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EFFECT OF SELECTION ON THE STRUCTURE OF A 2,4-DICHLOROPHENOXYACETATE (2,4-D) DEGRADING GUILD IN SOIL

By

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ABSTRACT

EFFECT OF SELECTION ON THE STRUCTURE OF A 2,4-DICHLOROPHENOXYACETATE (2,4-D) DEGRADING GUILD IN SOIL

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The effect of selection on the genetic diversity among 2,4-D degrading (2,4-D$^+$) organisms in soil which had no previous history of exposure to this herbicide was assessed. To sample indigenous populations, a direct plating method was developed in which colonies exhibiting the 2,4-D catabolic phenotype were sensitively detected amidst nontarget colonies by autoradiography. Comparison of enrichment culture techniques with the direct plating method demonstrated a significant bias in the genetic diversity of organisms obtained by enrichment techniques. Using the direct plating method, a high degree of genetic diversity was observed in the 2,4-D$^+$ guild in soil. Multiple catabolic groups defined by hybridization patterns with 2,4-D gene probes, a large number of species, and an even larger number of genetically distinct populations were identified. The dominant 2,4-D$^+$ species in the untreated soil was identified as *Variovorax paradoxus*. Analysis of isolates representing this species identified 14 populations which differed dramatically in the physical organization of their genomes. Variation was also observed within populations suggesting that in the field site we examined, most *V. paradoxus* cells were genetically distinct. Following addition of 2,4-D to the soil, only a fraction of the 2,4-D$^+$ populations present increased to high frequencies. One species predominated, despite the fact that 2,4-D$^+$ members of this species were initially rare in the soil. These results indicate that microbial guilds in disturbed soils can be rapidly restructured during short periods of growth. Competitive fitness among the 2,4-D$^+$ populations strongly correlated with specific alleles for 2,4-D catabolism and *rrn* operon copy number. It is not known whether the association between these genetic factors is

causal or merely correlative. However, it is possible that substrate transformation (growth rate) and lag time may be primary determinants of fitness among 2,4-D^+ populations in response to pulses of substrate.

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INTRODUCTION

Competition is considered one of the most important interactions among bacteria [16]. In natural environments, the reproductive success of an organism is thought to be frequently determined by its ability to compete with other organisms for a limiting resource [54]. For example, the availability of carbon often limits the extent of reproduction of heterotrophic bacteria in soil [47, 59]. Populations which obtain the most carbon in soil experience the most growth. Thus, differences in competitive fitness can result in dominance of some populations while others remain rare or become extinct. Since competition shapes the relative abundance of bacterial populations, identifying the determinants of competitive fitness provides a key to predicting the success of individual populations as well as understanding the structure and evolution of microbial communities. However, at present, the factors which determine competitive fitness and the dynamics of competition in natural microbial communities are poorly understood.

Competition dynamics in simple environments.

Current knowledge of competitive fitness and competition dynamics is derived from nutrient-based competition experiments in chemostats. Chemostats provide a homogeneous liquid environment for steady-state bacterial growth. In these simple environments, one population typically becomes dominant and less competitive populations become extinct due to "washout". During steady-state conditions in a chemostat, the competitive success of populations competing for a single limiting nutrient is based on a simple relationship between specific growth rate, μ, and substrate concentration, S, described by the Monod growth equation as follows:

$$\mu = \mu max \left(\frac{S}{Ks + S}\right)$$

where μmax is the maximum specific growth rate, and Ks is the substrate concentration at which 1/2 μmax is achieved [42]. Comparison of the μ vs. S curves for different organisms identifies the range of substrate concentrations in which an organism will be competitively superior (Figure 1). Generally, organisms with high μ_{max}/Ks values are most competitive at low nutrient concentrations while organisms with high maximum growth rates, μ_{max}, tend to become dominant at high nutrient concentrations.

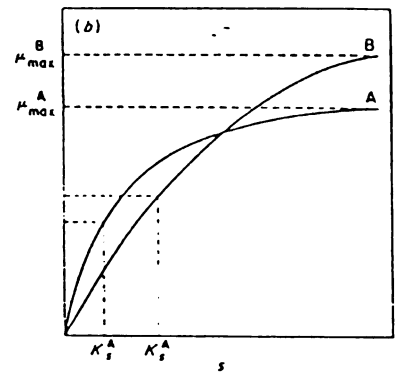

Figure 1. Hypothetical μ vs. S curves for two different organisms, A and B. Organism A is more competitive at high substrate concentrations. Organism B has a higher μ_{max}/Ks value and is more competitive during steady state growth than organism A at low nutrient concentrations. [From 53]

In actual practice, the specific growth rate of a pure culture in a chemostat is controlled by the dilution rate while the residual substrate concentration is a variable determined by the physiology of the organism being cultivated. Thus, the outcome of competition in chemostats can be forecast based on the steady-state substrate concentration values obtained from individual pure cultures [22, 53]. The most competitive population at a particular growth rate is the one which yields the lowest steady-state substrate concentration (reflecting a high substrate affinity). As the substrate concentration is driven

down by the most competitive population, competing populations with less affinity for the substrate grow slower and are washed out. Instead of comparing entire μ vs. [S] curves, the competitive success of populations grown in chemostats can theoretically be forecast based on a single index. Among the indices which have been proposed , the J parameter (a.k.a. R* [53]) most accurately predicts the competitive fitness of individual populations [17, 22, 54]. This index reflects the steady state substrate concentration in a chemostat containing a pure culture growing at a fixed rate and is expressed as:

$$J = Ks\left(\frac{D}{\mu_{max} - D}\right)$$

where D is the dilution rate or death rate due to washout, μ_{max} is the maximum specific growth rate, and Ks is the substrate concentration at which 1/2 μ_{max} is achieved. Ranking the J parameters of different organisms describes the predicted competitive outcomes in chemostats under steady state conditions. The organism having the lowest J parameter would be expected to become dominant and exclude competing populations. These simple dynamics are described by classical ecological theory which states that two or more competitors cannot coexist in a single niche.

Competition dynamics in complex environments.

More complex outcomes can occur in chemostats as a result of increasing the ecological complexity of the environment. For example, attachment of bacteria to the walls of chemostats results in coexistence of populations competing for a single limiting nutrient [23]. Dykhuizen and Hartl [9] reported that the initial ratio of competitors is retained among wall populations while dramatic changes in competitor ratios occur in the liquid medium. Immigration of cells from the walls to the liquid ultimately preserves the inferior population at a constant frequency in the liquid medium. The frequency of the inferior competitor in the liquid medium would presumably increase as the ratio of solid

surface area to liquid volume in the chemostat increased. In soil, most bacteria grow attached to surfaces [19]. Thus, competing populations in soil may retain their initial ratios, similar to wall populations observed in chemostats, and coexist at high population densities even though they may differ in fitness.

Growth of bacteria in chemical gradients can also result in coexistence of competing populations. It is known that temperature, pO2, pH, Eh, salinity, and nutrient concentrations can affect the rates of nutrient uptake and the kinetic constants of growth (Ks and μ_{max}) upon which competitive fitness are based [58]. Thus, it can be imagined that organisms with different physiological optima can coexist when competing for a single limiting nutrient by occupying different positions along a gradient. Gause [14] demonstrated coexistence of two *Paramecium* species competing for a bacterial prey in static liquid cultures; agitation of the cultures disrupted the oxygen gradient created by bacterial respiration and resulted in competitive exclusion of the more anaerobic *Paramecium*. Similarly, Wimpenny *et al.* [61] reported the coexistence of *E. coli*, *Pseudomonas aerogenes*, and *Clostridium acetobutyricum* in opposing oxygen and glucose gradients during growth in a linear array of five interconnected chemostats [61]. Competition for glucose resulted in *P. aerogenes* becoming dominant in the aerobic end of the array while *C. acetobutyricum* became dominant in the anaerobic end of the array. Subtle microhabitat ranges described by pH and salinity have also been demonstrated [37, 41, 51, 60]. It has been shown mathematically that *n* species can coexist on a single limiting resource provided there are *n* microenvironments [11]. Surfaces (for attachment) and gradients foster coexistence by dividing the environment into a set of differentiated subhabitats in which different bacteria can successfully compete.

Similarly, coexistence of populations competing for a single resource can be enhanced by temporal variation. Fluctuating environmental conditions represent an intangible resource in which change itself becomes a dimension for bacterial

differentiation. For example, two species of sulfur bacteria, *Chromatium vinosum* and *Chromatium weissei*, were shown to coexist at a ratio of 3:2 when competing for sulfide in a chemostat under a fluctuating light regime, whereas *C. vinosum* outcompetes *C. weissei* at all sulfide concentrations when grown under continuous light [56]. The physiological basis for the ability of *C. weissei* to compete successfully under fluctuating conditions was revealed to be its higher rate of sulfide oxidation compared to *C. vinosum* [56]. Since sulfide oxidation is a light driven process, sulfide accumulates during periods of darkness. When illumination resumes, *C. weissei* rapidly oxidizes the majority of sulfide present in the medium and deposits sulfur internally while synthesizing glycogen. The ability of *C. weissei* to sequester a large fraction of the sulfide during periods of excess, and its high growth rate on glycogen allow it to coexist with *C. vinosum* despite the higher substrate affinity and higher specific growth rate of *C. vinosum* at all concentrations of sulfide [56]. More recently, Upton *et al.* [55] demonstrated that the diversity of bacteria in a chemostat subjected to diurnally fluctuating temperatures from 1-16°C was greater than the diversity of bacteria in a chemostat held constant at 8°C . The former contained at least four groups of bacteria that occupied different temperature niches whereas only one group was observed in the stable chemostat [55]. Futhermore, the authors showed that one of the strains from the non-steady-state chemostat was able to exploit the temperature fluctuations and outcompete a strain from the stable chemostat [44, 55]. Fluctuating environmental conditions, or non-steady-state conditions, are typical of natural environments and are expected to foster coexistence. Indeed, the diversity of some natural communities appears to decrease without periodic perturbations [18, 43], consistent with predictions from chemostat experiments.

Most of the studies which have been performed in chemostats have focused on competition for a single limiting resource. However, in natural environments bacteria may compete for multiple limiting nutrients. Theoretically, *n* species can stably coexist given *n*

limiting nutrients [46, 50]. For example, *Bacillus cereus* and *Candida tropicalis* coexisted up to 21 days in chemostat culture when fructose and glucose were supplied simultaneously as growth limiting substrates over a range of dilution rates [62]. At dilution rates between 0.1 hr^{-1} and 0.23 hr^{-1} the concentration of the bacterium was 2/5 the concentration of the yeast. When these organisms were grown in mixed culture on either fructose or glucose alone, *C. tropicalis* quickly excluded *B. cereus* at all dilution rates. Similarly, in a mixed culture isolated from estuarine mud, *Desulfobulbus propionicus* coexisted with *Desulfovibrio baculatus* at a ratio of 3:1 in chemostats in which iron and ethanol were both growth limiting [30]. When ethanol was the only growth limiting substrate, *D. baculatus* outcompeted *D. propionicus*. Although different models have been proposed to explain such results, all predict the same effect---multiple growth limiting substrates expand the set of equilibrium points at which coexistence of competitors can be achieved, even when all competitors strongly prefer the same substrates. The presence of a second limiting nutrient can either increase the growth rate (and thus, the competitive fitness) of a population which was competitively inferior during competition for a single limiting nutrient, decrease the growth rate of the superior population, or both. Non-steady state conditions may contribute to this occurrence [15, 53, 54].

Instead of being provided with multiple nutrients from an external source, competing populations in chemostats can coexist by converting a single limiting nutrient into multiple nutrients which can be partitioned among populations. Helling *et al.* [20] reported the stable coexistence of four mutant types which arose in an *E. coli* population cultivated for 765 generations in a chemostat. Two mutant types occurred at relatively high frequencies while another two occurred at lower frequencies. These mutants differed in both morphological and physiological features. Coexistence of the four subpopulations arose, in part, from mutations which allowed partitioning of metabolic intermediates

arising from glucose metabolism. Mutations in some clones resulted in only partial oxidation of glucose. Metabolic intermediates excreted by these clones were more efficiently utilized by other clones. This provides an interesting example of niche differentiation. Although all mutant types could utilize glucose when grown separately in pure culture, the evolved strains competed for different resources when grown as mixed cultures. Thus, although a single carbon source (glucose) was provided in the chemostat, partitioning of metabolites of glucose oxidation resulted in only partial niche overlap such that the strains could coexist.

Coexistence of chemostat populations can also result from predation if the inferior competitor is more resistant than the superior competitor. Jost *et al.* [24] examined the interaction of a protozoan, *E. coli*, and *Azotobacter vinelandii* in continuous culture with glucose as the growth limiting nutrient for the bacteria. *E. coli* displaced *A. vinelandii* at all dilution rates in the absence of the predator. Addition of the protozoan resulted in coexistence of the two species at densities of 10^8 (*E. coli*) and 10^7 (*A. vinelandii*). In this case, predation prevented *E. coli* from attaining its maximal density of 10^9 cells/ml thereby permitting the coexistence of *A. vinelandii*. Similar results have been observed using two bacterial competitors and a viral predator [5, 31, 33]. Inhibitory substances can affect competition dynamics in a manner similar to predation. If the inferior competitor is able to detoxify the inhibitor, stable coexistence of populations competing for a single resource can be achieved in chemostats [32].

The preceding examples illustrate factors which mitigate the stringent selective conditions normally occurring in simple chemostat environments and promote the coexistence of multiple populations during competition for a single limiting resource. Soil is a much more complex environment than chemostats [reviewed in 10, 45]. Surfaces for bacterial growth, chemical gradients, non-steady-state conditions, resource partitioning, predation, and metabolic inhibitors all contribute to the physical and ecological complexity

of soil. In view of this complexity, it is reasonable to wonder if simple competition dynamics ever occur.

An understanding of how competition affects species abundance and shapes community structure in natural environments can be achieved by observing the outcome of selection in these environments or in systems which approximate them. Xenobiotic compounds are useful substrates for studying competition among indigenous soil bacterial populations. In soils which have not been exposed to a particular xenobiotic compound, the number and abundance of populations able to degrade the compound is expected to be low such that significant changes can be observed in the abundance of individual populations following selection. Furthermore, since most xenobiotic compounds have entered the environment relatively recently, the genetic pathways which have evolved for dissemination of a particular compound may be relatively limited, and thus measurable. Likewise, these pathways are expected to be distributed among a relatively small number of populations. Based on these considerations, the herbicide 2,4-dichlorophenoxyacetate appears to be a useful substrate for examining competition dynamics in soil microbial communities.

Biochemistry and genetics of 2,4-D degradation

Although 2,4-D has been used for only ~50 years, genes encoding enzymes which transform 2,4-D are distributed worldwide [2, 13, 38]. In pristine environments, 2,4-D appears to be mineralized primarily by consortia in soil [13], suggesting that assembled 2,4-D catabolic pathways are rare in environments which have not been exposed to 2,4-D. In contrast, individual populations able to completely mineralize 2,4-D are easily obtained from agricultural areas exposed to 2,4-D. The abundance of 2,4-D degrading organisms in agricultural areas is generally low, ranging from a few cells per gram soil up to 10^4 cells/gram soil in areas receiving frequent applications of 2,4-D at commercially recommended field concentrations (1 μg 2,4-D g soil^{-1}, or 1 ppm) [21].

Three different biochemical pathways for 2,4-D degradation have been identified. The modified ortho-cleavage pathway was the first 2,4-D catabolic pathway identified and was elucidated by Tiedje and Alexander [52] and others [3, 4, 8, 27] As shown in Figure 3, the first step in the pathway is cleavage of the ether bond, yielding glyoxylate and 2,4-dichlorophenol (2,4-DCP). Subsequent cleavage of the aromatic ring results in the formation of dichlorocatechol which undergoes several additional enzymatic transformations to yield acetate and succinate. Other pathways have been identified, but these appear occur rarely among 2,4-D degrading bacteria. For example, Balajee and Mahadevan [1] described a slightly different ortho-cleavage pathway in an *Azotobacter chroococcum* strain in which a dechlorination reaction, not removal of the acetate side chain, was the first step in the pathway. This pathway has not been observed yet in any other strain. Kozyreva *et al.* [29] described an unusual 2,4-D pathway in a *Nocardioides simplex* strain 3E in which the first step was cleavage of the ether bond, but 2,4-DCP was subsequently converted to 2,4-dichloropyrocatechol and then to 3,5-dichloro-1,2,4-trihydroxybenzene. A hydroxyhydroquinone-1,2-dioxygenase appeared to be involved in ring cleavage [28]. The remaining steps in the pathway were not clearly established. However, growth on 2,4-D resulted in stoichiometric release of chloride ions [29]. Although three biochemical pathways have been identified, analysis of 2,4-D catabolic genes among 2,4-D degrading isolates has suggested that the modified ortho-cleavage pathway is the most common pathway for metabolism of 2,4-D [13].

Genes for 2,4-D catabolism have been extensively characterized in one bacterium, *Alcaligenes eutrophus* JMP134 (pJP4) that was first isolated in Australia [7]. The 2,4-D catabolic genes in strain JMP134 are encoded on plasmid pJP4, which is a self-transmissible, broad host range IncP1 plasmid [7]. The genes, designated *tfdA, B, C, D, E, F* are regulated by *tfdR/tfdS* [36, 63] and are organized into three transcriptional units consisting of *tfdA* , *tfdB* , and the *tfdCDEF* operons [39, 48]. The *tfdB* gene is adjacent

to *tfdCDEF*; however the *tfdA* gene lies about 13 kb apart. The pJP4 *tfd* genes encode enzymes which transform 2,4-D to glyoxylate, acetate, and succinate via the modified ortho-cleavage pathway (Figure 2).

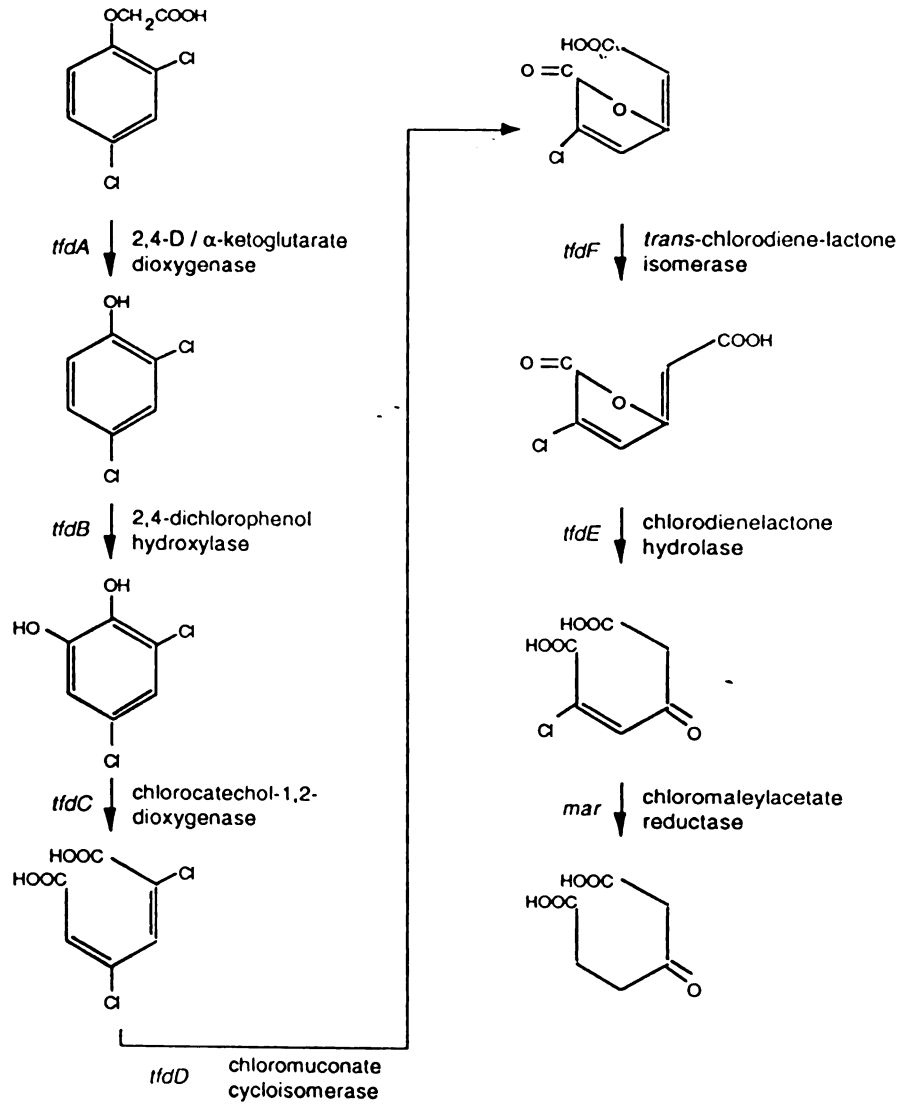

Figure 2. 2,4-D catabolic pathway encoded on pJP4. [Adapted from 7, 12, 40]

Genes highly similar, if not identical, to the pJP4 genes have been found in a variety of different species obtained from around the world [2, 6, 34], but primarily in species of *Alcaligenes* and *Burkholderia* [13]. While the pJP4-type *tfd* genes are commonly plasmid encoded in organisms other than *A. eutrophus* JMP134, they do not

always occur on IncP1 plasmids [2, 6, 26, 34], and the gene order observed on plasmid pJP4 is not always conserved [34]. Furthermore, the pJP4 *tfdABCDEF* genes do not always occur together, but instead appear distributed as mosaics with dissimilar 2,4-D genes which have varying degrees of DNA sequence similarity to the pJP4 borne genes [13]. Thus, migration, horizontal gene transfer, and recombination appear to have contributed to the spread of 2,4-D genes and the 2,4-D phenotype.

Recently, the *tfdA* and *tfdB* genes from *Burkholderia* sp. RASC have been cloned and sequenced [49]. The RASC *tfd* genes are chromosomally encoded, unlike the pJP4 *tfd* genes, but share >77% DNA sequence similarity with the corresponding pJP4 genes (Figure 3) and encode isozymes for the modified ortho-cleavage pathway. Despite their chromosomal location, the RASC genes are transmissible between species, although the mechanism of transfer is unknown [35]. At present, the *tfdA* and *tfdB* alleles from strains JMP134 and RASC are the only alleles with substantial sequence dissimilarities which have been cloned. The pJP4 and RASC *tfdA* alleles (or highly similar alleles) have been identified in most of the 2,4-D$^+$ isolates which have been examined to date by Southern hybridization analyses [13]. Among strains collected by various researchers from heterogeneous locations, Fulthorpe *et al.* [13] found that only 17% of the isolates possessed *tfdA* genes with less than 60% DNA sequence similarity to the pJP4 and RASC *tfdA* genes, and these isolates belonged almost exclusively to the genus *Sphingomonas*. Presently, it is unknown whether these alternative genes encode enzymes for a biochemically distinct pathway, or whether they encode new isozymes for the established ortho-cleavage pathway. However, it appears that the number of genes encoding enzymes which can initiate 2,4-D catabolism is quite limited.

