arose, and the mutants did not lose this phenotype even after repeated transfer through media without selection in the presence of the antibiotic. Rifampicin is bacteriostatic and inhibits RNA synthesis. Frequently resistance is obtained through a mutation in the β-subunit of RNA polymerase that provides resistance by blocking the rifampicin from complexing with the RNA polymerase. Trimethoprim allowed for growth of *B. cepacia,* and not *R. eutropha,* which would give a selective means of distinguishing between the two different host strains, and to allow for competition between *B. cepacia* populations resistant to trimethoprim or rifampicin. Trimethoprim is a drug that inhibits dihydrofolate reductase. Streptomycin, while allowing for the growth of *B. cepacia*, was appropriate for use in distinguishing between two populations *of R. eutropha*. Streptomycin is a bacteriocidal antibiotic that inhibits protein synthesis.

Substrate utilization profiles. Of the 95 carbon sources tested by the BIOLOG-GN plate, *R. eutropha* utilized 38 of the 95 substrates. *B. cepacia*

DB01 utilized 63 of 95 carbon sources. Only two substrates were used by the Ralstonia strains that were not used by Burkholderia: succinamic acid and L-leucine. On the other hand, Burkholderia utilized 30 substrates that were not used by Ralstonia. (Table 3.5). Utilization was based on development of a deep purple color in the wells of the microtiter plate. Inconclusive color formation was not scored.

Pure culture growth characteristics. *R. eutropha* colonies are smooth, shiny and translucent on R2A, agar plates, but small (< 0.2 mm in diameter) and translucent when grown on A+N plates without any carbon source after 48 h. Growth without an added carbon source is not completely unexpected, as the agar may contain impurities that could be used for growth. Also, *R. eutropha* is a chemoautotroph, and can grow slowly by fixing carbon from the atmosphere (Palleroni, 1987). As the colonies developed over a week, their edge became irregular. When grown on A+N plates with 2,4-D the colonies carrying a degradative plasmid (pJP4or pEMT3) did have a larger diameter (1 mm) than those on the no-carbon control plates, and they are much more robust (what is a robust colony?). In the presence of antibiotics no growth occurred on plates lacking a carbon source..

Table 3.5: BIOLOG substrate utilization by Ralstonia eutropha JMP228 and Burkholderia cepacia DB01.

	Used by all	Used by none	*B. cepacia* only	*R. eutropha* only
Carbohydrates	D-Fructose	N-acetyl-D-galactosamine Adonitol Cellobiose I-Erythritol Gentibiose Lactulose Maltose D-melibiose β-methyl-D-glucoside L-rhamnose Turanose Xylitol D-psicose	N-acetyl-D-glucosamine L-arabinose D-arabitol L-fucose D-galactose α-D-lactose D-mannitol D-mannose D-raffinose D-Sorbitol Sucrose D-trehalose	
Esters	Methyl pyruvate Mono-methyl succinate			
Aromatic	Urocanic acid Inosine	Uridine Thymidine		
Amines	Phenyl ehylamine		Putrecine 2-amino ethanol	
Amides	Alaninamide	Glucuronamide		Succinamic acid
Alcohols		2,3-butanediol	Glycerol	
Phosphorylated Compounds		Glucose-1-phosphate	D,L, α-glycerol phosphate Glucose-6-phosphate	
Brominated	Bromosuccinic acid			
Polymers	Tween 40 Tween 80	α-cyclodextrin Dextrin Glycogen		

	Used by all	Used by none	*B. cepacia* only	*R. eutropha* only
Carboxylic Acids	Acetic acid	D-galacturonic acid lactone	D-galacturonic acid	
	Cis-aconitic acid	α-hydroxy butyric acid		
	Citric acid	γ-hydroxy butyric acid		
	Formic acid	α-ketobutyric acid		
	D-gluconic acid	α-ketoglutaric acid		
	β-hydroxy butyric acid	α-ketovaleric acid		
	p-hydroxy phenylacetic acid			
	Itaconic acid			
	D-L-lactic acid			
	Malonic acid			
	Propionic acid			
	Quinic acid			
	D-saccharic acid			
	Sebacic acid			
	Succinic acid			
Amino Acids	D-alanine	Glycyl-L-aspartic acid	Hydroxy-L-proline	L-leucine
	L-alanine	Glycyl-L-glutamic acid	L-ornithine	
	L-alanyl glycine	D-serine		
	L-asparagine	D,L-Carnitine		
	L-aspartic acid	γ-amino butyric acid		
	L-glutamic acid			
	L-histidine			
	L-phenylalanine			
	L-proline			
	L-pyroglutamic acid			
	L-serine			
	L-threonine			

B. cepacia colonies were creamy white with an opaque appearance on R2A agar. Older colonies tended to develop lumpy edges, whereas younger ones were smooth. The trimethoprim resistant strain name it exhibited two distinguishable colony morphologies at a constant 1-to-1-ratio: smooth large mucoid colonies and flatter, smaller, colonies with a dry appearance. Neither morphology bred true. Mucoid colonies yielded both colony morphologies, as did the drier ones. Purity of the culture was confirmed microscopically, by repeated palting and transfer of well-isolated colonies, and by molecular analyses. Colonies grown in the absence of antibiotics tended to be larger than those grown in the presence of antibiotics. This was especially true for DB01T grown with trimethoprim. Colonies were consistently smaller when grown in the presence of the antibiotic, but still larger than the colonies of strain DB01r when grown on R2A plates. The colonies were transparent pinpoints on A+N plates without a carbon source. They remained the same size for 3 weeks. On 2,4-D minimal media, colonies appeared after a period of 72 hours, and developed a colony morphology that was indistinguishable from the *R. eutropha* type. A distinct difference occurred between the growth of JMP228 and DB01 on R2A plates. *R. eutropha* colonies became visible after approximately 24 hours, whereas at least 36-48 were needed for *B. cepacia* colonies to appear. However, colonies of the *B. cepacia* strains grew to be as large as

R. eutropha colonies within one week, in fact, the colonies continued to become even larger when incubated longer.

Plasmids pJP4 and pEMT3 both benefit their host strains Ralstonia eutropha and Burkholderia cepacia by supplying a catabolic pathway which can convert the herbicide 2,4-D to metabolic intermediates that can then be channeled into the TCA cycle. Plasmids pJP4 and pEMT3 carrying the 2,4_D degradative pathway and different antibiotic resistance genes were easily conjugated into strains JMP228R and JMP228S. Plasmid pJP4 readily conjugated into *B. cepacia* DB01, but pEMT3 could not be transferred to the same strain when the matings were performed on LB medium. Transconjugants were, however, obtained from matings on R2A agar.

The growth rates of the two host populations differed only slightly when succinate (5 mM) served as the source of carbon and energy. When grown in batch cultures, strains JMP228 and DB01 had very similar growth rates of 0.448 ±0.047 h^{-1} and 0.416 ±0.020 h^{-1}, respectively ($n = 18$, $P = 0.089$). When pure cultures were grown on succinate under 24 hour batch transfer conditions, the JMP228 strains attained densities of 1×10^9 ($\pm 0.8 \times 10^8$) CFU ml^{-1}. DB01 strains attained densities of 5×10^8 ($\pm 0.5 \times 10^8$) CFU ml^{-1} (Table 3.6). The results of a one-tailed T-test for comparing the strains using the batch culture data are shown in Table 3.7. Pairs 1 to 5

refer to the succinate-grown cultures, and pairs 6 to 10 are the 2,4-D-grown cultures.

Table 3.6. Growth rates of strains supplied with succinate or 2,4-D under batch culture conditions. Growth rates are in h^{-1}, ± SD.

strain	n=4 Succinate, microtiter (h^{-1})	n=6 Succinate, Flask (h^{-1})	n=12 2,4-D microtiter (h^{-1})	n=6 2,4-D flask (h^{-1})
JMP228	0.62 ± 0.11	0.45 ±0.02		
JMP228r	nd	0.47 ±0.03[a]		
JMP228s	nd	0.43 ±0.01[a]		
DB01	0.50 ± 0.15	0.36 ± 0.02		
DB01r	nd	0.46 ±0.05[a]		
DB01t	nd	0.43 ±0.03[a]		
JMP228R/pJP4	0.52 ± 0.11	0.54 ± 0.09	0.24 ± 0.07	0.22 ± 0.06
JMP228S/pJP4	0.54 ± 0.16	0.46 ± 0.03	0.23 ± 0.06	0.23 ± 0.05
JMP228R/pEMT3	0.36 ±0.03	0.37 ± 0.02	0.10 ± 0.02	0.10 ± 0.01
JMP228S/pEMT3	0.46 ±0.03	0.51 ± 0.05	0.10 ± 0.02	0.10 ± 0.01
DB01R/pJP4	0.52 ±0.23	0.49 ± 0.16	0.13 ± 0.02	0.16 ± 0.04
DB01T/pJP4	0.47 ±0.09	0.53 ± 0.22	0.13 ± 0.02	0.15 ± 0.04
DB01R/pEMT3	0.48 ±0.04	nd	0.09 ± 0.02	0.09 ± 0.03
DB01T/pEMT3	0.44 ±0.04	nd	0.09 ± 0.02	0.08 ± 0.03

[a] For these growth rate calculations, n= 3.

JMP228/pJP4 grew rapidly (μ = 0.23 ± 0.05 h^{-1}) when 2,4-D was supplied as a limiting resource. A lag period of 1 to 2 hours was observed

when succinate-grown cells were inoculated into medium amended with 2,4-D. In contrast, no lag time was observed when a culture exponentially growing with 2,4-D served as the inoculum. When stationary phase was reached, *Ralstonia* strains attained a population size of 2×10^8 CFU ml^{-1} (Fig. 3.5). In the slow growth rate conditions in the chemostats with dilution rates equal to 0.03 h^{-1}, the populations attained cell densities of about 8×10^8 CFU ml^{-1}.

In contrast to JMP228/pJP4, the growth rate of strain JMP228/pEMT3 was much slower ($\mu = 0.10 \pm 0.01$ h^{-1}). These rates significantly differed from those of JMP228/pJP4 (Pair 8, table 3.7 ($n = 24$, $P < 0.0001$). JMP228/pEMT3 grown with succinate displayed a lag time of 15 to 20 hours when transferred to 2,4-D medium. A stationary phase population size of 3×10^6 CFU ml^{-1} could be maintained in culture vessels sustaining a 10 % volume transfer to fresh 2,4-D minimal medium every 24 hours, a maximum density of 2 orders of magnitude less than that of JMP228/pJP4. When a similar experiment was carried out using 10% transfer every 72 h, JMP228/pEMT3 carrying strains obtained and maintained a population density equal to that of JMP228/pJP4 (fig. 3.5). In the slow growth rate chemostats with dilution rates equal to 0.03 h-1, the populations attained a size of 1×10^9 CFU/ml, similar to JMP228/ pJP4.

Table 3.7. One-tailed t-test on growth rates of strains determined under batch culture conditions. Column n shows the number of culture vessels used to calculate the P value. Boldface print indicates a significant difference between the growth. A P value <0.001 is considered to represent a significant difference between growth rates.

succinate	strain	Marker	plasmid	n	P
1. Antibiotic	JMP228	rif		18	0.9852
	JMP228	str			
2. Plasmid	JMP228		pJP4	24	0.0024
	JMP228		pEMT3		
3. Antibiotic	DB01	rif		12	0.3455
	DB01	tri			
4. Plasmid	DB01		pEMT3	12	0.6179
	DB01		pJP4		
5. Strain	JMP228			36	0.5415
	DBOr				
2,4-D	**strain**	**Marker**	**plasmid**	**n**	**P**
6. Antibiotic	JMP228	rif	pJP4	12	0.4190
	JMP228	str	pJP4		
7. Antibiotic	JMP 228	rif	pEMT3	18	0.0647
	JMP 228	str	pEMT3		
8. Plasmid	**JMP 228**	**rif or str**	**pJP4**	**24**	**< 0.0001**
	JMP 228	**str or rif**	**pEMT3**		
9. Antibiotic	DB01	rifR	pJP4	18	0.5376
	DB01	triR	pJP4		
10. Antibiotic	DB01	rifR	pEMT3	12	0.3559
	DB01	triR	pEMT3		
11. Plasmid	**DB01**	**rif or tri**	**pJP4**	**36**	**< 0.0001**
	DB01	**tri or rif**	**pEMT3**		
12. Strain	**JMP 228**	**rif or str**	**pJP4**	**36**	**< 0.0001**
	DB01	**tri or rif**	**pJP4**		
13. Strain	**JMP 228**	**rif or str**	**pEMT3**	**36**	**< 0.0001**
	DB01	**tri or rif**	**pEMT3**		
14. Strain/ Plasmid	**JMP 228**	**rif or str**	**pJP4**	**24**	**< 0.0001**
	DB01	**tri or rif**	**pEMT3**		
15. Strain/ Plasmid	**JMP 228**	**rif or str**	**pEMT3**	**36**	**< 0.0001**
	DB01	**tri or rif**	**pJP4**		

DB01/pJP4 had a fairly rapid growth rate (0.15 h^{-1}) on 2,4-D that is significantly lower than that of JMP228/pJP4 (0.22 h^{-1}) and higher than that of JMP228/pEMT3 (0.10 h^{-1}) (Table 3.7, pairs 12 and 15). Under batch transfer conditions and 200 μg ml^{-1} 2,4-D, strain DB01/pJP4 attained cell densities of only 1 x 10^6, similar to the population maintained for JMP228/pEMT3. Under 72 hour transfer conditions, the strains grew to $5x10^7$ CFU ml^{-1}, similar to the maximum cell density of JMP228/pJP4 under these conditions (Fig. 3.5). In chemostats, DB01/pJP4 attained densities of up to 5 x 10^9 CFU ml^{-1}, higher than for the JMP228 strains. DB01/pEMT3 was the slowest grower of the four host/plasmid combinations (μ= 0.08 h^{-1}). In the 24 hour batch transfer environment with 2,4-D as the limiting substrate, DB01/pEMT3 attained densities of only 1 x 10^4 CFU ml^{-1}, two orders of magnitude less than the cell densities observed with DB01/pJP4. Both of these *B. cepacia* strains reached and maintained high densities similar to that of JMP228/pJP4 under 72 h transfer conditions (Fig. 3.5). All four strains grew at different rates on 200 μg ml^{-1} 2,4-D.

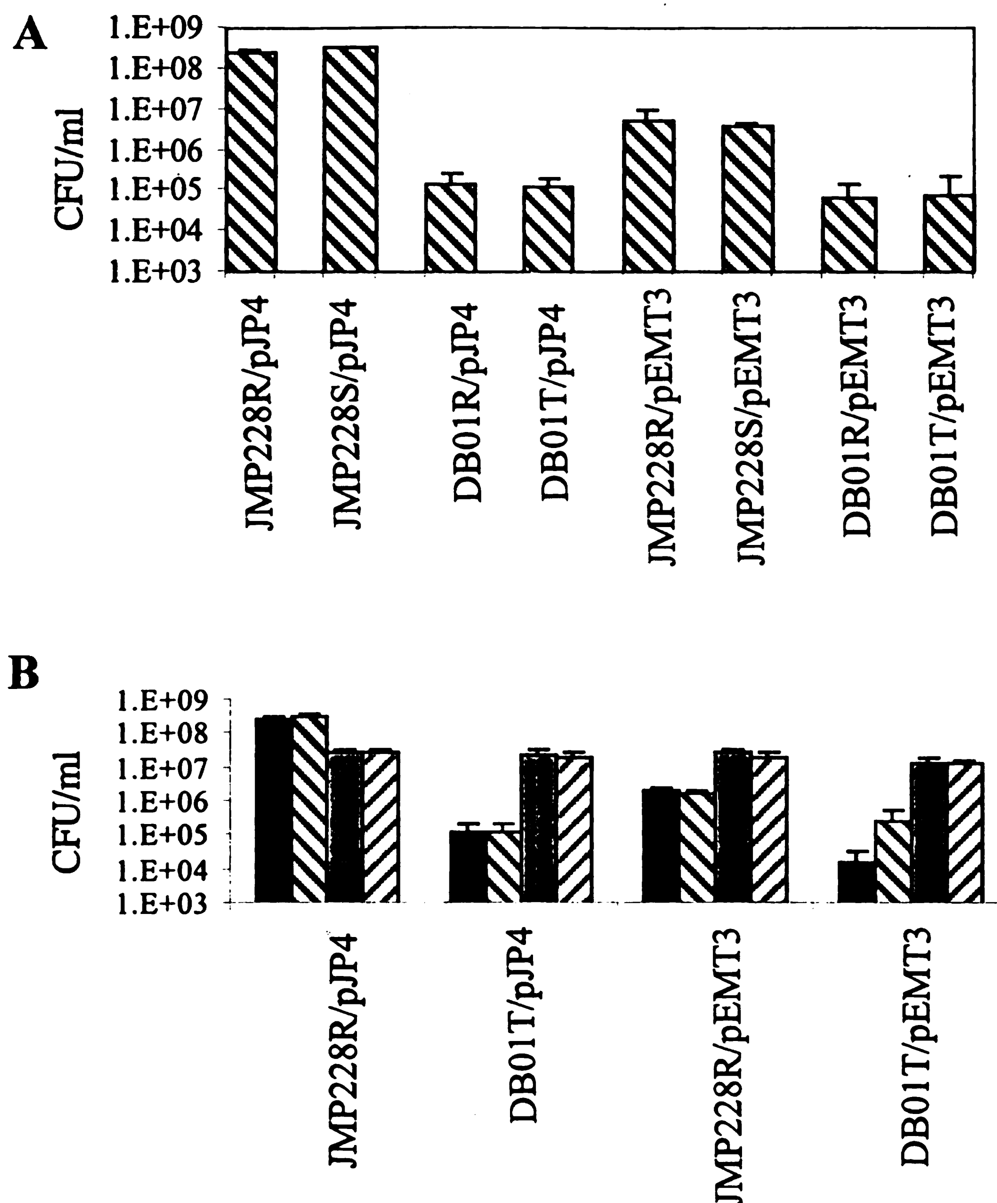


Figure 3.3. Average population sizes for selected strains grown in batch culture under 24 and 72 hours Transfer conditions. Panel A: Population sizes attained in 24 hour batch transfer experiments. The antibiotic resistance markers did not affect the maximum population sizes in 24 hour transfer, batch culture conditions. Panel B: Population sizes with 24 and 72 hour transfer experiments. Black columns represent the 24 hour transfer cultures, and the gray columns represent the 72 hour transfers. Colonies were counted on R2A agar plates and 2,4-D minimal medium plates amended with antibiotics (striped). Error bars indicate the standard deviation among three replicates. Because DB01T/pEMT3 formed fewer colonies on R2A plates, the population sizes in experiments were determined on 2,4-D agar only.

From the utilization of 2,4-D it was calculated that JMP228/pJP4 strains produce $3.7 \times 10^6 \pm 6 \times 10^5$ cells per mg of 2,4-D, JMP228/pEMT3 strains produce $3.7 \times 10^6 \pm 6.5 \times 10^5$ cells per mg of 2,4-D, DB01/pJP4 strains produce $3.4 \times 10^7 \pm 3.1 \times 10^5$ cells per mg of 2,4-D, and DB01/pEMT3 strains produce $3.5 \times 10^7 \pm 6.1 \times 10^5$ cells per mg of 2,4-D. These were used as the efficiency values in the chemostat models.

The BIOLOG plate reader was also used to assess whether there were differences in the growth rates of the strains on different concentrations of 2,4-D. While the 2,4-D was exhausted much more rapidly at lower resource concentrations, the strains grew at the same rate at 2,4-D concentrations between 10 and 500 ppm. Hence, a concentration of 200 ppm 2,4-D was chosen for physiological tests. This concentration allowed the cells to attain sufficient densities, without subjecting the bacteria to toxic levels of 2,4-D.

K_S values for 2,4-D utilization were not significantly different for JMP228R/pJP4, JMP228R/pEMT3, DB01/pJP4 or DB01/pEMT3, and ranged from 0.8 to 1.2 μg ml^{-1} 2,4-D. Additional information was gained while measuring K_S. The sum of the ^{14}C-2,4-D in the cell pellet and the supernatant were subtracted from the initial amount of 2,4-D to give a measurement of the quantity of 2,4-D presumably converted to CO_2 by each strain during the course of the experiment. At a 2,4-D concentration of 10 ppm, all of the cultures showed similar distributions of 2,4-D.

Interestingly, as the 2,4-D concentration dropped to below 1 ppm, a greater proportion of the 2,4-D was metabolized to CO_2 by *B. cepacia* strains relative to *R. eutropha* strains at the final timepoint (after 42 minutes). There appeared to be no noticeable difference between the two plasmids, pJP4 or pEMT3, in the *R. eutropha* host. *B. cepacia* with pJP4 mineralized a greater proportion of 2,4-D than the same host with pEMT3 (Fig. 3.6).

Chemostat growth. Pure cultures of the different populations were grown under chemostat conditions. The chemostats contained a total volume of 75 ml, and a constant flow rate of 0.33 h^{-1} was maintained. Mathematical models to predict the outcome of these experiments were run using the Stella Research Software. The graphs presented in Figure 3.7 and 3.8 shows the experimental data and the data obtained from the model. Table 3.8 shows the values that were used in the model as described in the Materials and Method section.

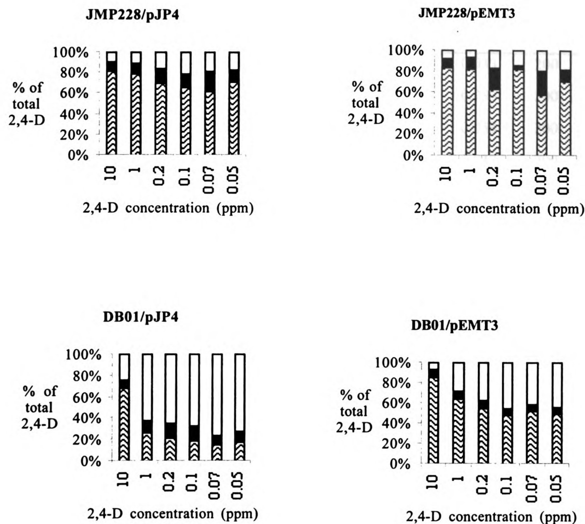


Figure 3.6. Allocation of ring-labeled ^{14}C-2,4-D in growing cultures of JMP228/pJP4, JMP228/pEMT3, DB01/pJP4 and DB01/pEMT3. The values were obtained from the final time point of the experiment taken after 41 minutes. 2,4-D concentration on the X axis is the initial amount of 2,4-D supplied. Bars represent the percentage of labeled 2,4-D remaining in each fraction. Wavy (slang) lines indicate the liquid fraction, the black lines represent the cell fraction, and the white lines show the fraction released as CO_2.

Table 3.8. Parameters used to calculate expected growth curves based on the Monod model, using Stella Research Software.

