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A MOLECULAR APPROACH TO UNDERSTANDING THE GENETIC RESPONSE OF 2,4-D DEGRADING MICROBIAL POPULATIONS IN SOIL UNDER SELECTION

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ABSTRACT

A MOLECULAR APPROACH TO UNDERSTANDING THE GENETIC RESPONSE OF 2,4-D DEGRADING MICROBIAL POPULATIONS IN SOIL UNDER SELECTION

By

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The diversity of 2,4-dichlorophenoxyacetic acid (2,4-D) degrading bacteria, their population ecology and genetic changes under selective pressure were analyzed at the DNA level. A total of 47 predominant 2,4-D degrading bacteria were isolated from Kellogg Biological Station (KBS) soils. The isolates were diverse in fatty acid methyl ester profiles, REP fingerprint patterns, substrate utilization patterns and hybridization patterns using DNA probes. The *tfdA* gene and a 6.5 kb probe (cloned from a 2,4-D$^+$ *Pseudomonas paucimobilis*) hybridized to the DNA of 70 % of the isolates which predominated in soil repeatedly treated with 2,4-D. These two DNA probes were useful in studying the population dynamics and genetic alterations in dominant 2,4-D degrading bacteria in soil under 2,4-D selection. Bacterial populations with homology to one or the other of these probes rapidly increased in soil treated with 2,4-D and changes in banding patterns (the position of hybridization signal) were observed in Southern blot analyses with total soil bacterial DNA. These data suggested that population changes or possibly genetic rearrangement in 2,4-D degrading microbial populations occurred in this soil. Strains responsible for the hybridization signals observed in total soil bacterial DNA were identified by comparing

DNA fingerprints of the isolates to the pattern observed in total soil bacterial DNA.

Four different 2,4-D degrading strains (*Pseudomonas cepacia* DBO1/pJP4 ; and three KBS soil isolates identified as *Pseudomonas pseudomallei*, *Pseudomonas paucimobilis*, and *Pseudomonas pickettii*) were inoculated into a native Kansas prairie soil to study competition among and between the indigenous and the inoculated 2,4-D degrading bacteria under 2,4-D selection. The hybridization patterns from total soil bacterial DNA revealed that *P. cepacia* DBO1/pJP4 outcompeted the other added strains and indigenous 2,4-D degrading bacteria. *P. paucimobilis* was also detected as a secondary dominant, but *P. pseudomallei* and *P. pickettii* were not. Broth culture competition experiments were also performed. The results suggested that different factors affected competitive ability in soil vs broth and that the 2,4-D degradative plasmids were important determinants of competitiveness.

Among the 47 isolates, strain 2811P, identified as *Alcaligenes paradoxus*, was observed to have a genetically flexible plasmid. The plasmid appeared to integrate in its entirety into the host chromosome and was excised either precisely or imprecisely.

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Literature Review

Introduction

Large quantities of synthetic organic chemicals have been introduced to the environment. Among them, 2,4-dichlorophenoxyacetic acid (2,4-D) has received widespread use as a herbicide during the past several decades, because it is selectively toxic to broadleaf plants but not to monocotyledonous plants including cereal crops (14, 41).

However, apart from its desired toxic effects on the weeds, a herbicide could have harmful effects on nontarget organisms. First, it could influence soil microflora and microbial processes that play a vital role in maintaining soil fertility. Second, the persistent herbicide could damage the sensitive crops, thus reducing the yield. Third, the runoff and leaching of herbicide applied to field could contaminate rivers, lakes, and groundwater, which is a potential danger to animals and to man.

Therefore, it is important to study the behaviour of herbicides in soil, their degradation by microorganisms, adaptation behaviour and population ecology of the responsive microorganisms, and the degradative pathways and genes. The information obtained from these studies may be also useful in understanding the degradation mechanisms of various other haloaromatic compounds currently of interest.

Microbial adaptation to 2,4-D

2,4-D applied to soil could affect the indigenous microbial populations in two ways : either it is toxic to them or it serves as additional growth substrate for microorganisms able to degrade it. It has been reported that while this chemical significantly stimulated fungal populations at high

application rate (30 kg/ha), it could significantly reduce the bacterial populations (14, 49). However, 2,4-D had no significant effect on soil microflora at agronomic rates (0.3 - 5 kg/ha) (61). The breakdown of 2,4-D in soil was demonstrated by radioactive release as $^{14}CO_2$ from ^{14}C-labelled 2,4-D (36, 48, 76). Microbial involvement in 2,4-D degradation was observed from the comparison of 2,4-D persistence in autoclaved and normal soils (15) and also by using metabolic poison such as sodium azide that inhibits cytochrome oxidase (7).