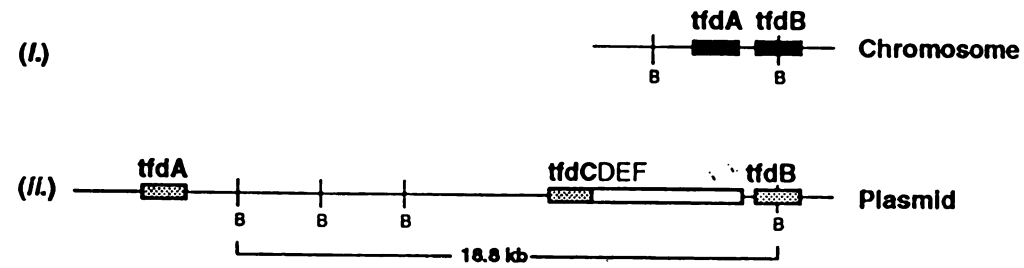

Figure 3. Arrangement of *tfdA* and *tfdB* genes in *Burkholderia* sp. RASC and *A. eutrophus* JMP134 (pJP4). (*i*) Strain RASC. (*ii*) pJP4. [Adapted from 40, 48, 49, 57]

Research Focus

The extant diversity of 2,4-D degrading organisms occuring within soil at a single location before and after selection is not well understood. Recently, Ka *et al.* [26] described the diversity of axenic 2,4-D degrading bacterial cultures obtained during four consecutive summers from eight field plots (~33m^2 each) that were either untreated or treated with 2,4-D at concentrations of 1, 10, or 100 ppm. A total of twelve 2,4-D degrading species were detected among the eight plots over the three year period. The 2,4-D degrading species obtained from treated plots differed from the strains obtained from untreated plots suggesting that the 2,4-D degrading guild had been restructured as a result of competition for 2,4-D. However, an average of only 1-2 strains per plot were obtained each year. With such a small sample size, the extent to which competition reshaped the 2,4-D degrading guild was impossible to evaluate. Due to the lack of other reports describing the diversity of indigenous populations in soil before and after competition for a limiting resource, the dynamics of competition in soil in response to 2,4-D amendment remain unknown.

To elucidate the dynamics of competition in soil, I assessed genetic diversity within the 2,4-D degrading guild in soil microcosms by intensive sampling before and

after selection. Soil was obtained from a 2 x 3 meter subsection of one of the untreated plots examined by Ka *et al.* The research was founded on the following two hypotheses:

1) Only a small number of 2,4-D degrading populations occur in soil obtained from a single location.

2) The physical, chemical, and biological complexity of soil will enable most 2,4-D degrading populations to increase to a similar abundance.

To assess genetic diversity within the 2,4-D degrading guild, pure cultures of bacteria able to degrade 2,4-D were obtained from soil by direct plating and then were characterized. The direct plating method employs autoradiography to identify bacterial colonies exhibiting a specific phenotype and is described in chapter 1. Previously, 2,4-D degrading strains have been obtained primarily by use of enrichment culture techniques [2, 6, 7, 13, 34, 38, 52] or extinction dilution in most probable number (MPN) enrichment tubes [25, 26]. Chapter 2 describes the effectiveness of direct plating compared to enrichment culture techniques in assessing the diversity of 2,4-D degrading bacteria in a single gram of soil.

The structure of the 2,4-D degrading guild in soil before and after selection was evaluated in terms of the diversity and relative abundance of 2,4-D catabolic groups (defined by hybridization patterns with 2,4-D genes), species diversity within catabolic groups, and population diversity within species. In chapter 3, the genetic variation observed among clones within populations representing a single 2,4-D degrading species obtained from untreated soil is described. The structure of the 2,4-D degrading guild in untreated and treated soil is documented in Chapter 4 illustrating the dynamics of competition in soil and potential genetic factors associated with competitive fitness.

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CHAPTER 1

Autoradiographic Method for Isolation of Diverse Microbial Species with Unique Catabolic Traits

Abstract

A novel autoradiographic method for isolation of bacteria with unique catabolic traits was developed to overcome many of the limitations of traditional selective enrichment techniques. The method consists of five steps: (1) An environmental sample is directly plated (without enrichment) on a microporous filter atop a solid medium that allows cultivation of diverse kinds of microorganisms. (2) Once colonies form, two replicas of the filter are prepared, and the colonies are regrown. (3) The replica filters are starved 24 to 72 hours to deplete intracellular carbon reserves then (4) placed on solid media containing $Na_2{}^{35}SO_4$ with and without a target compound. (5) Following an incubation period, the replica filters are exposed to film in order to identify colonies that incorporate more ^{35}S into cell biomass in the presence versus the absence of the target compound, providing presumptive evidence for metabolism of the compound. Target colonies identified in this manner can be recovered from the master filter. To demonstrate this technique, bacteria capable of degrading benzoate were isolated from a single soil slurry by traditional enrichment as well as by autoradiography. From the enrichment culture, a single isolate able to degrade benzoate was obtained. In contrast, 18 distinct strains were obtained by purifying 19 putative benzoate degrading colonies identified by autoradiography. Each of the 18 strains was able to completely transform the substrate based on HPLC analyses. The doubling times of a subset of the isolates grown in benzoate medium ranged from 1.4 to 17.1 hours, whereas the doubling time of the isolate obtained by enrichment was 2.0 hr. These data demonstrate that the method described can be used to obtain a collection of diverse organisms able to metabolize a specific compound.

Introduction

Selective enrichment culture techniques have traditionally been used to isolate bacteria in studies of microbial ecology and physiology. These techniques typically involve incubation of mixed populations (from soil or activated sludge, for example) in a medium designed to foster only the growth of microorganisms exhibiting a particular phenotype. Serial transfers of the enrichment culture to fresh medium are continued until the microbial culture appears to consist mainly of one or very few types of microorganisms exhibiting the desired properties. Undesired organisms are eliminated from the culture through competitive exclusion or dilution. Although selective enrichment techniques have historically been used with great success, there are two major limitations to these techniques which hamper their effectiveness to study and exploit the metabolic diversity and ecology of microorganisms.

First, the diversity of isolates within a sample and their frequencies in the environment are difficult to assess with the use of enrichment culture techniques. Batch cultures are competitive environments which typically select organsims with rapid growth rates. Thus, organisms with inherently slower growth rates or growth rates which are lower under the conditions used are excluded. Organisms with nutritional requirements are often overlooked as well since minimal media are frequently used for selective enrichments in an effort to limit or prevent the growth of undesired organisms. Thus, the diversity of organisms obtained from a single enrichment culture is low.

Second, enrichment procedures make it difficult to rapidly obtain organisms able to degrade recalcitrant substrates. Reports of isolation times from several months to a year are not uncommon [1, 11, 18, 23]. With enrichment cultures, the length of time required to purify bacteria is determined primarily by bacterial growth rates. Substrates which are metabolized inefficiently, toxic at high concentrations, or slightly soluble can substantially

increase the time required for organisms of interest to become established as dominant members of a culture.

Because of the limitations described above, we developed a simple method that does not rely on the use of selective enrichment techniques for screening and isolating microorganisms with particular phenotypes. The method involves cultivation of bacteria on filters atop nonselective agar medium that fosters the growth of diverse bacterial strains, subsequent incubation on radioactive test media, and the use of autoradiography to identify colonies with desired phenotypes amidst a background of nontarget colonies. Since this method permits the use of rich media and spatially separates otherwise competing populations, simultaneous recovery of organisms with different growth rates and nutritional requirements is possible, and large numbers of bacteria can be screened rapidly at a time.

Materials and Methods

Media and Reagents. R2A agar (Difco Lab., Detroit, Mich.), which is a general purpose medium for cultivation of heterotrophs, was used for initial cultivation of bacteria from soil. After isolation, bacterial strains were routinely cultured using 1/4 strength trypticase soy agar (TSA). Bacterial phenotypes were assayed in Defined Aerobic Basal medium (DAB) described in Table 1 with or without benzoate (3mM).

Soil. Soil for isolation of benzoate degraders was collected in October 1993 from an agricultural plot at Kellogg Biological Station (KBS), Michigan and stored at 4°C. A single soil slurry consisting of 1 g of soil and 9 ml of 10mM phosphate buffer (pH 6.8) was used as a common inoculum source for most probable number (MPN) assays and for isolation of organisms able to degrade benzoate (benzoate$^+$) by enrichment or by autoradiographic plating. The phosphate buffer was identical to the buffer used in DAB medium. After shaking the soil slurry for 30 minutes at 30°C (225 rpm rotary shaker), the slurry was serially diluted by transferring 1 ml to 9 ml of sterile phosphate buffer.

Enumeration of bacteria. The most probable number (MPN) of culturable heterotrophs and benzoate$^+$ organisms in the soil sample was determined according to Cochran [2]. Serial dilutions of the soil slurry were used to inoculate replicate tubes (five per dilution) containing either R2A liquid medium (for heterotrophs) or DAB-benzoate medium. MPN tubes were incubated at 30°C for two weeks. Cultures which became visually turbid were considered positives. The number of positive tubes at each dilution was scored, and estimates of microbial numbers were obtained from statistical tables [2]. Bacterial numbers were also estimated by counting colonies grown on R2A agar for seven days. The total number of colonies was used as an estimate of heterotrophic bacterial numbers. The fraction of colonies putatively identified as benzoate$^+$ by autoradiography (described below) was used as an estimate of benzoate$^+$ organisms in the soil sample.

Batch culture enrichment. An enrichment culture for benzoate$^+$ organisms was established by adding 0.1 ml of the soil slurry to a 50 ml flask containing 20 ml of DAB-benzoate (3mM) medium. The culture was shaken at 30°C in a rotary incubator shaker (225 rpm) and 0.2 ml were transferred to fresh medium every four days. After three transfers, the enrichment culture was streaked on R2A agar medium. Colonies with unique morphologies were transferred to 3 ml of DAB-benzoate medium. Cultures which became turbid were tested by HPLC for benzoate degradation. Positive cultures were streaked on 1/4 strength TSA to check for purity, then regrown in DAB-benzoate medium to establish 15% glycerol stocks for storage at -80°C.

HPLC analyses. The concentration of benzoate in liquid media was routinely measured using a Hewlett-Packard series 1050 HPLC with a LiChrosorb RP-18 (10μm) column (E. Merck, Darmstadt, Germany) and a 50% methanol:50% phosphoric acid mobile phase. One ml samples of broth cultures were filtered through 0.45 Acrodisc filters (Gelman, Ann Arbor, MI) prior to analysis.

Direct plating method. *(i) Production of master filters.* For isolation of benzoate degrading bacteria by direct plating, 0.1 to 0.5 ml of various dilutions of the soil slurry (described above) were spread on sterile, detergent free, 82mm nitrocellulose filters (Millipore HATF, 0.45 μm pore size) atop R2A agar plates. Prior to use, filters were soaked in MilliQ water, sandwiched (in single layers) between squares of Whatman #1 filter paper, then wrapped in aluminum foil and autoclaved for 25 minutes. Orientation marks were drawn on the "topside" of filters with permanent ink just before transferring the filters to R2A plates. Filters spread with soil dilutions ("master filters") were incubated on R2A plates for seven days at 30°C then replicated.

(ii) Production of replica filters. Replica filters were produced essentially as described by Hanahan [9] by pressing the master filters against sterile, HATF nitrocellulose filters. To accomplish this, a master filter was removed from an agar plate and placed colony-side up on a sterile square of Whatman #1 paper. A sterile filter was placed on the master filter, both were covered with another square of Whatman #1 paper, then the stack was compressed with a surface-sterilized plexiglass weight. Before separating the master and replica filters, the reference marks on the master filter were copied onto the back of the replica filter to record the orientation of the two filters relative to one another. From each master filter, two or more replicas were produced. Following replication, all filters were transferred to fresh R2A plates and regrown for 24 hrs at 30°C. The master filter was then photographed and stored at 4°C for further use. The replica filters were transferred to sterile DAB agarose (1%) plates and starved for 72 hrs at 30°C to deplete intracellular carbon reserves.

(iii) Substrate assay with replica filters. Following the starvation period, replica filters were transferred to DAB-agarose plates containing either 1 μCi ml^{-1} $Na_2{}^{35}SO_4$ (NEN Dupont) (control plate) or 1 μCi ml^{-1} $Na_2{}^{35}SO_4$ with 3mM benzoate (test plate). After 3 days incubation at room temperature, the filters were transferred to 10mM

phosphate buffered Bacto agar (1%) medium containing 0.2 M Na_2SO_4 to remove unincorporated $Na_2{}^{35}SO_4$ from the filters. Following a 60 min incubation on the wash medium, the filters were taped onto 20x25 cm sheets of paper, air-dried, wrapped with plastic wrap, and exposed to Kodak XAR film (Eastman Kodak Comp., Rochester, New York) overnight at -80°C. Film was developed according to the manufacturer's recommendations. Orientation marks on the replica filters were then copied onto the developed films.

(iv) Identification and isolation of colonies from master filters. Putative benzoate degrading colonies were identified by comparing the intensity of signals from filters incubated on DAB-$Na_2{}^{35}SO_4$-agarose medium with and without benzoate. The location of these colonies on a master filter was determined by alignment of a given autoradiogram with the corresponding master filter. Colonies corresponding to signals on the film were picked and restreaked on TSA agar to check for purity. Several colonies apparently unable to metabolize benzoate were also picked and restreaked. Purified cultures were then inoculated into sterile DAB-benzoate liquid medium to confirm their phenotypes. Benzoate disappearance was monitored by HPLC, and 15% glycerol stocks of positive cultures were established for storage at -80°C. Cultures unable to degrade benzoate were regrown in 1/10 strength trypticase soy broth (TSB) in order to establish -80°C glycerol stocks.

Sensitivity of detection. Two bacterial cultures, one benzoate$^+$ strain and one benzoate$^-$ strain were separately grown to stationary phase in 1/10 TSB then mixed together at a variety of ratios. From each mixed culture, 0.1 ml was spread on nitrocellulose filters atop R2A agar. Plates were incubated at 30°C for three days, then replicated and processed by autoradiography as described above.

rep-PCR. Fingerprints of the genomes of the isolates were obtained using consensus primers for Repeated Extragenic Palindromic sequences [24] and the polymerase chain

reaction (PCR) conditions described by de Bruijn [6]. Each strain was grown to stationary phase in DAB-benzoate medium or in 1/10 TSB, then cells were disrupted by four freeze-thaw cycles. For each strain, 1 µl of thawed cell lysate was added to 24 µl of PCR reaction mix. PCR reactions were performed in a Perkin-Elmer 9600 thermalcycler. Reaction products were visualized by electrophoresis in 1.5% agarose gels containing 1/2X TAE and ethidium bromide [20]. PCR amplification was performed twice for each strain to confirm the reproducibility of fingerprint patterns.

16S rDNA restriction analysis. Amplified ribosomal DNA restriction analysis (ARDRA) was carried out as follows. PCR amplification of 16S rDNA was performed using primers fD1 and rD1 described by Weisburg [25] and the reaction conditions described by Laguerre [17]. For each 100 µl reaction, 1 µl of thawed cell lysate (described above) was used as template. Approximately 10 µl of PCR product was subsequently used for each restriction digest. Amplified 16S rDNA was digested separately with *Hae*III, *Cfo*I, *Msp*I, and *Hinf*I according to the manufacturer's recommendations. Restriction fragments were visualized by electrophoresis in 2.75% Metaphor agarose (FMC) gels containing TAE and ethidium bromide.

Growth rates. Growth rates of organisms were determined in two ways. A rough comparison of the growth rates of all isolates was performed by determining the number of days required for pinpoint colonies to form on TSA plates. In these experiments, isolates were streaked directly from glycerol stocks. A more precise comparison of growth rates was performed with a subset of isolates representing the three growth rate categories (fast, medium, and slow) observed on TSA plates. Single colonies on TSA plates were transferred to 5 ml of DAB-benzoate medium and grown at 30°C, at 225 rpm, to mid-log phase. Portions of these cultures were diluted in 25 ml of sterile, prewarmed, DAB-benzoate medium in 125 ml flasks to an optical density of approximately 0.001 (l=600 nm). Each isolate was tested in triplicate. Optical density was monitored with a

Hewlett Packard diode array model 8452A spectrophotometer, and doubling times were calculated from least-squares regression analysis of transformed data [16].

Results

Bacterial numbers. Estimates of bacterial numbers in soil were obtained from viable counts on nitrocellulose filters atop R2A agar and from MPNs. According to plate counts, the number of heterotrophs and benzoate$^+$ organisms in the soil was approximately 4.9 x 10^6 cfu/g soil and 1.1 x 10^5 cfu/g soil, respectively. Similarly, according to MPN tubes which were incubated 1 week longer than the plates, the number of heterotrophs and benzoate$^+$ organisms was approximately 7 x 10^7 cells/g soil and 3.3 x 10^6 cells/g soil. Thus, among the culturable microorganisms in the soil, benzoate$^+$ organisms represented less than 5% of the heterotrophic community.

Isolation of benzoate$^+$ organisms. Bacteria able to degrade benzoate were obtained by enrichment and by direct plating with autoradiography. The benzoate enrichment culture was serially transferred four times, streaked on 1/4 TSA, and three prominent colony morphotypes were observed. Only one of the three types was capable of transforming benzoate according to HPLC analyses of cultures grown in DAB-benzoate medium (data not shown). In contrast, a large number of benzoate$^+$ isolates was identified by autoradiography of a single filter spread with a 2.5 x 10^{-5} soil dilution (Figure 1). Of the 125 colonies which grew on the filter, 27 colonies incorporated ^{35}S and produced autoradiographic signals when incubated in the presence of benzoate and not in the absence of benzoate. Thus, these 27 colonies were identified as putative benzoate$^+$ organisms. Of these, 19 were picked and purified by restreaking. All 19 isolates completely transformed benzoate according to HPLC analyses (Table 2). An additional five colonies identified by autoradiography as benzoate$^-$ (no incorporation of ^{35}S in the presence of benzoate) were purified. These five isolates differed from one another in colony morphology and rep-PCR pattern. None of them were able to transform benzoate

in DAB-benzoate medium. Thus, for the 24 colonies tested, the HPLC results confirmed the phenotypes indicated by autoradiography.

Diversity of benzoate$^+$ isolates. Genetic diversity among the benzoate$^+$ isolates was assessed by comparison of their rep-PCR fingerprints. Among the 19 benzoate$^+$ isolates obtained by direct plating, 18 distinct patterns were observed (Figure 2). The fingerprints of the 19 direct plating isolates also differed from the rep-PCR pattern of the enrichment isolate, E1. It is interesting to note that these 20 benzoate$^+$ isolates represent at least 14 different species based on restriction analysis of 16S-rDNA (data not shown).

These isolates were divided into three groups (Fast, Medium, Slow) based on the length of time required for pinpoint colonies to develop on 1/4 strength TSA plates (Table 2). The growth rate of an isolate chosen from each group was measured in DAB-benzoate medium. As illustrated in Figure 3, the doubling times ranged from 1.4 hrs (s = 0.082) to 17.1 hrs (s = 0.098). The doubling time of the isolate obtained by enrichment was 1.97 hrs (s = 0.032). These data demonstrate that a collection of phenotypically and genotypically diverse isolates was efficiently obtained by autoradiographic plating, in sharp contrast to the enrichment culture which yielded a single benzoate$^+$ isolate.

Sensitivity. The sensitivity of the method was measured by detection of strain DP5 (benzoate$^+$) on plates with a lawn of DP24 (benzoate$^-$). The frequency of DP5 was held constant at 500 cfu/ml while the frequency of DP24 varied from 0 cfu/ml to 10^8 cfu/ml. Strain DP5 was detected in all mixtures, thus the method is able to identify target organisms in a large background of nontarget organisms (Figure 4).

Discussion

The autoradiographic method is based on the detection of radionuclide incorporation as an indicator of cell growth. Previous investigators have used the rate of sulfur incorporation into protein as a measurement of bacterial growth rate, and the total amount of sulfur incorporated as a measure of total microbial growth [4, 7, 15]. We have

extrapolated this accepted technique to the growth of colonies on nitrocellulose filters on a solidified growth medium. Approximately 1.1% of the dry weight of bacterial cells is sulfur [5] that is primarily in the form of sulfur-containing amino acids that have been incorporated into proteins. Although both organic (amino acids and thiols, for example) and inorganic (sulfate and thiosulfate, for example) forms of sulfur can be assimilated and used for protein synthesis by bacteria [8], most bacterial species are capable of using sulfate as a sole source of sulfur [5, 8]. Consequently, radiolabeled sulfate can be effectively used with a broad range of bacterial species. Growth of bacterial cultures on a solid medium containing $^{35}SO_4^=$ yields radioactive colonies that can be detected by autoradiography. Other radiolabeled elements such as phosphorus that are incorporated into cellular macromolecules (e.g. nucleic acids and phospholipids) can also be successfully used (data not shown).

The data show that the direct plating method facilitates the isolation of diverse bacterial strains that have a specific catabolic phenotype in common. The genetic diversity of benzoate$^+$ isolates obtained was evident from the differences in rep-PCR genomic fingerprints (18 of 19 strains were different from one another) and ARDRA analyses. Differences between benzoate$^+$ strains were also reflected in their growth rates which varied 12.2 fold. These data indicate there is a high degree of functional redundancy with regard to benzoate degradation among bacterial populations in this soil and that this redundancy could not easily have been determined using enrichment culture techniques.

The fact that only two isolates among the 19 obtained using the autoradiographic method share the same rep-PCR pattern suggests that the total diversity of benzoate$^+$ populations in this soil is very high and greatly exceeded the number of strains sampled. The likelihood of detecting any one specific population would therefore be small unless a larger number of strains were examined. Thus, it is not necessarily surprising that strain E1 isolated using enrichment culture techniques differed from all 19 isolates obtained

using the autoradiographic technique. It could be that strain E1 was initially present in the soil in very low number but increased to become numerically dominant in the liquid batch culture. Use of the autoradiographic method provides a way to obtain a greater diversity of isolates than might be obtained using enrichment cultures; however, some strains with the desired phenotype will not be obtained if they are initially rare relative to other populations with the same phenotype.

The lack of diversity observed in the benzoate enrichment culture is typical of this technique. By design, an enrichment culture is intended to yield only one organism exhibiting a particular catabolic phenotype although the original sample may have contained a diverse group of organisms possessing the same phenotype. Furthermore, the high substrate concentrations ($>>K_m$) commonly used in batch culture enrichments generally select for fast-growing organisms [10]. However, cultivation of bacteria on solid medium spatially separates populations and thereby avoids the competition for resources that would otherwise occur in liquid enrichments. Populations with different growth rates such as isolate DP3 (doubling time = 1.4 hr) and DP17 (doubling time = 17.1 hr) can therefore be isolated simultaneously.

Various indicators have been used previously for presumptive identification and differentiation of bacterial colonies on solid medium. Metabolism of some compounds (e.g., cellulose or *p*-nitrophenol) visibly alters the opacity or color of the culture medium and the resulting zones of clearing can be used to identify colonies expressing a specific phenotype (e.g., cellulose or *p*-nitrophenol degradation) [3, 12-14]. Use of pH indicators has provided a more general method for identifying colonies which specifically metabolize a target compound [3, 14, 19]. Acid production during metabolism produces color changes in and around colonies on a medium with a suitably balanced pH indicator. Although these approaches are suitable in many circumstances, they suffer from a lack of sensitivity. Nonspecific bacterial metabolism (of agar impurities, for example) can

produce many false positives with pH-based detection methods, and diffusion of color can obscure many true positives. Detection of small colonies can also be problematic since they produce proportionately smaller visual effects. These problems are overcome with the use of autoradiography. False positives resulting from nonspecific growth are easily detected by comparison of replica filters incubated with and without a test substrate. Likewise, strains that form small colonies as a result of low rates of cell growth or activity can be readily identified and not overlooked using the autoradiographic method since small amounts of incorporated radionuclides can be sensitively detected. Furthurmore, since target colonies can be detected in a lawn of nontarget colonies, selective media are not required for general cultivation of bacteria.