Strain	N_0 (cfu ml^{-1})	μ (h^{-1})	K_S (mg L^{-1})	Y (g cfu^{-1})	S_0 (cfu ml^{-1})	D (h^{-1})	SR (cfu ml^{-1})
JMP228R/pJP4	3×10^7	0.23	1	2.7×10^7	200	0.033	200
JMP228S/pJP4	8×10^7	0.23	1	2.7×10^7	200	0.033	200
JMP228R/pEMT3	7×10^7	0.10	1	2.7×10^7	200	0.033	200
JMP228S/pEMT3	2×10^7	0.10	1	2.7×10^7	200	0.033	200
DB01R/pJP4	4×10^7	0.15	1	2.9×10^7	200	0.033	200
DB01T/pJP4	4×10^7	0.15	1	2.9×10^7	200	0.033	200
DB01R/pEMT3	5×10^7	0.08	1	2.9×10^7	200	0.033	200
DB01T/pEMT3	4×10^7	0.08	1	2.9×10^7	200	0.033	200

Abbreviations: N_0 = initial population size, μ = maximum growth rate (h $^{-1}$), K_S = half saturation constant (mg/L), Y = efficiency (mg 2,4-D/CFU), S_0 = initial 2,4-D (mg/L), D = dilution rate (h^{-1}), and SR = substrate concentration in the reservoir (mg/L)

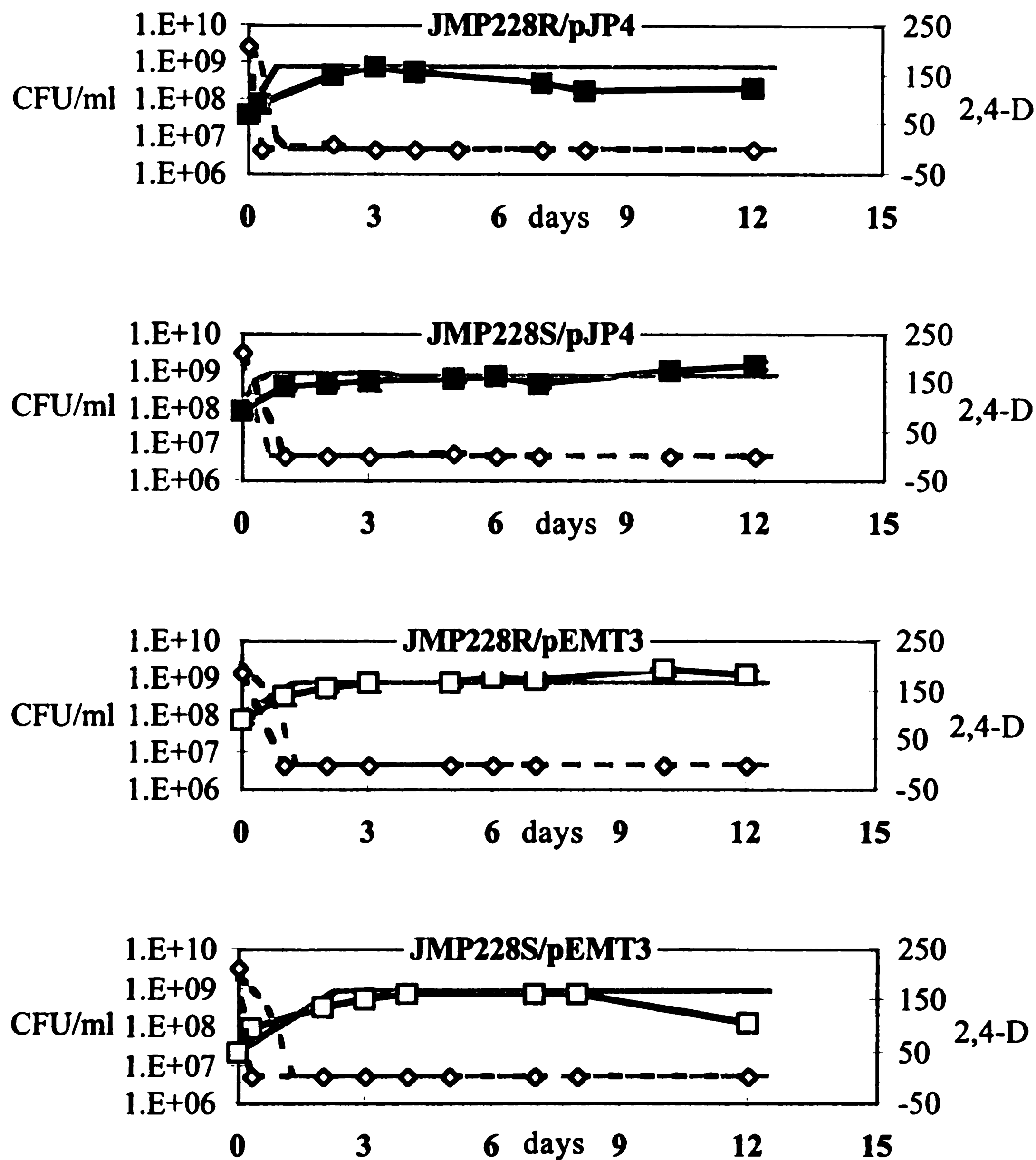


Figure 3.7. Chemostat growth of JMP228R/pJP4, JMP228S/pJP4, JMP228R/pEMT3 and JMP228S/pEMT3. The dilution rate was 0.03 h^{-1}, and the initial inoculum densities were 5 x 10^7 CFU ml^{-1}. Grey lines indicate modeled values, the black lines show the experimental data. Solid lines represent culture growth and the dashed lines show the 2,4-D concentrations in ppm.

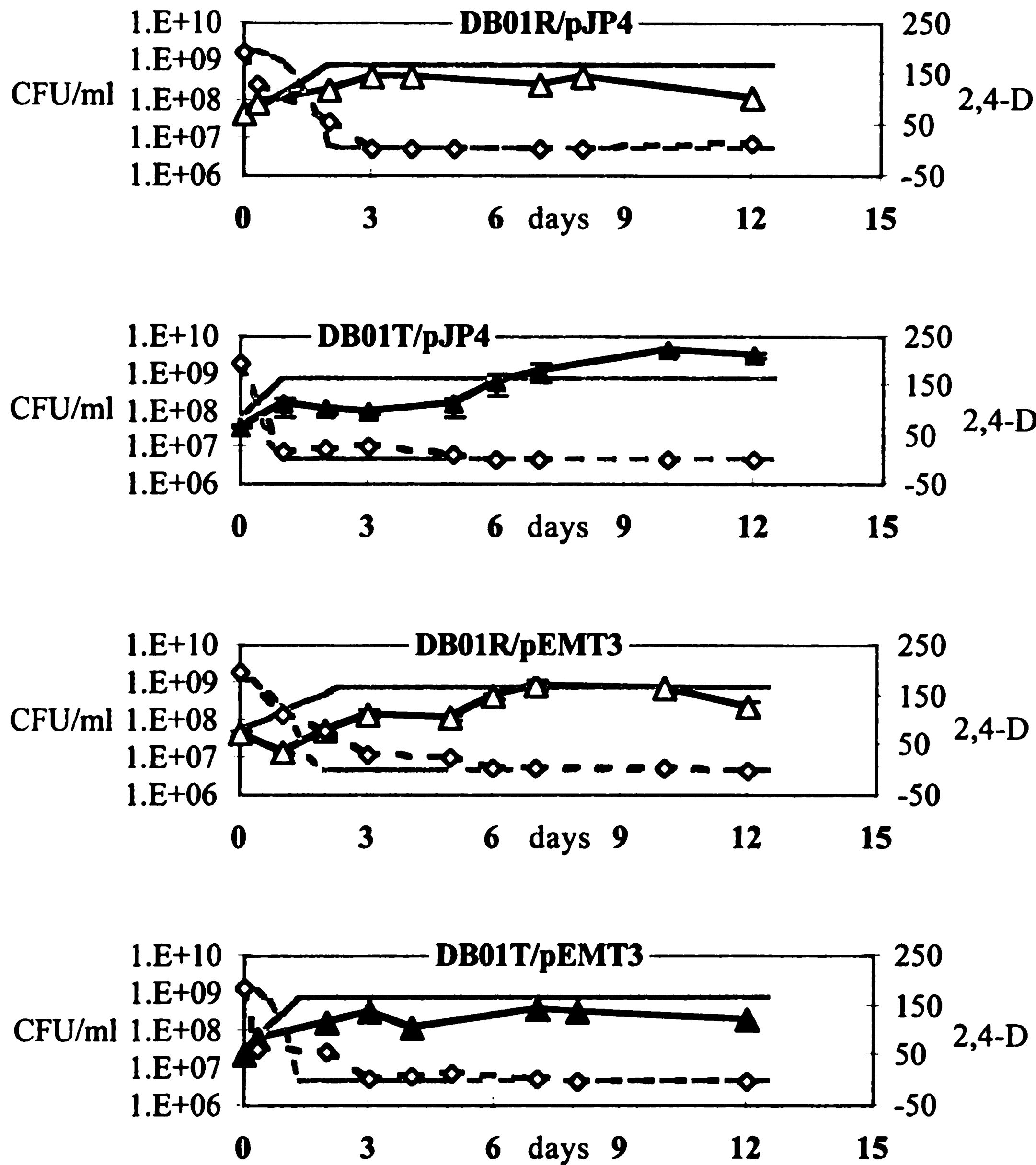


Figure 3.8. Chemostat Growth of DB01R/pJP4, DB01T/pJP4, DB01R/pEMT3 and DB01T/pEMT3. The dilution rate was 0.03 h^{-1}, and initial inoculum densities were 5 x 10^7 CFU ml^{-1}. 2,4-D is in ppm. Grey lines indicate modeled values, the black lines show the experimental data. Solid lines represent culture growth and the dashed lines show the 2,4-D concentrations in ppm.

JMP228S/pJP4 attained cell densities of 5 x 10^8 to 8 x 10^8 CFU ml^{-1}, and the concentration of 2,4-D in the chemostat decreased to undetectable levels within the first day. JMP228R/pEMT3 attained cell densities of 2 x 10^8 to 5 x 10^8 CFU ml^{-1}, and the concentration of 2,4-D in the chemostat also decreased to undetectable levels within the first day. These results implied that the pJP4 and pEMT3 containing *R. eutropha* populations did not differ much in their growth in chemostats, as they did in batch culture. All of the populations followed the trends predicted by the model closely. The decline in population numbers for JMP228S/pEMT3 was due to invasion by a fungal strain at the terminal portion of the experiment. Actual degradation of 2,4-D by the pEMT3 carrying strains was slightly faster than expected by the model (Fig. 3.7).

DB01T/pJP4 attained a density of 1 x 10^8 CFU ml^{-1}, with the concentration of 2,4-D in the chemostat at 20 μg ml^{-1} for the first 5 days. Then the population rose to 1 x 10^9 CFU ml^{-1} during days 6 to 12, and the 2,4-D concentration decreased to undetectable levels. Following a similar growth profile, DB01T/pEMT3 grew to 1 x 10^8 CFU ml^{-1}, with the concentration of 2,4-D in the chemostat at 20 to 50 μg ml^{-1} for the first 5 days. Eventually, this culture also attained a final density of 8 x 10^8 CFU/ml, and the concentration of 2,4-D in the chemostat decreased to about 5 ppm by day 7. 2,4-D levels gradually decreased to the limit of detection

by days 8 to 12. The growth curves of the *B. cepacia* strains did not follow the growth curves predicted by the model of Monod kinetics. By the time the 2,4-D was depleted to the values predicted in the model, the population sizes also reached the expected values. The reason for this oddity is as yet unknown (Fig. 3.8), but may be a reflection of di-auxic growth where the bacteria degrade 2,4-D to one of the intermediates, and then degrade the intermidiate to carbon dioxide.

DISCUSSION

Ralstonia eutropha and *Burkholderia cepacia* are phylogenetically related bacterial populations who are opportunists and abundant in a wide variety of environments. Their physiology and their genetics are well understood (Cheng et al., 1994; Chakrabarty et al., 1976; Palleroni, 1992; Yabuuchi et al., 1992). Both are nutritionally diverse, *Burkholderia* particularly so, as indicated by the wide range of substrates that can be utilized as a resource. These bacteria are not only limited to the carbon sources encoded within their chromosome(s), but can mutate or acquire additional catabolic capabilities carried on plasmids or other extrachromosomal genetic elements. The occurrence of catabolic genes on insertion sequence (IS) elements and on catabolic plasmids give them the ability to utilize a wide range of substrates, including aromatic compounds.

Many of the catabolic plasmids have a wide host range, and the genes they carry are composed of cassettes and organized in operons that may be independently assembled in different hosts, then rearranged in new hosts, before continuing on to subsequent bacterial populations. The consequence of this promiscuous genetic exchange is a shuffling of possible gene types and plasmids, as well as the possible lateral transfer of chromosomal segments (Fulthorpe et al., 1995; Top et al., 1995; McGowan et al., 1998; Jacoby, 1986; Haugland et al. 1990).

When a bacterial population acquires a new set of catabolic genes, the ability to fully mineralize an additional compound is determined by the host population's innate metabolic capabilities, as well as by the additional catabolic capabilities of the plasmid carrying the degradative genes. Since both of the investigated species are wealthy in terms of their metabolic options, they have a higher probability of acquiring additional metabolic pathways that will result in intermediates that can enter one of the existing central metabolic cycles (e.g. the TCA cycle). My research took advantage of two naturally occurring 2,4-D degradative plasmids, pJP4 and pEMT3, which utilize different sets of degradative alleles. Pure cultures with either of the two plasmids were examined for a range of characteristics. It was hoped that this data, together with what was already known for both species, would help to more accurately predict the outcome of competition experiments among constructed host-plasmid combinations.

Plasmids pJP4 and pEMT3 both benefit their host strains by supplying a catabolic pathway which can convert the herbicide 2,4-D to intermediates of the cell's central metabolism. These conjugative plasmids were both obtained from natural environments and can shuttle between a wide range of bacterial hosts. Plasmids pJP4 and pEMT3 were conjugated into *R. eutropha* JMP228. Plasmid pJP4 was also easily transferred into the recipient strain *B. cepacia* DB01. All of the transconjugants carrying pJP4 exhibited a relatively fast growth rate with 2,4-D. When JMP228R/pEMT3 served as the donor strain for plasmid pEMT3, it was challenging to obtain *B. cepacia* DB01/pEMT3 transconjugants. The low frequency of transfer was surprising since two similar plasmids, pKA2 and pKA4, with nearly identical restriction patterns and 2,4-D gene hybridization profiles, were found in a bacterial strain identified as *B. cepacia*. (Ka et al. 1994). The difficulty in introducing pEMT3 to *B. cepacia* DB01 was further reflected in poorer (slower) growth of DB01/pEMT3 transconjugants, while utilizing the degradative pathway from the plasmid. In addition, when pEMT3 was isolated from the transconjugants, in more than in more than half of the preparations additional restriction fragments were visible after gel electrophoretic separation (Fig. 3.1, lanes, 14-16 and 20). The unusual restriction profiles were in agreement with previous research supporting the high levels of genomic plasticity in *B. cepacia* populations (Hendrickson et

al., 1996). Interestingly, this level of rearrangement was not observed when inserting pJP4 into the *Burkholderia* strains..

The different plasmids confer different growth rates to a host population when 2,4-D is supplied as the limiting substrate. These genes, however, may also have other effects and influence growth and reproductive success of these strains. A relevant factor affecting growth rate, and therefore competitive outcome, is the substrate concentration.

Theoretically, a medium to which a substrate is added that allows the population to grow at the maximum growth rate would favor a population with the faster growth rate. This strategy is typically used to enrich for populations exhibiting high growth rates, and has been observed in several experiments, including those involving bacteria isolated from environmental (Fulthorpe et al., 1994), domestic and heavily polluted sites such an alkaline salt lake in Oregon (Maltseva and Oriel, 1997) or sewage sludge (Greer et al., 1990). The diversity of 2,4-D degradative alleles that are recovered from a site initially was thought to be low, however, this number increased significantly over the past decade. When soils were challenged with the frequent application of 2,4-D, particular microbial populations and degradative gene mosaics become enriched and dominant. Several different investigators have added 2,4-D to untreated soils in proximity to those that were treated regularly with 2,4-D. Enrichment studies using materials from these sites yielded different dominant species despite enrichment in a similar

fashion. Experiments utilizing high concentrations of 2,4-D (>50 μg ml^{-1}) usually resulted in the enrichment of populations exhibiting high growth rates and carrying plasmids of the pJP4 type. Typically, in enrichments with high 2,4-D concentrations members of the genera *Alcaligenes*, *Ralstonia* and *Burkholderia* will become dominant (Fulthorphe, 1995, McGowan, 1998).

Ka and Holben found that a *Sphingomonas* species became dominant in agricultural soils that were repeatedly treated with 2,4-D (Ka et al., 1994). Asuming-Brempong (1998) showed that a *B. cepacia* strain became dominant in agricultural soils with repeated 2,4-D application. In the cases where β–proteobacteria predominated, they tended to carry pJP4-like plasmids and genes. In soil microcosms and field studies, frequently identified isolates often cluster within the β–proteobacteria, largely belonging to the genera *Burkholderia* or *Ralstonia* (Fulthorpe et al., 1995).

Enrichment with low concentrations of 2,4-D shows a very different profile of the 2,4-D degrading community. Interestingly, Dunbar et al. found that the method of strain isolation correlated with the number of different bacterial species and 2,4-D degradative alleles that were recovered from a site. This study also presented evidence that enrichment-independent, direct isolation methods resulted in a much greater diversity of 2,4-D degrading populations. Furthermore, these organisms belonged to a

variety of taxonomic groups with a majority of their 2,4-D alleles being different from those of the frequently studied plasmid pJP4 (Dunbar et al., 1997).

It has also been recognized that soils without direct application of the herbicide 2,4-D yield many populations capable of degrading 2,4-D. These organisms frequently harbor genes involved in 2,4-D degradation that differ from the commonly found pJP4-like genes (Dunbar et al., 1997; Kamagata et al., 1997). Interestingly, pJP4-like and non-pJP4-like genes seem to coexist in environments that have not been subject to enrichment of certain populations due to the application of 2,4-D for agricultural purposes. Supplying a nutrient, such as 2,4-D, skews the population toward β–proteobacteria and genes of the pJP4 type.

Growth rate, however, is not the sole factor in determining how well a population will perform under different growth conditions. Frequently, environmental conditions are such that organisms spend much of their existence deprived of resources, and must eke out a living either on very low concentrations of a substrate, or have a means of avoiding starvation. This study showed that strains of *R. eutropha* and *B. cepacia* performed similarly in terms of growth rates when grown with succinate. The presence of the catabolic plasmid did not affect the growth rate relative to the plasmid free strain. In contrast, plasmid-harboring strains of the same species showed a much larger difference in the growth rates when grown

with 2,4-D. Under high substrate concentration conditions, greater than 50 ppm, the *R. eutropha* strains always grew faster and *B. cepacia*. This high growth rate at high substrate concentrations occurred at a cost of lesser efficiency at the lower concentrations. While this evidence is not conclusive, the results of the ^{14}C distribution experiment suggest that at less than 1 ppm 2,4-D, *B. cepacia* is more efficient at low resource concentrations. This observation is further supported by the chemostat findings, which showed that the *B. cepacia* populations, carrying either plasmid (pJP4 or pEMT3), attained population levels higher than those of *R. eutropha* under resource-limited conditions

ACKNOWLEDGMENTS

This research was funded by a grant from the National Science Foundation (BIR 912006)

REFERENCES

Ballard, R.W., N.J. Palleroni, M. Doudoroff, R.Y. Stanier, and M. Mandel. 1970. Taxonoy of the aerobic *Pseudomonads*: *Pseudomonas cepacia, P. marginata, P. allicola* and *P. caryophylli.* J. Gen. Microbiol. 60:199-214.

Burkholder, W.H. . 1950. Sour skin, a bacterial rot of onion bulbs. Phytopahtyology. 40:115-117.

Burns, J.L., L.A. Hedin, and D.M. Lien. 1989. Chloramphenicol resistance in *Pseudomonas cepacia* because of decreased permeability. Antimicrobial agents and Chemotherapy 33:136-141.

Chakrabarty, A.M. 1976. Plasmids in Pseudomonas. Annual Review of Genetics 10:7-30.

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

Daubaras, D.L., C.D. Hershberger, K. Kitano, and A.M. Chakrabarty. 1995. Sequence analysis of a gene cluster involved in metabolism of 2,4-5-trichlorophenoxyacetic acid by *Burkholderia cepacia* AC1100. Appl. Envrion. Microbiol. 60:4100-4106

Davis, D. H., M. Doudoroff, R.Y. Stanier, and M. Mandel. 1969. Proposal to reject the genus Hydrogenomonas: Taxonomidc implications. Int. J. Bacteriol. 19:375-390.

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

Dunbar, J., S. White, and L. J. Forney. 1997. Genetic Diversity through the looking glass: effect of enrichment bias. Appl. Environ. Microbiol.63:1326-1331.

Dykhuizen, D. E. and D. L. Hartl, 1983. Selection in chemostats Microbiol. Rev. 47:150-168.

Fulthorpe, R.R., C. Mcgowan, O.V. Maltseva, W.E. Holben, and J.M. Tiedje. 1995 2,4-Dichlorophenoxyacetic acid-degrading bacteria contain mosaics of catabolic genes. Appl. Environ. Microbiol. 61:3274-3281

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

Haugland, R.A.. U.M.X. Sangodkar, and A.M. Chakrabarty. 1990. Repeated sequences including RS1100 from *Pseudomonas cepacia* AC1100 function as IS elements. Mol. Gen. Genet. 220:222-228

Hendrickson, W, A. Hübner, and A. Kavanaugh-Black. 1996. Chromosome multiplicity in *Burkholderia cepacia* . pp. 259-269. In: Nakazawa, T, K. Furukawa, D. Haas, and S. Silver (eds). Molecular Biology of Pseudomonads. Washington, DC. Proceedings of the fifth international symposium on Pseudomonads. American Society of Microbiology

Isles, A., I. Maclusky, M. Corey, R. Gold, C. Proper, P. Fleming and H. Levison. 1984. *Pseudomonas cepacia* infection in cystic fibrosis: and emerging problem. Journal of Pediatrics. 104: 206-210

Jacoby, G.A. 1986. Resistance plasmids of *Pseudomonas*. P 265-293. In: Sokatch .J.R. (ed.), The bacteria, volume 10, The biology of *Pseudomonas*. Orlando: Academic Press.

Ka, J.O. ,W.E. Holben, and J.M. Tiedje. 1994. Analysis of Competition in Soil among 2,4-dichlorophenoxyacetic acid-degrading bacteria. Appl. Environ. Microbiol. 60:1121-1128

Kamagata, Y., R.R. Fulthorpe, K. Tamura, H. Takami, L. J. Forney and J.M. Tiedje. 1997. Pristine environments harbor a new group of oligotrophic 2,4-dichlorophenoxyacetic acid-degrading bacteria. Appl. Enviorn. Microbiol. 63: 2266-2272.

Keyser, P, B.F., Pujar, R. W. Eaton, and D.W. Ribbons. 1976. Biodegradation of the phthalates and their esters by bacteria. Environ. Health Perspect. 18:159-166

Lewis, D.M., E.S.P. Bloomfield, and L. R. Barran. 1987) Effect of rifampicin resistance on nodulating competitiveness of *Rhizobium meliloti.* Can. J. Microbiol. 33:343-345.

Löffler, F.E., J.E. Champine, K.M. Ritalahti, S.J. Sprague adn J.M. Tiedje. 1997. Complete reductive dechlorination of 1,2-dichloropropane by anaerobic bacteria. Appl. Environ. Microbiol. 63:2870-2875.

Maltseva, O. And P. Oriel. 1997. Monitoring of an alkaline 2,4,6-trichlorophenol-degrading enrichment culture by DNA fingerprinting methods and isolation of the responsible organism, haloalkaliphilic *Nocardioides* sp. Strain M6. Appl. Environ. Microbiol. 63:4145-4149.

McGowan, C., R. Fulthorpe, A. Wright, and J.M. Tiedje. 1998. Evidence for interspecies gene transfer in the evolution of 2,4-dichlorophenoxyacetic acid . Appl. Environ. Microbiol. 64:4089-4092.

Monod, J. 1949. The growth of bacterial cultures. Annu. Rev. Microbiol. 3:371-394.

Palleroni, N.J. 1992a. Introduction to the *Pseudomonads*. pp 3071-3085. In: J. Balows, A, H.G., Trüper, M. Dworkin, W. Harder, K-H Schleifer (eds.), *The Prokaryotes*: *A Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications.* 2nd ed. New York. Springer-Verlag,

Palleroni, N.J. 1992b. Present situation in the taxonomy of aerobic pseudomonads. pp 105-115. In: Galli, E., S. Silver, and B. Withold (eds.), *Psudomonads: Molecular Bioloty and Biotechnology.* Washington DC. American Society for Microbiology,

Palleroni, N.J. and B. Holmes. 1981. *Pseudomonas cepacia* sp. nov.,nom. rev. International Journal of Systematic Bacteriology. 31:479-481.