Lag period. Substantial 2,4-D degradation usually occurs after a lag time of 0 to 4 weeks (6) which was shown to be dependent on the concentration of 2,4-D added (27, 43). The lag period appears to be the time required for the development of substantial soil microflora able to degrade 2,4-D (5). Two mechanisms, induction and mutation, were proposed to explain the adaptation phenomenon during the lag period. The induction mechanism suggests that the responsive soil microbial populations which already have the degradative genes require time to induce enzymes before degradation begins, while the mutation mechanism postulates that the ability to degarde 2,4-D arises through a mutation during the lag period (5, 6, 8, 71). Alternatively, it was proposed that the adaptation period in soil is dependent on the population level of the 2,4-D degrading bacteria, the concentration of 2,4-D (2, 43), and the transfer rates of 2,4-D degradative plasmids (73). Although induction was suggested to be the most siginificant mechanism (41), the origin of the effective population is not yet clear.

Enrichment of soil. Once the active microbial populations build up to a level where appreciable degradation occurs, degradation of 2,4-D

subsequently added occurs more rapidly without a noticeable lag period (7, 46, 56, 74). It has been reported that the 2,4-D degradation rate of the second treatment was three- to five-fold higher in pretreated soils than in unadapted soils and repeated annual applications of 2,4-D resulted in a reduction in degradation time from 10 to 4 weeks (27). However, this enrichment effect was not observed in soil treated with 2,4-D at low application rates (27, 71), suggesting that low amount of 2,4-D may be metabolized by pre-existing microbial enzymes before the sufficient development of the active populations (29). It was also reported that 2,4-D was degraded faster in pretreated soil than in unadapted soil one year after the first application, suggesting that the enrichment effect persists for a long period in the absence of the herbicide (5, 6, 27, 45). Since most of the soil microflora live under starvation conditions (9), the loss of their adaptation caused by alternative metabolism would not be extreme (9, 27). Alternatively, a trace of 2,4-D (58) or naturally occurring, structurally related compound was suggested to maintain the active population for a long time (35).

Isolation of 2,4-D degrading bacteria

It is necessary to amplify the population level of 2,4-D degrading bacteria before attempting any isolations. For this purpose, a high amount of 2,4-D was added to the original environmental samples and whenever the 2,4-D disappears, successive 2,4-D was added (10, 34, 74) or an aliquot of samples was inoculated into 2,4-D mineral medium (3) for further enrichment. Then 2,4-D degrading bacteria were isolated from the enriched samples by streaking on 2,4-D minimum medium. With this enrichment culture technique, some colonies able to degrade 2,4-D were readily obtained (34),

while others required 17 days before they produced enough biomass on plates (74).

Identification of 2,4-D degrading bacteria

A variety of different species of bacteria able to degrade 2,4-D have been isolated and identified. The 2,4-D degrading bacteria isolated from soils in 1950s and 1960s were identified mainly by their colony characteristics, cell morphology, and biochemical characteristics. On the other hand, the bacteria isolated from soils and water samples in 1980s were identified by their growth property, pigment formation, flagella arrangement, and G+C content of chromosomal DNA. 2,4-D degrading microorganisms include *Achromobacter* sp. (10), *Alcaligenes eutrophus* JMP134 (18), *Alcaligenes paradoxus* JMP116 (25), *Arthrobacter* sp. (42), *Arthrobacter globiformis* (4), *Aspergillus niger* (24), *Corynebacterium* sp. (56), *Flavobacterium aquatile* (34), *Flavobacterium peregrinum* (65), *Mycoplana* sp. (74), *Nocardia coeliaca* (33), *Nocardia* sp. (57), *Pseudomonas* sp. (22), *Pseudomonas* sp. HV3 (35), *Pseudomonas* sp. NCIB 9340 (22), and *Streptomyces viridochromogenes* (13). Although the identification of the 1950s and 1960s exhibited more diverse and uncommon genera of 2,4-D degrading bacteria than those of the 1980s, this could be attributed to the relative imprecision of the earlier identification methods.

Enumeration of 2,4-D degrading bacteria

The estimation of 2,4-D degrading populations has been reported by several researchers. A bromocresol purple indicator liquid medium and an eosin-

methylene blue agar medium were developed to detect and isolate microorganisms able to degrade 2,4-D (39). The production of hydrochloric acid resulting from the oxidation of 2,4-D by organisms either changes the color of the medium from purple to yellow or forms red colonies on the eosin-methylene blue agar. This technique was further improved to estimate 2,4-D degrading bacteria by a most probable number (MPN) method (38). The MPN determination using indicator is more convenient and rapid than the MPN method monitoring the disappearance of the ultraviolet absorption spectrum of 2,4-D (57). It was also reported that the color change resulting from the reaction between chlorine ions released from 2,4-D degradation and added reagents and the release of $^{14}CO_2$ from MPN medium containing labelled 2,4-D could be utilized in enumerating the 2,4-D degrading microorganisms by MPN methods (26).