Use of nonselective media for cultivation of bacteria can substantially reduce the length of time required to isolate specific organisms. Selective media employing carbon sources that are inefficiently metabolized, slightly toxic, or poorly soluble require relatively long incubation periods to generate visible biomass. Slow growth combined with the need for numerous serial transfers can result in months of incubation before the target organism(s) becomes established as a dominant member of the culture and can be efficiently isolated. In contrast, the autoradiographic method employs a nonselective, general purpose medium for rapid cultivation of bacterial colonies ($\geq 10^6$ cells/colony). Bacterial phenotypes can subsequently be assayed on selective medium. Athough we have used DAB medium for screening the catabolic phenotypes of bacterial colonies, other media could be employed. Media with higher nutrient concentrations are possible as long as differences in the amount of incorporated radionuclide can be detected in the presence and absence of a test substrate. Since bacterial biomass is initially produced on a nonselective medium, extensive metabolism of a specific target compound is not neccessary. An estimate of the required amount of growth in the presence of the target compound can be calculated based on the medium composition and a cellular content of

1.1% sulfur [5]. For a small bacterial colony with 1 x 10^6 cells, one doubling on DAB medium containing 1 μCi/ml $Na_2{}^{35}SO_4$ would incorporate 18.2 nCi (4 x 10^4 dpm) which is at least twice the amount neccessary to produce a signal on Kodak XAR film in 18 hours. Thus, organisms can be rapidly detected which are able to transform compounds that are recalcitrant, toxic, or only slightly soluble.

In summary, we have developed a simple and effective autoradiography method for isolating diverse microbial species with unique catabolic traits. Modifications of the basic autoradiographic technique have also been used to isolate and characterize mutants [21, 22]. The method is most advantageous when isolating diverse populations which occur at low frequencies in a large background of nontarget populations. Under other circumstances, traditional plating on selective media may be more suitable such as when the populations of interest are very abundant relative to others and the target substrate is easily metabolized.

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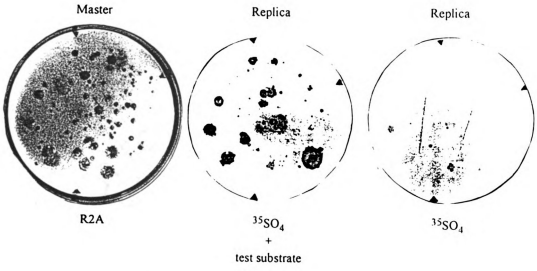

Figure 1. Autoradiographic detection of benzoate$^+$ organisms. Shown are a master filter (left) containing heterotrophic bacterial colonies from soil and autoradiograms of two replica filters. Colonies which produce darker autoradiography signals in the presence of the benzoate (middle) than in its absence (right) are putative benzoate$^+$ colonies.

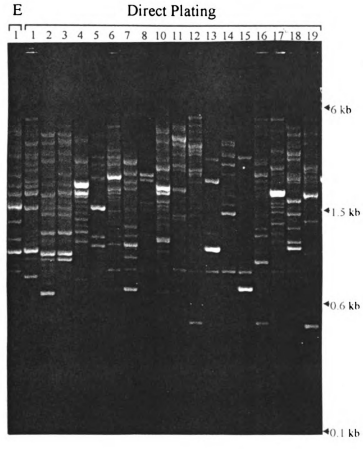

Figure 2. rep-PCR fingerprint patterns observed among isolates obtained by enrichment and by direct plating. Lane 1 - Enrichment isolate E1; lanes 2-20 - Direct Plating isolates DP1-19 (isolate DP9 was omitted; its rep-PCR pattern is identical to DP8).

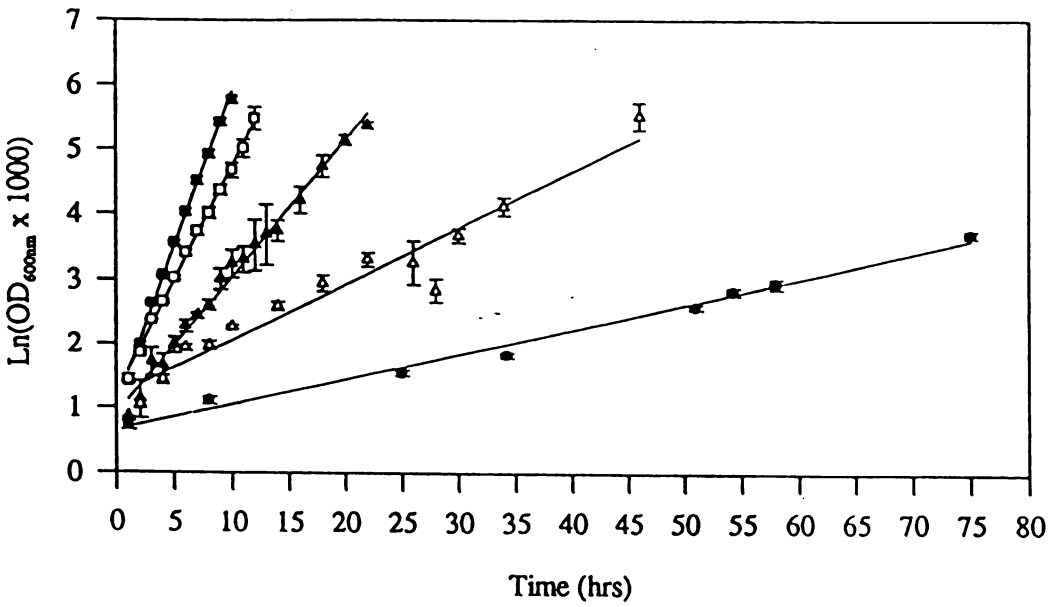

Figure 3. Growth rates of 5 benzoate$^+$ isolates. Closed squares (■), strain DP3; open squares (□), strain E1; closed triangles (▲), strain DP11; open triangles (Δ), strain DP16; closed circles (●), strain DP 17. Error bars are standard deviations of data from three replicate cultures.

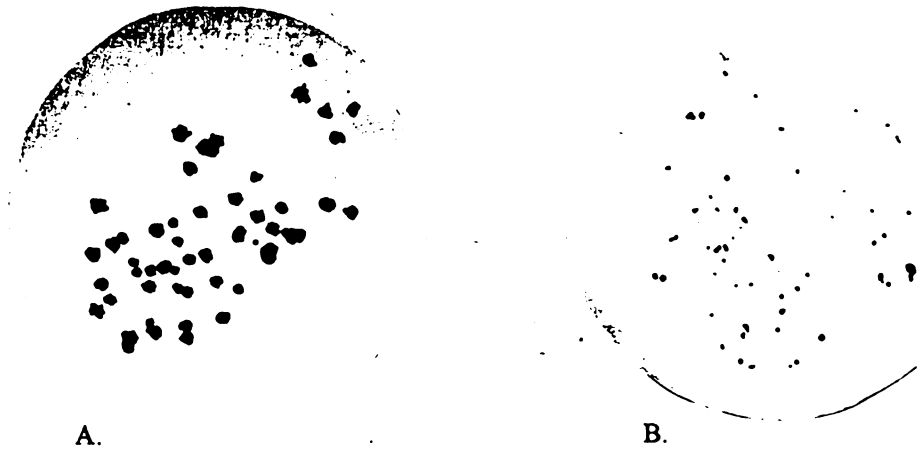

Figure 4. Sensitivity of autoradiographic detection. Shown are autoradiograms of filters containing a 10^{-1} dilution of (a) strain DP3 (benzoate$^+$) at 500 cfu/ml and (b) strain DP3 and strain DP20 (benzoate$^-$) at 500 cfu/ml and 5 x 10^8 cfu/ml respectively.

Table 1. Composition of Defined Aerobic Basal (DAB) medium

Compound	Concentration (mg/l)
Na_2HPO_4	866
KH_2PO_4	531
$(NH_4)_2SO_4$	0.3
$MgSO_4$	50
$CaCl_2 \cdot 2H_2O$	5.88
Na_2HPO_4	3.2
$FeSO_4 \cdot 7H_2O$	2.78
$ZnSO_4 \cdot 7H_2O$	1.15
$MnSO_4 \cdot H_2O$	1.69
$CuSO_4 \cdot 5H_2O$	0.375
$Co(NO3)_2 \cdot 6H_2O$	0.233
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.1236
Pyridoxine·HCl	0.05
Thiamine·HCl	0.025
Nicotinic acid	0.025
p-aminobenzoic acid	0.025
Biotin	0.01
Folic acid	0.01
Pyridoxal phosphate	0.0005
Riboflavin	0.025
Thioctic acid	0.025
Pantothenic acid	0.025
L-glutamate	0.94
L-leucine	0.38
L-lysine	0.30
L-serine	0.30
L-isoleucine	0.26
L-tyrosine	0.26
L-valine	0.26
L-aspartate	0.25
L-aline	0.22
L-phenylalanine	0.2
L-arginine	0.18
L-threonine	0.16
L-methionine	0.14
L-asparagine	0.1
L-histidine	0.08
L-tryptophan	0.04
L-glycine	0.02
L-proline	0.02

Table 2. Comparison of Isolates Obtained by Enrichment and by Direct Plating

Isolate	REP-PCR pattern	Putative phenotype (autorad)	Confirmed phenotype (HPLC)	Growth on 1/4 TSB agar[c]	Generation time (s)[d]
E1	1	NA[a]	+	F	2.0 hr (0.082)
DP1	2	benzoate$^+$	+	F	ND[b]
DP2	3	benzoate$^+$	+	F	ND
DP3	4	benzoate$^+$	+	F	1.4 hr (0.032)
DP4	5	benzoate$^+$	+	F	ND
DP5	6	benzoate$^+$	+	F	ND
DP6	7	benzoate$^+$	+	F	ND
DP7	8	benzoate$^+$	+	M	ND
DP8	9	benzoate$^+$	+	M	ND
DP9	9	benzoate$^+$	+	M	ND
DP10	10	benzoate$^+$	+	M	ND
DP11	11	benzoate$^+$	+	M	3.4 hr (0.366)
DP12	12	benzoate$^+$	+	M	ND
DP13	13	benzoate$^+$	+	M	ND
DP14	14	benzoate$^+$	+	M	ND
DP15	15	benzoate$^+$	+	S	ND
DP16	16	benzoate$^+$	+	S	6.5 hr (0.311)
DP17	17	benzoate$^+$	+	S	17.1 hr (0.098)
DP18	18	benzoate$^+$	+	S	ND
DP19	19	benzoate$^+$	+	S	ND
DP20	20	benzoate$^-$	-	F	ND
DP21	21	benzoate$^-$	-	M	ND
DP22	22	benzoate$^-$	-	M	ND
DP23	23	benzoate$^-$	-	M	ND
DP24	24	benzoate$^-$	-	S	ND

[a]NA = Not Applicable
[b]ND = Not Determined
[c]F, M, and S = Fast, Medium, and Slow.
[d] Doubling times were caculated by least-squares analysis of transformed data; s indicates standard deviation.

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CHAPTER 2

Genetic Diversity Through the Looking Glass: Effect of Enrichment Bias

Abstract

The effect of enrichment bias on the diversity of 2,4-D degrading (2,4-D$^+$) bacteria recovered from soil was evaluated by comparing the diversity of isolates obtained by direct plating to the diversity of isolates obtained from 85 liquid batch cultures. Using the two methods, a total of 159 isolates were purified from a single gram of soil and divided into populations based on rep-PCR genomic fingerprints. Approximately 42% of the direct plating isolates hybridized with the *tfdA* and *tfdB* genes from *Alcaligenes eutrophus* JMP134 (pJP4), 27% hybridized with the *tfdA* and *tfdB* genes from *Burkholderia* sp. RASC, and 30% hybridized with none of the probes. In contrast, the enrichment isolates not only represented fewer populations than the isolates obtained by direct plating but also exhibited, almost exclusively, a single hybridization pattern with 2,4-D catabolic gene probes. Approximately 98% of the enrichment isolates possessed pJP4-type tfdA and tfdB alleles. An isolate containing RASC-type tfdA and tfdB alleles was obtained from only two of the 85 enrichment cultures. The skewed occurrence of the pJP4 genotype among the isolates obtained by enrichment suggests that the competitive fitness of 2,4-D$^+$ populations during growth with 2,4-D may be influenced either by specific *tfd* alleles or other genetically linked or phylogenetically associated factors. Moreover, the results indicate that evaluation of the diversity and distribution of catabolic pathways in nature can be highly distorted by use of enrichment culture techniques.

Introduction

Our understanding of the genetic diversity underlying specific bacterial phenotypes in nature is shaped by the organisms used for study. The use of culturable bacteria instead of nonculturable bacteria is a well known source of potential bias. Culturable bacteria represent only 0.1-5% of the viable bacteria in microbial communities. Since culturable bacterial strains are the ones used for biochemical and genetic studies, it is possible that only a small portion of the biochemical and genetic diversity underlying particular phenotypes has been documented. Even within the culturable fraction of bacteria, obtaining organisms which are representative of the diversity in nature can be difficult as a result of inherent biases in the methods used for isolation. Batch culture enrichment is the most widely used method for isolation of bacteria expressing specific phenotypes. It has long been recognized that enrichment limits the number and relative growth rates of organisms obtained from the environment [12]. These limitations may also affect the diversity of catabolic pathways obtained from the environment.

By design, enrichment in liquid batch culture yields only one or a few strains possessing a specific phenotype. This limitation is potentially overcome by establishing numerous enrichment cultures, but to our knowledge this has not been systematically evaluated. A more serious limitation is the fact that liquid batch culture enrichments typically select for fast growing organisms [12]. A number of researchers have demonstrated that both fast and slow growing organisms possessing the same phenotype occur simultaneously in natural communities and can be isolated from the same inoculum source by use of chemostats with different dilution rates [15, 27] or by direct plating [10]. However, due to the high substrate concentrations (>>Ks) commonly used in batch culture enrichments, slower growing organisms are typically out competed by organisms with a higher maximum specific growth rate, μ_{max}. The genetic factors responsible for differences in growth rates and competitive fitness are poorly understood. Thus, the bias

which enrichment might impose on the diversity of a particular catabolic pathway detected in environmental samples is unknown.

To evaluate the potential for bias due to the use of enrichment cultures, we compared the genetic diversity of 2,4-D catabolic pathways among 2,4-D degrading bacteria obtained by enrichment and by direct plating. All of the isolates originated from a single gram of soil. Gene probes representing different alleles for the first two genes in the 2,4-D catabolic pathway were used in Southern hybridization analyses to evaluate diversity among the isolates.

Materials and Methods

Media and Reagents. Defined Aerobic Basal (DAB) medium amended with 2,4-D at a concentration of 250 μg/ml was used for specific cultivation of 2,4-D degrading bacteria. DAB medium consists of a mineral salts base supplemented with amino acids and vitamins at low concentrations and has been previously described [10]. Reagent grade 2,4-D and ^{14}C-UL-2,4-D were purchased from Sigma (Sigma Chemical Co., St. Louis, MO).

Soil. Soil was obtained from an experimental field plot at Kellogg Biological Station (Hickory Corners, MI) which had been treated with 2,4-D (100 μg/g soil) from 1989 to 1992 [17]. One kg of soil was collected in October 1993 and stored at 4°C until used. For isolation of 2,4-D degrading bacteria, one gram of soil was mixed with 9 ml of 10 mM phosphate buffer (pH 7.0) containing 6.1 mM Na_2HPO_4 and 3.9 mM KH_2PO_4, shaken for 20 minutes at 250 rpm on a rotary shaker, then aliquots were used for enrichment or direct plating.

Enrichment cultures. Enrichment cultures were established by transferring 0.1 ml of soil slurry to each of 74 test tubes containing 3 ml of DAB medium amended with 250 μg/ml 2,4-D. After 1 wk incubation at 30°C on a rotary shaker set at 250 rpm, disappearance of 2,4-D in each enrichment culture was confirmed spectrophotometrically using a Hewlett Packard model 8452A diode array spectrophotometer. A 0.03 ml aliquot

of each culture was then transferred to 3 ml of fresh DAB-2,4-D medium, incubated for 1 wk, and monitored spectrophotometrically for 2,4-D disappearance. After three serial transfers, each culture was streaked on 1/4 strength trypticase soy agar (TSA). 2,4-D degrading bacteria on each plate were identified by transferring colonies representing different morphologies into 3 ml of DAB-2,4-D medium and monitoring the disappearance of 2,4-D. Cultures which tested positive were streaked on 1/4 strength TSA to check for purity. Pure cultures were fingerprinted by rep-PCR (see below), then representatives of different rep-PCR patterns were regrown in DAB-2,4-D medium to establish 15% glycerol stocks that were stored at -80°C.

Direct Plating. To isolate 2,4-D degrading bacteria by direct plating, the soil slurry was serially diluted and appropriate dilutions were spread directly on HATF nitrocellulose filters (Millipore, Bedford, MA) atop R2A agar medium (Difco, Detroit, MI). After one week of incubation at 30°C, colonies able to degrade 2,4-D were identified by autoradiography based on their ability to incorporate ^{14}C from [U-^{14}C]2,4-D. This procedure has been described previously using $^{35}SO_4^=$ instead of a ^{14}C-labeled carbon source [10]. The procedure was modified for [U-^{14}C]2,4-D as follows. Each master filter was replicated once, and each replica filter was incubated on DAB-2,4-D solid medium containing 1.5% agar and 0.1 μCi/ml of [U-^{14}C]2,4-D. After 3 days of incubation, the replica filters were transferred to 1% agar medium containing 10 mM phosphate buffer and 750 μg 2,4-D ml^{-1} and incubated 30 min in order to remove unincorporated [U-^{14}C]2,4-D. This wash step was repeated once. The replica filters were dried, mounted on paper, covered with a layer of Saran, then exposed to Kodak X-OMAT XAR (Eastman Kodak, Rochester, ME) film overnight at -70°C. The film was developed according to the manufacturer's recommendations. The autoradiographic films were aligned with appropriate master filters, and colonies corresponding to film signals were considered putative 2,4-D$^+$ colonies. Since the filters used for isolation of 2,4-D$^+$

bacteria were crowded with 2,4-D$^-$ degrading bacterial colonies, putative 2,4-D$^+$ colonies were transferred to 3 ml of DAB-2,4-D medium to reduce the relative abundance of 2,4-D$^-$ bacterial contaminants. Cultures were transferred 2-3 times, and purified as described above for enrichment cultures.

Most probable number (MPN) estimation. The most probable number of 2,4-D degrading bacteria in the soil was determined according to standard methods [5] using five tubes per dilution. For each 10-fold dilution of the soil slurry, one ml was transferred to each of five tubes containing 3 ml of DAB-2,4-D medium. MPN tubes were incubated two weeks at 30°C. Disappearance of 2,4-D in the tubes was determined spectrophotometrically and estimates of the most probable number of 2,4-D degrading bacteria were obtained from statistical tables [5]. 2,4-D degrading bacteria were isolated from MPN tubes as described above for enrichment cultures.

rep-PCR. Genomic fingerprints of 2,4-D degrading isolates were obtained using consensus primers for the Repeated Extragenic Palindromic (REP) sequence [28]. PCR reactions were performed according to de Bruijn [7] using colonies of pure cultures grown on 1/4 strength TSA as template. Each 25 µl PCR reaction contained 5 µl Gitschier buffer, 2.5 µl DMSO, 12.15 µl milliQ water, 1.25 mM dNTPs (Pharmacia Biotech), 0.3 µM primers, 0.2 µl BSA (Boehringer Mannheim, Indianapolis, IN), and 2 U *Taq* polymerase (Gibco BRL, Grand Island, NY) and approximately 1 µl of template. PCR products were electrophoresed through 1.5% agarose gels containing 1/2 strength TAE [22] and 0.25 µg/ml ethidium bromide then photographed. After obtaining fingerprints of all isolates, the reproducibility of the rep-PCR patterns was confirmed by retesting representative isolates regrown in DAB-2,4-D medium.

16S rDNA restriction analysis. 16S rDNA was amplified using the primers rD1 and fD1 [29] and the reaction conditions described by Laguerre *et al.* [20]. PCR reactions were performed in a Perkin-Elmer model 9600 thermal cycler. Cells from broth cultures

subjected to four freeze-thaw cycles were used as template for PCR amplifications. For each 2,4-D$^+$ strain, amplified 16S rDNA was separately digested with *Cfo*I, *Hae*III, *Msp*I, *Rsa*I, and *Hinf*I (Gibco) using 8 µl of PCR product per reaction. After 3 hours incubation, digested 16S rDNA was electrophoresed through 2.75% Metaphor agarose gels (FMC Bioproducts, Rockland, ME) containing TAE buffer and 0.25 µg/ml ethidium bromide, and the gels were photographed.

2,4-D gene probe analysis. Genomic DNA was obtained according to standard procedures [2]. Bacterial strains were grown overnight in 5 ml of 1/10 strength TSB, then 1.5 ml were transferred to an eppendorf tube for DNA extraction. For each strain, approximately 5 µg of genomic DNA were digested with 2 units of *Eco*RI for 4 hours, then electrophoresed in ethidium bromide-stained, 0.8% agarose gels. Digested DNA was transferred to Hybond-N nylon membranes (Amersham, Arlington Heights, IL) by capillary blotting [25], and crosslinked using a UV stratalinker 1800 (Stratagene, La Jolla, CA) for subsequent hybridization with gene probes.

Gene probes were derived from 2,4-D catabolic genes encoded on plasmid pJP4 from *A. eutrophus* JMP134 and from the chromosomally encoded 2,4-D genes in *Burkholderia* sp. strain RASC. Four probes were used that consisted of internal fragments of *tfdA* and *tfdB* from pJP4 [13], and of *tfdA* [26] and *tfdB* from strain RASC. The RASC *tfdB* probe consisted of a 1 kb *Sac*I-*Kpn*I fragment from a 1.2 kb *Sac*I-*Sac*I fragment which had been cloned into pUC9. The RASC *tfdA* and *tfdB* genes have 73% and 65% DNA sequence similarity to the corresponding pJP4 alleles [26]. Gel-purified probes were labeled with digoxigenin-dUTP using the DIG DNA Labeling and Detection Kit (Boehringer Mannheim, Indianapolis, IN). Prehybridization and hybridization solutions were prepared essentially as described in the Genius System User's Guide for Membrane Hybridization (Boehringer Mannheim) and contained 5X SSC [22], 0.1% N-lauroylsarcosine, 0.02% SDS, 5% blocking reagent, and 50% formamide.

Hybridizations were conducted at 62°C to achieve high stringency conditions (90-100% similarity). Following hybridization, membranes were washed twice in 2X SSC then processed for probe detection.

Results

Population diversity of 2,4-D$^+$ isolates. A total of 159 2,4-D$^+$ isolates were obtained from a single gram of soil. One 2,4-D$^+$ strain was obtained from each enrichment culture yielding a total of 74 isolates, an additional 74 isolates were obtained by direct plating, and one 2,4-D$^+$ isolate was obtained from each of 11 MPN tubes representing 10^{-3}, 10^{-4}, and 10^{-5} dilutions of soil. These isolates exhibited 30 distinct rep-PCR patterns (Figure 1). Isolates with identical rep-PCR patterns were considered to be members of a single population. Thus, at least 30 different 2,4-D$^+$ populations occurred in the original gram of soil.