Top E.M., W.E. Holben and L.J. Forney (1995) Characterization of diverse 2,4-Dichlorophenoxyacetic acid-degrading plasmids isolated from soil by complementation. Appl. Environ. Microbiol. 61:3274-3281

Stanier R.Y., N.J. Palleroni, and M. Doudoroff, 1966. The aerobic pseudomonads: a taxonomic study J. Gen. Microbiol. 43: 159-271.

Tabacchioni, S., P. Visca, L. Chiarini, jA. Bevivino, C. Di Serio, S. Fancelli, and R. Fani. 1995. Molecular characterization of rhizosphere and clinical isolates of *Burkholderia cepacia*. Res. Microbiol. 146:531-542

Wyndham R.C. 1986. Evolved aniline catabolism in *Acinetobacter calcoaceticus* during continuous culture of river water. Appl. Environ Microbiol. 51:781-789

Yabuuchi E., Y. Kosako, H. Oyaizy, I. Yano , H. Hotta, and Y. Nishiuchi. 1992. Transfer of two *Burkholderia* and an *Alcaligenes* species to *Ralstonia* gen. nov. : Proposal for *Ralstonia picketii* . (Ralston, Palleroni, Doudoroff 1973) comb. nov., *Ralstonia solanacerum* (Smith 1896) comb. nov. and *Ralstonia eutropha* (Davis 1969). comb. nov. Microbiol. Immunol. 39:897-904.

CHAPTER 4

Two Hosts and Two Plasmids in Simple Environments: The influence of 2,4-D Degradative Plasmids on the Competitive Fitness of a Host Strain

ABSTRACT

The contribution of different plasmid-encoded degradative alleles to the competitive fitness of two host populations was investigated. Natural variation in the catabolic gene cassettes for the degradation of aromatic compounds has resulted in the evolution of different, but functionally similar degradation pathways for the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D). Frequently, these degradation pathways are carried on broad host range plasmids that are horizontally transferred among a variety of bacterial populations. In this study we utilized two such plasmids, pJP4 and pEMT3, that confer the ability to utilize 2,4-D upon two bacterial host species, *Ralstonia eutropha* JMP228 and *Burkholderia cepacia* DB01. Each of the plasmids has a similar replication origin and belongs to the Inc-P incompatibility group. The plasmids have different alleles for the catabolism of 2,4-D. Pure cultures were characterized by maximum growth rates and $K_{S(2,4\text{-}D)}$, as described in the previous chapter. The values for these

two parameters for each host/plasmid combination were used to predict the outcome of competition experiments performed under batch and chemostat conditions with 2,4-D as the limiting resource. Pairwise combinations of the two different bacterial species , *R. eutropha* JMP228 and *B. cepacia* DB01, carrying the two different plasmids, pJP4 or pEMT3, were carried out in the two culture conditions. In Chapter 3, it was determined that the fastest growth and most rapid 2,4-D degradation were observed for the JMP228/pJP4 combination, followed by DB01/pJP4, and JMP228/pEMT3. Population DB01/pEMT3 grew the slowest. There was no measured difference in the K_S values as determined for any of the strain/plasmid combinations. Hence, in batch culture competitions where resources were replenished periodically at a high concentration, a faster growth rate should lead to dominance, and thus the following order of dominance was predicted: JMP228/pJP4 > DB01/pJP4 > JMP228/pEMT3 > DB01/pEMT3. As expected, possessing plasmid pJP4 was advantageous, as it offered a faster growth rate than pEMT3, and the expected order of dominance was observed. In chemostat experiments conducted at a very low dilution rate of 0.03 h^{-1}, it was also expected that the faster growth rate should predominate if the populations have the same K_S. The host/plasmid combinations should reflect the same rank order as the batch competitions.

Surprisingly, the following order of dominance was observed: DB01/pJP4 ≥ DB01/pEMT3 > JMP228/pJP4 = JMP228/pEMT3. This result was unexpected. When the substrate was supplied at constant but low concentrations, *Burkholderia*, rather than *Ralstonia* predominated, regardless of the plasmid. Possible causes for the different dominance patterns under the two growth conditions are discussed, as are possible ecological implications for maintaining different catabolic plasmids and host species in the environmental gene pool.

INTRODUCTION

Among bacterial populations, most genes are transferred vertically, from "mother" to "daughter". The chromosomal genes encode for all of the basic requirements of the cell, and determine much of the population's physiological diversity. Additional characteristics to expand a population's substrate range or genetic diversity may reside on extrachromosomal elements, such as conjugative plasmids, that are transferred horizontally and vertically. Naturally occurring broad host range plasmids have been identified, and can carry genes for metabolic functions such as the degradation of aromatic compounds and resistance to heavy metals or

antibiotics. Plasmids that carry pathways for 2,4-D, 3-chlorobenzoate, napthalene and toluene utilization have been isolated, and their horizontal transfer by conjugation has been demonstrated (Di Giovanni et al., 1996; Ravatin et al., 1998; Herrick et al., 1997; Wright et al., 1994). For a degradation pathway involving any of these compounds, there exists some amount of inherent variation in the gene sequences or pathways (Ghosal and You, 1988; Koh et al., 1997).

For example, in order to mineralize phenol to carbon dioxide and to use it as a source of carbon and energy, a bacterial population may utilize a catabolic pathway that hydroxylates phenol to catechol followed by an ring-cleavage event to form muconic acid (Rochind-Dubinsky et al., 1987; Häggblom et al., 1992). There may be several alleles for each of the genes involved in phenol degradation. Different enzymes may have slightly different substrate specificities and different kinetic constants (V_{max} and K_m). Thus, one set of enzymes may prefer chlorinated substrates, while another prefers the non-substituted analogues, but both have the capacity to degrade either, albeit with different kinetics. When required to grow by utilizing one substrate as a sole carbon source, a population will do so by means of whatever suitable pathway it may possess.

The products of those different degradation pathways enter into the organism's intermediary metabolic pathways, where they are used for generating energy and biomass. The ability to utilize additional carbon sources has obvious advantages. Hence, plasmid-encoded and chromosomally-encoded genes contribute to the reproductive success of the population capable of utilizing the new resource. Figure 4.1 illustrates the integration of plasmid-encoded and chromosomally-encoded genes when 2,4-D is utilized as a carbon source. The portion of the pathway depicted in gray is plasmid encoded, and determines the rate of metabolizing the substrate (2,4-D) into intermediates, in this case chloromaleylacetate (CMA), that can be channeled into the organism's intermediary metabolism. The host bacterium's genome encodes for the transformation of CMA to succinate, a common intermediate of central metabolism. Consequently, what we measure is reproductive success: which population leaves more offspring, and why? Is greater success due to the genes of the chromosome, or those of the plasmid?

For pure cultures we can use growth rate and the half-saturation constant (K_S) as indicators of reproductive success. In competition studies, the more successful competitor is the one that achieves numerical dominance.

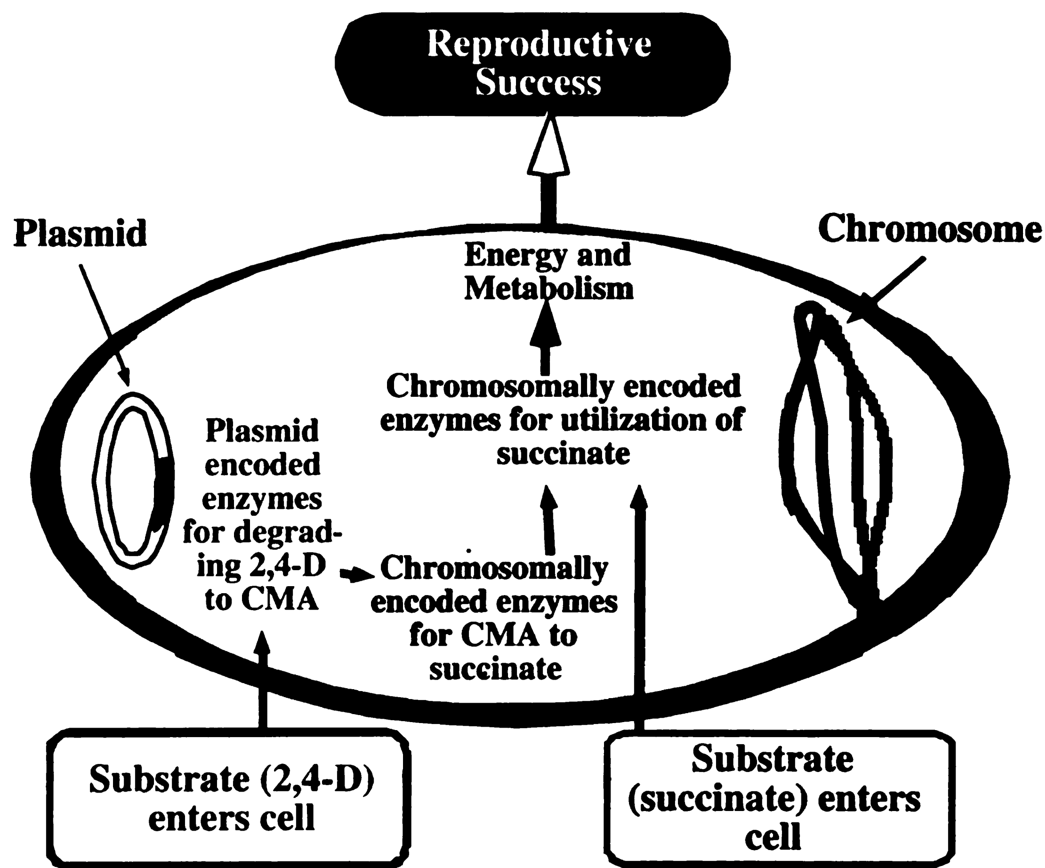


Figure 4.1. An overview of the genetic contribution of different genes in the competition experiments. Black regions are encoded by the bacterial chromosome, the gray portions are controlled by the alleles for the 2,4-D degradative pathway that are encoded on the plasmid.

Bacteria in the environment may compete for a limiting resource and the population that can most rapidly utilize the resource tends to become enriched. The enrichment effect, in which the fastest growing phenotype becomes dominant, is commonly observed during the enrichment and isolation of bacteria with the ability to degrade recalcitrant substrates (Dunbar et al., 1996; Dunbar et al., 1997; Fulthorpe et al., 1995). The observations by Dunbar et al. (1997) correlated 2,4-D application with enrichment for the pJP4-like genotype among several different bacterial populations. As a result of enrichment and isolation techniques, it was initially believed that the bacterial utilization of 2,4-D proceeded through a very conserved and related set of catabolic genes. The genes encoded by plasmid pJP4 were chosen to represent the model system to which newly isolated degradative genes were compared, since they represented the most frequently described set of 2,4-D utilization genes. Over time, it was discovered that when alternative isolation methods were utilized, the environment held a greater diversity of catabolic pathways that degraded this herbicide (Ka. and Tiedje, 1994; Top et al., 1995). The emerging trend was that of a high natural diversity in catabolic plasmids, 2,4-D degradation genes, and suitable populations capable of metabolizing 2,4-D.

Plasmids pJP4 and pEMT3 were chosen for this investigation, because both encode a pathway for 2,4-D degradation, however, they carry different alleles. Residing on pJP4 are genes which encode for the most extensively studied 2,4-D degradative pathway; they encode for six enzymes that sequentially break down 2,4-D to chloromaleylacetate (Don and Pemberton, 1981; Don and Pemberton, 1985; Don et al., 1985). The canonical pathway from plasmid pJP4 is described in Chapter 1. Although the first enzyme of the pathway (*tfd*A) is homologous for both plasmids, pEMT3 carries a different set of 2,4-D gene alleles for the *tfd*CDFE operon and for the *tfd*B gene. Host strains carrying pEMT3 show slower growth rates when 2,4-D is supplied as a sole source of carbon and energy. However, the genes of pEMT3 encode for enzymes with a higher activity toward non-chlorinated substrates than the enzymes encoded on pJP4 (Maltseva 1994).

Ralstonia eutropha JMP228 and *Burkholderia cepacia* DB01 served as hosts for the two plasmids, pJP4 or pEMT3, described above (Don and Pemberton, 1981; Top et al., 1995; Ka et al., 1994). Both bacterial species are members of the β-proteobacteria, and have been shown to degrade 2,4-D when they acquire the upper 2,4-D degradation pathway on a catabolic plasmid (Ka et al., 1994; Top et al., 1995). This study evaluated the contribution of the two different plasmids and their allelic genes encoding

for the enzymes involved in 2,4-D catabolism, to the fitness of their host strains in two simple habitats (batch culture versus chemostat). The physiological characteristics of the different strain/plasmid combinations are described in Chapter 3. In high nutrient environments, the presence of plasmid pJP4 resulted in a higher growth rate in both host strains and outcompeted the same strain carrying pEMT3. When both hosts had the same plasmid, *Ralstonia eutropha* had a higher growth rate on 2,4-D than *Burkholderia cepacia*. There was no measured difference in the K_S for any of the four host/plasmid combinations.

Batch conditions represent a high nutrient environment selecting for fast growth-rate, whereas chemostats simulate a constant, low nutrient environment that selects for a lower K_S. Based on models of substrate limited growth in the batch cultures, if abundant 2,4-D is supplied, populations should grow at their maximal rate until 2,4-D is depleted. If a portion of this culture is transferred to fresh medium, the population that had grown to a larger size should again produce more offspring in a successive spurt of growth, leading to an ever widening chasm in population sizes. A stable coexistence could be maintained if the population that grew more slowly at the high substrate concentration could grow more rapidly after the substrate was nearly exhausted. While it may not become dominant, it

would not become extinct. From these theoretical considerations it was predicted that the strain with the faster growth rate would dominate. In competition experiments with the same bacterial species with either of the two plasmids, pJP4 and pEMT3, it was expected that the strain with pJP4 would dominate, since this plasmid bestows a higher growth rate on its host.

Under continuous flow chemostat conditions, a high maximum growth rate is desirable, but in addition, the affinity of the organisms to the limiting resource becomes relevant. Populations with a lower half saturation constant (K_S) should outcompete populations with lower affinities to the limiting resource. Using the substrate limited growth kinetics described by Monod (1949), the following predictions can be made. First we assert that the natural logarithm of the ratio of the competing populations (Ln (N_A/N_B)) plotted against time results in a line with the slope representing the rate of difference at which the populations grow. N_A and N_B represent the population sizes of population A and B respectively. A positive slope indicates that population A became numerically dominant, and a negative slope indicates dominance by population B. Next consideration is given to the influence of substrate concentration on the Monod equation (Chapter 3). If the substrate is present at a concentration much higher than the K_S of either competitor then the population with the higher maximum growth rate

should predominate (slope = $\mu_{max\,A}$ - $\mu_{max\,B}$). If the substrate concentration in the chemostat equals the K_S for the strains, then the slope of the line created by (Ln (N_A/N_B)) = 1/2 ($\mu_{max\,A}$ - $\mu_{max\,B}$), and again the population with the faster growth rate should win. In the third case, when the substrate is present at concentrations well below the K_S of the strains, then it is the ratio of the μ_{max}/K_S for each strain that determines fitness, so the slope becomes ($\mu_{max\,A}$ / $K_{S\,A}$)- ($\mu_{max\,B}$ / $K_{S\,B}$), and therefore the strain with the lower value for K_S should have the long term advantage. In this system, it was expected that the pJP4 bearing populations predominate over the pEMT3 bearing strains, since the measured K_S for the four populations did not differ. To predict the outcome in competition experiments, hypothetical curves for each competitor using the Monod model were generated, and average growth rate and K_S values for each pure culture host/plasmid combination were determined. By comparing modeled growth curves to our observed outcomes of competition, we describe the contribution of the two 2,4-D degradative plasmids with different alleles to the fitness of the two host populations under two contrasting environmental conditions.

MATERIALS AND METHODS

Bacterial strains. *Ralstonia eutropha* JMP228 (JMP228), the pJP4 cured strain of *R. eutropha* JMP134 (Don and Pemberton, 1981) and *Burkholderia cepacia* DB01 (Ka et al., 1994) were used as the recipient host strains for the two 2,4-D degradative plasmids. Descriptions of the plasmids and the construction of the transconjugants used in the competition experiments are described in Chapter 3. Strain characteristics are summarized in table 4.1

Table 4.1. Pure culture characteristics for strains grown on mineral salts medium with succinate or 2,4-D under batch culture conditions.

strain	Succinate, $\mu \pm SD$[a]	2,4-D $\mu \pm SD$	K_S (2,4-D) (mg L^{-1})	Yield[b] (CFU g^{-1})
JMP228R/pJP4	0.538 ±0.082	0.223 ± 0.056	1.2	$3.7x10^6$
JMP228S/pJP4	0.456 ±0.031	0.238 ± 0.050	nd	nd
JMP228R/pEMT3	0.371 ±0.020	0.094 ± 0.014	0.8	$3.7x10^6$
JMP228S/pEMT3	0.509 ±0.054	0.106 ± 0.014	nd	nd
DB01R/pJP4	0.489 ±0.160	0.159 ± 0.038	1.0	$3.4x10^6$
DB01T/pJP4	0.534 ±0.228	0.152 ± 0.039	nd	nd
DB01R/pEMT3	nd	0.085 ± 0.027	1.2	$3.5x10^6$
DB01T/pEMT3	nd	0.075 ± 0.028	nd	nd

[a] SD = Standard deviation

[b] Yield is the number of colony forming units per gram of 2,4-D.

Cell cultivation. Cells were grown in A+N mineral salts medium to which carbon sources and antibiotics were added (Wyndham et al., 1986). The preparation of A+N medium was described previously for the pure culture competitions in Chapter 3. For each independent experiment, cultures were started from a loopful of frozen culture stock. The cell cultures were grown to late log phase in 16-ml test tubes with 3 ml A+N minimal amended with 2,4-D (200 ppm) and the appropriate antibiotic (rifampicin, streptomycin or trimethoprim). A 2.5% (vol/vol) inoculum to fresh A+N medium with succinate (5 mM) or 2,4-D (200 ppm) was used to start any other culture used in the competition experiments. Population identification in the competition experiments was carried out using 2,4-D minimal medium plates supplemented with antibiotics. Additionally, morphological differences in colony types on R2A agar could distinguish between *Ralstonia* and *Burkholderia* species.

2,4-D concentration. 2,4-D concentration was determined using High Performance Liquid Chromatography (HPLC), as described in Chapter 3.

Batch culture competition experiments. Cultures were initially grown to late log phase in minimal medium with succinate (5 mM) or 2,4-D (200 ppm), depending on whether the competition would take place in medium amended with succinate or 2,4-D. Competitive pairs consisted of pair-wise combinations of strains with different antibiotic susceptibilities carrying either plasmid pJP4 or pEMT3. Competitions with succinate were used to determine if plasmids pJP4 and pEMT3 affected the competitive ability of JMP228 when the plasmid was not required for growth. Succinate was also used to determine which host, JMP228 or DB01, would become dominant in the batch environment. Competition experiments using 2,4-D as the limiting resource were much more exhaustive. Reciprocal mating pairs between each of the antibiotic resistant strains were used to account for the effect of the chromosomal marker, rifampicin versus streptomycin and rifampicin versus trimethoprim for JMP228 and DB01 respectively. Cells were harvested by centrifugation, rinsed with sterile medium, and suspended to an optical density of 0.1, in minimal medium without 2,4-D. To determine if ten-fold differences in the initial ratio of competitors affected the outcome, 1:10, 1:1 and 10:1 ratios of the populations were combined. The CFU/ml was determined on selective medium prior to inoculating (10 % [vol/vol])

triplicate cultures with the mixture in 2 ml minimal medium with 300 ppm 2,4-D. Alternating wells of a 24-well microtiter plate were used to perform many replicates while avoiding cross-contamination. The cultures were incubated at 30°C in a rotary shaker (200 rpm). Every 24 hours, 10% of the culture volume was transferred to fresh medium, as well as diluted in PBS, and plated to selective medium for determining viable bacterial population sizes. Additional inter-species competition experiments were carried out with equal numbers of starting populations of the competing strains. Instead of transferring the culture every 24 hours, 10% (vol/vol) of the culture was transferred every 72 hours. With a longer time allotted for growth, slower growing strains were able to complete their entire growth cycle and use all the available 2,4-D. Competitive pairs and treatments are listed in table 4.2.

Table 4.2. Competition experiments

Competition	Strain 1	Strain 2	Batch competition Succinate 24 h	Batch competition 2,4-D 24 h	Batch competition 2,4-D 72 h	Chemostat
Same strain, Same plasmid	JMP228R/ pJP4	JMP228S/ pJP4	nd [c]	3[a]	nd	nd
	JMP228R/ pEMT3	JMP228S/ pEMT3	nd	3	nd	nd
	DB01R/ pJP4	DB01T/ pJP4	nd	3	nd	1
	DB01R/ pEMT3	DB01T/ pEMT3	nd	3	nd	nd
Same strain, Different. plasmid	JMP228/ pJP4	JMP228/ pEMT3	8	6[b]	nd	3
	DB01/ pJP4	DB01/ pEMT3	nd	9[b]	nd	2
Different. strain, Same plasmid	JMP228/ pJP4	DB01/ pJP4	8	6[b]	3	2
	JMP228/ pEMT3	DB01/ pEMT3	8	9[b]	3	2
Different strain, Different. plasmid	JMP228/ pJP4	DB01/ pEMT3	nd	9[b]	3	2
	JMP228/ pEMT3	DB01/ pJP4	nd	6[b]	3	2

[a] number of replicates.
[b] the two antibiotic resistant populations bearing the same plasmids are being considered as the same population, and the marker designation has been omitted.
[c] not done

The dominant strain can be detected using the natural log (Ln) of the ratio of population sizes (Malthusian parameters) plotted over time. The resulting curve represents the rate of difference at which the populations grow. The slope of the line away from zero indicates dominance by one of the populations (Lenski et al., 1992).

Chemostat Competition Experiments. Chemostats based upon the design of Chao et al. (1977) were used, and were described in Chapter 2. Cultures were initially grown as described for the batch competition experiments in 200 ml of A+N minimal salts medium with 200 μg ml^{-1} 2,4-D as the sole carbon source. When the cultures attained late-log phase, which correlated to an optical density of about 0.1 at 600 nm, the cells were gently collected by centrifugation (4,000 rpm for 20 min in a Sorvall SS34 rotor). After rinsing with A+N minimal medium, cultures were suspended to an OD of 0.1 in A+N medium containing 200 μg ml^{-1} 2,4-D. Initially, 37.5 ml of each competitor population was combined in the chemostat, to give approximately the same total number of cells per vessel as in pure culture controls (Chapter 3). 2,4-D was added to an initial concentration of 200 μg ml^{-1}. Each chemostat was sampled and incubated for 8 hours at 30° C before a constant flow of medium was begun. The dilution rate was set to 1 volume exchange (75 ml) per day (3 ml h^{-1}) with a reservoir 2,4-D concentration of

200 μg ml^{-1}. Chemostats were operated for 10 to 12 days. Under these flow conditions, the populations should all grow at doubling times of 17.5 hours. To sample the chemostats, the ports were rinsed with 70% ethanol. Samples (1 ml) were removed with a plastic syringe, and the 2,4-D concentrations, OD_{600} and the CFU/ml of each competing strain were determined. Additional 1-ml samples were withdrawn from each chemostat to determine the CFU/ml over time. A+N agar with 200 μg ml^{-1} 2,4-D and the appropriate selective antibiotics was used to count the population sizes of the competing bacterial strains. In addition, the CFU/ml were also determined on R2A agar amended with the appropriate antibiotics. R2A agar without antibiotics was used to monitor for possible contaminants. As with the batch competition experiments, the Malthusian parameter of the competing strains was plotted over time to determine the relative fitness as described by Lenski et al., (1992). Competitive pairs and treatments are listed in Table 4.2.