Soils not previously treated with 2,4-D usually contained fewer 2,4-D degrading microorganisms than those in the corresponding soils treated with 2,4-D, the latter population densities range from 1.2 (38) to 8.8×10^6/g soil (36). Numbers of 2,4-D-co-metabolizing microorganisms in soils not amended with 2,4-D ranged from 1.7×10^4 (26) to 3.0×10^5 (27), but their populations in the corresponding treated soils were slightly to markedly increased. The population of 2,4-D degrading microorganisms in soil was maintained at higher levels than that of the 2,4-D-co-metabolizing organisms with relatively high concentrations of 2,4-D (> 5 mg/l), but the former population was not much higher than that of the latter when the concentration was 1.2 μg/l in the MPN medium (26).

2,4-D degradative plasmids

It was suggested that 2,4-D degradative gene was evolved from plasmid borne phenoxyacetate degradative gene through gene duplication and subsequent mutation (53).

In spite of the extensive use of 2,4-D in agriculture, it seldom persists in the environments because of the widespread occurrance of a variety of soil microflora capable of degrading it. The involvement of a conjugative plasmid in the 2,4-D degradation was first reported in *Pseudomonas* sp. (52) and in *Alcaligenes paradoxus* (25), providing a mechanism for the widespread distribution of those genes among microbial populations. Since then, six other 2,4-D degradative plasmids were described from *Alcaligenes* species isolated from soil (18). Among them, one plasmid from *Alcaligenes paradoxus*, pJP4, has been well characterized physically and genetically by using cloning, transposon mutagenesis, and restriction endonuclease analysis (16). The plasmid belongs to the P1 incompatibility group, has broad host range, encodes resistance to mercuric chloride, and degradation of 2,4-D, 2-methyl-4-chlorophenoxyacetic acid (MCPA), and 3-chlorobenzoate (16).

2,4-D degradative pathway

Most of the research on the 2,4-D degradative pathway has been performed with aerobic cultures and not much attention has been given to possible anaerobic degradation, since ring cleavage following aerobic hydroxylation seems to be the major mechanism of 2,4-D dgradation.

Several different 2,4-D degradative pathways were suggested in studies with *Pseudomonas* species (23), *Achromobacter* species (65), and *Nocardia*

species (5) prior to 1967, but the pathways were based solely on indirect evidences and were not well characterized. Since then, details of the pathway was revealed by the extensive studies with growing or resting whole cells, enzyme preparations, transposon mutagenesis, and gene cloning in *Arthrobacter* sp. (11, 12, 21, 40, 42, 70), *Pseudomonas* sp. (22), and *Alcaligenes eutrophus* JMP134 (17).

Induction of 2,4-D enzymes. 2,4-D degrading enzymes of *Flavobacterium peregrinum* were induced when grown on 2,4-D, MCPA, or 2-chloro-4-methylphenoxyacetic acid, while MCPA was not degraded by this 2,4-D degrading enzyme system (65). In the *Achromobacter*, the 2,4-D-degrading enzyme system was induced by 2,4-D and 2,4-dichlorophenol (65). In *Alcaligenes eutrophus* JMP134, the 2,4-D degradative pathway was induced in the presence of 2,4-D or 3-chlorobenzoate plus Casamino acids, but Casamino acids alone could not induce the 2,4-D enzymes (31).

Metabolic pathway of *Arthrobacter* sp. and *Alcaligenes eutrophus* JMP134. The pathway of 2,4-D degradation observed from these bacteria involves the removal of the acetic acid side chain to yield 2,4-dichlorophenol, followed by ortho hydroxylation of the phenol to produce 3,5-dichlorocatechol. The catechol is then converted to a muconic acid by ortho cleavage of the aromatic ring. The muconic acid is lactonized to form 2-chlorodiene-lactone, during which one of the chlorines is lost from the aromatic ring. The chlorodiene-lactone is delactonized to produce maleylacetic acid which is subsequently metabolized to succinic acid in *Arthrobacter* sp.