Most of the 30 populations were identified among the isolates obtained by direct plating, and not from enrichment cultures (Figure 2). Among the 74 isolates obtained by direct plating, 25 distinct populations were detected while only 7 were identified among the 74 enrichment isolates. Similar results were obtained in replicate experiments in which 2,4-D degrading isolates were obtained from a single gram of soil either by direct plating or by enrichment (Table 1). The estimated population diversity, E(S), for a standardized sample size of 50 isolates was calculated by rarefaction [14, 24] and used to compare the diversity of isolates obtained in different experiments since the actual number of isolates in each experiment varied. The average population diversity, E(S), among isolates obtained by direct plating from each of three separate grams of soil was 19 populations (S.D. = 1.026). In contrast, the average value of E(S) for isolates obtained from two separate grams of soil by enrichment was 8.6 populations (S.D. = 3.182). These results demonstrate that numerous 2,4-D$^+$ populations can be obtained by establishing multiple

enrichment cultures, but the population diversity of isolates from enrichment cultures is still significantly lower than that obtained by direct plating.

Significant differences were also observed in the diversity of 2,4-D catabolic genotypes obtained by enrichment and by direct plating (Figure 3). Hybridization of 2,4-D gene probes with genomic DNA from each of the 25 populations obtained by direct plating identified three catabolic groups among the isolates obtained by direct plating. Nine populations representing approximately 42% of the isolates belonged to catabolic group A_1B_1 since these populations hybridized with the *tfdA* and *tfdB* genes from *Alcaligenes eutrophus* JMP134 (pJP4), nine populations representing 27% of the isolates belonged to group A_2B_2 based on hybridization with the *tfdA* and *tfdB* genes from *Burkholderia* sp. RASC, and seven populations representing 30% of the isolates belonged to group A_xB_x since these populations hybridized with none of the probes. In contrast, 6 populations representing 98% of the isolates obtained by enrichment belonged to group A_1B_1. A population exhibiting the A_2B_2 hybridization pattern was obtained from only one of 74 enrichment cultures. Similarly, a population representing group A_2B_2 was obtained from only one of 11 MPN tubes; the remaining tubes yielded populations exhibiting the A_1B_1 hybridization pattern. These data demonstrate a marked bias in the catabolic groups obtained from soil by use of enrichment.

To determine if enrichment of the pJP4 genotype was due to outgrowth of populations representing a single species, species diversity was estimated by restriction analysis of 16S rDNA amplified from each population. The 25 populations obtained by direct plating represented at least 16 different species which were rather evenly distributed among the 3 catabolic groups (Figure 4). The most abundant species (species 1) accounted for no more than 24% of the isolates obtained by direct plating and did not hybridize with any of the 2,4-D gene probes. In contrast, the frequency distribution of the species obtained from enrichment cultures and MPN tubes was much more skewed. At

least six species were obtained, but one of them (species 11) accounted for 72% of the enrichment and MPN isolates (combined). Five of the species belonged to group A_1B_1. The enrichment of multiple species exhibiting, almost exclusively, pJP4-type *tfd* genes suggests that the competitive fitness of these species during competition for 2,4-D is enhanced by pJP4-type alleles or genetically linked or phylogenetically associated traits.

Discussion

Bacteria expressing specific phenotypes are most commonly obtained from natural samples by enrichment. Competition among populations during the enrichment process typically results in dominance of one or a few populations with the highest growth rates. This phenotypic bias of enrichment culture techniques is well known. We have demonstrated the genetic consequences of this phenotypic bias among 2,4-D degrading bacteria. Enrichment of 2,4-D degrading bacteria from a single gram of soil containing a large number of 2,4-D^+ species with diverse 2,4-D genes resulted in the almost exclusive isolation of species possessing *tfdA* and *tfdB* genes highly similar, if not identical, to the genes from *A. eutrophus* JMP134 (pJP4). These results suggest there is an association between specific alleles of 2,4-D catabolic genes and the competitive fitness of the species obtained by enrichment. More importantly, the data demonstrate the limitations of enrichment culture techniques in assessing not only the redundancy of 2,4-D degrading populations in soil, but also the diversity and distribution of 2,4-D genes.

It is not clear whether the association between specific 2,4-D alleles and competitive fitness is coincidental or causal. Preferential selection of specific *tfd* alleles may simply be a coincidental function of host range. It is conceivable that the pJP4-type *tfdA* and *tfdB* alleles are distributed among species which, in general, are better adapted to rapid exploitation of new resources compared to species harboring other *tfd* alleles. In previous studies, the pJP4-type alleles have been identified primarily in species of *Alcaligenes* and *Burkholderia* [1, 3, 4, 8, 9, 11]. However, the RASC-type alleles also

occur primarily in *Burkholderia* species [11, 23, 26]. Although the phylogenetic identities of the species obtained in the present study were undetermined, restriction analysis of amplified 16S rDNA identified one case in which the host range of pJP4-type alleles and RASC-type alleles overlapped (species 12). In this cases, the pJP4-type alleles and RASC-type alleles appeared in different populations belonging to the same (or very closely related) species (Figure 4). The fact that these two sets of alleles occur in the same or very similar organisms would suggest that both sets have a similar chance of being obtained by enrichment if, in fact, competitiveness during enrichment is primarily a result of host-specific factors. However, the abundance of the pJP4-type alleles relative to the RASC-type alleles among the enrichment isolates in the present study suggests that competitiveness is not simply a result of host-specific factors.

The predominance of the pJP4-type *tfdA* and *tfdB* alleles among the six species obtained by enrichment suggests that the competitive fitness of 2,4-D degrading species may be strongly influenced by one or more of the pJP4-type alleles or by other genetic factors linked to these alleles. Plasmids encoding different sets of *tfd* alleles have been shown to confer different growth rates on *B. cepacia* DBO1 transconjugants [16]. The growth rate of a transconjugant harboring pJP4 was 2 fold greater than the growth rate of a transconjugant containing a pJP4-type *tfdA* allele and alleles for *tfdB* and *tfdC* with <90% DNA sequence similarity to the pJP4 alleles [16]. At maximum growth rates, TfdB, the second enzyme in the pJP4 pathway, appears to be rate limiting based on the observed accumulation of 2,4-dichlorophenol in a chemostat [6]. The fact that 2,4-dichlorophenol is toxic to bacterial cells would tend to magnify the effects of catalytic differences between tfdB isozymes. Although the kinetics of TfdB isozymes have not been measured, the deduced amino acid sequences of TfdB (pJP4) and TfdB (RASC) are 80.5% identical and 95.5% similar (Y. Suwa, personal communication). The isozymes encoded by different *tfdB* alleles may thus differ in catalytic efficiency and affect the

growth rates of 2,4-D degrading populations. If the pJP4 *tfdB* allele is in fact optimal for rapid growth, genetic linkage with other pJP4 2,4-D genes could account for the frequent appearance of the genetic pathway encoded on pJP4 following selection for maximum growth rates. Alternatively, other genetically linked factors, such as *tfd* gene copy number per cell or elements involved in the regulation of transcription or translation of *tfd* genes, may affect the fitness of different *tfd* genotypes.

During the past 15 years of investigation of the genetic basis of 2,4-D metabolism, the pJP4 alleles have been the most frequently encountered. The prototypical strain, *Alcaligenes eutrophus* JMP134 containing the broad host range 2,4-D degradative plasmid pJP4, was obtained in 1980 from an enrichment culture in Australia [9]. Genes highly similar, if not identical, to the pJP4 alleles were subsequently identified in isolates obtained from India [3], Estonia [21], Florida [4], and Oregon [1] by standard enrichment techniques. While these studies established the widespread occurrence of pJP4-type *tfd* alleles, enrichment bias may have obscured the presence of other 2,4-D catabolic genes in the environment. The existence of different alleles for 2,4-D catabolic genes was established only recently. Hybridization of the pJP4 alleles with DNA from pure cultures and from entire microbial communities known to contain 2,4-D degrading populations has indicated that numerous other alleles occur in nature for each of the 2,4-D catabolic genes and may be widespread [11, 17, 18, 30]. Recently, the RASC *tfdA* and *tfdB* alleles were cloned and sequenced [19, 23, 26]. These alleles have 77.2% and 78.5% sequence similarity to the pJP4 alleles. Other alleles with even lower similarities have also been identified [11].

Although populations with different 2,4-D catabolic alleles have recently been obtained by enrichment, our data indicate that this method severely distorts the apparent diversity of 2,4-D degrading populations in individual samples from the environment. The limited number of populations obtained in a single enrichment culture can be partially

overcome by establishing multiple enrichment cultures. However, although a higher number of populations may be obtained, the population diversity is still substantially lower than that obtained by other methods such as direct plating. Furthermore, specific alleles of catabolic genes may be exclusively obtained as a result of the enrichment bias for organisms with relatively rapid growth rates. Future use of alternative methods to obtain isolates exhibiting a specific phenotype may identify novel biochemical pathways or, at least, a broader array of isozymes. With a better understanding of the diversity and distribution of particular catabolic pathways, new insights into the evolution of catabolic pathways may be obtained.

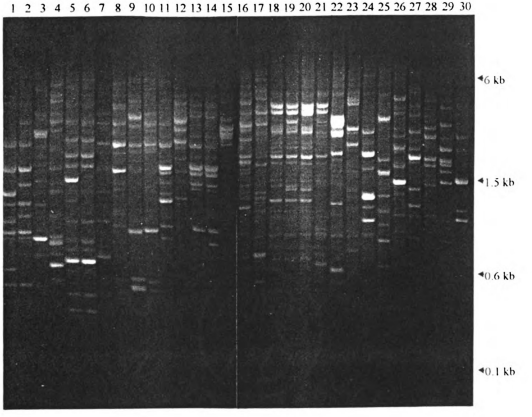

Figure 1. Unique rep-PCR patterns of 2,4-D$^+$ strains obtained by direct plating and by enrichment.

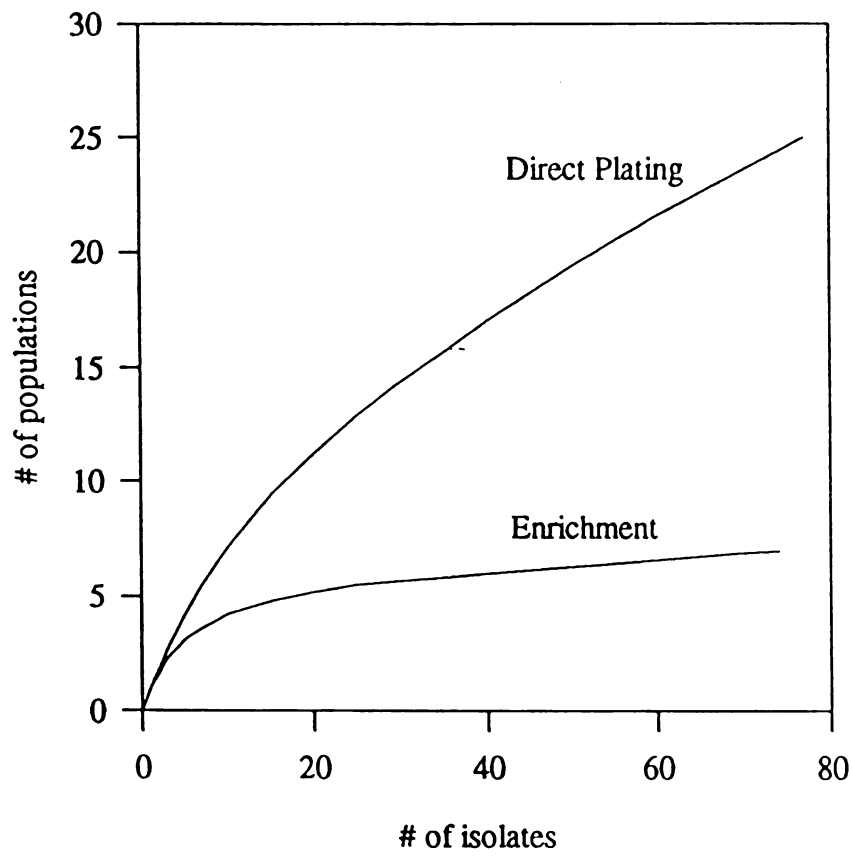

Figure 2. Cumulative diversity curves for isolates obtained by direct plating and by enrichment. Rarefaction (12, 22) was used to estimate population diversity for numerous samples sizes between 0 and 74. The estimates were then plotted in order to construct the curves shown.

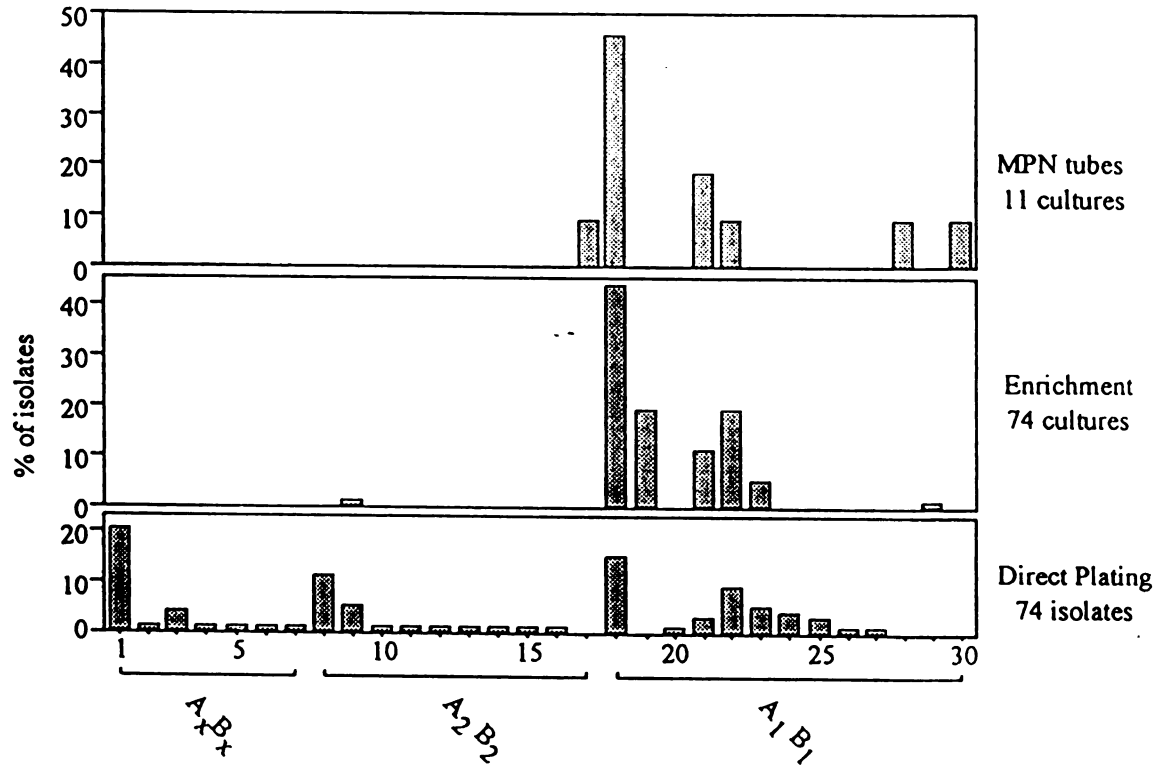

Figure 3. Frequency distribution of populations obtained by direct plating and by enrichment. The numbers below the *x* axis correspond to lane numbers in Figure 1. The hybridization pattern of each population with 2,4-D catabolic gene probes is indicated by the brackets below the *x* axis. Genotype A_1B_1 indicates hybridization with *tfdA* and *tfdB* gene probes from *A. eutrophus* JMP134 (pJP4), genotype A_2B_2 indicates hybridization with *tfdA* and *tfdB* probes from *Burkholderia* sp. RASC, and genotype A_xB_x indicates lack of high stringency (90-100% sequence similarity) hybridization with any of the probes.

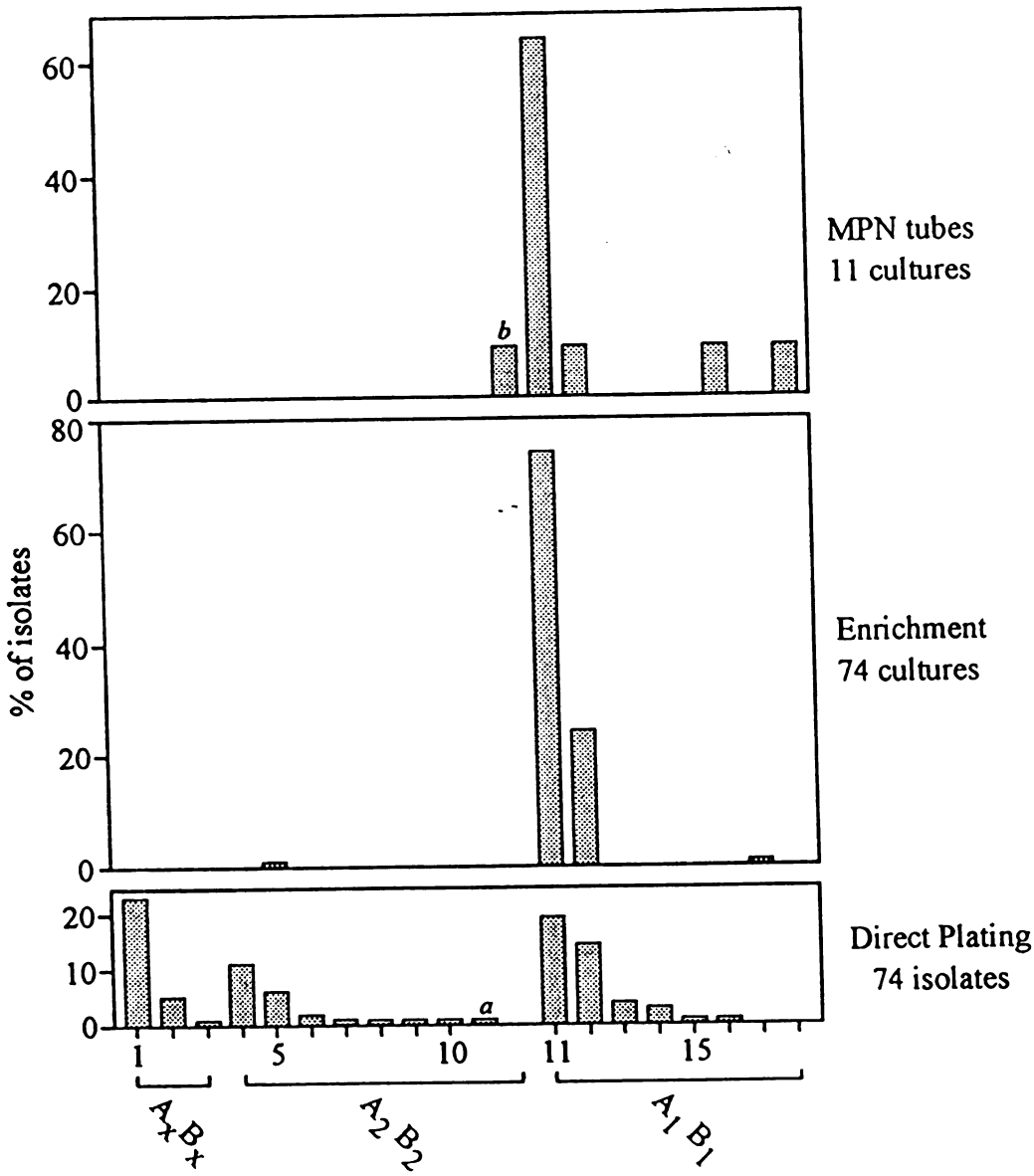

Figure 4. Frequency distribution of species obtained by direct plating and by enrichment. The bars marked with *a* and *b* were identified as members of species 11 and 12, respectively, based restriction analysis of 16S rDNA despite the fact that these populations exhibited different hybridization patterns with *tfd* gene probes.

Table 1. Populations Diversity of 2,4-D+ Isolates Obtained by Enrichment and by Direct Plating

			Enrichment		Direct Plating	
Expt.	Amt. Soil	No. isolates[a]	E(S)[b]	Var.[c]	E(S)	Var.
1	1 g	50	--	--	18	0.745
2	1 g	50	10.8	0.146	20	0.000
3	1 g	50	6.3	0.440	19.4	2.871

[a] The actual number of isolates collected in each trial ranged from 51 to 77. The number indicated was chosen as a standardized sample size.

[b]The standardized population diversity, E(S), was obtained by rarefaction (12, 22).

[c]The variance arises from inherent statistical uncertainty in the estimation of population diversity, E(S), for sample sizes lower than the actual sample size.

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CHAPTER 3

Variation in genome organization among *Variovorax paradoxus* clones isolated from a single environment

Abstract

The genetic diversity within a population of *Variovorax paradoxus* was investigated by examining the genome organization and metabolic characters of strains isolated from soil. From a 6 m^2 fallow agricultural plot, 145 isolates of *V. paradoxus* were collected. These isolates were taxonomically indistinguishable by colony morphology, substrate utilization profiles, cellular fatty-acid profiles, and 16S-rDNA restriction patterns. Despite these similarities, 14 subpopulations that differed in the physical organization of their chromosomes were identified by fingerprinting the genome of each strain using consensus primers for Repetitive Extragenic Palindromic (REP) sequences and PCR. Three populations were most abundant, present in approximately equal numbers, and accounted for 74% of the total strains. Based on analyses of genomic DNA that had been restricted with a rare-cutting enzyme, *Xba*I, and separated by pulse field gel electrophoresis, the differences in genome organization were apparently not localized but occured throughout each genome. The restriction patterns not only confirmed the differences between subpopulations seen by REP-PCR fingerprint analysis, but revealed differences among strains within individual subpopulations. Further variation among strains having identical REP-PCR patterns was demonstrated by isolation of plasmids that varied in size and restriction pattern. The combination of these analyses demonstrated extensive genetic variation in the isolates examined. These data indicate that in the field site we examined, *V. paradoxus* is a heterogeneous population comprised of strains with highly polymorphic genomes.

Introduction

Natural selection, and therefore evolution, can only occur if genetic variation exists in traits that affect the survival and reproductive success of organisms. Numerous processes that give rise to genetic diversity within species of bacteria have been identified [19]. These processes can be broadly classified in two groups, namely: point mutations that occur during DNA replication and repair, and genetic variation arising from recombination. The significance of the former to the diversification and evolution of specific gene sequences has been well documented. However, the role and importance of the latter to the evolutionary divergence and speciation of asexually reproducing bacteria is less well understood. Recombination between homologous sequences may occur in bacteria following horizontal transfer of DNA by transformation, transduction, or conjugation between related or unrelated strains of bacteria. These events can significantly alter the genetic structure of a bacterial population by altering the frequency of various alleles among strains of the population and by introducing novel genes that confer new phenotypic traits. Alternatively, intragenomic recombination (asexual recombination) events may occur without the involvement of horizontal gene transfer leading to evolution of gene sequences and phenotypes by duplication, deletion, inversion, or transposition of sequences within a genome.

Previous studies have shown that the frequency of horizontal gene transfer and recombination varies widely among bacterial species [13, 14, 17, 29-31, 37, 41, 44, 45, 47]. These studies have largely relied on determining the extent of linkage disequilibrium based on the the use of multilocus enzyme electrophoresis (MLEE) and more recently on alignment of homeologous gene sequences. The frequency of sexual recombination in some bacterial species (such as *Escherichia coli*) is low, and these populations have a clonal structure [41]. Even though the frequency of sexual recombination in *E. coli* is comparatively low, Guttman and Dykhuizen [14] have shown that it occurs at a frequency

of ~5.0 $X10^{-9}$ changes per nucleotide per generation which is 50-fold greater than the point mutation frequency. In other bacterial species, sexual recombination occurs at a high frequency such that linkage disequilibrium among alleles at different loci is virtually undetectable [36, 45].