Modeling the Monod equation. In order to evaluate the outcome of the chemostat competitions, the expected population sizes and 2,4-D concentrations for each combination of competitors were estimated using the Monod equation. Models were run using the Stella Research Software v 5.0 (High Performance Systems Inc. Hanover, NH). Values determined for each host/plasmid combination were inserted into the model. The resulting curves

give the predicted population size for each competitor, given that the growth rate, growth yield, and K_S are known for each strain. The Monod equation for each strain is shown in equation (a). The Monod equation describing competition in chemostats was previously described by Dykhuizen and Hartl (1983). dN/dt is the rate of change in population size, N is the population size, μ_{max} is the maximum growth rate on the resource, and S is the substrate concentration. K_S is the half-saturation constant, a value that describes the concentration of resource at which the growth rate is one half the maximum. D is the dilution rate of a chemostat. In a chemostat, where continuous growth is the goal, the dilution rate (D) is the rate at which culture volume and cells are removed with the equivalent input of fresh media. The dilution rate D determines the growth rate of the bacterial population in a chemostat, and equals the growth rate.

Equation (a)

$$\frac{dN}{dt} = N \mu_{max} \frac{S}{K_S + S} - DN$$

Considering there are two competitor populations, we can set up the equation for each population A and B, and call their population sizes N_A and N_B. The values for the μ_{max}, K_S and efficiency are run through a model. Equations (b) describe the growth of population A and population B.

Equations (b)

$$\frac{dN_A}{dt} = N_A \, \mu_{max(A)} \frac{S}{K_{S(A)}+S} - DN_A$$

and

$$\frac{dN_B}{dt} = N_B \, \mu_{max(B)} \frac{S}{K_{S(B)}+S} - DN_B$$

For each competitor there is a value for S, the substrate concentration, at which the cell obtains just enough energy to avoid being washed out of the chemostat. The winner of competition, then, is the strain with a lower break-even concentration of substrate. Populations that obtain enough energy to replace their cell numbers are not washed out, and cells that starve (do not make sufficient replacement numbers) are washed out. The break-even concentration (S*) is the same value as the resource threshold value (R*) described in Tilman's resource ratio theory discussed in Chapter 1 (Tilman 1981). If one of the populations is able to reduce the level of resource in the chemostat to below the break-even concentration of its competitor, the competitor will be diluted out. For each strain the break-even concentration can be calculated using equation (c).

Equation (c)

$$S^* = \frac{DK_{S(A)}}{\mu_{max\,(A)} - D}$$

The lower S* can be achieved in two main ways. If both strains have a very similar K_S, then the population with the faster growth rate will have the lower break-even concentration for the substrate, and the strain with the lower growth rate will be diluted out. On the other hand, if both populations have a very similar μ_{max}, then the population with the lower K_S value should attain the lower value for S*. In this way, a population with a lower growth rate could become dominant under low substrate concentrations, and it would become extinct in a high growth rate selective environment.

Similar to the batch culture competitions, the natural log of the ratio of the population sizes was determined during the course of the experiment. The natural logarithms of the ratio of the two populations (Malthusian parameters) of the competing strains were plotted over time to determine their relative fitness (Lenski 1992). A significant deviation in the slope of this line away from zero indicated that one strain had become competitively dominant to another. Using the equations in (b) the slope of the line can be described by the equations in (d) and (e). Equation (d) is the slope of the

line as represented by rearranging the equations in (b) as the rate of growth of the population sizes.

Equation (d)

$$\frac{dN_A}{N_A\,dt} - \frac{dN_B}{N_B\,dt}$$

The same equation can be represented in Equation (e) using the other half of equation (b). By substituting values for K_S and μ_{max}, for the two different populations in equation (d), the slope of the line can be described under three widely different substrate (S) concentrations.

Equation (e)

$$\mu_{max(A)}\frac{S}{K_{S(A)}+S} - \mu_{max(B)}\frac{S}{K_{S(B)}+S}$$

By making the value of S smaller than the K_S, the half saturation constant becomes the main determinant of the slope of the line. In the first case stated below in equations (f), the population with the lowest value of K_S will be the one that determines the direction of the slope in its favor. For the second case, we find that if S is sufficiently larger than K_S, then the half-saturation constant can be ignored, and the population with the higher

growth rate controls the direction of the slope of the line. Even when the value of S is nearly equal to the values of K_S for the two populations, the growth rate still determines the slope of the line, although it will be less steep.

Equations (f)

If $S \ll K_{S(A)}$ and $K_{S(B)}$

$$\text{slope} = \left(\frac{\mu_{max(A)}}{K_{S(A)}} - \frac{\mu_{max(B)}}{K_{S(B)}} \right) S$$

If $S \gg K_{S(A)}$ and $K_{S(B)}$

$$\text{slope} = \mu_{max(A)} - \mu_{max(B)}$$

If $S \sim K_{S(A)}$ and $K_{S(B)}$

$$\text{slope} = 1/2(\mu_{max(A)} - \mu_{max(B)})$$

Toxicity of DB01 chemostat filtrate. To determine if the reason that DB01 became dominant in chemostats, the possibility of the production of an inhibitory compound toward JMP228 was briefly explored. At the conclusion of one chemostat experiment, the vessels containing pure cultures of DB01/pJP4 and DB01/pEMT3 were each filtered through a 0.22 µm filter to obtain two cell-free spent media stocks. A dilution series of the spent media and phosphate buffered saline were prepared, so that final concentrations of the spent media were 100 %, 75 %, 50 %, 25 % and 0% by

volume. 2,4-D (100 ppm) was added to each dilution. Three ml of each dilution was put into three 16-ml test tubes per susceptibility test (JMP228/pJP4 and JMP228/pEMT3). 200 μl of chemostat grown cultures of the two strains of *R. eutropha* were inoculated into each tube, and the tubes were incubated in a rotary shaker (30 °C, 200 rpm, 24hours). The final optical density of each tube was recorded spectrophotometrically, and a decrease in turbidity with an increase in the concentration of filtrate would warrant further investigation into the presence of growth inhibiting compounds produced by DB01. No difference in the turbidity would indicate that there was little possibility of inhibitor production by DB01.

RESULTS

Succinate batch competitions. Competitions in minimal medium supplemented with succinate, indicated that for host strain JMP228, there was a greater difference in the effect of the antibiotic resistance marker, rifampicin or streptomycin, than the catabolic plasmid (pJP4 or pEMT3) in 24 hour batch transfer environments. In either reciprocal combination of JMP228R/pJP4 vs JMP228S/pEMT3 (four replicates), or JMP228S/pJP4 vs JMP228R/pEMT3 (four replicates), the two host populations coexisted at a summed population size of about 1 x 10^9 CFU ml^{-1}. The total population

size was equivalent to the pure culture's population sizes when grown alone under these conditions. Figure 4.2 shows the average population sizes of the two reciprocal sets of competitors

The slope of the regression line (-0.0042) drawn through the points derived from the Malthusian parameter, did not deviate significantly from zero, as indicated by a very poor r^2 value when all data were considered together ($r^2 = 0.0155$) (Fig. 4.3). On the other hand, there was always a slight, but significant difference in slope of the lines in favor of the rifampicin resistant strain over the streptomycin resistant one. For each of the four replicate reciprocal competitions, all of the points were plotted on the same graph, to generate a slope for all the points. The best fit line yielded slopes of 0.33, and 0.50 with r^2 values of 0.7896 and 0.9252, respectively, indicating a slight advantage of the rifampicin resistant population.

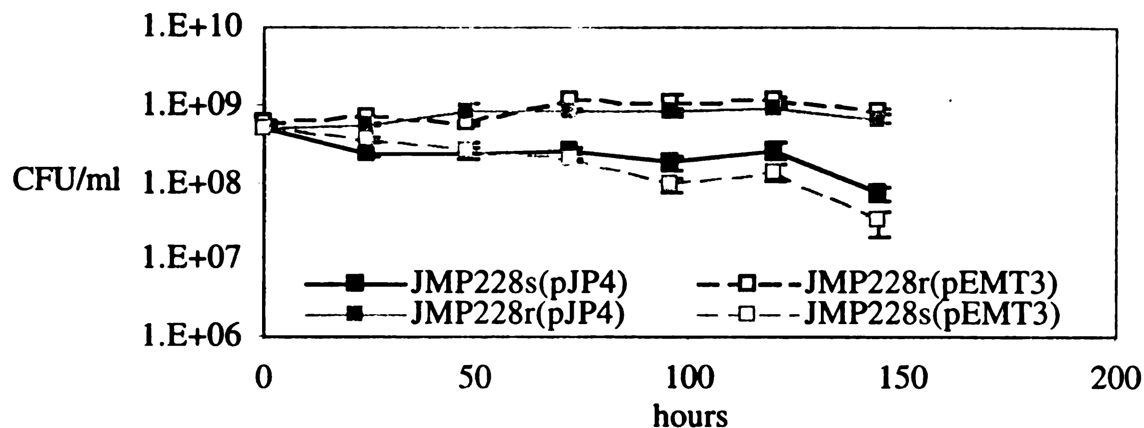


Figure 4.2. Growth of JMP228/pEMT3 and JMP228/pJP4 competing for 5 mM succinate as a limiting resource. Grey and black lines indicate two separate experiments and each symbol represents the average of four replicates with error bars indicating the standard deviation around the mean.

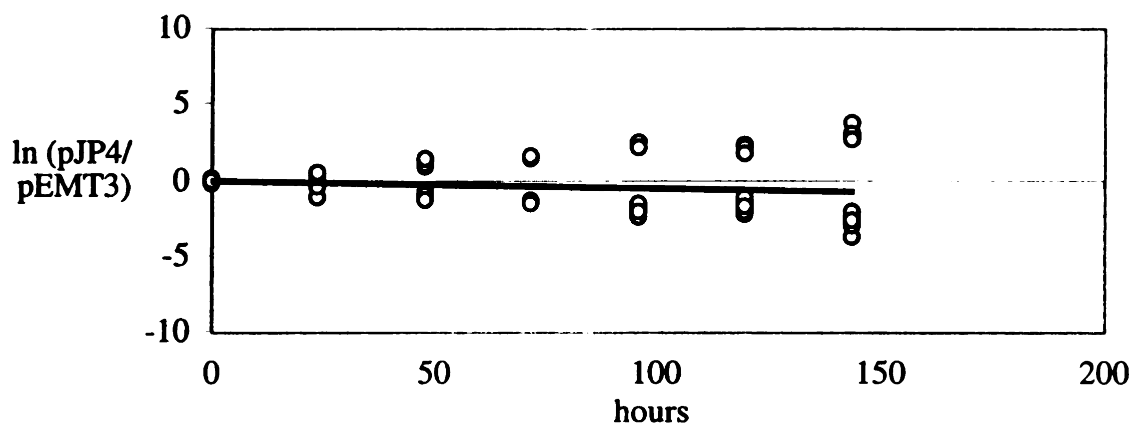


Figure 4.3. Plasmids pEMT3 and pJP4 do not govern fitness of *R. eutropha* competing for succinate. With succinate-grown cells the slope of the line generated by the ln of the ratio of JMP228/pJP4 / JMP228/pEMT3 is not different from zero, as indicated by the low r^2 value (0.0155). The mirror image appearance is due to the replicate competitions performed for each antibiotic resistance, and the sloping away from zero is due to a slight advantage for the rifampicin resistant population.

In contrast, the four replicate competition experiments between JMP228R/pJP4 vs DB01R/pJP4 and JMP228R/pEMT3 vs DB01R/pEMT3, showed that the *R. eutropha* populations always dominated over the *B. cepacia* competitors when both rival strains had the same plasmid (Fig. 4.4). The slope of the regression line of Malthusian parameters was positive in favor of JMP228 (0.055), and the r^2 value was high (>0.9761).

The DBO1R/pJP4 population was decreased to values lower than those of the pure cultures, while the JMP228R/pJP4 and JMP228R/pEMT3 populations attained sizes equal to those of their non-competing controls (Figure 4.5).

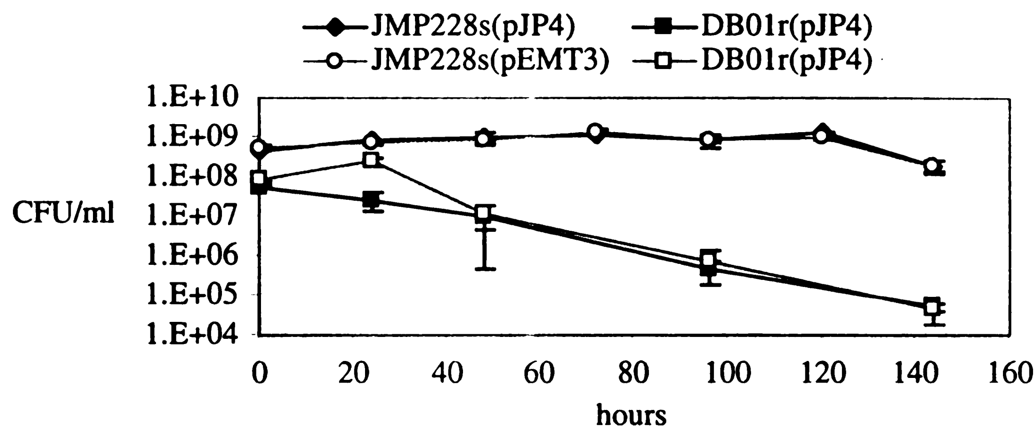


Figure 4.4. JMP228 is a superior competitor to DB01 when succinate is supplied as the limiting resource.

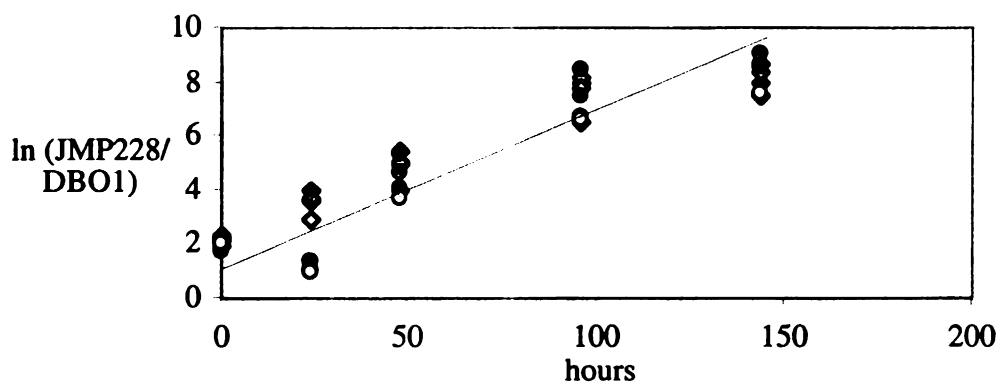


Figure 4.5. ln of the population sizes of JMP228/DB01 when succinate is supplied as the limiting resource. The positive slope of the line (0.055) and the r^2 value of 0.9761, indicate dominance by JMP228. Filled diamonds are competitions of DB01/pJP4 with JMP228/pJP4, and circles are competitions of DB01/pJP4 with JMP228/pEMT3.

Plasmid pJP4 conferred dominance in batch culture intra-specific competitions. All competitors were able to maintain a viable population size in minimal medium with 200 μg ml^{-1} 2,4-D and a daily transfer of 10% culture volume, although only JMP228/pJP4 was capable of completely utilizing the provided 2,4-D in that time period. No significant differences were noticed in pair-wise combinations of the same host population with different antibiotic resistance markers that were carried on the same plasmid. For example, in 12 independent, replicate, batch culture competitions among JMP228R/pJP4 and JMP228S/pJP4, the effects of host strain antibiotic resistance determinants did not have a large effect on the dominant population (Fig. 4.6).

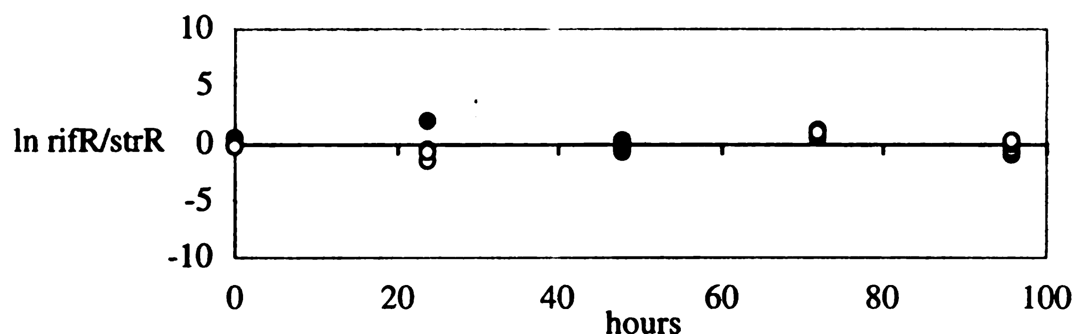
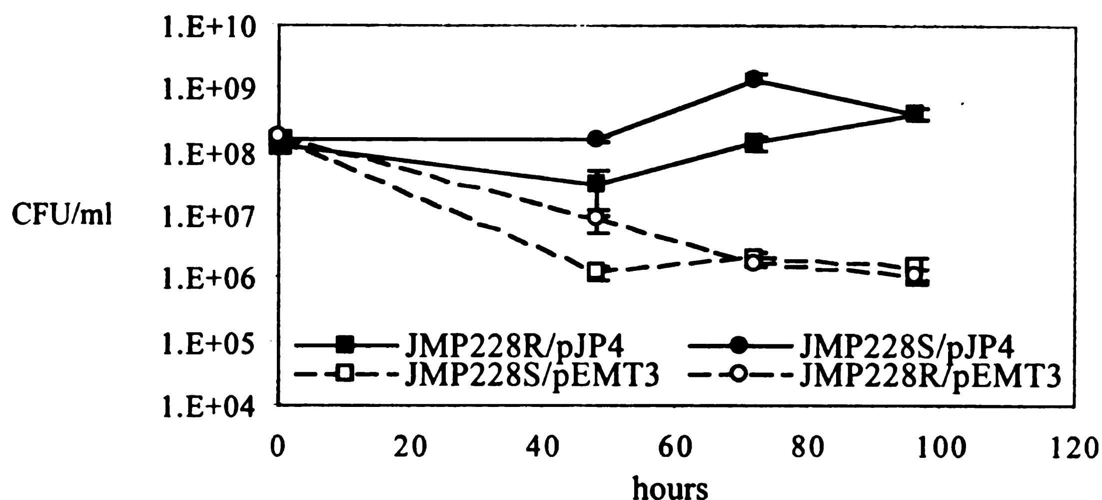


Figure 4.6. Effect of antibiotic resistance markers to fitness of JMP228/pJP4 and JMP228/pEMT3. ln of the population sizes of JMP228R/pJP4 to JMP228S/pJP4 (solid circles), and JMP228R/pEMT3 to JMP228S/pEMT3 (open circles) when 2,4-D (200 ppm), was supplied as the limiting resource. The slope of the line is 0.002 with an r^2 value of –0.5465, indicating that the population coexisted.

The results of 12 independent replicates also indicated that JMP228/pJP4 was competitively dominant over JMP228/pEMT3 when grown on 200 μg ml^{-1} 2,4-D. These findings confirm the predictions made using the pure culture growth kinetics, in which the strain with the faster growth rate predominated. There was a significant increase in the proportion of JMP228S/pJP4 relative to JMP228R/pEMT3 (Figure 4.7). The same trend was evident even if the pEMT3 carrying strain was given a ten-fold greater initial population size (data not shown).



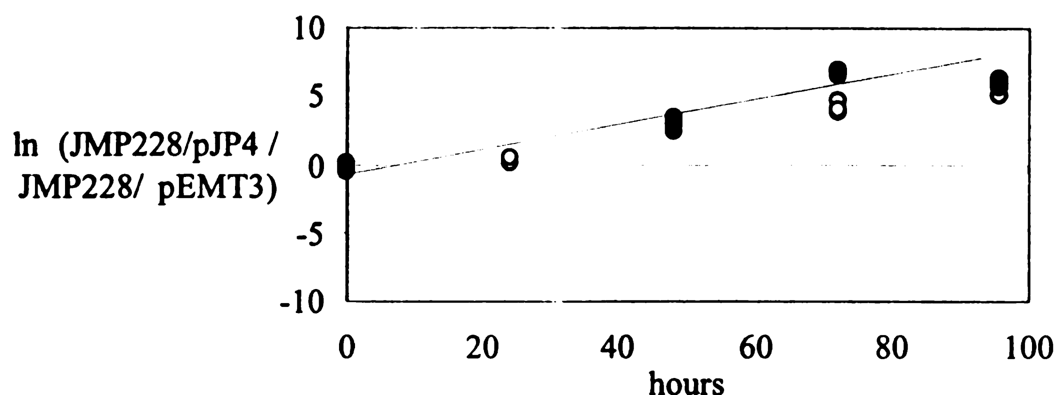


Figure 4.7. Competition between JMP228/pJP4 and JMP228/pEMT3 for 2,4-D in batch culture. Top panel: the CFU/ml of each competitor JMP228/pJP4 to JMP228/pEMT3 in 24 hour batch transfer experiments. Squares are one experiment, and circles are another, each with three replicates. Solid symbols represent pJP4 carrying strains, and the open symbols are pEMT3 carrying JMP228. Bottom panel: the ln of the population sizes of JMP228R/pJP4 to JMP228S/pEMT3 (open circles), and JMP228S/pJP4 to JMP228R/pEMT3 (closed circles) when 2,4-D (200 ppm) was supplied as the limiting resource. The slope of the line (0.07) with an r^2 of 0.9232, suggested dominance by the pJP4-bearing strain of *R. eutropha*.

Although the two antibiotic resistant populations of DB01/pJP4 exhibited similar growth characteristics when in pure culture, an advantage was noted for the rifampicin resistant populations relative to the trimethoprim resistant populations when they were grown together (Figure 4.8). While the effect of antibiotic resistance cannot entirely be ignored, it did not have a profound effect on competition when the strains carried different plasmids or when DB01 strains competed with JMP228/pJP4 or JMP228/pEMT3. As with the JMP228 strains, intra-species competition experiments between DB01R/pJP4 and DB01T/pEMT3 or DB01T/pJP4 and DB01R/pEMT3) lead to dominance by the pJP4 carrying population (Fig. 4.9).

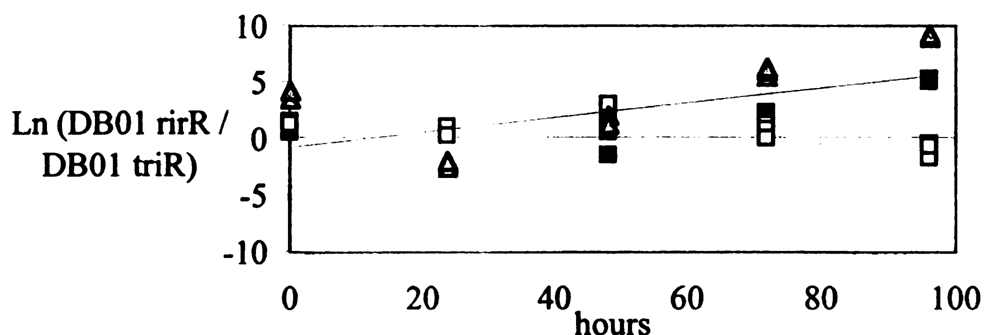


Figure 4.8. Effect of antibiotic resistance markers on fitness of DB01/pJP2 and DB01/pEMT3. ln of the population sizes of DB01R/pJP4 to DB01T/pJP4 (triangles), and DB01R/pEMT3 to DB01T/pEMT3 (squares). The slope of the line (0.034) and the r^2 value (0.7360) indicate that the rifampicin resistant population may have an advantage over the trimethoprim resistant population.