Metabolic pathway of *Pseudomonas* sp. The pathway observed from two *Pseudomonas* sp. by Evans et. al. (22) was similar to that for the *Arthrobacter* sp. However, other related compounds, such as 2-chlorophenol and 2,4-dichloro-6-hydroxyphenoxyacetate, were detected during growth on 2,4-D medium. Since these organisms were capable of oxidizing a variety of mono- and dichlorophenols and phenoxyacetates, different 2,4-D degradative pathways were also suggested to operate in these bacteria.

2,4-D degradative genes. 2,4-D degradative genes, such as *tfdA*, *tfdB*, *tfdC*, *tfdD*, *tfdE*, and *tfdF* were identified, localized, and cloned in *Alcaligenes eutrophus* JMP134 (17, 68). Four genes, *tfdB* to *tfdE*, encode *2,4-dichlorophenol hydroxylase*, *dichlorocatechol 1,2 -dioxygenase*, *chloromuconate cycloisomerase*, and *chlorodienelactone hydrolase*, respectively, but the function of *tfdF* gene is not yet known (17). These five genes were observed to constitute a cluster on plasmid pJP4, while the *tfdA* gene, encoding 2,4-D monooxygenase, has been localized to a region 13 kb away from the cluster. Recently a second 2,4-D monooxygenase, designated as *tfdAII*, has been observed on a different region of pJP4 (54). The *tfdAII* gene was clustered with the five genes *tfdBCDEF* on plasmid pJP4 and substantial difference was observed between the *tfdA* gene and the *tfdAII* gene from DNA-DNA hybridization experiments. It was also suggested that two different genes encoding chlorocatechol 1,2-dioxygenase were present on plasmid pJP4 (28).

Environmental factors affecting 2,4-D degradation

Soil moisture and temperature. Soil moisture and temperature were observed to affect significantly the microbial activity. The optimum moisture tension and temperature was 0.1 bar and 27°C, respectively, in a sandy loam soil (51). At moisture above the optimum (37) and at temperatures above the optimum (51), 2,4-D was degraded less rapidly. In broth cultures, 2,4-D degradation was faster at 25°C than at warmer temperatures (72) and in river water studies, the optimum temperature range was 21 - 25°C (44).

pH. The optimum pH range for 2,4-D degradation in broth culture was 6.2 - 6.9 (72). The 2,4-D degradation in soil was slower in the acid range (pH 3.8 - 5.6) than in the neutral range (pH 6.6 to 7.4) (67). In the neutral pH range, more 2,4-D is thought to be present as its dissociated form which could be less toxic. 2,4-D is degraded mainly by fungi in acid soils (41), while its degradation in neutral soils is catalyzed mainly by bacteria and actinomycetes (1).

Oxygen. Since 2,4-D degradation is mainly by an aerobic process, oxygen concentration is suggested to be an important factor. 2,4-D degradation rate was much lower in flooded soils than that in non-flooded soils (57, 77) and an other study shows that the degradation rate generally increased with increasing oxygen supplies (59).

Nutrient. While the addition of glucose plus urea to soil (48) was found to have no significant effect on the 2,4-D degradation rate, most of the studies (19, 20, 44, 48) suggested that the organic amendment or the addition of

fertilizer could stimulate the degradation rate. It was suggested that the added compounds could increase the corresponding populations, thus enhancing the degradation rate (55).

Soil type and adsorption. The adsorption of 2,4-D and microorganisms to soil particles was found to be controlled by the soil organic content and the sorbed 2,4-D was completely protected from biological degradation (47). Clay was observed to have low adsorptive capacity for 2,4-D (30). It was reported that 2,4-D was degraded more slowly in a fine sand, which had a lower pH (5.2) and a higher organic content, than in a sandy clay loam (63, 69). The mobility of 2,4-D is relatively high (32) and it leaches readily in sandy and low organic soils (75), thus being not metabolized by microorganisms.

Rate of application. A high concentration of 2,4-D (500 μg/g soil) was observed to be toxic to the soil microflora in a loam and a sandy loam, causing a long lag prior to substantial degradation (67). Lag time was proportionally increased with increasing 2,4-D concentration (50) and 2,4-D degradation rate was reduced due to a high 2,4-D concentration (49). However, it was suggested that a different 2,4-D application rate (0.33 to 330 μg/g) did not influence co-metabolizing soil microflora as long as the 2,4-D concentration was not toxic and the co-substrate was not depleted (27).

2,4-D formulations. The amine or ester formulations of 2,4-D were shown to rapidly dissociate to the anion in soils (62, 64, 66). The formulated dimethylamine salt was degraded more rapidly, probably due to the presence of additional nutrients in this salt, than that for technical grade at high application rates (49).