The methods used to measure the incidence of sexual recombination do not necessarily reflect intragenomic recombination (asexual recombination) that alters the physical arrangement of the genome. Events that restructure the genome may not alter the frequency of alleles within a population but nonetheless can lead to genetic diversification. Indeed, asexual recombination events such as the duplication of regions within the chromosome may be key processes in the evolution of novel genotypes [53], bacterial chromosomes [24, 38], and the speciation of bacteria [24]. The significance of these processes over long evolutionary periods has recently been illustrated by Labaden and Riley [20] who have estimated that 38-45% of the genes in *E. coli* may have arisen from gene duplications, and a similar value (30%) has been suggested for ***Haemophilus influenzae*** [5]. These findings suggest that studies employing methods which examine the physical organization of bacterial genomes might provide new perspectives on the extent of genetic diversity in bacterial populations from nature.

Differences in the physical organization of bacterial genomes can be readily detected using pulse field gel electrophoresis of restricted genomic DNA or by one of several methods for "fingerprinting" genomic DNA. An example of the latter, REP-PCR fingerprinting, utilizes oligonucleotide primers based on the consensus sequence for the Repeated Extragenic Palindromic (REP) sequence [52] to amplify DNA fragments that are flanked by REP elements which can then be resolved by gel electrophoresis. The number and size of the amplified DNA fragments reflects the organization of REP sequences in a bacterial genome, and thus can be used to distinguish strains which differ in genome structure. Using REP-PCR fingerprinting and pulse-field gel electrophoresis as two

independent methods, we assessed the variation in genome organization among clones from a population of *Variovorax paradoxus* isolated from soil. The strains isolated could be grouped into 14 cohesive subpopulations that apparently have diverged extensively from one another.

Materials and Methods

Media. The mineral salts base used for minimal media consisted of 6.1mM Na_2HPO_4, 3.9 mM KH_2PO_4, 2.5 mM $(NH_4)_2SO_4$, 0.2 mM $MgSO_4$, 50 mM $CaCl_2 \cdot 2H_2O$, 8.6 mM EDTA, 10 mM $FeSO_4 \cdot 7H_2O$, 4 mM $ZnSO_4 \cdot 7H_2O$, 10 mM $MnSO_4 \cdot H_2O$, 1.5 mM $CuSO_4 \cdot 5H_2O$, 0.8 mM $Co(NO_3)_2 \cdot 6H_2O$, and 0.1 mM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$. For minimal medium containing yeast extract and 2,4-D (MS-YE-2,4-D medium), the mineral salts base was supplemented with 10 mg/l yeast extract (Difco, Detroit, MI) and 250 mg/l 2,4-D (Sigma Chemical Co., St. Louis, MO). A 20 g/l 2,4-D stock solution (pH 7.0) was prepared by dissolving 2,4-D in 0.1 M NaH_2PO_4 and adjusting the pH with 5N NaOH. Reagent grade chemicals were purchased from Sigma.

Bacterial isolates. *V. paradoxus* isolates (formerly *Alcaligenes paradoxus*; [55]) were obtained from a large collection of bacterial isolates purified from soil on the basis of their ability to metabolize the herbicide 2,4-D. Soil was collected at Kellogg Biological Station, Hickory Corners, MI. Eight soil samples from the top 10 cm of an uncultivated 6 m^2 agricultural plot were combined and seived through a 2 mm pore-size seive into a polypropylene bag, then homogenized by mixing. The soil was stored in the lab for 1 week at 25°C, then 3 independent samples (10g each) were removed on each of 4 sampling days (days 8, 11, 13, and 15) for isolation of organisms capable of degrading 2,4-D.

Putative 2,4-D degrading organisms were identified and purified as follows from each soil subsample: 10 g of soil were added to 90 ml of phosphate buffer (pH 7.0) containing 6.1mM Na_2HPO_4 and 3.9 mM KH_2PO_4 then agitated at 25°C for 30 minutes

on a rotary shaker (200 rpm). A 0.5 ml aliquot of the soil slurry was spread on a nitrocellulose filter atop R2A agar (Difco, Detroit, MI) medium which is a general purpose growth medium for isolation of heterotrophs. Following a 9 day incubation period at room temperature, a replica of the filter containing bacterial biomass was prepared and regrown on R2A agar medium containing ^{14}C-UL 2,4-D. Colonies able to degrade 2,4-D were identified by autoradiography based on their ability to incorporate ^{14}C from ^{14}C-UL 2,4-D into cell biomass [12].

For each of the 12 soil subsamples (10g each), between 20 and 40 2,4-D degrading isolates were purified from colonies identified by autoradiography. Putative 2,4-D degrading colonies were picked from a "master" filter and transferred to 2.5 ml of MS-YE-2,4-D medium. After 4 days of incubation at 25°C, 25 µl of each culture were transferred to new tubes containing 2.5 ml of fresh medium. After approximately 4 cycles of enrichment, the cultures were streaked on MS-YE-2,4-D agar medium, and predominant colony morphotypes on each plate were tested for their ability to degrade 2,4-D by monitoring the disappearance of 2,4-D spectrophotometrically in broth cultures. Positive cultures were streaked on 1/2 strength trypticase soy agar (TSA) to check for purity, and -70 °C glycerol stocks of the purified isolates were established.

REP-PCR Fingerprints. Primers derived from a consensus sequence for eubacterial Repeated Extragenic Palindromic (REP) sequences were used for amplification of DNA fragments lying between direct repeats of REP elements [52]. PCR reactions were performed using the conditions and reagents described by de Bruijn [9]. For each 25 µl reaction, 1 µl of cells from frozen glycerol stocks was added directly to 24 µl of reaction mixture. Amplifications were performed using a Perkin-Elmer Gene Amp 9600 PCR thermocycler (Perkin-Elmer, Norwalk, CT). Reaction products were separated by electrophoresis in 1.5% agarose gels containing 1/2 strength TAE and ethidium bromide [39]. After obtaining REP-PCR fingerprints of all isolates, isolates representative of each

pattern observed were retested using both whole cells from glycerol stocks as well as purified genomic DNA to confirm reproducibility of the fingerprints.

Similarity coefficients of REP-PCR patterns and restriction fragment patterns were calculated by the following formula:

% similarity = [(# of comigrating bands)x2/(total # bands)] x 100%

Substrate utilization profiles. Substrate utilization profiles for *V. paradoxus* isolates representing several different REP-PCR fingerprints were determined using BIOLOG (GN) plates (Biolog, Hayward, CA). For each isolate, the assay was performed in triplicate. Assays were conducted according to the manufacturer's recommendations using carefully standardized conditions.

Fatty Acid Methyl Ester (FAME) profiles. Fatty acid profiles were determined for isolates used for BIOLOG (GN) assays. Cultures were grown overnight at 30°C in 1/2X TSB and cells were harvested by centrifugation. Fatty acids were saponified, methylated, and extracted according to standard methods [40]. Fatty acids were identified from gas chromatography profiles using analytical software (version 3.8) from Microbial Identification Systems, Inc. (MIS, North Newark, DEL). For each isolate the assay was performed in triplicate.

Restriction fragment analysis of 16S rDNA. Amplification of 16S rDNA from *V. paradoxus* isolates representing unique REP fingerprints was performed using consensus primers [54], reagents, and reaction conditions described previously [21]. Amplified 16S rDNA was cut with the following 5 restriction enzymes: *Alu*I, *Cfo*I, *Hae*III, *Msp*I, *Sau*3A, *Rsa*I, and*Taq*I (Gibco BRL). All of these enzymes have 4-base recognition sites. Digested DNA was visualized by electrophoresis in 2.5% Metaphor (FMC Bioproducts, Rockland, ME) agarose gels containing TAE and ethidium bromide.

Restriction analysis of genomic DNA. Genomic DNA in SeaKem (FMC Bioproducts) agarose gel plugs was prepared for pulse-field gel electrophoresis according

standard techniques [42]. Cultures grown to an optical density of 0.5-0.7 at 600nm in 1/10 TSB medium were used for preparation of DNA/agarose plugs. Genomic DNA in gel plugs was digested with 40 U of *Xba*I at 37°C for 16 hours in 150 µl H_2O and 20 µl of 10X buffer. A BIORAD CHEF mapper™ was used for electrophoresis of restriction fragments in 1% SeaKem agarose (FMC Bioproducts) gels containing 1/2 strength Tris Borate EDTA buffer. Electrophoresis conditions were as follows: 6 V/cm, 120° angle, and switch times ramped linearly from 12.55 to 58.69s for 29 hours 57 minutes.

Restriction analysis of plasmid DNA. Plasmid DNA was isolated by alkaline lysis and column chromatography using QIAGEN columns (QIAGEN Inc., Chatsworth, CA). Strains were grown in 500 ml LB medium at 30°C to an optical density of 0.4-0.5 (λ = 600 nm). For each column, 250ml of culture was harvested and processed according to the manufacturer's recommendations. Purified plasmid DNA was digested separately with *Hin*dIII, *Eco*RI, *Bam*HI, *Bgl*II, and *Pst*I.

Growth curves. Batch cultures used for measuring the growth rate of bacterial strains were established as follows: Glycerol stocks were streaked on 1/2 strength TSA plates and incubated at 30°C. A single colony arising on each plate was used as inoculum in 10 ml of mineral salts-succinate medium (5 mM succinate) in 50 ml flasks. Incubation was at 25°C in a shaking water bath (200 rpm). At mid-log phase (OD_{600nm} = 0.25-0.33), the cultures were diluted to 0.001 OD in 60 ml of fresh medium in 125 ml flasks. Incubation was continued as before, and at various timepoints, 0.6 ml of culture was removed for measurement of OD using a Hewlett Packard diode array spectrophotometer (model 8452A). Growth curves are based on data from three independent cultures. Doubling times were calculated from optical density data using least-squares regression analysis of transformed data [18].

Results

Phylogenetic relatedness and taxonomy of strains. A previous study assessed the species diversity of bacteria within an agricultural soil that share the ability to degrade the herbicide 2,4-D [11]. In total, 364 strains were isolated from microcosms constructed with soil taken from a 6 m^2 fallow agricultural plot. Among these, 145 strains shared a distinctive colony morphology on trypticase soy agar plates. The colonies were yellow, glistening, slightly mucoid, concave with entire margins, and 4-5 mm in diameter after 7 days incubation at 30°C. Spreading variant colonies were occasionally observed.

The phylogentic relatedness of the strains was determined by comparison of restriction analyses of PCR-amplified 16S rDNA genes. This analysis was done on a representative strain from each of 14 subpopulations (based on REP-PCR fingerprints; see below). The restriction patterns obtained with seven different endonucleases, namely *Alu*I, *Cfo*I, *Hae*III, *Msp*I, *Sau*3A, *Rsa*I, and *Taq*I, were identical. For example, digestion of the 16S rDNA with *Cfo*I yielded 4 fragments that were 540, 440, 370, and 140 bp in size (Figure 1). Thus, it appears the 14 subpopulations are members of a phylogenetically coherent group.

The taxonomic relationships of 12 isolates representing four subpopulations were determined based on their ability to utilize 95 carbon sources using Biolog (GN) plates and their cellular fatty acid profiles. The substrate utilization profiles of the strains were nearly identical and differed in their response to only 5 of the 95 substrates tested. The cellular fatty acid profiles of the strains were also very similar and the strains were determined to be taxonomically identical to each other using the pattern matching alogorithm of the MIDI software (version 3.8, Microbial Identification Systems Inc., Newark, DEL). By comparison of the results obtained to those in the Biolog and MIDI databases, these strains were identified as *V. paradoxus* (data not shown).

Variation in genome organization. Genetic diversity among the *V. paradoxus* isolates was determined by comparing the REP-PCR fingerprints of their genomes. Differences in the number and physical location of REP elements in the genomes would result in PCR amplification of DNA fragments that differed in size thus altering the fingerprints produced. This analysis showed there were 14 distinct subpopulations among the 145 strains based on differences in the REP-PCR fingerprints (Figure 2). Subpopulations 7, 11, and 13 accounted for most (27, 25, and 22%, respectively) of the strains (Figure 3) with fewer strains distributed among the remaining 11 subpopulations. The high degree of genetic diversity among the subpopulations was reflected in the observation that no single fragment was found to comigrate in the fingerprints of all 14 subpopulations (Figure 2). Subpopulations 12 and 13 were the most similar and shared five fragments of similar size but differed in the presence of two fragments. Subpopulations 6 and 7 were the most divergent and had only one fragment in common and differed in the presence of 31 fragments.

It is possible that the differences observed in the genomic fingerprints resulted from limited differences that were clustered in the genome or were due to some unknown bias in the procedure. To determine if this was the case, we restricted total genomic DNA from twelve strains representing four subpopulations with *Xba*I and resolved the DNA fragments using pulse field gel electrophoresis. Each subpopulation showed a characteristic restriction fragment pattern that differed substantially from the patterns of other subpopulations (Figure 4). Similar results were obtained when the genomic DNA of these strains was restricted with *Vsp*I (data not shown). Subpopulations 7 and 11 were most similar to each other and shared 11 *Xba*I fragments of similar size but had 27 fragments that did not comigrate; this yielded a similarity coefficient of 45%. This is a higher degree of similarity than estimated from REP-PCR fingerprints, where these subpopulations shared two fragments of similar size but differed in the presence of 22

fragments to give a similarity coefficient of 15%. Subpopulations 4 and 9 were the most different based on polymorphisms in restricted genomic DNA and had a similarity coefficient of only 18%. The extensive differences in the size of restriction fragments between subpopulations demonstrated that genetic variation between subpopulations was not confined to a small region of the genome. Thus, both REP-PCR fingerprints and analysis of restriction fragment length polymorphisms in genomic DNA indicated there were substantial differences in the genome organization among strains of the same species isolated from a single location.

Genetic variation within subpopulations. The restriction fragment patterns of genomic DNA were consistent with the placement of strains within a given subpopulation based on REP-PCR fingerprints. However, for three of the four subpopulations examined, differences among strains within a given subpopulation were also evident. For example, the pattern of restriction fragments observed for three strains of subpopulation 4 all differed from one another. Strain 4A contained one unique fragment that was 325 kb, but lacked two fragments, 252 kb and 231 kb, that were present in strains 4B and 4C. Strain 4C contained a unique 316 kb fragment, but lacked a 250 kb fragment found in strain 4B. Similar variation was also found among the strains of subpopulations 7 and 9. Thus, pulse field analyses of restricted genomic DNA revealed genetic diversity among strains of the same subpopulation that was not evident from the REP-PCR fingerprints.

These 12 strains also differed in the number and size of the plasmids they carried (Figure 5). Strains A and B of subpopulation 4 each had single plasmids that were 57 and 58 kb in size, respectively, whereas plasmid DNA was not detected in strain 4C. In contrast, strain 7A had three plasmids that were 5, 8, and 51 kb, but strains 7B and 7C had single plasmids that were 52 and 58 kb. There were no differences in the size or number of plasmids among strains within subpopulations 9 and 11. Strains 9A-C carried a 52 kb plasmid, whereas strains 11A-C carried a 58 kb plasmid.

The 51, 52, 57 and 58 kb plasmids of these strains appear related to one another based on similarities in restriction fragment patterns and hybridization to 2,4-D catabolic genes. For example, the 51 kb plasmid of strain 7A yielded seven *Pst*I fragments (Figure 5). Of these, all but one were also found upon digestion of the 52 kb plasmid of strains 9A-C. Similarly, all of the *Pst*I fragments of the 52 kb plasmid were present in *Pst*I digests of the 58 kb plasmid along with three unique fragments that were 1.1, 1.7, and 2.8 kb in size. Among the *Pst*I fragments from the 57 kb plasmid, five were similar in size to fragments from the 51, 52, and 58 kb plasmids. In addition, the 51, 52, 57, and 58 kb plasmids of these strains encode a gene on a common 5 kb *Pst*I fragment that was homologous to *tfdA* (2,4-D etherase gene) from *A. eutrophus* JMP134 (pJP4) under high stringency hybridization conditions (data not shown). Likewise, these four plasmids share 5.0 and 3.6 kb *Pst*I fragments which hybridize to *tfdB* (2,4-D hydroxylase) from pJP4 under medium stringency conditions. All of the strains tested except strain 4C carried one of these four plasmids. Thus, six different plasmids were found among the 12 strains, and four of the six appear to differ from one another as a result of recombination events that have altered the size and restriction pattern of the plasmids.

Phenotypic variation within subpopulations. Growth rates in a mineral-salts succinate medium were measured to determine if the genetic variation observed among the of the 12 *V. paradoxus* strains was reflected in phenotypic differences (Table 1). The mean generation time of strains from subpopulations 4 and 9 were similar to each other (4.1 h) and were less than the mean generation times of subpopulations 7 and 11, which were 4.8 and 5.0 h, respectively. However, an ANOVA test [46] of the mean generation times showed that the differences between subpopulations were not significant ($P > 0.1$). In contrast, differences in the generation times of individual strains were highly significant ($P < 0.01$). Strain 4C grew most rapidly of those tested and had a generation time of 3.5 h, whereas strain 7A grew slowest with a generation time of 5.7 h (Figure 6). Thus, the

growth rates of the strains differed by as much as 38%. The large difference in the mean growth rates between individual strains suggests the strains may differ in relative mean fitness.

Discussion

Differences in the physical organization of genomic and plasmid DNA were observed among 145 strains of *V. paradoxus* isolated from soil. Based on REP-PCR fingerprint patterns, 14 genetically distinct subpopulations were identified. Three subpopulations accounted for 74% of the strains, and the remaining strains were distributed among the other 11 subpopulations. More extensive analyses were done on three strains from each of four subpopulations by comparing restricted chromosomal DNA separated by pulse field gel electrophoresis, the number and sizes of plasmids, and restriction fragment length polymorphisms of plasmid DNA. Of the 12 strains examined, 8 differed from one another based on the criteria examined. These data suggest that sexual and asexual recombination events are common in *V. paradoxus* and lead to extensive genetic diversification among strains of the population.

The REP-PCR fingerprint pattern observed for any given strain reflects the number and distribution of REP elements in the *V. paradoxus* chromosome. Although the specific distribution of REP elements within the *V. paradoxus* chromosome is unknown, in *E. coli* K12, there are approximately 500 copies of the REP element distributed rather evenly throughout the chromosome [10, 27]. The production of a specific DNA fragment by PCR amplification requires that two REP elements be in relatively close proximity to one another in the chromosome (~ 6 kb or less). Any event that alters the number or relative spacing of REP elements could potentially result in the gain or loss of fragments from the PCR pattern. The substantial differences observed in the REP-PCR fingerprints of the *V. paradoxus* subpopulations suggest there are marked differences in the physical organization of these genomes.

We can not rule out the possibility that some differences in REP-PCR fingerprints might be due to point mutations within REP elements of the *V. paradoxus* genome. Divergence of the REP sequence through the accumulation of point mutations could result in failure of the oligonucleotide primers to anneal to the REP elements during PCR amplification and preclude the amplification of specific fragments. However, we think this is unlikely to account for most (if any) of the differences in fingerprints observed for several reasons. First, the sequences of the primers used were degenerate and a low stringency annealing temperature was used. Thus, slight alterations of the sequences of REP elements would not be expected to prevent amplification of the DNA flanked by REP elements. Second, the REP element sequence is conserved among eubacterial species [9, 48, 52] and apparently under strong selection. This makes it unlikely that point mutations would accumulate in REP elements. Finally, we observed the gain (as well as the loss) of DNA fragments in the fingerprints, an event that would require numerous point mutations to create a REP sequence in the genome where one did not previously exist. This seems improbable. Instead, we postulate that recombination events including insertions and deletions that either increase or decrease the relative spacing of REP elements have altered the genome organization and the REP-PCR fingerprint that is obtained. Such recombination events have been shown to cause changes in REP-PCR fingerprints [34]. This postulate is consistent with analyses of the restriction fragment patterns of genomic DNA from the strains which corroborated the placement of strains within a given subpopulation based on REP-PCR fingerprints. Hall [15] found that most of the restriction fragment polymorphisms examined in *Enterococcus faecalis* strains were the result of recombination events such as deletions or insertions, not point mutations. Thus, in view of the differences in the *V. paradoxus* REP-PCR fingerprints and in the *Xba*I restriction patterns, strains belonging to different subpopulations have apparently diverged by extensive recombination that has affected genome organization.

Previous studies have shown that the frequency of recombination can vary among different regions of the bacterial chromosome [51], and are particularly common among regions that have significant nucleic acid sequence homology such as *rrn* operons [1, 22], the terminal recombination zone (TRZ) [6, 25], or recombination 'hot spot' (*rhs*) regions [23]. However, the differences observed in REP-PCR fingerprints of *V. paradoxus* strains suggest that these recombination events are not restricted to any particular region of the genome but rather occur throughout the chromosome. REP-PCR amplification of genomic DNA "samples" only a small portion of the chromosome. The genome of *V. paradoxus* is >5,000 kb, and yet the sum of DNA fragments in the REP-PCR fingerprint amounts to <50 kb. This suggests that recombination events which alter genome organization may be relatively common since they can be seen in a presumably random, small sample (< 1%) of the total genome. We can not exclude the possibility there is a nonrandom distribution of REP elements within the *V. paradoxus* genome, and the elements responsible for the fingerprints observed may be clustered within a region that exhibits a high degree of plasticity. However, since extensive differences were observed in restriction fragment patterns of genomic DNA, it seems as though the former explanation is more likely.

The relative contribution of sexual and asexual recombination to the genetic variation observed in *V. paradoxus* is unknown. In populations of *Burkholderia cepacia*, *Rhizobium leguminosarum*, *R. meliloti*, and *Bacillus subtilis* isolated from soil [17, 47, 56], sexual recombination has been shown to cause extensive genetic variation. In these studies, MLEE analyses showed there was low linkage disequilibrium among strains of the same species thus reflecting high genetic variation within each species. However, in the case of *V. paradoxus*, it seems unlikely that sexual recombination is a major cause of the variation observed. Horizontal gene transfer and sexual recombination among members of the same species typically results in gene conversion in which homologous

recombination occurs with a "foreign" sequence of similar length and nucleotide composition [28, 43]. These events would usually conserve the physical organization of the genome.

There is increasing evidence that the physical and genetic maps of bacterial strains of the same species can significantly vary. This is true even in species such as *E. coli* and *Salmonella enterica* [4, 24]. where genome organization is generally thought to be highly conserved. Bergthorsson and Ochman [3] determined the genome size of 14 strains of *E. coli* selected from each of the five major phylogenetic subgroups of the ECOR collection and found the genome size ranged from 4660 kb to 5300 kb. (Interestingly, Louws et al. [26] have shown that the REP-PCR fingerprints of these strains were also markedly different.) Genome organization within other species varies even more drastically. For example, Carlson and Kolsto [7, 8] found the genomes of several *Bacillus cereus* strains ranged from 2400 kb to 6300 kb, with some regions appearing to be common to all the chromosomes although numerous rearrangments had resulted in a shuffling of gene order. Likewise, alignment of a 1.2 Mb segment from the genome of each of three *Rhodobacter capsulatus* strains revealed numerous translocations, deletions and insertions ranging from 1-30 kb in length, and a mosaic of 15-80 kb segments containing clustered restriction site polymorphisms [35]. Sundin et al. [49] used genome fingerprinting by REP-PCR and amplification of DNA flanked by IS50 sequences to compare isolates from two epiphytic populations of *Pseudomonas syringae* inhabiting two orchards 26 km apart. There were 84 unique fingerprint patterns found among 100 strains examined suggesting abundant recombination. Two major subgroups were apparent with less than 50% similarity, although within each subgroup most of the isolates were more than 80% similar. Thus, there may be greater plasticity in genome organization among strains of the same bacterial species than is generally believed to exist.