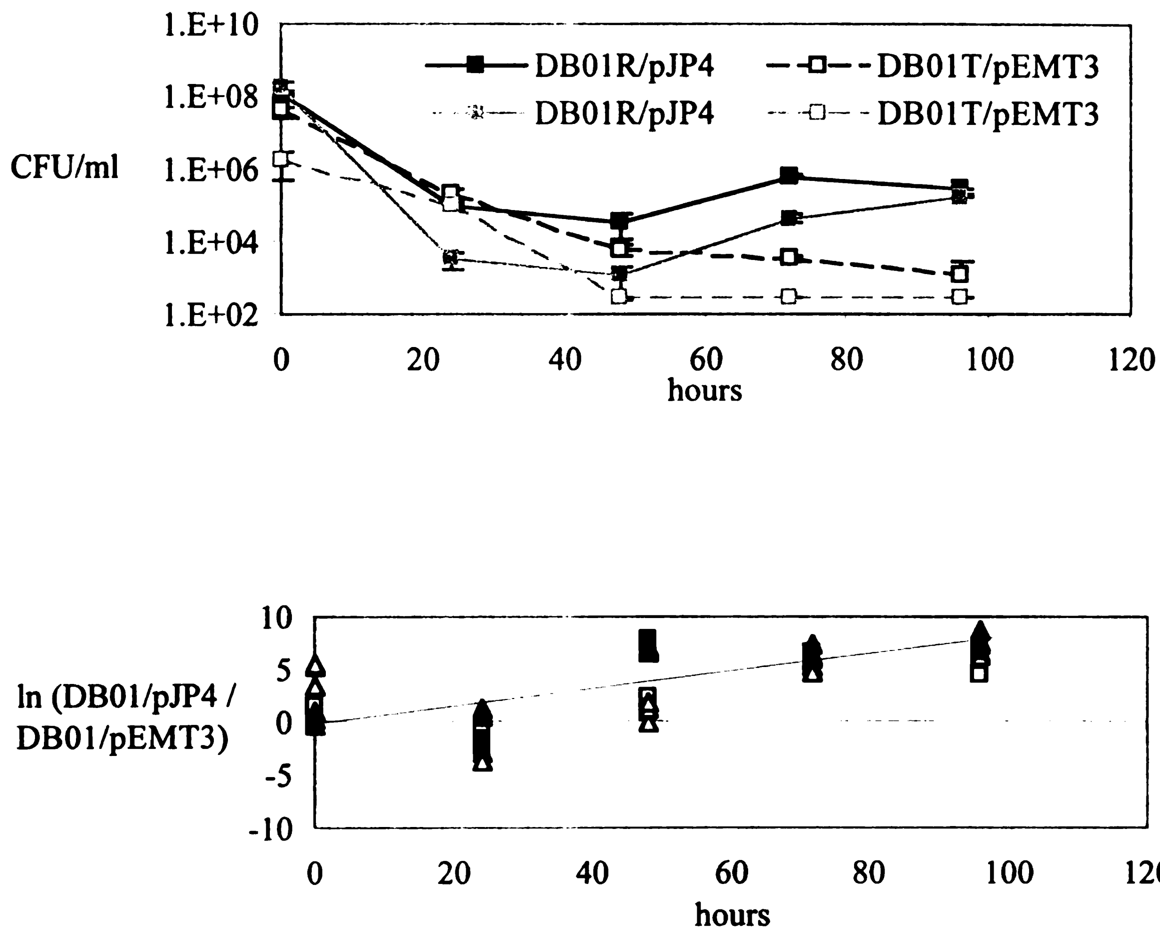


Figure 4.9. Competition between DB01/pJP4 and DB01/pEMT3 for 2,4-D in batch culture. Top panel: the CFU/ml of each competitor DB01/pJP4 to DB01/EMT3 in 24 hour batch transfer. Black and gray lines indicate two independent experiments, each run in triplicates. Closed symbols represent pJP4 carrying strains and open ones are pEMT3 carrying DB01 strains. Bottom panel: the ln of the population sizes of DB01R/pJP4 to DB01T/EMT3 (solid symbols), and DB01T/pJP4 to DB01R/EMT3 (open symbols). The slope of the line for DB01T/pJP4 to DB01R/EMT3 had r^2 values of 0.72, and DB01R/pJP4 to DB01T/EMT3 had r^2 values equal to 0.94.

Inter-species batch competition between *R. eutropha* JMP228 and *B. cepacia* DB01, resulted in dominance by the JMP228 strains when both competitors had the same plasmid, whether transferred at 24 hour or 72 hour intervals. In the 24-hour competitions, three replicate flasks for each antibiotic resistance combination were performed. In the 72-hour competitions, only one of the two possible antibiotic resistance combinations was combined, but three replicates at three different starting ratios (1:10, 1:1 and 10:1) of each competitor strain was utilized. Regardless of the ratio of competitors, the same strain from the competitive set became dominant. Results of the 1:1 ratio are presented, because they are representative of all three starting ratios. The outcome of competition for both 24 and 72 hour transfer experiments were similar. DB01/pJP4 strains were outcompeted by JMP228/pJP4 and similarly DB01/pEMT3 strains were outcompeted by JMP228/pEMT3 (Figures 4.10 and 4.11).

When alternate host/plasmid pairs were subjected to interspecies competition, again, the population with the fastest growth rate became dominant. Intra-species competition among the pairs DB01/pJP4 vs JMP228/pEMT3 and DB01/pEMT3 vs JMP228/pJP4 were carried out

(Figures 4.12 and 4.13). The dominant population in each case was the strain with the faster growth rate; the one that carried pJP4.

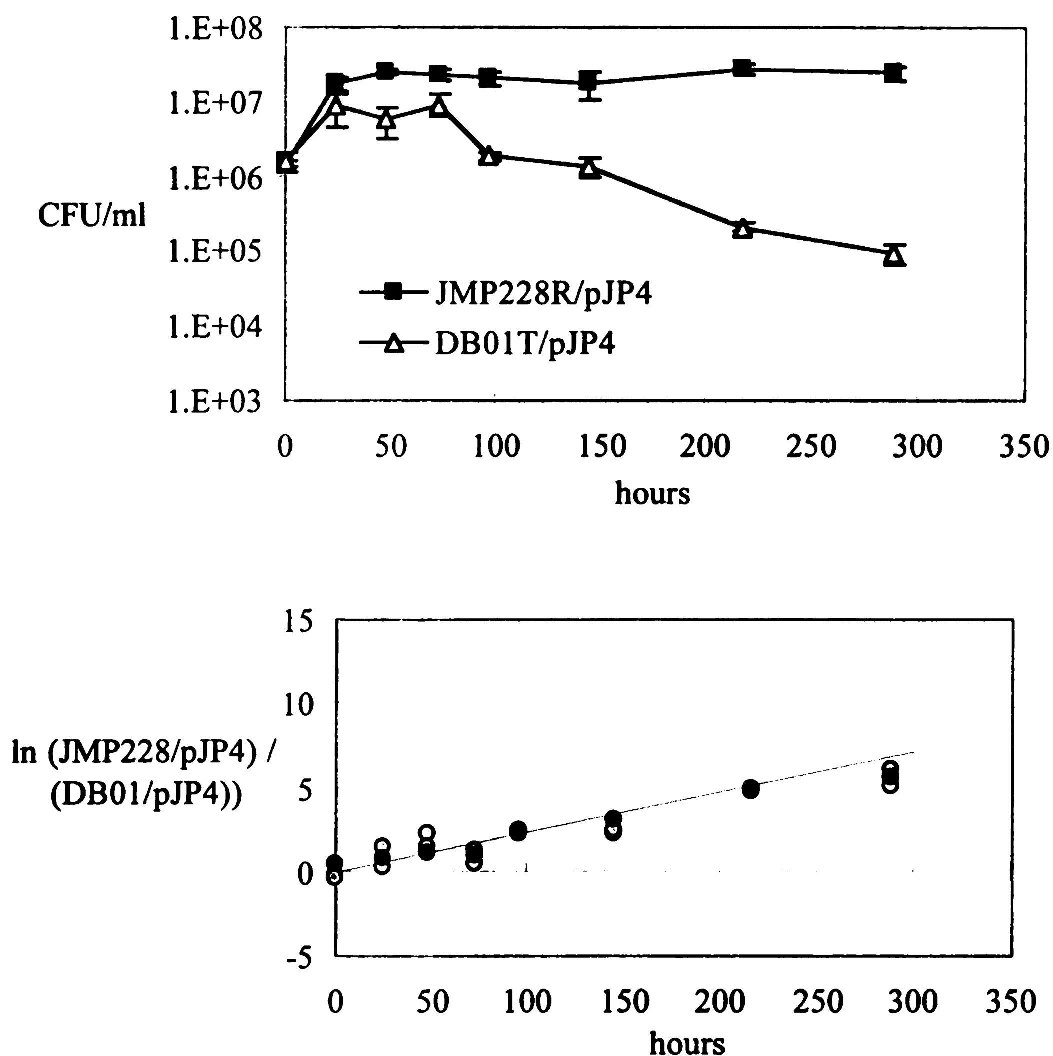
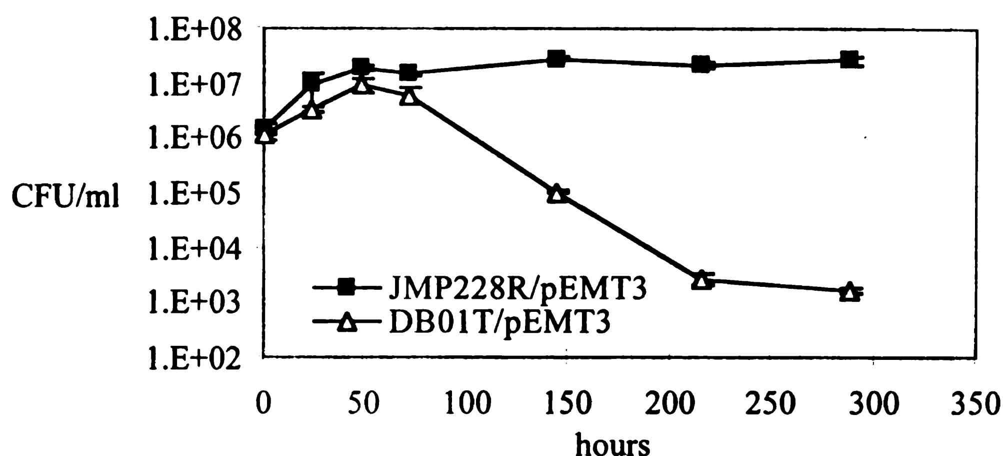


Figure 4.10. Competition between JMP228/pJP4 and DB01/pJP4 for 2,4-D in batch culture. Top panel: the CFU/ml of competitors JMP228R/pJP4 (solid symbols) and DB01R/pJP4 (open symbols). Bottom panel: the ln of the population sizes of JMP228R/pJP4 to DB01T/pJP4. The slope of the line, 0.02 had an r^2 of 0.9366, indicating that *R. eutropha* populations increased significantly relative to *B. cepacia*.



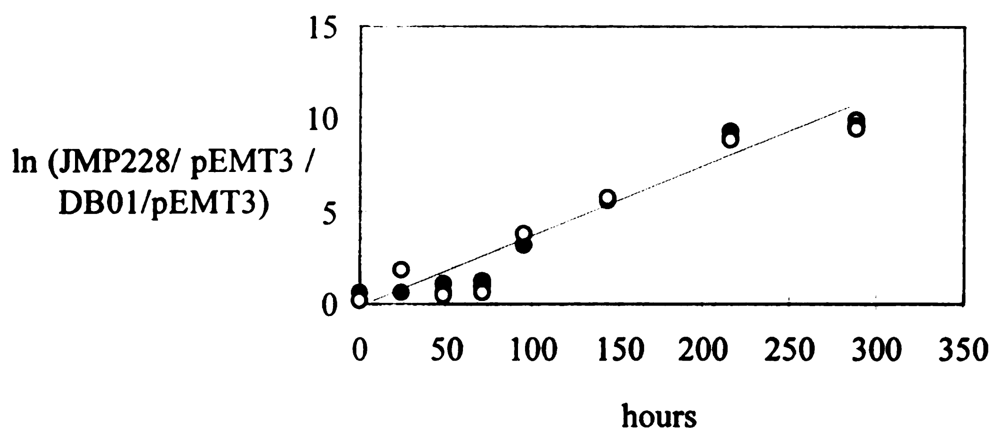


Figure 4.11. Competition between JMP228/pEMT3 and DB01/pEMT3 for 2,4-D in batch culture. Top panel: the CFU/ml of competitors JMP228R/pEMT3 (solid symbols) and DB01T/pEMT3 (open symbols). Bottom panel: the ln of the population sizes of JMP228R/pEMT3 to DB01T/pEMT3. The slope of the line, 0.036 had an r^2 of 0.963, indicating that *R. eutropha* again was dominant to *B. cepacia.*

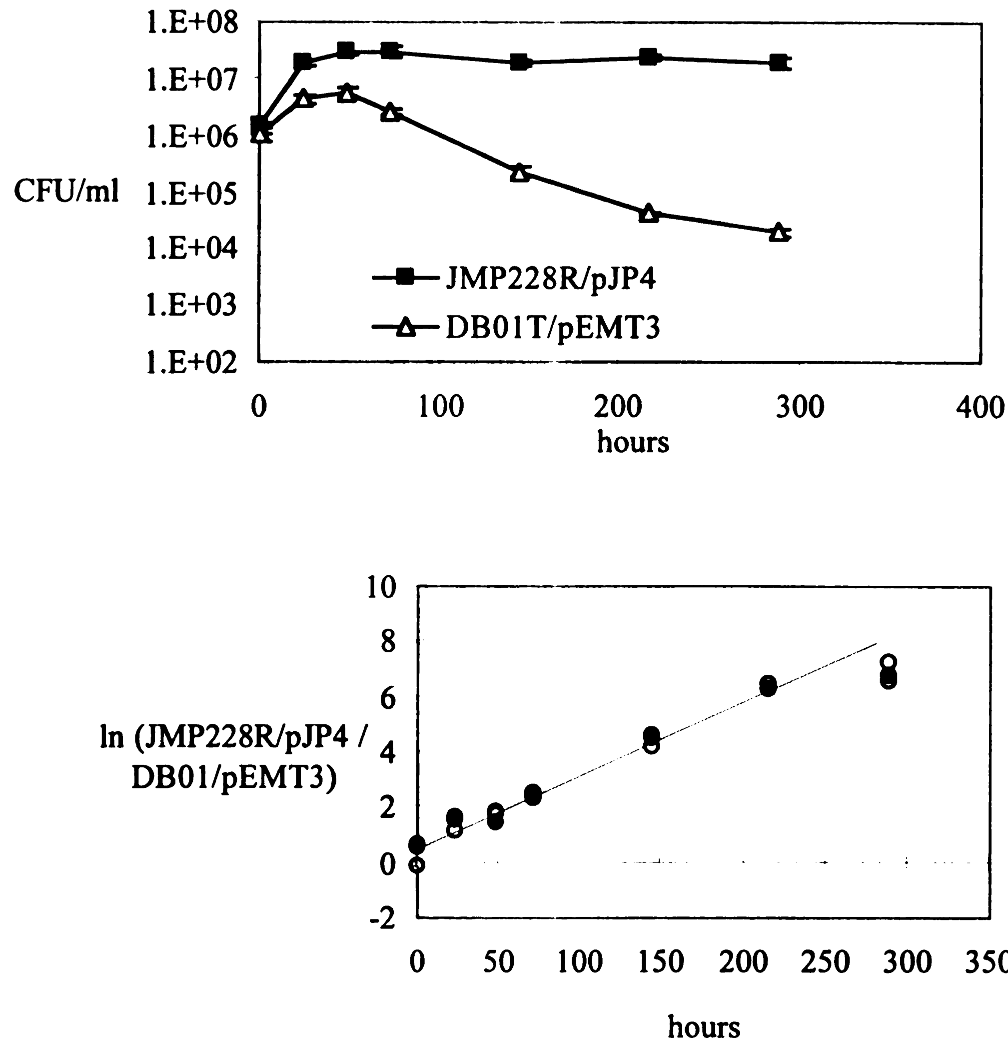
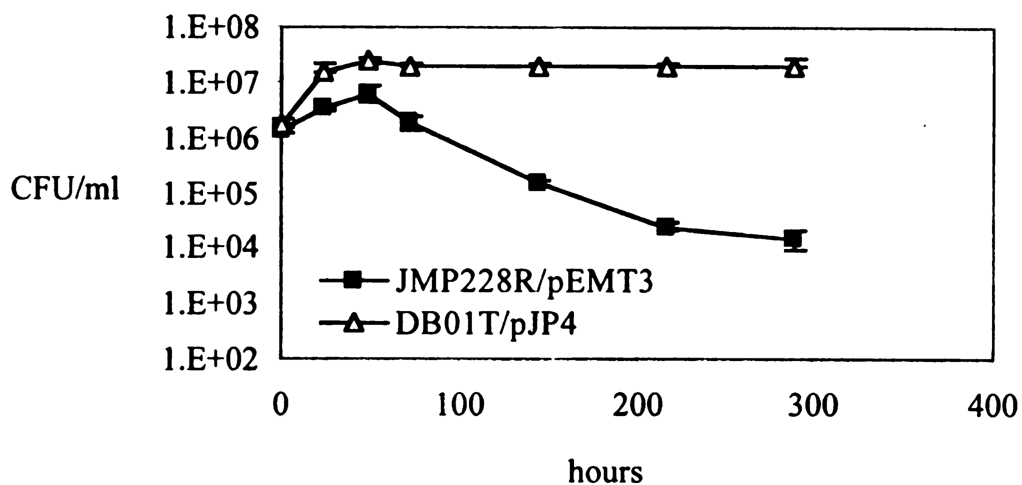


Figure 4.12. Competition between JMP228/pJP4 and DB01/pEMT3 for 2,4-D in batch culture. Top panel: the CFU/ml of competitors JMP228R/pJP4 (solid symbols) and DB01T/pEMT3 (open symbols). Bottom panel: the ln of the population sizes of JMP228R/pJP4 to DB01T/pEMT3. The slope of the line, 0.023 had an r^2 of 0.926, indicating the faster growth conferred by pJP4 results in dominance.



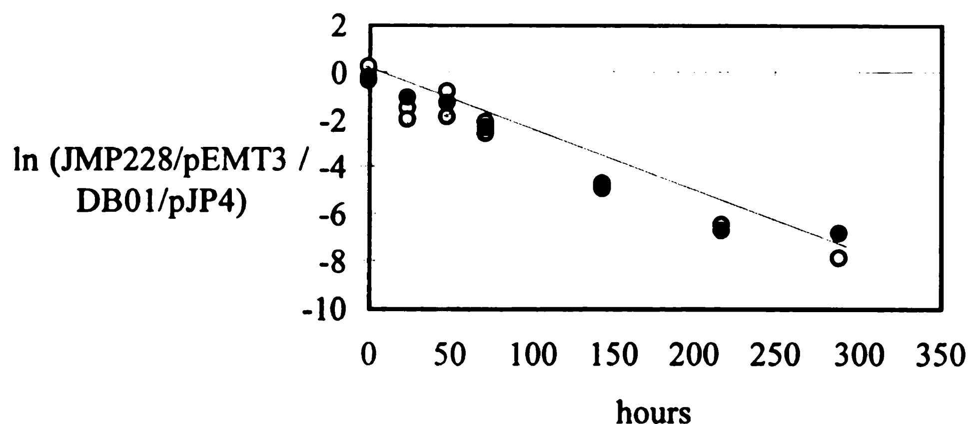


Figure 4.13. Competition between JMP228/pEMT3 and DB01/pJP4 for 2,4-D in batch culture. Top panel: the CFU/ml of competitors JMP228R/pEMT3 (solid symbols) and DB01T/pJP4 (open symbols). Bottom panel: ln of the population sizes of JMP228R/pEMT3 to DB01T/pJP4. The slope of the line, -0.025 had an r^2 of 0.950, again supporting that the faster growth conferred by pJP4 resulted in dominance.

Modeling the chemostat competitions. Chemostat competitions were modeled using the Stella Research Software Package (v 5.0). The software is designed to run mathematical models, when the appropriate constants and equations are introduced to the various parts of the model. A drawing of the model for a two species competition is shown in figure 4.14. In this figure, the large black rectangles represent the population sizes of each competing bacterial strains (1 and 2), and the concentration of 2,4-D remaining in the chemostats. Initially this value was equal to each original population size, and 200 ppm 2,4-D, as in the experimental populations. The thick arrows connected to clouds with a gray circle describe rates, and all of them are represented by an equation that influences the item in the black box. In the case of bacterial growth, there is one input and one output. Both K_S and μ_{max} are needed as constants for the input, and the Monod (1954) equation can solve for the population size over time. For example, "growth Bacteria 1" is equal to the change in population size over time described by equation (g). The output is equal to the population size (N) times the dilution rate (D). So, the population size of "Bacteria 1" is described by equation (a).

Equation (g)

$$\frac{dN}{dt} = N \mu_{max} \frac{S}{K_S + S}$$

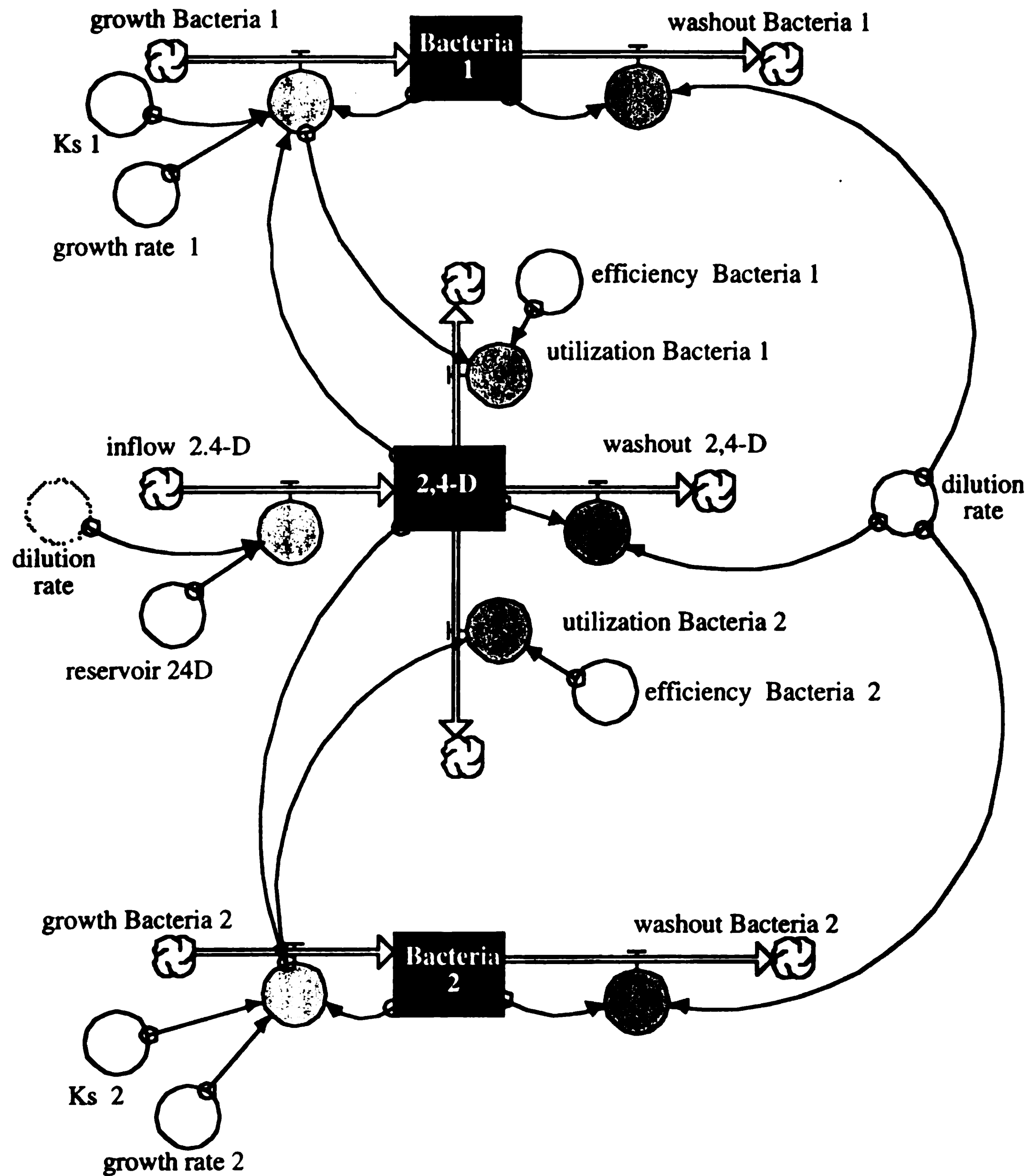


Figure 4.14. The model that was used by the Stella Research Program to generate expected population growth curves for two competing populations. The constants for K_S, growth rate, and efficiency were chosen using an average value determined for each host/plasmid combination (Table 4.3).