The reasons for different degrees of genetic diversity within different species are unclear [45]. Differences in the severity of natural selection may account for some of the variation observed among bacterial species [33, 50]. Nass *et al.* [33] found that genetic variation arising from recombination within populations of *E. coli* increased with the length of time spent in stationary phase. Analysis of subclones from a 30 year old stab indicated that the majority of cells in the stab population comprised a heterogeneous pool of variants compared to the parent strain [32]. In contrast, the population in a 1 year old stab was highly homogeneous. These data suggest that genetic heterogeneity may be common among organisms in terrestrial and marine environments since the mean generation times of these organisms are significantly longer than commonly observed in laboratory cultures. For example, the *in situ* doubling times of heterotrophic bacteria in grassland soils range from 50 to 100 days [2, 16]. With only 4 to 7 doublings per year, bacterial populations in soil may either grow slowly at a relatively constant rate or experience a short, episodic period of exponential growth and spend most of the time in stationary phase. Mutations that would be lost by competitive exclusion in a rapid growth environment might be retained and detected in starving populations; thus leading to higher genetic diversity. Additional studies are needed to clarify the effect of nutrient availability on the the genetic diversity found in bacterial populations.

Acknowledgements

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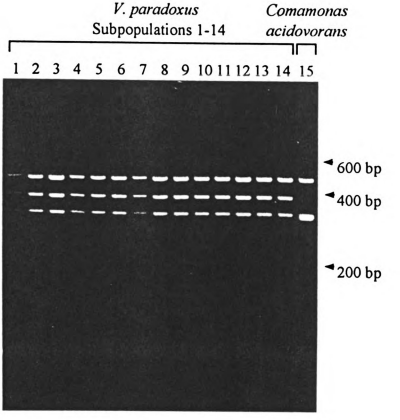

Figure 1. *Cfo*I digest of PCR-amplified 16S-rDNA. 16S-rDNA was obtained from *V. paradoxus* strains representative of 14 subpopulations and from a closely related organism, *Comamonas acidovorans* ATCC 15668.

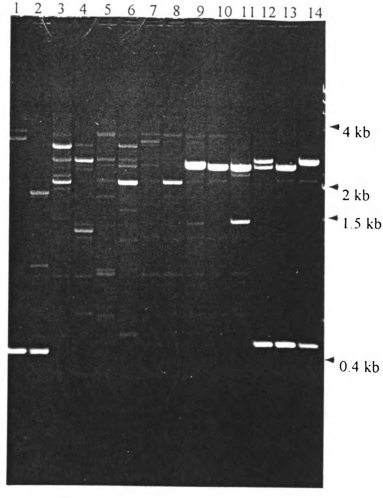

Figure 2. REP-PCR DNA fingerprint patterns found among 145 V. paradoxus strains. Each pattern identifies a distinct *V. paradoxus* subpopulation.

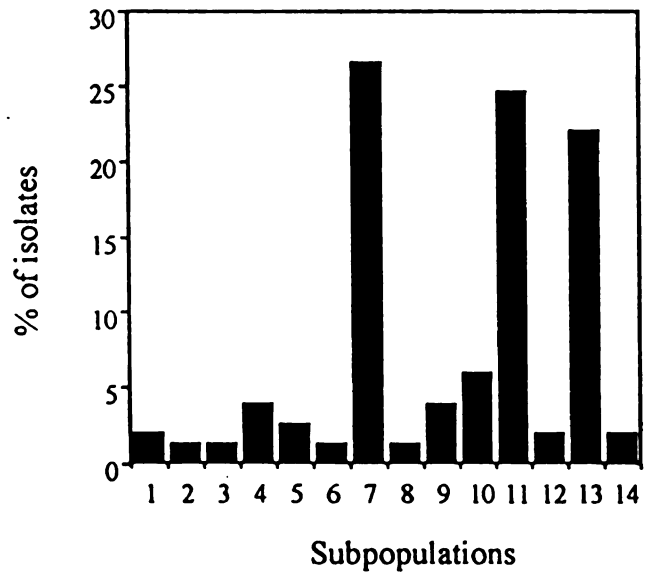

Figure 3. Distribution of 145 *V. paradoxus* isolates among 14 subpopulations. Subpopulation numbers correspond to lane numbers in Figure 2.

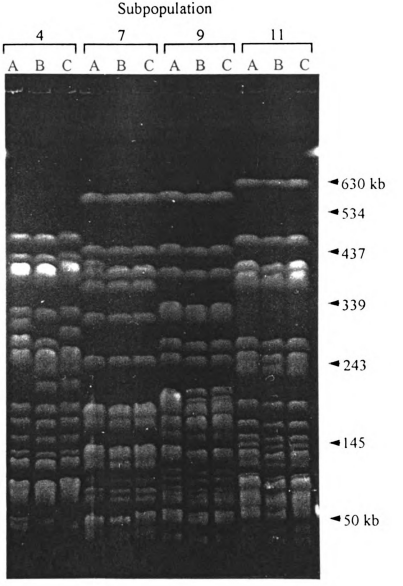

Figure 4. *Xba*I digest of genomic DNA from 12 *V. paradoxus* isolates. Three clones selected from each of four different subpopulations (4, 7, 9 and 11) were analyzed.

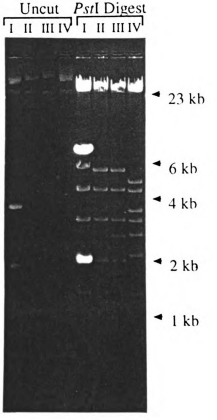

Figure 5. Unique plasmid profiles found among 12 *V. paradoxus* isolates selected from four different subpopulations.

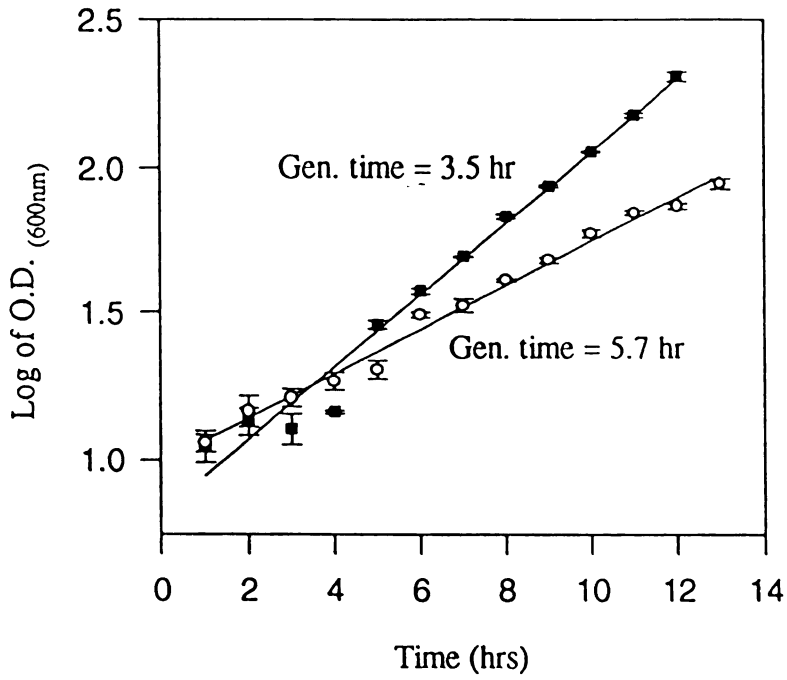

Figure 6. Maximum growth rate differences observed among 12 *V. paradoxus* strains. Strain 4C, closed squares (•); strain 7A, open circles, (o).

Table 1. Genetic diversity and growth rates of 12 *V. paradoxus* isolates.

Isolate	REP-PCR pattern	Genomic RFLP[a]	Plasmid Size	Doubling Time (SD)
4A		I	ND[c]	4.5 hr (0.05)
4B	#4	I'	46 kb	4.2 hr (0.13)
4C		I"	44 kb	3.5 hr (0.12)
7A		II	41, 8, 5 kb	5.7 hr (0.04)
7B	#7	II	42 kb	4.7 hr (0.05)
7C		II'	46 kb	4.1 hr (0.05)
9A		III		4.1 hr (0.11)
9B	#9	III	42 kb[d]	4.0 hr (0.03)
9C		III'		4.3 hr (0.08)
11A				5.0 hr (0.37)
11B	#11	IV[b]	46 kb[e]	5.0 hr (0.23)
11C				5.1 hr (0.29)

[a] Roman numerals indicate a "core" *Xba*I RFLP pattern while the asterisks indicate minor variations in the core pattern.

[b] Isolates 11A-11C had identical RFLP patterns.

[c] ND = Not Detected

[d] Isolates 7B and 9A-9C had identical plasmid profiles.

[e] Isolates 4B, 7C, and 11A-11C had identical plasmid profiles.

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CHAPTER 4

Effect of Selection on the Structure of an Indigenous 2,4-Dichlorophenoxyacetic Acid (2,4-D) Degrading Guild in Soil

Abstract

The short-term effect of selection on the frequencies of bacterial populations able to degrade 2,4-D (2,4-D$^+$)was examined by comparing the 2,4-D degrading guild in 9 replicate microcosms treated with either 0, 10, or 100 ppm 2,4-D. These microcosms contained soil which had no known exposure to 2,4-D. In the control microcosms, the number of 2,4-D$^+$ organisms remained at 10^3 cfu/g soil during the experiment while the number increased to approximately 10^5 and 10^6 cfu/g soil, respectively, in microcosms treated with 10 or 100 ppm 2,4-D. Bacteria representing numerically dominant members of the 2,4-D degrading guild in each microcosm were isolated. Genetic diversity among these isolates was evaluated at several levels; populations were identified by rep-PCR genomic fingerprints of each isolate, species were identified based on restriction analysis of 16S-rDNA obtained from each population, and 2,4-D catabolic groups were identified based on hybridization of 2,4-D gene probes with genomic DNA from each population. These analyses revealed a high degree of genetic diversity within the 2,4-D guild. A total of 66 populations representing at least 35 species and 5 hybridization groups were detected. Only a small number of these populations increased to high frequency upon addition of 2,4-D. The average richness, E(S), per microcosm was 25.8 populations (S.D. = 3.05) per 75 isolates in the untreated microcosms, and 8.7 populations (S.D. = 3.45 and 0.24, respectively) following treatment with either 10 or 100 ppm 2,4-D. Thus, the guild became more skewed following selection as a subset of the 2,4-D$^+$ populations rapidly increased in frequency relative to others. These results indicate that microbial guilds in disturbed soils can be rapidly restructured in response to pulses of substrate.

Introduction

It has been suggested that functional redundancy within microbial communities may contribute to the stability of ecosystem processes. Greater genetic diversity within a functional group, or guild, potentially extends the range of environmental conditions under which a particular functional trait will be expressed. Diversity may also contribute to the efficiency of resource utilization [7] as well as the resiliency of the guild and the general community in response to disturbance [60]. Microbial communities in agricultural soils are thought to be extremely diverse. Indeed, DNA reassociation kinetics suggest there are as many as 10,000 species per gram [61]. However, very little is known about the extent of functional redundancy or how the redundancy associated with a particular function contributes to the overall activity. In order to model the activity of natural microbial communities, it is necessary first to understand the dynamics of competition, which structures genetic diversity within microbial guilds and communities.

Current views of competition dynamics in physically complex environments such as soil are based on results from simple environments such as liquid batch cultures and chemostats. In batch cultures and chemostats, competition for a single growth limiting nutrient typically results in the dominance of one population and extinction of other competitors. However, coexistence of competing populations in batch cultures or chemostats can be achieved by introducing factors such as surfaces for bacterial attachment and growth [20], chemical gradients [69], resource fluctuations [55, 64, 65], chemical inhibitors [39], predators [11, 31, 38, 40], or multiple growth limiting nutrients [27, 58, 59] to increase the ecological complexity of the environment. These factors are natural components of soil microbial communities. Consequently, the view has arisen that coexistence may be common in natural environments such as soil [27]. Based on this view, genetic diversity should be maintained within guilds in soil microbial communities despite the purging effect of natural selection. Indeed, dominance of one or a few

competing populations may occur only at microscales that are difficult to observe while coexistence of a large number of populations might be observed at scales of millimeters and greater.

Competition dynamics in soils would be understood best by evaluating dynamics among indigenous populations and not by inoculation of strains into a foreign environment. The effect of competition on indigenous genetic diversity in soil microbial communities has previously been examined by tracking the frequency of specific genes following selection [29, 32, 71]. Hybridization of gene probes with extracted community DNA has been used to estimate the allelic variation and abundance of specific catabolic genes and biochemical pathways in a community for the degradation of particular compounds [29, 34]. Similarly, PCR amplification has been used to detect specific metabolic gene sequences in community DNA [28, 63, 67, 72]. However, these approaches do not reveal the diversity of hosts among which a biochemical pathway or gene is distributed. Thus, the effect of competition on the frequency distribution of indigenous competing populations in soil is poorly understood.

We investigated the genetic diversity underlying 2,4-D degradation and the short term effect of selection on the structure of an indigenous 2,4-D degrading guild in soil microcosms. A total of 840 2,4-D$^+$ isolates collected from the microcosms were examined at three genetic levels: populations (or subspecies), species, and catabolic groups. The criteria used for identification of populations and species in this study were merely operational. Although the criteria may have understimated the true diversity of populations (clonal lineages) or species, they clearly established different levels of genetic similarity adequate for the analysis of competition dynamics in soil. Soil with no documented previous exposure to 2,4-D was used in order to evaluate the distribution of diversity within the 2,4-D catabolic guild before and after competition for 2,4-D.

Materials and Methods

Media. 2,4-D degrading bacterial cultures were routinely cultivated in mineral salts-yeast extract-2,4-D medium (MS-YE-2,4-D). The mineral salts base consisted of 6.1 mM Na_2HPO_4, 3.9 mM KH_2PO_4, 2.5 mM $(NH_4)_2SO_4$, 0.2 mM $MgSO_4$, 50 mM $CaCl_2 \cdot 2H_2O$, 8.6 mM EDTA, 10 mM $FeSO_4 \cdot 7H_2O$, 4 mM $ZnSO_4 \cdot 7H_2O$, 10 mM $MnSO_4 \cdot H_2O$, 1.5 mM $CuSO_4 \cdot 5H_2O$, 0.8 mM $Co(NO_3)_2 \cdot 6H_2O$, and 0.1 mM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$. Yeast extract was added at a concentration of 10 mg/l and 2,4-D at a concentration of 250 mg/l. A 20 mg/ml 2,4-D stock solution (pH 7.0) was prepared by dissolving 2,4-D in 0.1 M NaH_2PO_4 and adjusting the pH with 5N NaOH. 2,4-D and [ring-UL ^{14}C]2,4-D were purchased from Sigma (St. Louis, MO).

Soil microcosms. Nine soil microcosms were established from homogenized soil collected from the top 10 cm of a fallow agricultural plot which had no previous documented exposure to 2,4-D. The field site was an experimental research plot at Kellogg Biological Station, Hickory Corners, MI. Ten to fifteen soil samples from a $6m^2$ area were combined, sieved through a 2 mm pore-sized sieve, homogenized by mixing in a polyethylene bag, and stored at room temperature in the laboratory for one week prior to use. For each microcosm, 243 g of soil (8% moisture content) were transferred to a polyethylene bag while 27 g of soil were oven-dried (100°C) overnight to serve as a carrier for liquid amendments. Each 27 g portion of carrier soil was mixed with 2,4-D dissolved in 0.1 M Na_2HPO_4 buffer (pH 7.0) or Na_2HPO_4 buffer alone such that each microcosm would receive identical concentrations of sodium phosphate but either 0, 10, or 100 μg 2,4-D/g soil (final concentration). After mixing the carrier soil with 243 g of bulk soil in a polyethylene bag, the soil moisture content was adjusted to 25% (wt/wt) with sterile, distilled water. All nine microcosms were incubated at 25°C for one week during which time samples were periodically removed in order to measure the 2,4-D concentration and to isolate 2,4-D^+ strains.

Sampling. At various timepoints, 1 g soil samples were removed from each microcosm for determination of 2,4-D concentration by extraction with 0.1 M NaH_2PO_4 (pH 7.0) and analysis by high pressure liquid chromatography [29]. A 10 g soil sample from each microcosm was collected at various timepoints for isolation of 2,4-D^+ strains. Each sample consisted of approximately 10 subsamples. The samples were added to 90 ml of sterile, 10 mM phosphate buffer shaken at 25°C for 20 min at 200 rpm on a rotary platform shaker, then serially diluted. Aliquots from appropriate dilutions were plated on R2A (Difco, Detroit, MI) agar for enumeration of heterotrophs and on HATF nitrocellulose filters (Millipore, Bedford, MA) atop R2A agar for enumeration of 2,4-D degrading colonies.

Identification of 2,4-D degrading colonies. Colonies able to degrade 2,4-D were identified by autoradiography based on their ability to incorporate ^{14}C from [^{14}C]2,4-D into biomass [17]. Nitrocellulose filters spread with soil dilutions were incubated 4 or 9 days depending on the dilution, then replicated. The master filters were re incubated on fresh R2A agar medium while the replicas were placed on R2A agar medium containing [ring-UL-^{14}C]2,4-D (Sigma). After 4 days incubation, the replica filters were mounted on paper, dried, covered with saran, and exposed to Kodak X-OMAT™ film (Eastman Kodak, Rochester, NY) for 12-18 hours. Colonies which produced signals on film were counted as putative 2,4-D degrading colonies. The location of these colonies on the master filters was determined by aligning autoradiograms with the master filters.

Purification of 2,4-D isolates. Putative 2,4-D degrading colonies were picked from master filters and transferred to 2.5 ml of MS-Y-2,4-D medium. In most cases, 2,4-D degrading colonies were retrieved from filters crowded with non-target organisms. Therefore, each broth culture initiated from a "colony" was serially transferred in order to reduce the background of non-target organisms. After about 4 transfers, cultures were streaked on MS-Y-2,4-D medium solidified with 1.5% agar. The dominant colony types

on each plate were picked and tested in MS-Y-2,4-D liquid medium for their ability to transform 2,4-D. Disappearance of 2,4-D was monitored using a Hewlett Packard model 8452A diode array spectrophotometer. Cultures able to transform 2,4-D were streaked on 1/2 strength trypticase soy agar to check for purity, then single colonies were picked and regrown in MS-Y-2,4-D medium to establish 15% glycerol stocks at -80°C.

rep-PCR analysis. The genome of each isolate was fingerprinted by using degenerate primers to amplify DNA lying between repeated extragenic palindromic (REP) sequences [66]. The primers and reaction conditions were the same as those described by DeBruijn [14]. Each 25 µl PCR reaction contained 5 µl Gitschier buffer, 2.5 µl DMSO, 12.15 µl milliQ water, 12.5 mM dNTPs (Pharmacia Biotech), 0.3 µM primers, 0.2 µl BSA, and 2 U *Taq* polymerase (Gibco BRL, Grand Island, NY). PCR reactions were performed in a Perkin-Elmer GeneAmp 9600 thermal cycler. From each amplification reaction, 10 µl were electrophoresed in a 1.5% agarose gel containing 1/2 strength TAE buffer [47] and 0.4 µg/ml ethidium bromide. Rep-PCR patterns of different isolates were visually compared in order to identify groups of isolates with identical patterns. Isolates representative of the unique rep-PCR patterns were then retested to confirm the reproducibility of the patterns.

16S restriction analysis. 16S rDNA was amplified using the primers rD1 and fD1 [68] and the reaction conditions described by Laguerre *et al.* [37]. Each 100 µl reaction contained 10 µl PCR reaction buffer (Gibco), 1.5 mM $MgCl_2$, 0.1 µM primer rD1 and fD1, 2.5 mM dNTPs (Pharmacia), 2.5 U *Taq* DNA polymerase (Gibco), and 1.5 µl of bacterial broth culture lysed by several freeze-thaw cycles. Amplified DNA from isolates representing different rep-PCR patterns was digested separately with *Msp*I, *Cfo*I, *Alu*I, and *Hae*III. Digested DNA was electrophoresed through ethidium bromide stained, 2.75% Metaphor (FMC Bioproducts, Rockland, ME) agarose gels containing TAE, and the restriction fragment length polymorphism (RFLP) patterns of all isolates were

compared. Isolates whose 16S rDNA restriction patterns differed with at least one enzyme were counted as different species.

2,4-D gene probe analysis. Genomic DNA was isolated according to standard procedures [2]. Bacterial strains were grown overnight in 5 ml of 1/10 strength trypticase soy broth (TSB), then 1.5 ml were transferred to a microfuge tube for DNA extraction. For each strain, approximately 5 µg of genomic DNA were digested with 2 units of *Eco*RI for 4 hours, then electrophoresed through ethidium bromide-stained, 0.8% agarose gels with TAE buffer. Digested DNA was transferred to Hybond-N nylon membranes (Amersham, Arlington Heights, IL) by capillary blotting [53], and cross linked using a UV stratalinker 1800 (Stratagene, La Jolla, CA) for subsequent hybridization with gene probes.

Gene probes were derived from 2,4-D catabolic genes encoded on plasmid pJP4 from *Alcaligenes eutrophus* JMP134 and from the chromosomally encoded 2,4-D genes in *Burkholderia* sp. strain RASC. Five probes were used consisting of internal fragments of *tfdA*, *tfdB*, and tfdC from pJP4 [29], and of *tfdA* [56] and *tfdB* from strain RASC. The RASC *tfdB* probe consisted of a 1 kb *Sac*I-*Kpn*I fragment from a 1.2 kb SacI-SacI fragment which had been cloned into pUC9. The RASC *tfdA* and *tfdB* genes have 73% and 65% sequence similarity to the corresponding pJP4 alleles [56]. Gel-purified probes were labeled with digoxigenin-dUTP using the DIG DNA Labeling and Detection Kit (Boehringer-Mannheim). Prehybridization and hybridization solutions contained 5X SSC [47], 0.1% N-lauroylsarcosine, 0.02% SDS, 5% blocking reagent, and 50% formamide. Hybridizations were conducted at 62°C to achieve high stringency conditions (90-100% similarity). Following hybridization, membranes were washed twice in 2X SSC then processed for probe detection.

16S rRNA gene copy number. The 16S probe used for determining gene copy number was derived from a 574 bp *Eco*R1 restriction fragment of 16S rDNA amplified

from species 1. The restriction fragment was cut from a 0.8% agarose gel, re-electrophoresed through a 0.8% Sea Plaque (FMC) gel containing TAE and 0.25 μg/ml ethidium bromide, then purified using a Gene Clean kit (Biolabs 101). The fragment labeled with digoxigenin-dUTP. The probe was hybridized under medium stringency conditions with *Eco*R1 digested genomic DNA from each 2,4-D$^+$ population as described above as well as *Pvu*II digested genomic DNA from a population representing each species. Prehybridization and hybridization solutions contained 5X SSC [47], 0.1% N-lauroylsarcosine, 0.02% SDS, 2% blocking reagent, and 20% formamide. The formamide concentration was reduced from 50% to 20% in order to achieve medium stringency (80-100% sequence similarity) hybridization conditions.

Results

Guild dynamics. Following addition of 2,4-D, an increase in the number of 2,4-D degrading organisms was observed in treated microcosms while no significant change was observed in untreated microcosms (Figure 1). The size of the 2,4-D degrading guild in the control microcosms remained at ~10^3 cfu/g soil. In the microcosms treated with 10 or 100 ppm 2,4-D, the guild size increased to approximately 10^5 and 10^6 cfu/g soil, respectively. Consistent with the increase in the number of 2,4-D$^+$ organisms in the treated microcosms, the concentration of 2,4-D in the soil decreased and was mostly depleted in the microcosms treated with 10 or 100 ppm 2,4-D by day 5 and day 7, respectively (Figure 2). The total number of culturable heterotrophs detected in each of the 9 microcosms remained relatively stable at ~10^7 cfu/g soil.

Guild diversity. To examine the composition of the 2,4-D degrading guild in each microcosm, numerically dominant 2,4-D degrading organisms were isolated by direct plating from soil collected at various timepoints. An average of 27 isolates were purified from each sample yielding a total of 840 isolates. Isolates were grouped into populations

based on rep-PCR fingerprint similarity (compared by visual inspection) and into species based on similarity of 16S rDNA RFLPs.

The species composition detected before and after selection differed dramatically. Species 1 was the most abundant 2,4-D$^+$ species in the soil prior to addition of 2,4-D and was the only species consistently detected in every sample from the untreated microcosms (Figure 3A). Extensive genetic variation was observed within this species and has been described elsewhere [19]. In contrast, species 30 which was rare in the untreated soil was consistently detected in all samples (Figure 3B and C) from microcosms treated with 10 or 100 ppm 2,4-D. Although other species were occasionally more abundant within a single sample than species 30, no other species was detected more than once in the same microcosm at a frequency higher than species 30. Based on its consistent appearance relative to other species, species 30 was considered the dominant 2,4-D$^+$ species in the treated microcosms. These results demonstrate a qualitative effect of selection on diversity within the 2,4-D$^+$ guild. That is, the most abundant 2,4-D$^+$ species in the soil prior to selection (species 1) was replaced by another species (species 30) which was initially rare.

The quantitative effect of selection on guild structure was evaluated by comparing cumulative diversity in each microcosm. Population richness (measured as the total number, S, of different populations) was greater among isolates collected from the untreated microcosms (Figure 4). Since the total number of isolates obtained from different microcosms ranged from 75 to 135 isolates, estimates of population richness, E(S), within a standardized sample size of 75 isolates were obtained by rarefaction [30, 51]. The average population richness, E(S), among the untreated microcosms was 25.8 populations. This value was significantly different from the average of 7.7 and 8.7 populations in the microcosms treated with 10 or 100 ppm 2,4-D according to a Student t-test ($P < 0.003$ and $P < 0.001$, respectively). Clustering isolates into species instead of populations reduced the apparent diversity in the untreated microcosms by approximately

50% (Figure 4). Nonetheless, the average species richness (per 75 isolates) among the untreated microcosms was also significantly different from species richness in the microcosms treated with 10 or 100 ppm 2,4-D ($P < 0.05$ and $P < 0.03$, respectively). These reductions in richness indicate that only a fraction of the 2,4-D$^+$ populations initially present in soil increased to high frequencies in the treated microcosms. Thus, the distribution of diversity within the 2,4-D$^+$ guild became more skewed following selection.

The breadth of 2,4-D$^+$ populations detected and the limited number which increased to high frequency are shown in Figure 5. A total of 66 distinct 2,4-D$^+$ populations representing at least 35 species were detected in the microcosms. Among the 327 2,4-D$^+$ isolates obtained from the untreated microcosms, 55 populations representing at least 27 species were identified. An additional 11 populations (representing 11 species) were found only among isolates from treated microcosms.

Diversity of *tfd* catabolic groups. To assess the diversity of 2,4-D catabolic genes carried by these 66 populations, genomic DNA from each population was hybridized under high stringency with available 2,4-D gene probes. The probes were derived from two alleles of the first gene, *tfdA*, in the 2,4-D catabolic pathway, two alleles of the second gene, *tfdB*, and one allele of the third gene, *tfdC*. This analysis divided the 68 populations into five groups, or groups. As shown in Figure 5a, 45 of the 55 populations detected in the untreated microcosms hybridized with the pJP4 *tfdA* allele only (group $A_1B_xC_x$). This group accounted for 87% of the isolates. Thus, group $A_1B_xC_x$ was the most abundant group in the soil prior to addition of 2,4-D. Only 3 of the 55 populations (5% of the isolates) hybridized with pJP4 *tfdA*, *tfdB*, and *tfdC* (group $A_1B_1C_1$), and 1 population hybridized with RASC *tfdA* and *tfdB* (group $A_2B_2C_x$). Following treatment with 10 ppm 2,4-D, group $A_1B_1C_1$ became dominant, accounting for 79% of the isolates. In the microcosms treated with 100 ppm 2,4-D, groups $A_1B_1C_1$ and $A_2B_2C_x$ became more abundant and represented 48% and 47% of the isolates, respectively. These results

demonstrate replacement of an initially dominant group by one that was more competitive under the conditions imposed. Moreover, the observation that groups $A_1B_1C_1$ and $A_2B_2C_x$ accounted for 9 of the 14 species detected in microcosms treated with 100 ppm 2,4-D suggests that these alleles themselves may contribute to competitive fitness.

rrn copy number. The copy number of the 16S rRNA gene in each 2,4-D+ population was determined by hybridization experiments and used as a measure of the copy number of *rrn* operons. As illustrated in Figure 6, one to seven copies of the 16S rRNA gene were observed among the 2,4-D$^+$ species. Different populations belonging to the same species exhibited the same *rrn* copy number as expected (data not shown). Most of the 2,4-D$^+$ species detected prior to addition of 2,4-D had between 2-4 rrn operons. Only 5 of the 29 species had between 5 and 7 rrn operons. In contrast, among the species detected in microcosms treated with 10 or 100 ppm 2,4-D, 7 of 13 species and 11 of 14 species, respectively, had between 5 and 7 copies of the *rrn* genes. The average copy number of *rrn* genes among the species detected in each treatment is shown in Figure 7. An average of 3.3 copies of the *rrn* genes per genome was observed among the species obtained from the control microcosms. The mean *rrn* copy number among the dominant species obtained from the microcosms treated with 10 or 100 ppm 2,4-D was 3.9 and 4.9, respectively. An ANOVA test demonstrated a significant effect of 2,4-D treatment on the outgrowth of species with a higher *rrn* copy number ($P < 0.01$). The positive correlation between mean *rrn* copy and 2,4-D concentration illustrated in Figure 7 indicates an association between *rrn* copy number and competitive fitness. The upward trend indicates that larger pulses of substrate result in a greater resolution between superior and inferior competitors in soil.

Discussion

Genes for metabolism of 2,4-D have previously been identified in a large number of species obtained from around the globe [4, 12, 15, 16, 22, 23, 26, 36, 57]. However,

the genetic diversity underlying 2,4-D degradation in local environments is not well understood. The only previous analysis of 2,4-D guild diversity described the isolation of 2,4-D$^+$ bacteria from eight field plots receiving different 2,4-D treatments over a three year period [33]. Each year, approximately two isolates were obtained from each plot, and analysis of these isolates identified an average of four species per plot over a three year period. Using a more comprehensive approach, we examined the redundancy of 2,4-D$^+$ populations in soil from one of the field plots [33] and the effect of selection on the frequencies of these populations. At least 35 2,4-D$^+$ species were represented among isolates collected from the soil. Substantial genetic variation, indicated by differences in the rep-PCR genomic fingerprints of isolates, was detected within some of the species. As a result, the diversity of populations was approximately 2-fold higher than the diversity of species. This degree of diversity was unexpectedly high since the soil had no previous documented exposure to 2,4-D.

In contrast to the large number of 2,4-D$^+$ populations, only a small number of different *tfdA* groups were detected in the soil. Most (88%) of the 66 populations hybridized under high stringency with the *tfdA* allele from either *Alcaligenes eutrophus* JMP134 (pJP4) or from *Burkholderia* sp. strain RASC. The pJP4-type *tfdA* allele was the most common type encountered. The pJP4-type *tfdA* allele has also been the most frequently encountered allele among 2,4-D$^+$ isolates obtained worldwide [1, 6, 12, 16, 26, 29, 33, 46]. However, it is not clear whether the global distribution of the pJP4-type allele reflects a greater abundance of this type relative to others, or simply a bias resulting from the enrichment culture techniques commonly used to obtain 2,4-D$^+$ bacteria from the environment [18]. In the present study, the pJP4-type *tfdA* allele was identified in 81% of the populations, whereas the RASC-type *tfdA* allele was identified in only 7% of the populations. The RASC-type *tfd* alleles have been found thus far only on bacterial chromosomes [48, 56] unlike the pJP4-type alleles which appear to be commonly encoded

on plasmids [1, 6, 12, 16, 33, 46]. Since the frequency of horizontal transfer of chromosomally encoded genes is expected to be lower than the transfer rates of plasmid encoded genes such as the pJP4-type alleles, one could speculate that the relative frequencies of the pJP4-type and RASC-type alleles in untreated soil may reflect their modes of transfer.

Previous studies have demonstrated that horizontal gene transfer has contributed substantially to the genetic diversity underlying 2,4-D degradation and that 2,4-D genes have spread globally by migration [6, 12, 15, 26, 46]. The relative contributions of gene transfer and migration to the diversity of populations observed locally are unknown. Field studies of the movement of agriculturally important bacterial species have indicated that horizontal migration rates of bacteria (i.e. transport and successful establishment) are low [5, 10, 35, 42, 50]. Lowther and Patrick [42] found that field populations of *Rhizobium loti* and *Bradyrhizobium* sp. migrated upslope less than 20 cm/yr. The authors concluded that wind-blown dust contributed very little to the movement of these bacterial populations. Movement of bacteria in soil appears to be a largely passive process and is highly dependent on percolating water [8, 45, 62, 70] and plant roots which create channels for percolating water [3, 41, 45, 49, 62]. Horizontal migration rates reported for different bacterial species in the field vary [5, 10, 35, 42] and range from 15 cm in 11 months [10] to 160 cm in 1 month [5]. Genetic analysis of *Rhizobium meliloti* populations across several geographical scales also provided evidence of low migration rates [54]. These data suggest that migration of a large number of different 2,4-D$^+$ strains from distant sources is unlikely to account for the diversity of 2,4-D$^+$ populations observed in the present study. Fulthorpe and Wyndam [25] demonstrated the rapid spread of plasmid encoded catabolic genes into a variety of hosts following introduction of a plasmid-carrying population into lake mesocosms. Similarly, the genetic diversity underlying 2,4-D metabolism in the soil used in the present study may have arisen by

migration of a few 2,4-D$^+$ populations into the soil followed by local dissemination of 2,4-D genes into a large number of different hosts.

Only a fraction of the populations initially present increased to high frequency following addition of 2,4-D to the soil. These dynamics, and in particular the apparent predominance of a single species (species 30), were unexpected. The physical, chemical, and biological complexity of soil provide a multitude of micro environments for growth of different bacterial populations[reviewed by 24, 52]. With such heterogeneity, dominance of a single species following addition of a growth limiting nutrient would be expected only on a microscale. It is possible that moderate mixing of the soil prior to construction of the microcosms disrupted most of the heterogeneity which exists *in situ* and forced many populations which would otherwise have been physically or ecologically segregated to compete directly. Thus, greater diversity might be observed in undisturbed soil following selection. However, the data suggest that in agricultural soils which experience frequent mixing, short-term competition dynamics following the addition of nutrients which promote bacterial growth more closely resembles competition dynamics in liquid batch cultures.

Determinants of competitive success: The marked changes which occurred in the relative frequencies of specific 2,4-D catabolic groups suggest that either the alleles of different 2,4-D genes directly influence competitive fitness or the alleles hitchhike as a result of genetic linkage or phylogenetic association with other factors which determines fitness. Whereas the pJP4 and RASC groups (i.e. $A_1B_1C_1$ and $A_2B_2C_x$, respectively) appeared in only 4 of the 55 populations detected in untreated soil, these groups accounted for 10 of the 15 populations detected after exposure to 100 ppm 2,4-D. The non-random distribution of catabolic groups following selection is highly suggestive that an association exists between competitive fitness and the 2,4-D alleles present within a population. Similar results were obtained in a separate experiment [18], in which 2,4-D$^+$ soil

populations were obtained by enrichment in 74 liquid batch cultures. All but one of 74 isolates obtained by enrichment possessed the pJP4-type *tfdA* and *tfdB* alleles despite the fact that roughly two-thirds of the populations actually present in the soil possessed other alleles. As was discussed previously [18], different alleles may encode enzymes with altered substrate affinities or catalytic efficiencies and affect the rate of bacterial growth on 2,4-D by altering the rate of 2,4-D catabolism. This may explain the 2.3-fold difference in growth rates observed by Ka *et al.* [32] for *Pseudomonas cepacia* DBO1 transconjugants harboring either pJP4 or the 2,4-D catabolic plasmid pKA4 which carries the pJP4-type *tfdA* allele but dissimilar alleles for the other 2,4-D genes . It may be that one of the pJP4 genes (*tfdB*, *C*, *D*, or *E*) is particularly favored under conditions of rapid growth and that genetic linkage of alleles accounts for the appearance of the pJP4-type catabolic group.

Alternatively, the distribution of 2,4-D alleles following selection might be an accident of host range and hitchhiking. The pJP4 *tfdA* allele was distributed in both competitive and noncompetitive populations suggesting the host range of this gene is quite broad. However, the other pJP4 genes might be less mobile and largely confined to a small number of species which are generally adapted for rapid exploitation of new resources. The pJP4 genes have been observed primarily in species of *Alcaligenes* and *Burkholderia*.[1, 6, 12, 15, 16, 26] Likewise, the RASC genes seem to occur primarily in species of *Burkholderia* [26, 48, 56]. Based on substrate utilization patterns and membrane fatty acid profiles, the species in the present study exhibiting the pJP4 and RASC genotypes ($A_1B_1C_1$ and $A_2B_2C_x$, respectively) also appear to be *Alcaligenes* and *Burkholderia* species (data not shown). Given the limited host range of these genes, host-specific genetic factors unrelated to the 2,4-D catabolic pathway may be largely responsible for the observed competitive outcomes.

Fundamental genetic factors which shape the overall life-strategy of different populations could cause dramatic differences in competitive fitness. For example, the

copy number of *rrn* genes may affect the competitive fitness of microbial populations by limiting the availability of ribosomes and thus protein synthesis. The dosage of *rrn* operons may be most critical during periods when a bacterium shifts from a low growth rate to a higher growth rate [9, 13] such as would occur following a pulse of substrate in soil. As the growth rate of bacterial cells increases, the amount of DNA, RNA, and protein in the cells also increases [reviewed in 9]. In order to produce more protein in a shorter time span, bacterial cells increase the number of ribosomes per cell [9, 21, 43, 44]. A lower copy number of *rrn* genes may increase the time required to produce a new complement of ribosomes and thereby increase the transition, or lag, time to achieve a higher specific growth rate. Consistent with this theory, Condon *et al.* [13] provided preliminary evidence demonstrating a positive correlation between the number of functional *rrn* operons in *Escherichia coli* and the lag time associated with a shift-up in growth rate following nutrient addition.

Lag time has been shown to influence competitive outcomes among 2,4-D$^+$ populations in soil [32]. Ka *et al.* [32] demonstrated that a *Pseudomonas pickettii* strain carrying the 2,4-D catabolic plasmid pKA4 and a *P. cepacia* DBO1 transconjugant carrying the same plasmid exhibited nearly identical growth rates (0.078 hr-1 versus 0.077 hr^{-1}, respectively) but significantly different lag times in 2,4-D minimal medium. A causal relationship between lag time and *rrn* copy number has not yet been clearly established. However, we observed a statistically significant differences between the mean *rrn* copy number among dominant 2,4-D$^+$ populations in soil before and after selection (Figure 7). The positive correlation between the copy number of *rrn* genes and the competitive success of 2,4-D$^+$ populations in soil following a pulse of 2,4-D is consistent with the hypothesis that a higher *rrn* copy number permits more rapid exploitation of improved nutrient conditions. It may be that specific *tfd* alleles and *rrn* copy number both contribute to the competitive fitness of populations competing for 2,4-D

by determining growth rates on 2,4-D and the lag time required to achieve those rates, respectively.

Acknowledgments

We gratefully acknowledge Dr. Bill Holben and Dr. Yuichi Suwa for providing cloned *tfd* genes.

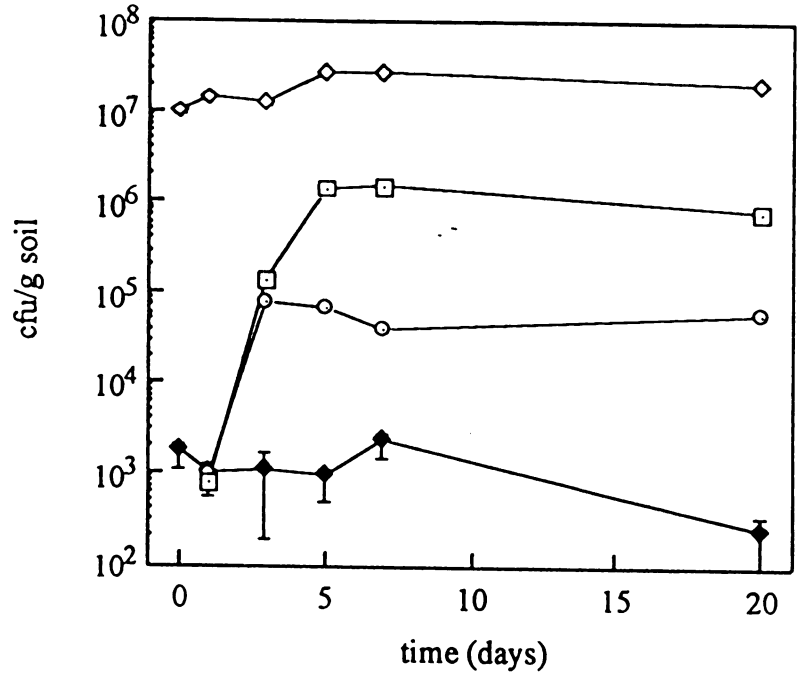

Figure 1. Response of the 2,4-D$^+$ guild in microcosms treated with 0, 10, or 100 ppm 2,4-D. Total culturable heterotrophs are shown for comparison. Error bars are standard deviations.

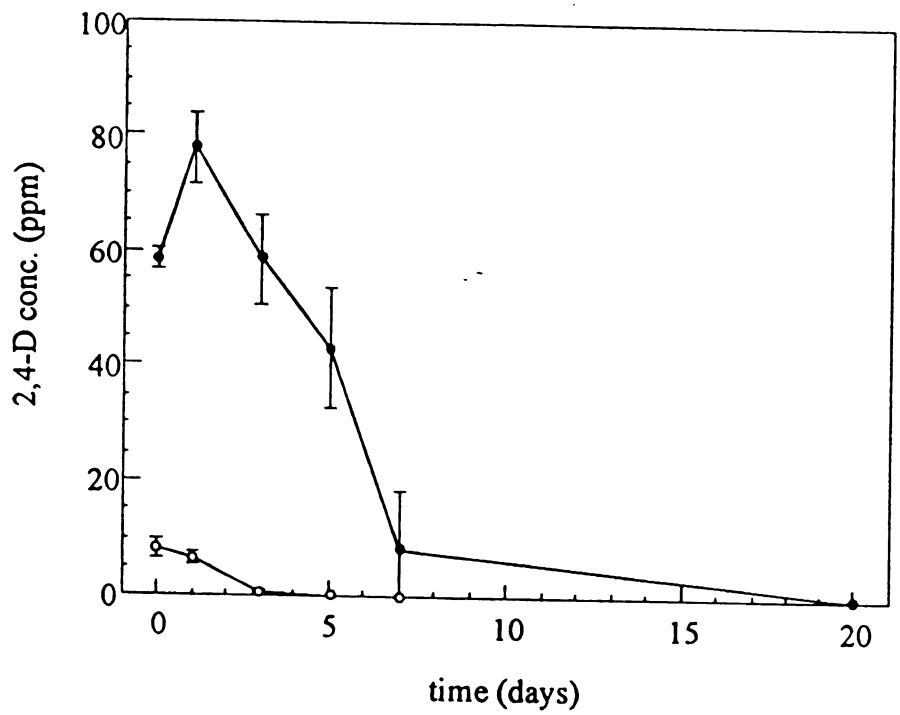

Figure 2. Depletion of 2,4-D in soil microcosms treated with 10 or 100 ppm 2,4-D.

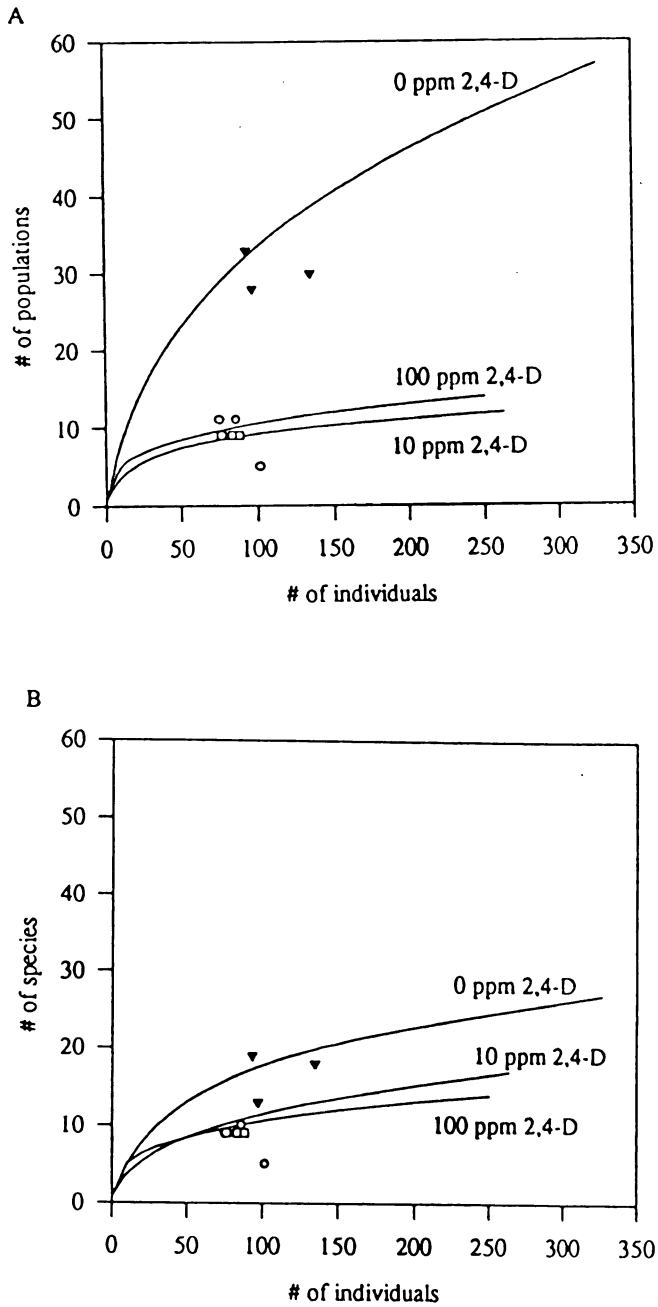

Figure 3. Richness of (A) 2,4-D$^+$ populations (B) 2,4-D$^+$ species in treated and untreated microcosms. Symbols indicate the number of species or populations identified in individual microcosms. Solid triangles (▲) represent microcosms treated with 0 ppm 2,4-D, open circles (o) represent microcosms treated with 10 ppm 2,4-D, and open squares (□) represent microcosms treated with 100 ppm 2,4-D. Cumulative diversity among replicate microcosms within treatments is illustrated by labeled curves (calculated by rarefaction [30, 51] of pooled data).