Substrate concentration (2,4-D) has one input and three outputs. Input is the reservoir concentration of 2,4-D (200 ppm) times the dilution rate. Outputs are the utilization of 2,4-D by the two bacterial strains (a product of the population size and the efficiency of 2,4-D utilization by each strain) and dilution (dilution rate times substrate remaining). The efficiency of utilization (g / CFU) is the inverse of the growth yield CFU/g. The curved arrows indicate that there is a relationship between one component and another.

Table 4.3: Values for constants in the Stella model.

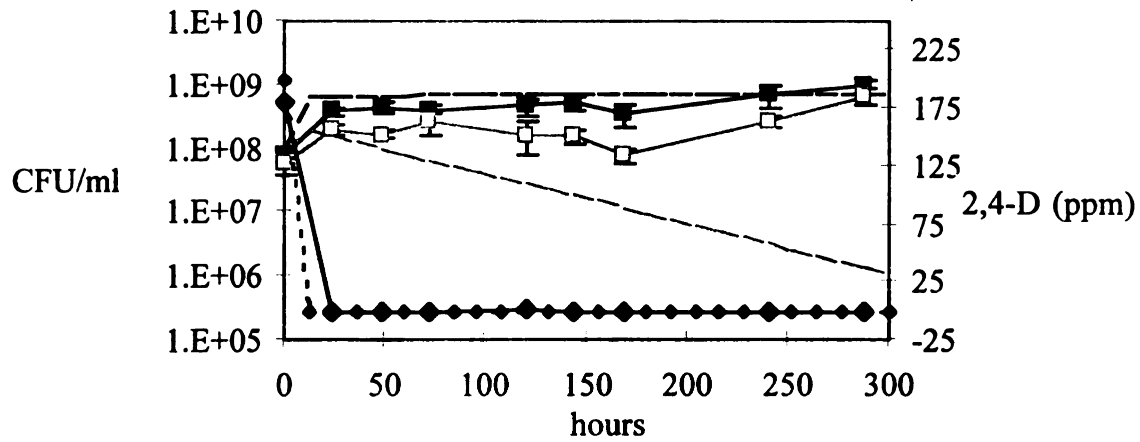
Variable	JMP228/ pJP4	JMP228/ pEMT3	DB01/ pJP4	DB01/ pEMT3
μ_{max} (h^{-1})	0.22	0.10	0.15	0.08
$K_{S\,1}$ (ppm)	1	1	1	1
$K_{S\,2}$ (ppm)	1	1	0.25	0.25
Starting population	2×10^7	2×10^7	2×10^7	2×10^7
	1×10^8	1×10^8	1×10^8	1×10^8
efficiency (g/CFU)	2.7×10^{-7}	2.7×10^{-7}	2.9×10^{-7}	2.9×10^{-7}
dilution rate (h^{-1})	0.033	0.033	0.033	0.033
reservoir (mg/ L)	200	200	200	200
original 2,4-D	200	200	200	200

For each host/plasmid pair that was competed in a chemostat, a model was run. These are graphically shown as dashed lines superimposed on each graph of the measured values in the chemostat. Two different values of K_S were used to generate models of competition with DB01 strains, in order to illustrate how a lower value of K_S could allow a population with a slower growth rate to predominate at these low nutrient concentrations. For the point to be made, only one graph of the DB01 strains with $K_S = 1$ ppm is shown. The reasons for using a K_S value of 0.25 ppm for the DB01 host, will become evident in the next section.

Chemostat competitions. Chemostat competitions were carried out simultaneously with pure cultures. The chemostats were fed very slowly (0.033 h^{-1}), so that nutrients (2,4-D) would become limiting. In the computer simulated models run at the above conditions, 2,4-D levels in the chemostats attained final concentrations of between 0.15 and 0.7 ppm, values that lie below the 2 ppm limit of resolution of the HPLC. The results of the competitions were compared with predicted population sizes that were determined using the Stella Research Software Package. Plasmid pJP4 plays a lesser role in chemostat competition than in batch culture, and also supports the theory that if the K_S of the two populations is the same, then the strain with the maximum growth rate would become dominant. First,

consider the case of the same host population with a different plasmid. In reciprocal combinations within chemostats between JMP228S/pJP4 and JMP228R/pEMT3 or between JMP228R/pJP4 and JMP228S/pEMT3 both competitors coexisted, but the JMP228/pJP4 combination was numerically dominant.. (Figure 4.15 and 4.16) A plot of the difference in the Malthusian parameters tracked over 300 h for each pair indicated a slope of 0.004 and 0.190 in favor of the pJP4 carrying strain. Since this is a small slope with an r^2 value of 0.566 and 0.621, there was a marginal deviation from a slope of zero. Carried out over a longer period of time, this may have lead to the exclusion of JMP228/pEMT3. Qualitatively, the strain that was expected to dominate, did. However, the magnitude of the difference was not nearly as great as expected by the model. The pEMT3 carrying strain was not excluded nearly as rapidly as one would have predicted.

In competition studies between DB01/pJP4 and DB01/pEMT3 it was observed that the experimental results were consistent with the modeled results, except that the final population sizes of the two competitors were less than predicted. (Figure 4.17). When the slope of the ln of the ratio of the pJP4 carrying strain to the pEMT3 carrying strain was graphed, it could be seen that the DB01/pJP4, with a faster growth rate, also became dominant over DB01/pEMT3.



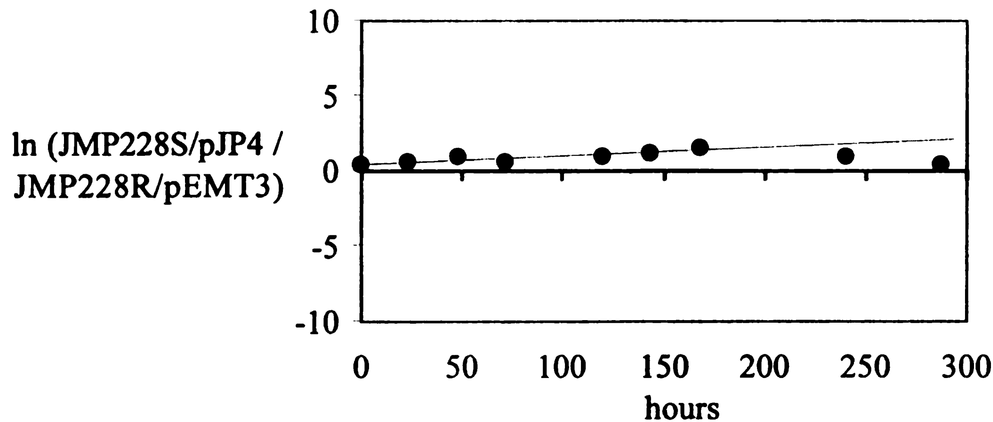


Figure 4.15. Chemostat competition between JMP228S/pJP4 and JMP228R/pEMT3. Top panel: the colony forming units of JMP228S/pJP4 (black squares) and JMP228R/pEMT3 (gray open squares) in chemostat competition. The expected growth curves for each strain are indicated with black and gray lines, respectively. Solid diamonds indicate measured 2,4-D concentration, open ones are predicted by the model. Bottom panel: the natural log of the ratio pJP4 carrying to pEMT3 carrying strains of JMP228. The slope of the line (0.004) and r^2 (0.567) indicate a marginal advantage by JMP228S/pJP4.

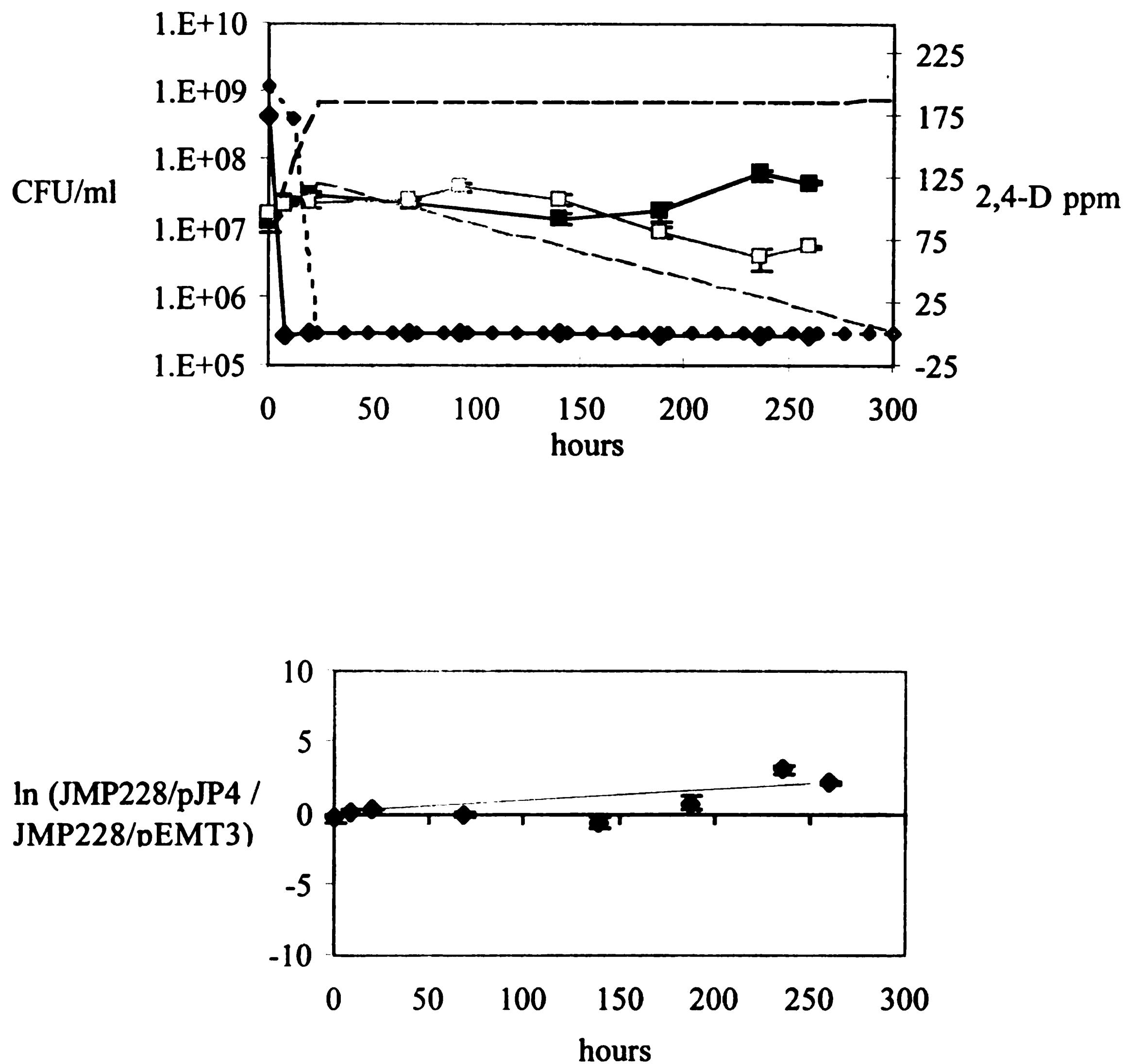


Figure 4.16. Chemostat competition between JMP228R/pJP4 and JMP228S/pEMT3. Top panel: the colony forming units of JMP228R/pJP4 (black squares) vs. JMP228S/pEMT3 (gray open squares) in chemostat competition. The expected growth curves for each strain are indicated with black and gray lines, respectively. Solid diamonds indicate measured 2,4-D concentration, open ones are expected. Bottom panel: the natural log of the ratio pJP4 carrying to pEMT3 carrying strains of JMP228. The slope of the line (0.190) and r^2 (0.621) indicate a marginal trend in favor of JMP228R/pJP4.

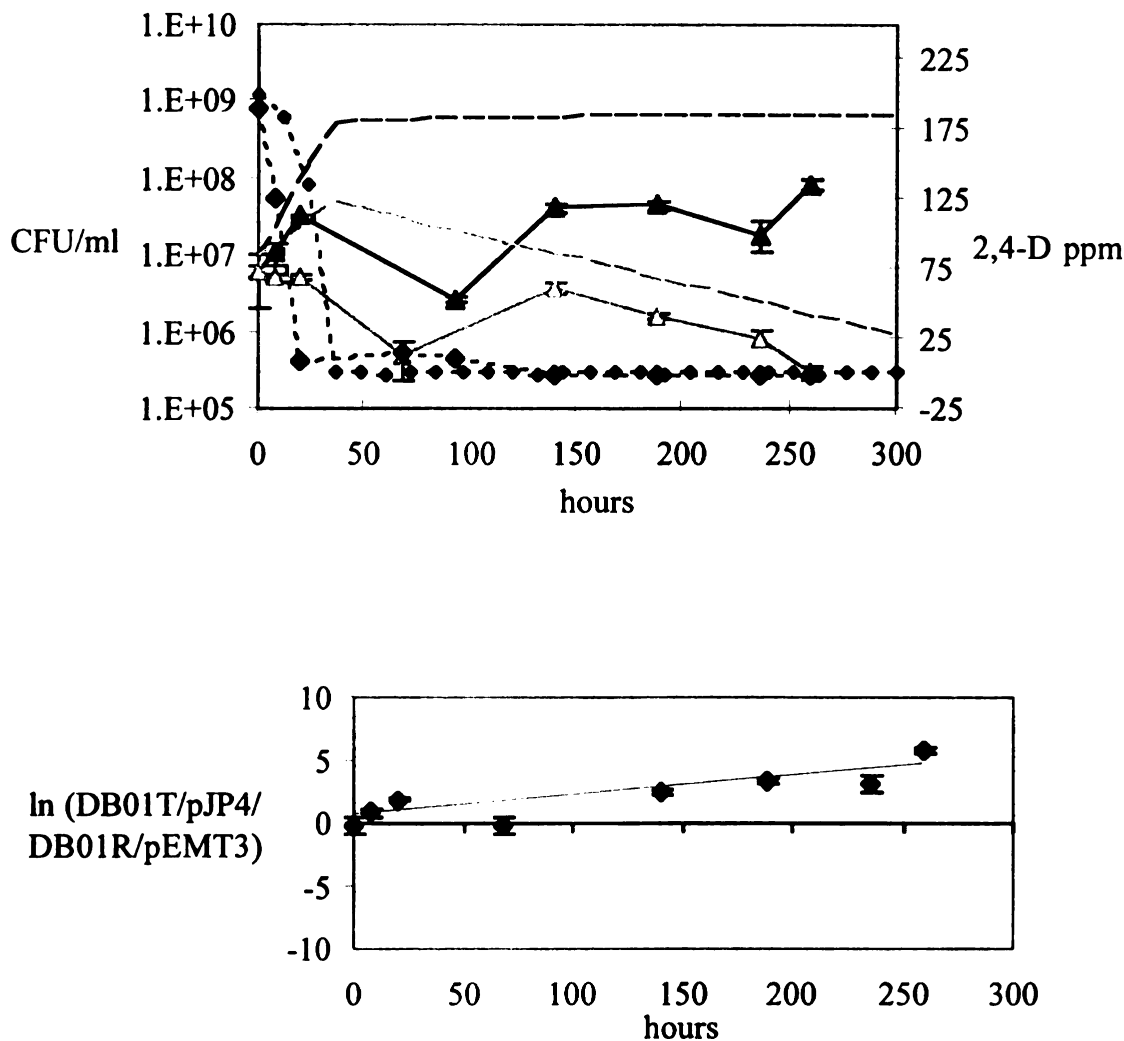


Figure 4.17. Chemostat competition between DB01T/pJP4 and DB01R/pEMT3. Top panel: the colony forming units of DB01T/pJP4 (black triangle) and DB01R/pEMT3 (gray open triangle) in chemostat competition. The expected growth of each strain is indicated with black and gray lines, respectively. Solid diamonds indicate measured 2,4-D concentration, open ones are obtained from the model. Bottom panel: the natural log of the ratio pJP4 carrying to pEMT3 carrying strains of DB01. The slope of the line (0.02) and r^2 (0.868) indicate a marginally significant trend in favor of DB01/pJP4.

In two independent replicate chemostat experiments with reciprocal antibiotic resistance markers on the populations, JMP228/pJP4 and DB01/pJP4 were competed. A surprising outcome was observed. While JMP228/pJP4 dominated over DB01/pJP4 in batch transfer competition, the DB01/pJP4 strain outcompeted JMP228/pJP4 in these slow growth rate chemostats (figure 4.18). Notice that the expected values for these strains did not correlate well with the observed values, when μ=0.22 for JMP228/pJP4 and μ= 0.15 for DB01/pJP4, and each has a K_S of 1 ppm. The prediction would be that the faster growing strain (JMP228/pJP4) should dominate. On separate occasions these two host/plasmid combinations were competed. The same outcome was observed: DB01/pJP4 became dominant over JMP228/pJP4. One representative graph of the competition between JMP228S/pJP4 and DB01R/pJP4 is shown (Figures 4.18 and 4.20). The slope and r^2 of the ratio of the population is reported here for comparison of the three experiments. The slope was calculated after the initially supplied 2,4-D was depleted, to eliminate the rapid growth period by *R. eutropha.* The slope of the difference in the Malthusian parameter of ln (JMP228S/pJP4)/ (DB01R/pJP4) was -0.072 and correlated to a r^2 value of 0.908 in the graphed example. The other two replicates resulted in a ln ratio or JMP228/DB01 with slopes = -0.045 and -0.01 with r^2 = 0.8924, and

0.8043 respectively. The negative slope to the line indicated dominance by DB01. The increase in DB01R/pJP4 occurred after 120 hours of incubation, and by 150 hours, the DB01 strain had increased to numbers higher than that of JMP228S/pJP4.

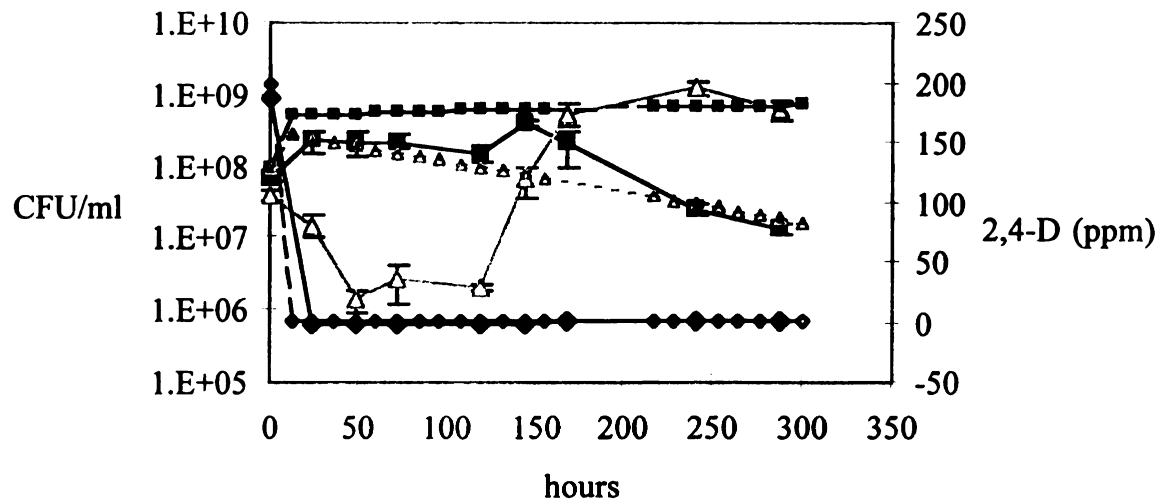


Figure 4.18. Chemostat competition between JMP228S/pJP4 and DB01R/pJP4. JMP228S/pJP4 (black square) and. DB01R/pJP4 (gray open triangle) in chemostat competition. The expected growth curves for each strain are indicated with black and gray lines, respectively. Solid diamonds indicate measured 2,4-D concentration, and open ones are expected. The modeled lines (black and gray for each strain as identified), were for JMP228S/pJP4, μ=0.22 h^{-1}, K_S=1 ppm and DB01R/pJP4, μ=0.15 h^{-1}, Ks=1 ppm. Notice that it is predicted that JMP228 should become dominant.

Since the observed population dynamics did not match the predicted curve, alternative explanations for why DB01 became dominant over JMP228 were explored. The first of these ties back into the pure culture experiments for determining K_S. The method used may not have been sensitive enough to accurately calculate the K_S for these strains at these low concentrations of substrate. Evidence from figure 3.6 suggests that the K_S being at least as low as 1 ppm, if not significantly lower. It is also possible that DB01 may be more efficient at utilizing very low concentrations of as described by the ^{14}C studies. DB01 appeared to completely mineralize a greater fraction of the total 2,4-D at concentrations below 1 ppm (Chapter 3).

In a set of hypothetical curves, also run on the Stella v 5.0 software, we can see that as little as a 50% decrease in the K_S of a strain with a lower growth rate, can be sufficient to change the outcome of competition. Figure 4.19, illustrates the consequence of a sequential decrease in the K_S of DB01/pJP4, which has $\mu = 0.15\ h^{-1}$, when it competes with JMP228/pJP4, which always has a K_S=1, and $\mu = 0.22\ h^{-1}$. Hypothetical case A, both strains have K_S =1. In case B, DB01/pJP4, has K_S= 0.1, in case C, K_S= 0.25 and in case D, K_S= 0.01 ppm.

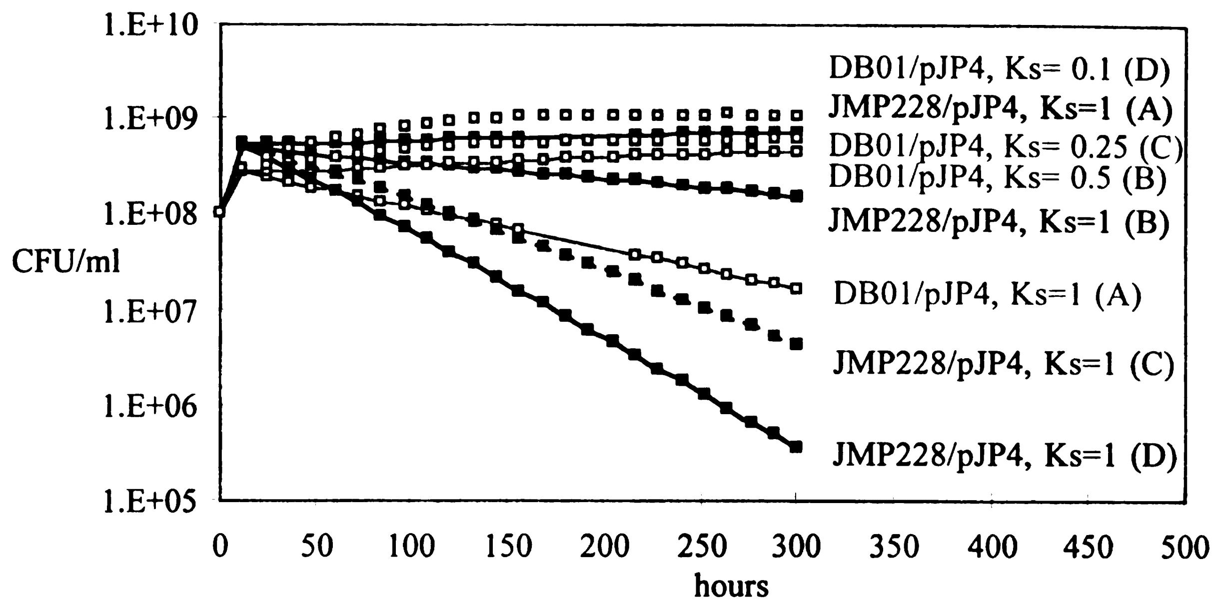


Figure 4.19. different K_S values for DB01 influence competition between JMP228S/pJP4 and DB01R/pJP4 in chemostats. Four hypothetical competitions between JMP228S/pJP4 (black filled square) and DB01R/pJP4 (gray open square). With a decrease of K_S to 0.5 ppm 2,4-D the slower growing DB01 is able to become dominant.