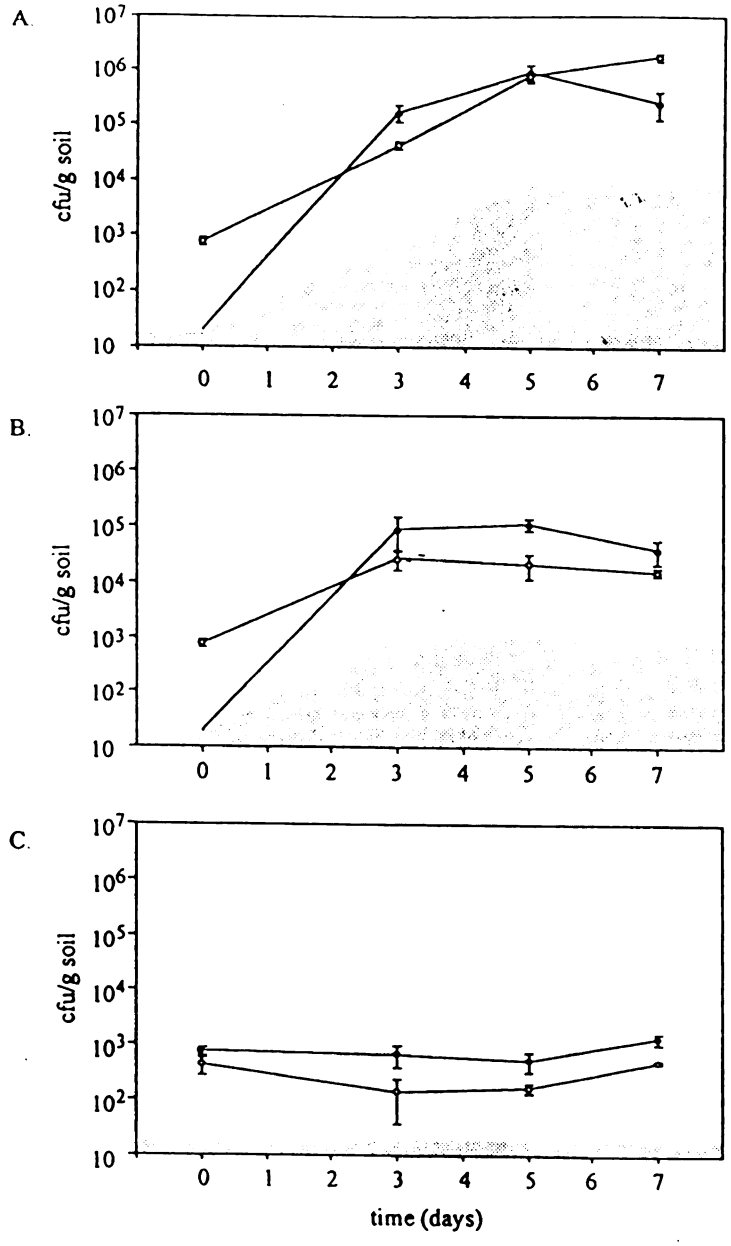

Figure 4. Frequencies of 2,4-D+ species in microcosms treated with (A) 0 ppm 2,4-D, (B) 10 ppm 2,4-D, and (C) 100 ppm 2,4-D. Closed circles (●) indicate the average frequency of the dominant species, species 1 (Panel A) or species 30 (Panels B and C). Open circles indicate the average maximum frequency of other species detected in addition to the dominant species. Error bars are the standard error of the mean for replicate microcosms. Hatched areas indicate the detection limit based on the soil dilution from which 2,4-D$^+$ isolates were obtained.

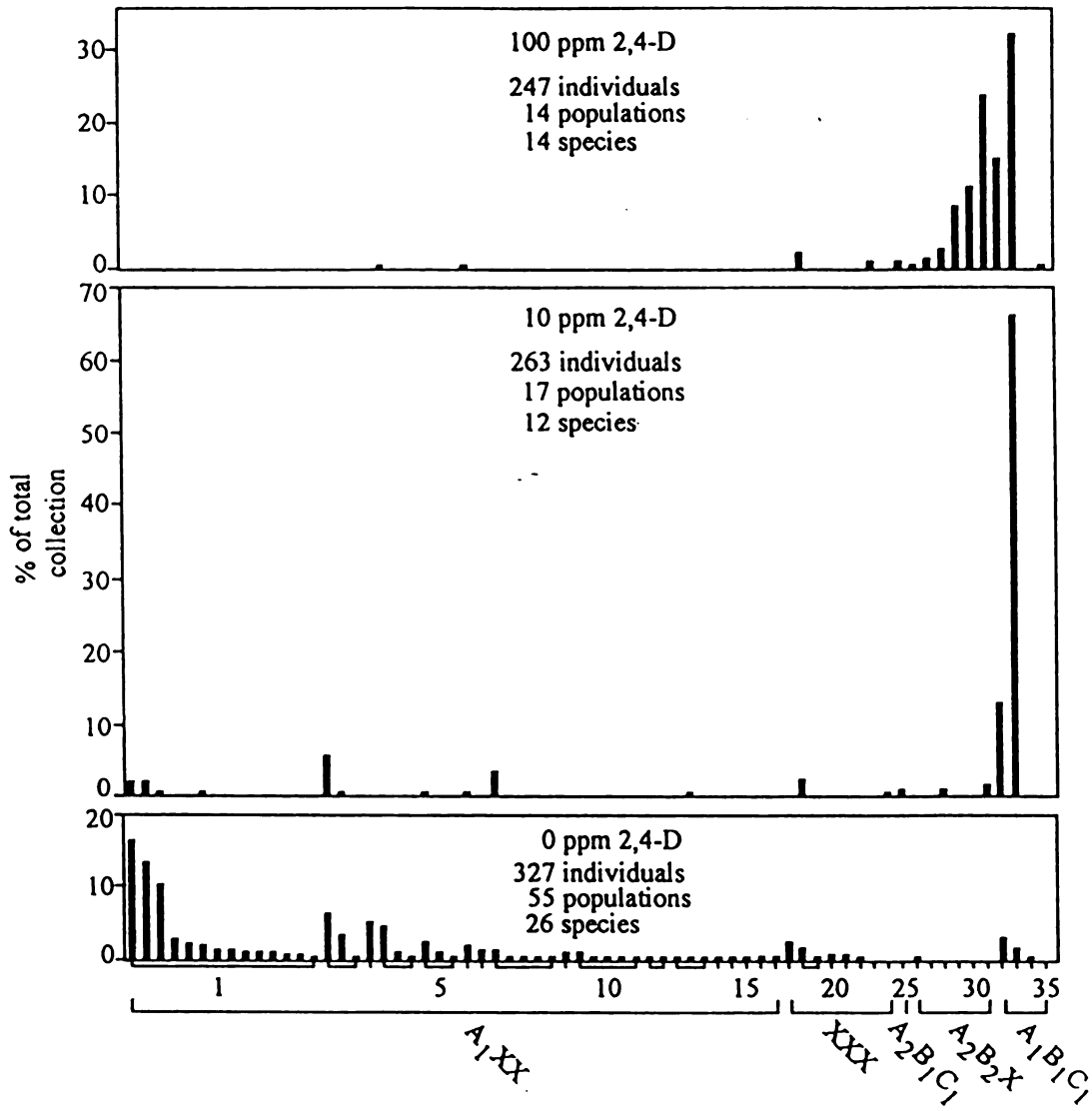

Figure 5. Frequency distribution of 2,4-D$^+$ populations among isolates collected from treated and untreated microcosms. Bars in graphs represent 68 different populations identified by REP-PCR fingerprints of individual isolates collected from treated and untreated microcosms. These populations represent 35 different species indicated by ticks (or brackets) below the x axis. Species were identified by restriction analysis of 16S rDNA from each population. The lowermost brackets identify populations which exhibit similar hybridization patterns with *tfdA*, *B*, and *C* genes (A_1, B_1, and C_1 respectively) cloned from *A. eutrophus* (pJP4) and *tfdA* and *B* (A_2 and B_2) from *Burkholderia* sp. strain RASC. Lack of high stringency hybridization to any cloned allele is indicated by an X.

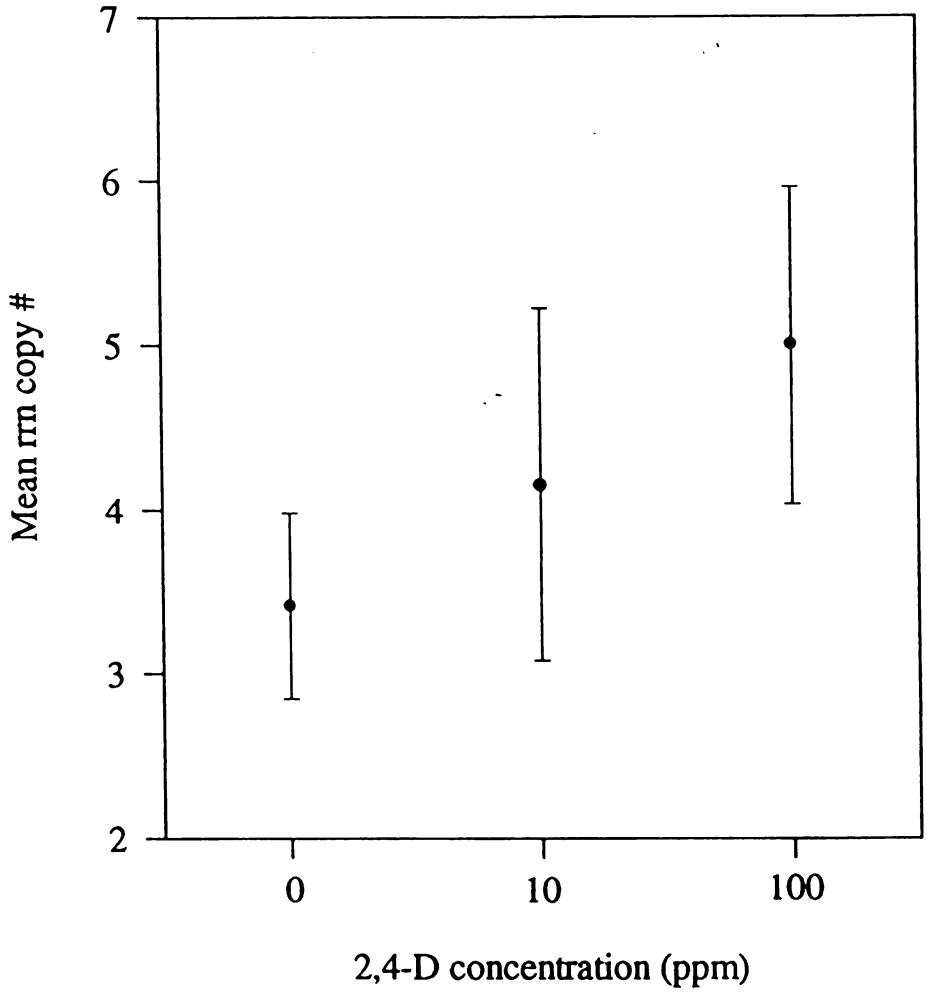

Figure 7. Mean 16S rRNA gene copy number species obtained from treated and untreated microcosms. Error bars are 95% confidence intervals.

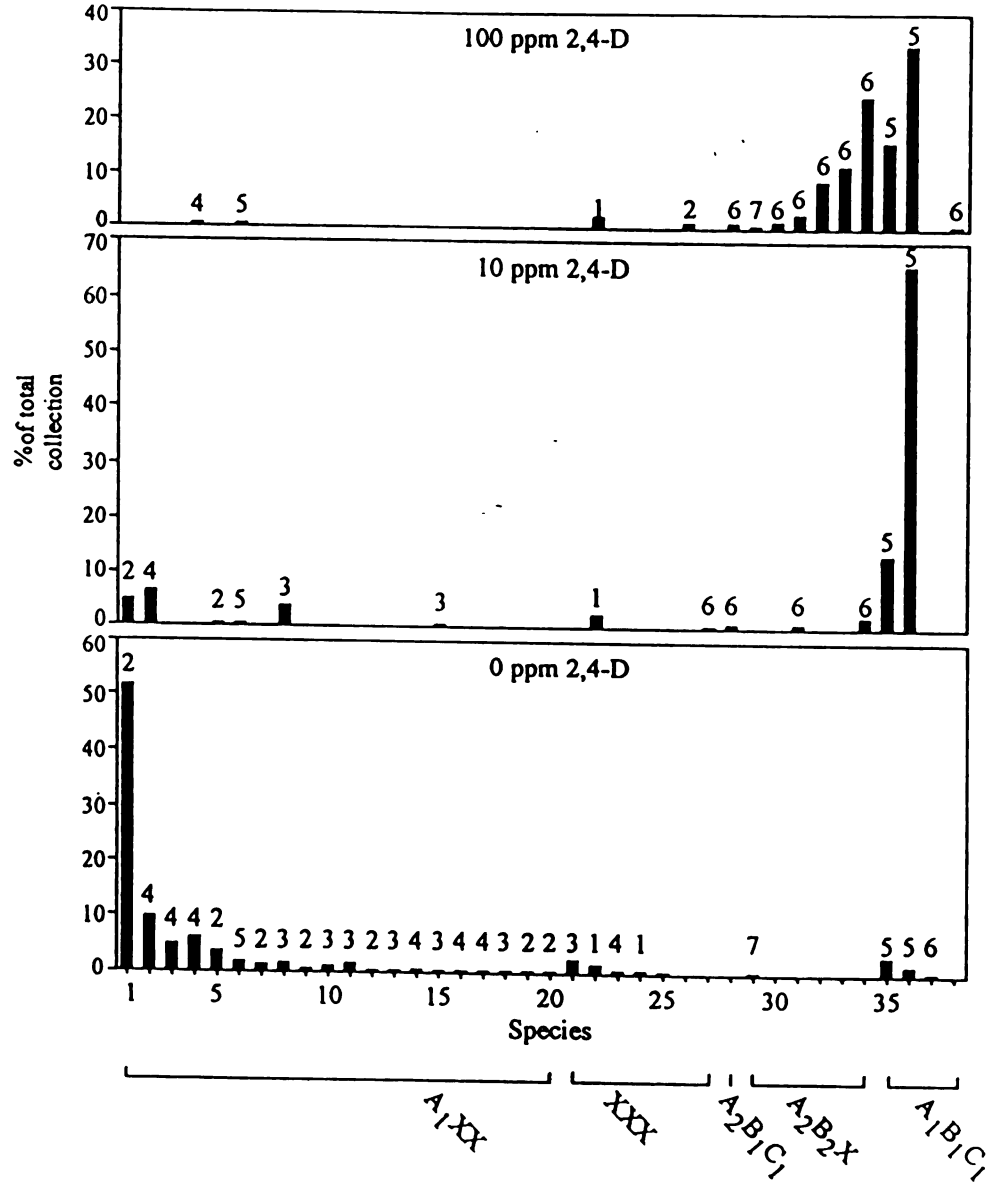

Figure 6. 16S rRNA gene copy number among 2,4-D+ species detected among isolates from treated and untreated microcosms. Each bar represents a different species. Numbers above bars indicate gene copy number.

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CHAPTER 5

Concluding Remarks

The structure of soil microbial communities is poorly understood. Estimates of species diversity obtained by DNA-DNA reassociation kinetics suggest that some soils contain as many as 10,000 bacterial species per gram [23]. The abundance of each species is determined by its reproductive success. Since bacterial reproduction in soil is frequently limited by the availability of nutrients [21, 25], the ability to compete for limiting nutrients can strongly influence the reproductive success of individual species. Thus, competition may be one of the key processes shaping the structure of microbial communities. There have been few attempts to evaluate the diversity of indigenous populations in soil that can compete for the same limiting nutrient [12] or to assess the outcome of competition among indigenous populations. In the preceding chapters, results of experiments to assess the diversity of 2,4-D degrading populations in soil before and after selection were described. Four significant findings were identified.

First, it was demonstrated that enrichment of 2,4-D degrading populations in liquid batch cultures imposes a significant bias on the types of 2,4-D genes that are recovered from environmental samples. It has long been known that organisms with rapid growth rates are typically obtained by enrichment culture techniques [8]. However, the results presented in Chapter 2 provide the first evidence illustrating the effect of selection for rapid growth rates on the diversity of catabolic genes recovered from the environment. Populations enriched from a single gram of soil possessed, almost exclusively, 2,4-D genes that were highly similar, if not identical, to the 2,4-D genes encoded on plasmid pJP4. Since enrichment has been the primary method used by previous researchers for obtaining 2,4-D degrading strains from nature, it is perhaps not surprising that genes similar or identical to the genes encoded on plasmid pJP4 have been the most frequently encountered [2, 4-6, 14]. The results presented in Chapter 2 provide another demonstration that the genetic variation observed in nature is strongly influenced by the techniques used to assess genetic diversity.

Second, a high degree of genetic diversity was detected within the dominant 2,4-D degrading species obtained from untreated soil. A relatively small number of studies have examined the genetic variation which exists within species obtained from a single environment [1, 9, 19, 20, 22, 26-28]. The aim of such studies is to determine the relative contribution of different mutational mechanisms to genetic variation in natural populations. Most studies of local populations have employed multilocus enzyme electrophoresis (MLEE) to evaluate the allelic diversity of housekeeping genes and the extent of sexual recomination between strains [1, 9, 19, 20, 26, 27]. The analysis of *V. paradoxus* strains (described in chapter 3), differed from previous studies in that genome organization among *V. paradoxus* clones was assessed. Comparison of REP-PCR fingerprints, genomic RFLPs, and plasmid RFLPs provided evidence suggesting that most of the *V. paradoxus* strains in soil were genetically distinct. However, the mutational mechanisms which contributed most to the observed variation have not been identified. It's possible that most of the variation was simply the result of divergence through the accumulation of point mutations. However, if the REP-PCR reactions are highly specific such that the PCR primers anneal only to REP sequences and not to arbitrary sequences during PCR amplification, then recombination is a more plausible explanation for most of the variation observed in REP-PCR patterns. The extent of variation observed among REP-PCR patterns of *V. paradoxus* populations would then suggest that different *V. paradoxus* populations diverged as a result of extensive recombination events which have altered the linear distance between loci if not the linear order.

Third, the number of 2,4-D degrading populations in soil obtained from a single location was unexpectedly large. The 66 populations identified in Chapter 4 represented at least 35 different species. This appears to be the first reported attempt to comprehensively evaluate the diversity of populations in soil competing for a single limiting nutrient. Since

the soil had no known exposure to 2,4-D, it is unknown why so many 2,4-D degrading species were detected. Due to the occurence of 2,4-D genes on plasmids and the fact that nearly identical 2,4-D genes have been found in different species, it has been suggested that much of the genetic diversity underlying the 2,4-D phenotype has arisen as a result of horizontal gene transfer [6, 15, 16]. Similar evidence for horizontal gene transfer was observed in the present study. However, the relative contributions of immigration versus local horizontal gene transfer are unknown.

Fourth, two genetic factors were identified which were associated with the competitive fitness of 2,4-D degrading populations in soil in response to a pulse of 2,4-D (Chapter 4). Only a small number of populations increased in abundance to high frequencies following addition of 2,4-D, and the dominant populations possessed 2,4-D genes highly similar, if not identical, to the 2,4-D genes encoded on plasmid pJP4 despite the fact that this catabolic group was initially rare. The copy number of *rrn* genes was also significantly correlated with competitive fitness. It is unknown whether the association of these two genetic factors with competitive fitness is causal or merely correlative as a result of genetic linkage or phylogenetic association with other factors which determine competitive fitness. However, it is reasonable to suspect that a causal relationship may exist. The hypothesis that different 2,4-D gene groups encode catabolic pathways which differ in catalytic efficiency is consistent with traditional views of competitive fitness which have emphasized the importance of substrate uptake and transformation kinetics [7, 10, 13, 18, 24]. Furthermore, evidence has been provided recently indicating that lag time may also be an important determinant of fitness [11] and that *rrn* copy number may significantly affect lag time [3]. Since the competitive fitness of bacterial populations in natural environments has previously been described only in terms of phenotypic characters such as μ_{max}, K_s, and lag time, presumptive evidence for potential genetic determinants represents a significant contribution to the field.

Numerous questions remain to be answered about the mechanisms which create genetic diversity in soil microbial communities. The observation that gene exchange and recombination appear to have contributed substantially to the diversity of genetic pathways encoding 2,4-D degradation [6] and that the extent of gene exchange and recombination is reasonably high in soil bacterial populations [9, 17, 20] suggest that recombination may profoundly contribute to the diversification and adaptation of soil microbial communities. The contribution of intragenomic recombination relative to intergenomic recombination in bacterial populations has not been assessed. Documenting the contribution of recombination to genetic variation among the *V. paradoxus* strains described in Chapter 3 requires furthur work. The extent of sexual recombination could be easily measured by use of MLEE. However, the contribution of recombination to the variation observed in genome organization requires sequence comparisons of homologous DNA fragments which yeild different RFLP patterns in different strains or which yeild REP-PCR amplification products in one strain but fail to yeild the same products in a second strain. Alternatively, the specificity of the REP-PCR reactions could be assessed with a set of templates which have predetermined degrees of divergence from the REP consensus sequence. Based on the results of such experiments, REP-PCR or related techniques might be established as a tool for assessing the contribution of inter- and intragenomic recombination events to genetic variation within populations.

The mechanisms which maintain diversity within microbial communities also require furthur investigation. Analysis of the 2,4-D$^+$ guild in untreated soil demonstrated a high degree of species diversity, population diversity within species, and diversity within populations. Extrapolation of results from chemostat experiments suggests that the large number of competitors would be maintained as a result of the physical, chemical, and biological complexity of soil. However, addition of 2,4-D resulted in competition dynamics similar to the dynamics observed in simple batch cultures. These dynamics

suggest that only a fraction of the populations present in soil are highly adapted to exploitation of pulses of substrate. It seems likely that most carbon sources encountered by soil bacteria are delivered in pulses as nutrients are episodically flushed through the soil column by percolating water. It is not apparent what conditions maintain and select for less opportunistic bacterial populations are unclear.

The genetic basis of competitive fitness in soil also requires additional investigation. A direct link between specific 2,4-D catabolic genes, *rrn* copy number, and the competitive fitness of 2,4-D degrading populations has not been established. Demonstrating a causal link requires highly controlled genetic experiments in which the specific 2,4-D genes or *rrn* copy number is varied in an isogenic background. Such experiments may reveal that a competitive fitness among bacteria is broadly determined by a small set of genetic factors. This outcome would provide a genetic basis for understanding patterns of r- and K-selection among bacterial populations.

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