For a combination of strains, if we assume that the K_S is the same, (1 ppm), the populations should diverge at a rate proportional to the difference in their maximum growth rates. This is not observed. *Burkholderia* outcompeted *Ralstonia*. To support the possibility that the K_S for strain DB01 is lower than that of strain JMP228, the upcoming graphs will show the expected ratios for DB01 calculated with a K_S of 0.25 ppm. Using values generated for the hypothetical population curves, when DB01/pJP4 was assigned a Ks=0.25 expected values did fit the observed data more

closely. Figure 4.20 plots the same empirical data as in figure 4.18, but the hypothetical curves were generated for DB01/pJP4 with a K_S = 0.25 ppm 2,4-D. While K_S differences can explain one way in which the DB01/pJP4 strain could become dominant, the fact that the shift appears much later than expected, and more rapidly may indicate the influence of another parameter. Evolution of the DB01/pJP4 strain to grow better on the low concentrations of 2,4-D or perhaps some other physiological change or pathway induction is responsible for the rapid reversal of the dominant strain in Figure 4.20.

Chemostat competitions between JMP228/pEMT3 and DB01/pEMT3 indicated that DB01/pEMT3 became dominant. The slope of the ln ratio of (JMP228/pMET3) / (DB01/pEMT3) was -0.013 and correlated with an r^2 value of 0.815. (Figure 4.21). Again a value of 0.25 was used for the hypothetical K_S of DB01/pEMT3. The emerging trend is that DB01 strains outcompete JMP228 under the low growth rate, nutrient poor conditions simulated in these chemostat conditions.

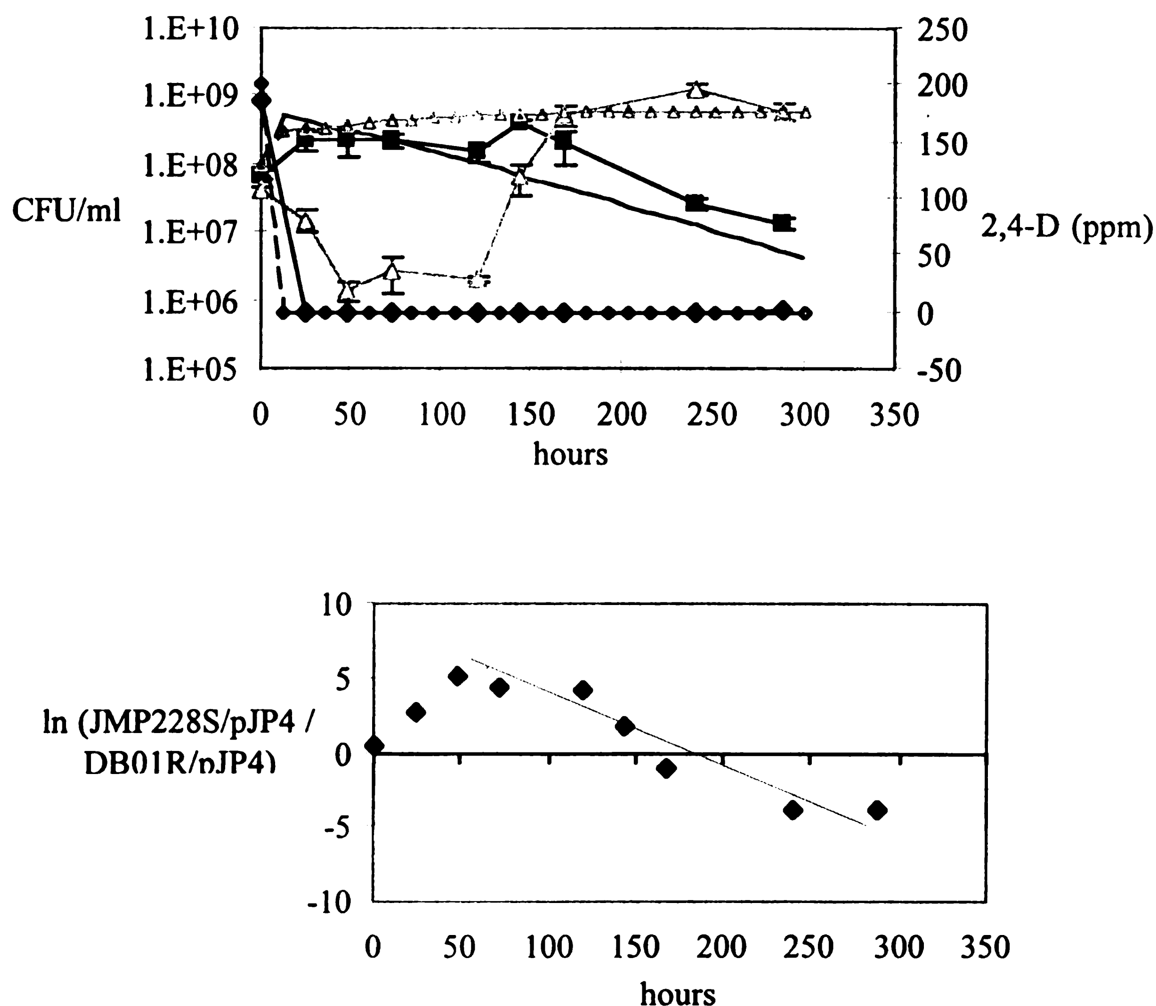


Figure 4.20. Chemostat competition between JMP228S/pJP4 and DB01R/pJP4. Top panel: population size of JMP228S/pJP4 (black solid square) vs. DB01R/pEMT3 (gray open triangle) in chemostat competition. The expected growth curves for each strain are indicated with black and gray lines, respectively. Solid diamonds indicate measured 2,4-D concentration, open ones are expected from the model. Bottom panel: the natural log of the ratio JMP228 to DB01. The slope of the line (-0.072) and r^2 (0.908) indicate a significant trend in favor of DB01/pJP4. This finding suggests that a lower K_S may be a factor in allowing the strain with the slower growth rate to become dominant.

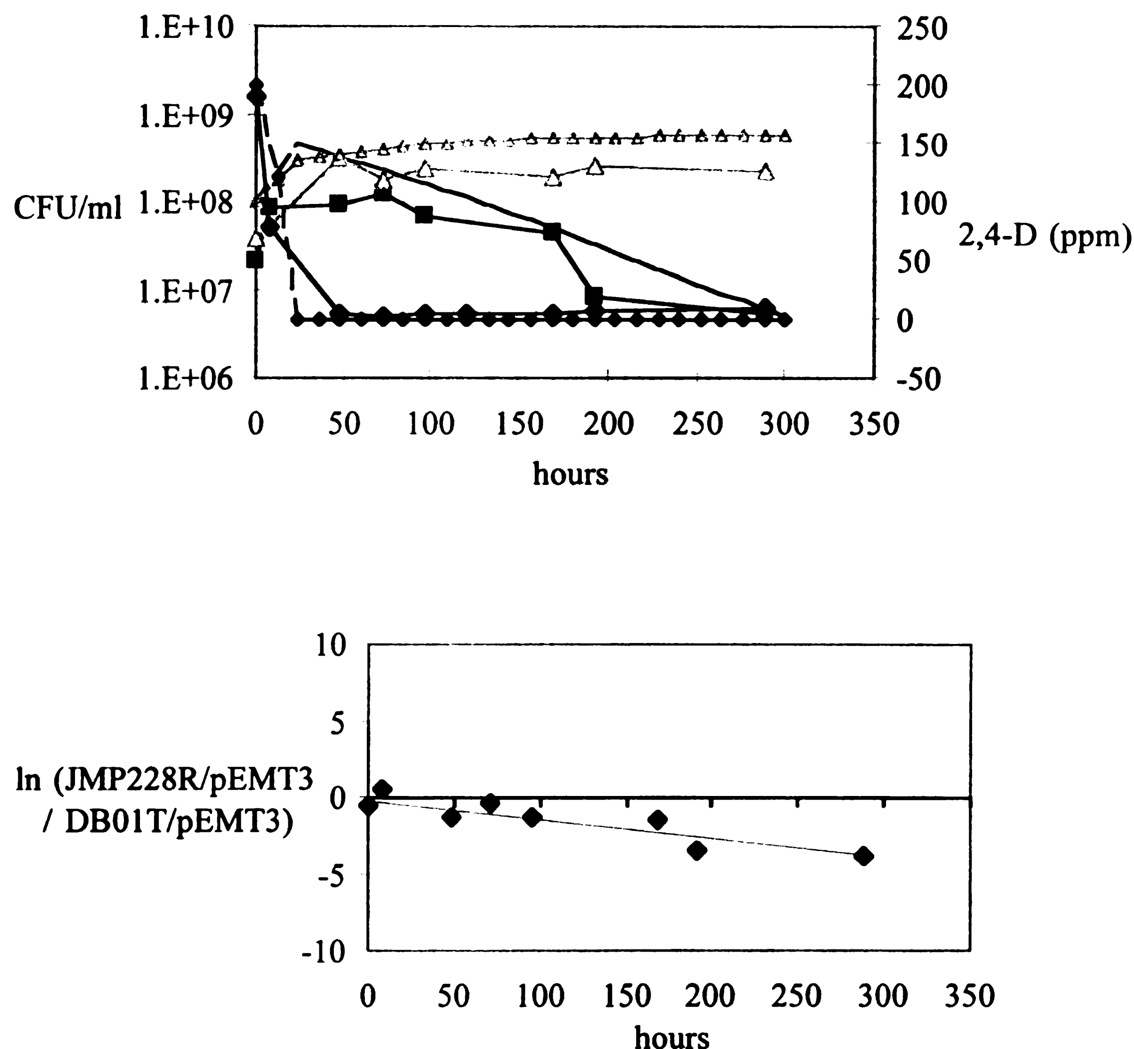
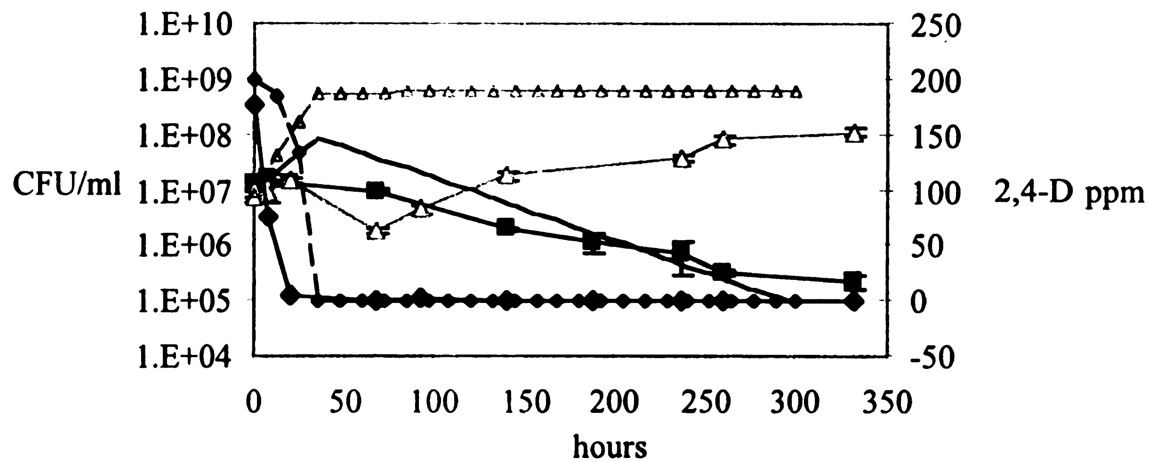


Figure 4.21. Chemostat competition between JMP228R/pEMT3 and DB01T/pEMT3. Top panel: the population size of JMP228R/pEMT3 (black square) vs. DB01T/pEMT3 (gray open triangle) in chemostat competition. The expected growth curves for each strain are indicated with black and gray lines, respectively. Solid diamonds indicate measured 2,4-D concentration, open ones are expected from the model. Bottom panel: the natural log of the ratio JMP228 to DB01. The negative slope of the line (-0.013) and r^2 (0.819) indicate a significant trend in favor of DB01/pEMT3.

The observation that strain DB01 became dominant held true for the observed population sizes for all of the chemostats of interspecies competition. Figures 4.22 and 4.23 show competition between populations of different species with different degradative plasmids: JMP228R/pEMT3 vs. DB01T/pJP4 and for JMP228R/pJP4 vs. DB01T/pEMT3. In both pairs, DB01 dominated at the end of the experiment. When JMP228R/pEMT3 competed with DB01T/pJP4, it was expected that DB01 should dominate. First, DB01/pJP4 had a faster growth rate, and additionally, it may have a lower value for K_S. The regression line generated by the natural log of the ratio of population sizes was negative (-0.48), and the r^2 of 0.923, indicating superior fitness of strain DB01.

On the other hand, the results were more surprising for the competition between JMP228R/pJP4 and DB01T/pEMT3. These strains have a very large difference in their μ_{max}. In fact, when the model is run with the values of μ=0.22 and K_S = 1 ppm for JMP228/pJP4 and μ= 0.08 and K_S = 0.25 for DB01/pEMT3, the predicted population dynamics would support coexistence of the two populations, with a greater proportion of the community being composed of strain JMP228R/pJP4. In theory the two populations could coexist. In reality, JMP228/pJP4 was adversely affected by competition with DB01/pEMT3 (figure 4.23).



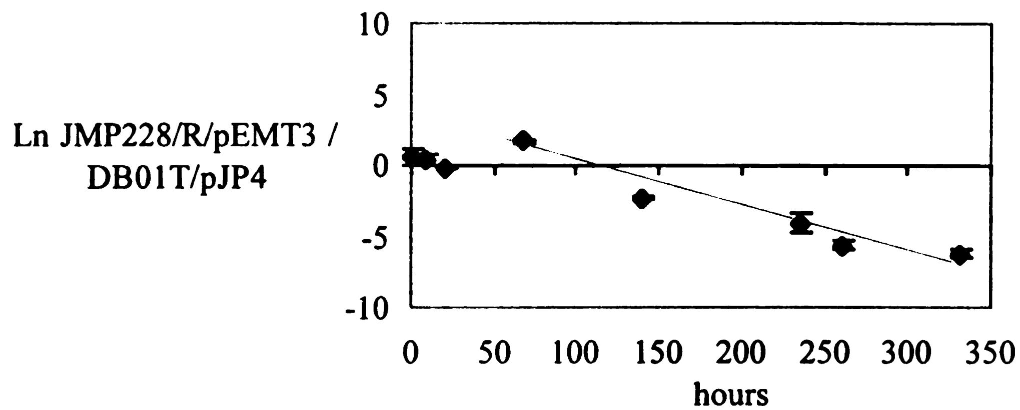


Figure 4.22. Chemostat competition between JMP228R/pEMT3 and DB01T/pJP4. Top panel: population size of JMP228R/pEMT3 (black square) vs. DB01T/pJP4 (gray open triangle) in chemostat competition. The expected growth curves for each strain are indicated with black and gray lines, respectively. Solid diamonds indicate measured 2,4-D concentration, open ones are expected from the model. Bottom panel: the natural log of the ratio JMP228 to DB01. The slope of the line (-0.48) and r^2 (0.923) indicate a significant trend in favor of DB01/pEMT3.

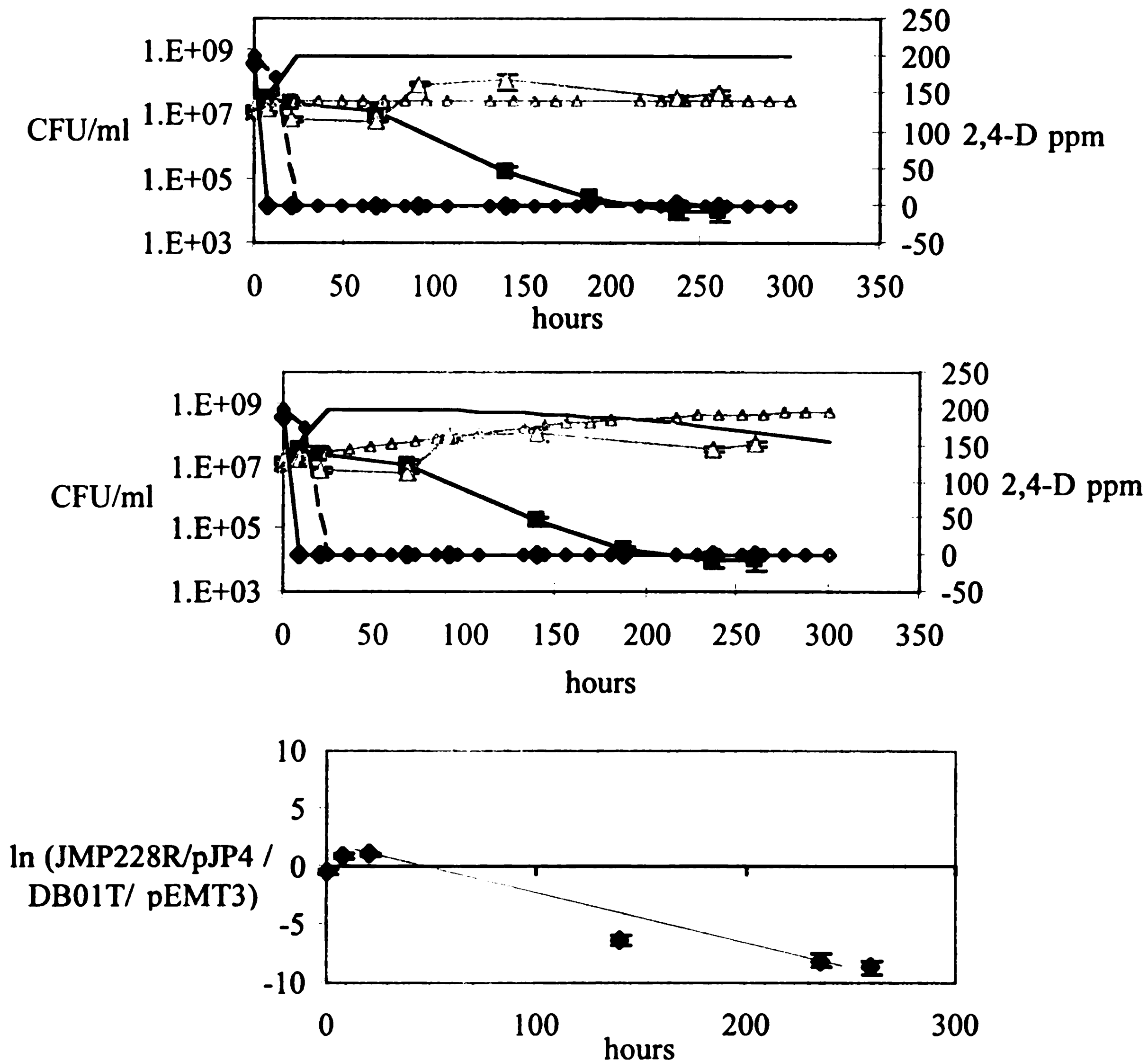


Figure 4.23. Chemostat competition between JMP228R/pEMT3 and DB01T/pEMT3. Top panel: the population size of JMP228R/pJP4 (black square) and DB01T/pEMT3 (gray open triangle) in chemostat competition when DB01/pEMT3 has a K_S value of 0.25 ppm. Middle panel: the same observed values as top panel, but with the expected outcome of competition calculated with the K_S of DB01/pEMT3 assigned to 0.1 ppm. The expected growth curves for each strain are indicated with black and gray lines, respectively. Solid diamonds indicate measured 2,4-D concentration, open ones are expected values. Bottom panel: the natural log of the ratio JMP228/pJP4 to DB01. The slope of the line (-0.039) and r^2 (0.9062) indicate a trend in favor of DB01/pEMT3.

Inhibitor production by DB01. Of three scenarios that could have resulted in the dominance of DB01 over JMP228, two were investigated. In the above examples, *B. cepacia* DB01 may indeed posses a lower K_S than *R. eutropha* JMP228, although it was not detected by empirical means, and additional factors may be involved as well. Another plausible cause for the decline in JMP228, would be if DB01 was producing an inhibitory compound. Using cell-free filtrates of the chemostat fluid of DB01 strains with supplemental 2,4-D as the substrate for the growth of JMP228 indicated two things. All of the different dilutions of the culture fluid supported the growth of the JMP228/pJP4 and JMP228/pEMT3 strains to the same extent, as measured by turbidity. All cultures reached comparable densities (0.1 - 0.14), at 600 nm. Less turbidity was seen in phosphate buffered saline with 2,4-D (0.09). Consequently, there was no indication of an inhibitory compound secreted to the medium by DB01 during chemostat growth that adversely affected the growth of JMP228. The experiment also suggested that there were sufficient micro-nutrients present in the "spent" media to support growth, especially if more of the primary nutrient (2,4-D) was supplied. These experiments did not address the possibility of evolution of fitness among the DB01 strains.

DISCUSSION

When populations compete for limited resources, whether for habitat, shelter, food or breeding grounds, the interaction may have different outcomes. The first may relate to Gause's Principle of Competitive Exclusion. Two competing species cannot coexist indefinitely in a single niche (Gause, 1934). A mutual requirement for the resource will result in competition for survival and the population that can utilize the resource more effectively will dominate. Consequently, populations of bacteria such as *R. eutropha* or *B. cepacia,* competing for one limiting resource (2,4-D), should have one winning population.

A competitive mechanism that could be used by bacteria is to interfere directly with a competitor through the production of antibiotics or toxins (Schroth 1992). This is analogous to "fighting it out" for territory or mates among land mammals or birds. Results from these experiments did not indicate the production of inhibitory compounds toward *R. eutropha* by *B cepacia* growing on 2,4-D in chemostats. However, the potential exists that the analyses performed were insufficient to detect toxins, particularly if they were lipid soluble, or did not pass through the membrane during filtration. Lipid soluble toxins, like cepalysins with hemolytic or antifungal properties,

are made by *B. cepacia* strains (Abe and Nakazawa, 1994). Biocidal activity toward bacteria and fungi has been reported to involve lipid associated compounds that are secreted through vesicles. (Hu and Young, 1998). Most of the toxic activity acts against eukaryotic cells, such as in the colonization of lung tissue of Cystic Fibrosis patients by *B. cepacia.* So far the activity of the cepalycins have not been shown to be active against members of *R. eutropha*.

In the absence of inhibitory compounds, the interaction is probably one of exploitative competition. Among bacterial populations, a competitive interaction that occurs by more efficient exploitation of a resource has been demonstrated in numerous experiments using different bacterial populations and different substrates (Duetz et al., 1994; Gisi et al., 1988; Hansen and Hubbell, 1980; Lenski et al. 1994).

Once it has been observed that a population becomes dominant over another, the exact mechanism by which a population becomes a more successful competitor must be defined. From ecological studies of succession and community evolution, it has been suggested that populations fall on a gradient of two basic life style groupings. Populations that are r-selected maximize their growth rates when resources are plentiful.

Pioneering species that have the capability to grow quickly utilize resources rapidly in order to fuel a rapidly increasing population size. Weedy plants (that are the targets of the 2,4-D used in this study) are just such an example. In bacteria, like in plants, rapid growth may have a price, and as the populations expand in size resources are exhausted and the environmental pressures change. Nutrient poor conditions arise, and competition for the resource becomes ferocious. Levels of utilizable substrate may drop below the detection capabilities of the r-selected bacteria, and they begin to starve. Under nutrient-poor conditions, a populations that can detect the lowest quantities of a resource will be more successful. These are the K-selected populations, and they are adapted to survival under conditions of crowding or resource limitation. Populations thought of as r-selected should have high growth rates, and those that are K-selected, should have low K_S values.

The two different growth conditions used in these experiments, batch and continuous culture, represent two environments that could favor growth by bacterial populations utilizing different life history strategies (Andrews, 1986). The r- and K- selection strategies were originally used to describe sexually reproducing populations, but also have some application to microbial ecology (Roughgarden, 1971, Kasahara,1991, Gorlach, 1994). In essence, bacterial populations that maximize their growth rates should be

good pioneering species and invaders. Both host strains, *R. eutropha* JMP228 and *B. cepacia* DB01 fall into this category of the continuum of survival strategies. Differences in how the strains compete with one another in batch and continuous culture suggest that *B. cepacia* DB01 is more K-selected and *R. eutropha* JMP228 is more r-selected.

Obviously, r- and K- selection theories can be tied directly to bacterial growth kinetics, the properties that have traditionally been used to predict the outcome of resource based competition among two populations (Monod, 1949; Hansen and Hubbell, 1980). In several experiments, populations were characterized based on measurable traits such as maximum growth rate (μ_{max}), and the half saturation constant (K_s) for a compound supplied as a sole source of carbon and energy. (Duetz et al. 1994, Greer et al. 1992, Graham, 1993). Previously, a kinetic comparison was performed among seven 2,4-D degrading strains of bacteria but this study did not determine how the different strains would interact when in competition (Greer et al., 1992). Growth parameters were estimated using the Monod model fitted to 2,4-D depletion data. This data indicated that there was generally very little difference among the examined strains. Since at least five of the isolates were obtained by enrichment from sewage sludge, the likelihood of comparing only fast growing phenotypes was quite high. The experiments

presented in this Chapter were designed to test not only the fastest growing 2,4-D degraders, but rather to examine combinations of alleles that would confer either a high growth rate or a low growth rate to the host population.. In order to predict dominance among two populations carrying either of two plasmids in terms of their 2,4-D degradative characteristics, the maximum growth rate and K_S of the bacterial strains were determined. Based on resource competition theory, under high nutrient concentrations and batch transfer conditions, the dominant strain would be the one with a faster growth rate. In contrast, the dominant strain under low nutrient continuous culture conditions would be the one to reduce the substrate to the lowest concentration. When resource levels are depleted to a level at which a competing population would be unable to maintain a positive growth rate or viable population size, the population with the lower threshold value should become dominant. (Tilman, 1981; Smith, 1993).

Differences in alleles encoding enzymes for the degradation of a compound can influence the fitness of the bacterial population that utilizes that compound as a growth substrate. The ability to fully mineralize a compound is determined by a combination of the effects of the host population and the plasmid carrying the degradative alleles. The research presented here, took advantage of the naturally occurring diversity in the

degradative plasmids, alleles and bacterial populations capable of the complete catabolism of the herbicide, 2,4-D. To test whether our observations matched our pure culture predictions, populations were combined in equal ratios and the relative fitness' of the strains were assessed using the slope of the line generated by the ratio of the natural log of the population sizes (Lenski et al. 1992). By competing isogenic host populations carrying different 2,4-D alleles, as well as different species with the same alleles, the contribution of the different plasmids, with different degradative genes to the fitness of the host populations were determined.

It was expected from pure culture growth kinetics and prior research, that supplying high concentrations of 2,4-D would result in the enrichment of the pJP4-like alleles (Dunbar et al., 1996). In all batch competition experiments the successful competitor was the one with the faster growth rate, regardless of whether it was in members of the same host strain or different host strains. Plasmid pJP4 conferred dominance over plasmid pEMT3 in isogenic host populations, in both JMP228 and DB01 strains. These results supported a correlation between plasmid borne genes and fitness of the host strain.

In order to evaluate the outcome of competition in batch culture, it was necessary to allow enough time for all of the 2,4-D to be degraded by the competing populations. In 24 hours, only JMP228/pJP4 could utilize all of the substrate, while all strains degraded the substrate in 72 hours. Of what significance is stationary phase dynamics to the competitive interaction among the populations? Once the substrate is depleted, the cultures entering into stationary phase may begin to die off. Mortality could be significant if death rates during stationary phase are high. Declines in the *R. eutropha* and *B. cepacia* populations used in these experiments were not seen during the first three days of stationary phase. In the 72 hour transfer experiments, population sizes were determined daily during each of the first 3 days (Figures 4.10, 4.11, 4.12 and 4.13). JMP228/pJP4 populations did not decline despite spending 48 hours in stationary phase, and the slower growing strains were not in stationary phase for more than 24 h.

Interestingly, in these experiments, the 2,4-D degradative plasmid-allele combination was most influential in intra-species interactions, and was most pronounced in the batch competition environments. These results contrasted those observed for dichloromethane (DCM) dehalogenases from two different species of methylotrophic bacteria. In the experiments by Gisi, (1998), two isofunctional dehalogenases supplied to *Methylobacterium* DM4

(without dehalogenase) using a plasmid vector, had different activities under batch and chemostat conditions. The dehalogenase of DM11 lead to dominance of its host strain in conditions of substrate excess, and that of DM4 lead to dominance under substrate-limited conditions. For different catabolic functions, such as the degradation of 2,4-D and DCM dehalogenases, it is conceivable that there is a different relative contribution of the donated and chromosomally encoded genes

While the batch competition experiments indicated that the plasmid and its corresponding genes did have an effect on intra-species competition, the properties of the host strain were also critical to interspecies competition in the two different growth environments. Batch culture, discussed above, was selective for populations with a fast growth rate, and would represent the r-selected environments. In fact, while very little difference was observed for the growth rate of *Ralstonia* and *Burkholderia* with succinate as a carbon source, JMP228 with either plasmid, out competed DB01/pJP4. The host strain *R. eutropha* had a distinct advantage over *B. cepacia*. The dominance of strain JMP228 over DB01 might have been due to a longer lag time for the *B. cepacia* strain, as well as to the slightly faster growth of *R. eutropha*. When grown on 2,4-D in batch conditions, *R.eutropha* also outcompeted *B. cepacia*, when both utilized the same plasmid.

The half-saturation constant (K_S) refers to the concentration of resource that allows for growth at one-half the maximum growth rate, and reflects an increased affinity of an organism for a substrate. Low K_S values and high population densities under nutrient limited conditions, are indicative of K-selected populations. It is most practical to examine low substrate conditions in continuous culture, where byproducts and cells are removed as new resource is supplied. The organism that displays a lower substrate threshold should be able to reduce the resource to a lower concentration than its competitor can utilize. The magnitude of the difference would be related to the amount of extra available resource the successful competitor will have access to after the other strain is no longer capable of responding to the presence of the nutrient.

K_S values obtained experimentally, were very similar among the four strain/plasmid combinations. With the same K_S, the same dominant population should have been observed in both batch and chemostat competitions, but surprisingly, different results were obtained. Intra-specific competition between JMP228/pJP4 and JMP228/pEMT3 strains resulted in coexistence of the two different plasmid-bearing populations, and the same was seen in competition between DB01/pJP4 and DB01/pEMT3. The pEMT3 carrying strain initially decrease relative to the pJP4 carrying strain,

due to the initial 2,4-D that was utilized by the faster growing strain. Eventually the two populations carrying different plasmids equilibrated, with slightly larger numbers of the pJP4 bearing population..

The most surprising result was obtained in the inter-specific chemostat competitions. During the first 8 hours of equilibration, *R. eutropha* outcompeted *B. cepacia*, as would be expected by its faster growth rate on the initial 2,4-D. After the original resource was depleted and both populations sought a new equilibrium, DB01/pJP4 and DB01/pEMT3 strains began to increase in numbers and slowly replace the JMP228 competitor populations. How did the slower growing DB01 dominate?

Another explanation for dominance of DB01 populations may lie in the differences in the resource uptake mechanisms or affinity for the substrate, despite the fact that there was little observed difference in the measured value for K_S. Perhaps the difference was in the manner in which the 2,4-D was utilized in the slow growth rate environments. In pure culture experiments (Chapter 3), the DB01 populations appeared to convert more of the 2,4-D to CO_2, which may have allowed them to more completely utilize more of the substrate than the JMP228 strains. Perhaps the K_S for DB01 was lower than that of JMP228, but the experiment to determine K_S was not sensitive enough to detect a difference between the strains. When models

were run with lower K_S values assigned to DB01, expected and observed population dynamics resulted in similar outcomes, supporting the hypothesis that DB01 does, indeed, possess greater affinity for the 2,4-D at low resource concentrations.

It is also possible that there are differences between JMP228 and DB01 in the portion of the degradation pathway involved with the conversion of CMA to succinate. The enzymes responsible for this transformation are chromosomally encoded, and may differ between the two species of bacteria. To date, it is not known how different the *mar* genes of *Ralstonia* and of *Burkholderia* are, or if variations in this portion of the pathway could be sufficient for influencing the outcome of the competitive interaction between the two strains.

Since antibiotic resistance markers were used to selectively identify the pJP4 and pEMT3 carrying strains, two scenarios could explain the coexistence of the same host with different plasmids. The first was that a pJP4 carrying strain, resistant to both antibiotics was being detected, rather than the pEMT3 carrying strain. Alternatively, pJP4 had transferred from JMP228/pJP4 to the JMP228/pEMT3, or from DB01/pJP4 to DB01/pEMT3. Initial experiments conducted on antibiotic resistant populations indicated that there was a very low frequency of occurrence of a doubly resistant

antibiotic phenotype (<10 CFU per 10^8 CFU plated), and colonies resistant to both antibiotics were obtained from the chemostats at < 10^2 CFU ml^{-1}. Resistance to both antibiotics could not account for the high numbers of the pEMT3-host type detected in the competition experiments. Could plasmid tranfer promote the replacement of pEMT3 due to incompatibility of the two Inc-P plasmids? Although the possibility for plasmid transfer exists, there was little evidence to suggest that this was the driving force behind the coexistence. Plasmid transfer was investigated by using a technique to determine which 2,4-D alleles for *tfd*A and *tfd*B were found in of 10 colonies of JMP228R/pEMT3 from a the 10^{-7} selective plate, as well as 10 colonies for each of the JMP229S/pJP4 strains from the 10^{-7} dilution. The colonies were analyzed using the Polymerase Chain Reaction (PCR) with primers specific for the *tfd*A and *tfd*B genes of pJP4 as described by (Vallaeys et al, 1993). Results indicated that since the JMP228/pJP4 and DB01/pJP4 yielded a product of the appropriate size with both primer sets, all those isolates maintained genes like those of plasmid pJP4. The JMP228/pEMT3 and DB01/pEMT3 colonies all yielded product with the *tfd*A primer set, but did not amplify with the *tfd*B set, suggesting that pJP4 had not been acquired by the population.

In conclusion, host encoded characteristics are crucial to inter-specific competition, particularly under different growth conditions. If a plasmid provides a faster growth rate to its host, that population will gain a competitive advantage when resources are plentiful, regardless of the strain hosting that plasmid. However, at the lower limits of substrate acquisition, small differences in resource utilization, such as the K_S for 2,4-D, may swing the pendulum in favor of a different, slower growing population.

ACKNOWLEDGMENT

This research was funded by a grant from the National Science Foundation (BIR 912006)

REFERENCES

Abe, M, and T. Nakazawa. 1994. Characterization of hemolytic and antifungal substance, cepalycin, from *Pseudomonas cepacia*. Microbiol. Immunol. 38:1-9

Andrews H. H. and R. F. Harris. 1986. r- and K- Selection and Microbial Ecology. In: ED. K.C. Marshall. Advances in Microbial Ecology. Vol.9. Plenum Publishing. pp 99-147.

Chao, L., B.R. Levin and F.M. Stewart (1977). Complex community in a simple habitat: Experimental study with bacteria and phage. Ecology. 58:369-378.

DiGiovanni G.D., J.W. Neilson, I.L. Pepper and N.A. Sinclair. 1996. Gene transfer of Alcaligenes eutrophus JMP134 plasmid pJP4 to indigenous soil recipients. Appl. Enviorn. Microbiol. 62:2521-2526

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

Don, R. H., A.J. Weightman, H.J. Knackmuss and K. N. Timmis. 1985. Transposon mutagenesis and cloning analysis of the pathways for degradation of 2,-4dichlorphenoxyacetic acid and 3-chlorobenzpate in Alcaligenes eutrophus JMP134 (pJP4). J. Bacteriol. 161:85-90.,

Duetz W. A., C. DeJong, P.A. Williams, and J. G. Van Andel. 1994. Competition in chemostat culture between *Pseudomonas* strains that use different pathways for the degradation of toluene. Appl. Environ. Microbiol. 60:2858-2863.

Dunbar, J., D.C. L. Wong, M.J. Yarus, and L. J. Forney. 1996. Autoradiographic mothod for isolation of diverse microbial species with unique catabolic traits. Appl. Envrion. Microbiol. 62:4180-4185.

Dunbar, J., S. White, and L. Forney. 1997. Genetic diversity through the looking Glass: effect of enrichment bias. Appl. Envrion. Microbiol. 63:1326-1331.

Dykhuizen, D. E. and D. L. Hartl, 1983. Selection in chemostats Microbiol. Rev. 47:150-168.

Fukumori F. and R.P. Hausinger. 1993. Alcaligenes eutrophus JMP134 "2,4-D dichlorophenoxyacetate monooxygenase" is an a-ketoglutarate-dependent dioxygenase. J. Bacteriol. 175:2083-2086.

Fulthorpe, R.R., C. Mcgowan, O.V. Maltseva, W.E. Holben, and J.M. Tiedje. 1995 2,4-Dichlorophenoxyacetic acid-degrading bacteria contain mosaics of catabolic genes. Appl. Environ. Microbiol. 61:3274-3281

Gause, G. F. 1934. The struggle for existence. Hafner, New York. 1964. Pp163-

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.

Gisi, D., L. Willi, H. Traber, T. Leisinger and S. Vuilleumier. 1998. Effercts of bacterial host and dichlromethane dehalogenase on the competitiveness of methylotrophic bacteria growing with dichloromethane. Appl. Environ. Microbiol. 64:1194-1202

Gorlach, K, R. Shingaki, H. Morisaki and T. Hattori. 1994. Construction of eco-collection of paddy field soil bacteria for population analysis. J. Gen. Appl. Microbiol. 40:509-517.Greer C.W., J. Hawari and R. Samson. 1990, Influence of environmental factors on 2,4-dichlorophenoxyacetic acid degradation by *Pseudomonas cepacia* isolated from peat. Arch. Microbiol. 154:317-322.

Graham, D. W. , J. A. Chaudhary, R. S. Hanson and R. G. Arnold. 1993. Factors affecting competition between Type I and Type II methanotrophs in two-organisms, continuous-flow reactors. Microbial Ecology, 25:1-17

Häggblom, M.M. 1992. Microbial breakdown of halogenated aromatic pesticides and related compounds. FEMS Microbiology Reviews 103:29-72

Hansen S. R. and S. P. Hubbell. 1980. Single-nutrient microbial competition: qualitative agreement between experimental and theoretically forecast outcomes. Science. 207:1491-1493.

Herrick J.B., K.G. Stuart-Keil, W.C. Ghiorse and E. L. Madsen. 1997. Natural horizontal transfer of a naphthalene dioxygenase gene between bacteria native to a coal tar-contaminated field site. Appl. Eviron. Microbiol. 63:2330-2337.

Hu, F. P., and J. M. Young. 1998. Biocidal activity in plant pathogenic Acidovorax, Burkholderia, Herbaspirilum, Ralstonia and Xanthomonas spp. J. Applied Microbiol. 84:263-271

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. 60:1121-1128

Ka, J.O. and J.M. Tiedje 1994b. Integration and Excision of a 2,4-dichlorophenoxyacetioc acid-degradative plasmid in Alcaligenes paradoxus and Evidence of its natural intergeneric transfer. J. BActeriol. 176:5284-5289,

Kasahara, Y. and T. Hattori. 1991. Analysis of bacterial populations in a grassland soil according to rates of development on solid media. FEMS Micobiol. Ecol. 86:95-102.

Koh,, S., M.V. McCullar, and D.D. Focht. 1997. Biodegradation of 2,4-dichlorophenol through a distal meta-fission pathway. Appl Environ. Microbiol. 63:2054-2057.

Kukor, J.J, R.H. Olsen and J.S. Siak. 1989. Recruitment of a chromosomally encoded maleylacetate reductase for degradation of 2,4-dichlorophenoxyacetic acid by plasmid pJP4. J. Bacteriol. 171:3385-3390.

Lenski, 1992, Relative Fitness: its estimation and its significance for environmental applications of microorganisms in Microbial Ecology (eds. M. A. Levin, Seideler R.J. and Rogul, M.) McGraw-Hill, Inc. pp183-198

Lenski, R.E., S.C. Simpson, and T.T. Nguyen. 1994. Genetic analysis of a plasmid-encoded host genotype-specific enhancement of bacterial fitness

Maltseva, O. And P. Oriel. 1997. Monitoring of an alkaline 2,4,6-trichlorophenol-degrading enrichment culture by DNA fingerprinting methods and isolation of the responsible organism, haloalkaliphilic *Nocardioides* sp. Strain M6. Appl. Environ. Microbiol. 63:4145-4149.

McGowan, C., R. Fulthorpe, A. Wright, and J.M. Tiedje. 1998. Evidence for interspecies gene transfer in the evolution of 2,4-dichlorophenoxyacetic acid . Appl. Environ. Microbiol. 64:4089-4092.

Monod, J. 1949. The growth of bacterial cultures. Annu. Rev. Microbiol. 3:371 - 394.

Ravatin R., A.J.B. Zehnder and J.R. Van Der Meer. 1998. Low-frequency horizontaltransfer of an element containing the chlorocatechol degradation genes from Pseudomonas sp. strain B13 to Pseudomonas putida F1 and to indigenous bacteria in laboratory-scale activated-sludge microcosms. Appl. Eviron. Microbiol. 64:2126-2132.

Rochkind-Dubinsky, M.L., G.S. Sayler, and J. W. Blackburn, *Microbiological Decompostition of Chlorinated Aromatic Compounds.* New Yorek, Marcel Dekker Inc. pp 108-123

Roughgarden, J. 1971. Density-dependent natural selection. Ecology. 52:453-468.

Schroth, M. N., D. C. Hildebrand and N. Panopoulos. 1992. Phytopathogenic pseudomonads and related plant-associated pseudomonads. Pp 3104-3131. In: J. Balows, A, H.G., Trüper, M. Dworkin, W. Harder, K-H Schleifer (eds.), *The Prokaryotes: A Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications.* 2nd ed. New York. Springer-Verlag,

Smith, V. 1993, Implications of Resource-Ratio Theory for microbial ecology. Advances in Microbial Ecolgy. 13:1-37.

Tilman, D. 1981. Tests of resource competition theory using four species of Lake Michigan algae. Ecology. 62:802-815.

Top E.M., W.E. Holben and L.J. Forney (1995) Characterization of diverse 2,4-Dichlorophenoxyacetic acid-degrading plasmids isolated from soil by complementation. Appl. Environ. Microbiol. 61:3274-3281

Vallaeys T., F. PerselloCartieaux, N. Rouard, C. Lors, G. Laguerre, and G. Soulas. 1993. PCR-RFLP analysis of 16S rRNA, *tfd*A and *tfd*B genes reveals a divresity of 2,4-D degraders in soil aggregates. FEMS Microbiology Ecology. 24:269-278

Vollmer M. D. K. Stadler-Fritzche and M. Schlömann. 1993. Conversion of 2-chloromaleylacetate in *Alcaligenes Eutrophus* JMP134. Microbiology.

Wright, A. and R.H. Olsen. 1994. Self-mobilization and organization of the genes encoling the toluene metabolic pathway of Pseudomonas mendocina KR1. Appl. and Environ. Microbiol. 60:235-242.

Wyndham R.C. 1986. Evolved aniline catabolism in *Acinetobacter calcoaceticus* during continuous culture of river water. Appl. Envrion. Microbiol. 51:781-789

CONCLUDING STATEMENT

WHY ARE 2,4-D DEGRADING MICROBES SO DIVERSE?

Microbes with the capacity to utilize 2,4-D as a substrate, are abundant in many environments, particularly in agricultural and urban areas where broad-leaf weeds are controlled. Table 1.3 illustrates the presence of great diversity in 2,4-D degrading microbes. Natural variation also exists in pathways for degrading 2,4-D, and in the vectors that move them among microbial populations. What is the advantage of such diversity in bacterial genes? Why do they persist and continue to diversify, when a particular set of alleles may offer a fitness advantage to its host? Why do we consistently find many populations carrying out similar activities in the same habitat?

At the root of this bacterial diversity, is environmental variation. The fact that most soils are composed of numerous abiotic and biotic components: moisture, rock, sand, silt, decaying organic matter, predators, which lead to numerous microhabitats. To a microbe with a size of only 1 μm length, a grain of sand is a great boulder. Decaying leaves provide a source of nutrients to many different microbes, each potentially suited to one very specific task in the degradation process. Among plant components that can be degraded by microbial populations, are the products of lignin degradation by fungi. The lignin products are many, and include compounds like chlorinated phenols. To degrade chlorinated phenols, is

only a step away from the ability to utilize 2,4-D. If the microbe possesses a means of breaking down 2,4-D to a chlorinated phenol, and already can degrade lignin-like compounds, it has acquired the ability to utilize a new source of carbon and energy. In environments where the entire 2,4-D degradative pathway is favorable, such as in maintained gardens and locations frequently treated with 2,4-D, the components may become assembled on mobile genetic elements that can be shared among microbial populations. In this way, familiar elements and patterns become recognizable in our analysis of 2,4-D degrading genes and plasmids.

Why is this diversity maintained in nature? Perhaps it is because of differences in the microhabitats that occur in close proximity to one another. At different times, under different resource limitations, a greater diversity of pathways in the environment can lead to either a more complete degradation of compounds or the maintenance of variants capable of degrading slightly different compounds with different affinities for each. Even the plasmids used in this study, pJP4 and pEMT3 differed in the specificity of their enzymes toward chlorinated or non-chlorinated substrates, respectively. Plasmid pJP4 provided greater efficiency toward chlorinated substrates, while pEMT3 provided more rapid degradation of non-chlorinated substrates. Depending on the natural fluctuation of the environment, the variant pathways may both be advantageous at different times.

Microhabitats provided by differences in resource concentration, may be as important as ones created in physical space. The research conducted in this dissertation examined two very simple habitats; one in which the substrate concentration was plentiful (batch transfer), and one in which the substrate was limiting (low flow rate chemostats). In each of the different habitats, there was a propensity for a different bacterial population to become dominant. *Ralstonia eutropha* tended to dominate under conditions of high 2,4-D concentrations, and the presence of pJP4 was important, as a faster growth rate was favored. To the contrary, the low flow rate chemostats, in which the concentration of the resource was maintained at levels below 1 ppm 2,4-D, *Burkholderia cepacia* was able to dominate over the *R. eutropha* strains. The dominance of *B. cepacia* strains appeared to be the result of slightly lower half-saturation constants relative to the *R. eutropha* strains. In environments where 2,4-D application is occasional, but at high concentrations, the pJP4 carrying strains may become more abundant shortly after application, but after the initial substrate is diminished, a different physiological parameter becomes more important. The ability to utilize lower concentrations of 2,4-D (or any other resource) will lead to an increase in the size of the populations with lower thresholds for a substrate. Variation in substrate concentrations over time, can therefore support a greater diversity of bacteria than any constant environment. Since natural habitats are much more complex than the

environmental parameters that were tested, the potential niches provided to microbial populations in nature provide sufficient variation to support a diverse range of genes, continuously adapting and evolving to suit its current habitat. Spatially separated micropopulations can begin to diversify, along their own evolutionary path, thus continuously increasing the diversity of microbial populations on earth.

MICHIGAN STATE UNIVERSITY LIBRARIES
3 1293 02092 5313