Conclusion

The herbicide 2,4-D is readily degraded by soil microorganisms. The lag time is usually observed in the first exposure, but it is not noticeable from subsequent doses. The adaptation effect is maintained for a long time in soil after disappearance of the herbicide. The number of 2,4-D degrading microorganisms in soil previously treated with 2,4-D is generally higher than that in soil not amended with 2,4-D. A variety of different bacterial species involve in the 2,4-D degradation and the degradative plasmids and genes have been isolated and described.

However, there are still many areas to be investigated regarding 2,4-D degradation. Although various different bacterial species able to degrade 2,4-D have been independently isolated and characterized, few studies have contributed to the systematic study on their diversity and not much information is available on the ecological significance and dynamics of an individual population in an ecosystem. The enumeration of 2,4-D degrading microorganisms by the MPN method has provided information on the population, but it can not furnish information on their competitive interactions and their population and genetic changes in the environments under the 2,4-D selective condition. Although numerous 2,4-D degradative plasmids have been isolated and identified, little attention has been given to their diversity and ecological contributions and further research is required to reveal the possibly diverse pathways of 2,4-D degradation. Finally, there has been little effort to investigate physiologic and genetic aspects of the adaptive power of 2,4-D degrading bacteria to grow more efficiently under 2,4-D selection.

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Chapter Two

Studies on Diversity of 2,4-D Degrading Bacteria Isolated from Kellogg Biological Station Soils.

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ABSTRACT

A total of 47 predominant 2,4-D degrading bacteria were isolated at different times from 1989 to 1992 from agricultural soils either not treated with 2,4-D or treated with 2,4-D at different rates. The isolates were analyzed by fatty acid methyl ester (FAME) profiles, patterns of the polymerase chain reaction (PCR) amplified repetitive extragenic palindromic (REP) sequences, and hybridization patterns with *tfd* genes from the plasmid pJP4 and a 6.5 kb probe subcloned from a 2,4-D degrading isolate (*Pseudomonas paucimobilis*). Substrate utilization abilities of selected isolates among the strains identified as the same species by FAME analysis were evaluated by using 2,4-D related compounds as sole carbon sources. FAME analysis revealed 12 FAME groups that cluster with Euclidian Distance of less than 10. 55.3 % of the isolates could be identified to the species level ; the remainder could not be identified due to their poor match to any profile within the MIDI (Microbial ID, Inc.) library and to lack of growth on laboratory medium. The REP PCR patterns of the isolates, identified as different species or genera by FAME analysis, were quite distinct from one another. Hybridization analysis revealed four hybridization groups. Group I which showed sequence homology with the four *tfd* genes clustered within a Euclidian Distance of 31.5 by FAME analysis, group II which had homology only with the *tfdA* gene clustered at 11.8, group III which had homology only with the 6.5 kb probe clustered at 6.4, and group IV (all others) which showed no homology to any of the available probes clustered at 31.5. Group I, II, and IV strains exhibited fairly diverse REP PCR patterns within groups, while group III strains yielded similar patterns. Strains of the same hybridization groups exhibited general similarity in substrate utilization abilities. Strains

of groups I and III were preferably detected from soils treated with higher rates of 2,4-D and were encountered more frequently with an increase in number of 2,4-D treatments.

INTRODUCTION

Bacterial diversity in a natural ecosystem reveals the structure of a microbial community arising from the complex interactions between microorganisms and their biotic and abiotic environments. It has been of general interest to microbial ecologists, in part because the composition of the microbial community plays an important role in the effective bioremediation and recyling of a variety of organic and inorganic contaminants.

2,4-D seldom persists in the environment due to the widespread occurrance of a variety of soil microflora capable of its degradation (2, 32). The degradation of 2,4-D by microorganisms has received substantial attention not only because it has been extensively used as a herbicide, but also because it could serve as a model compound in understanding the biodegradation mechanism of other haloaromatic compounds in general use. Numerous different bacterial genera, such as *Achromobacter*, *Alcaligenes*, *Arthrobacter*, *Corynebacterium*, *Flavobacterium*, *Pseudomonas*, and *Streptomyces* have been reported to be involved in 2,4-D degradation (3, 6, 12, 13, 18, 25, 30). Populations of microorganisms able to degrade 2,4-D were estimated by the most probable number method in soils (14, 22) and the 2,4-D degradative plasmids were isolated and biophysically and genetically characterized (8, 10).

However, little is known about the diversity of 2,4-D degrading bacteria isolated from environmental samples. Many researchers have isolated

different species of 2,4-D degrading bacteria in 1950s and 1960s, and the bacteria were identified based on their morphological and cultural characteristics. Recently, aquatic microorganisms able to degrade 2,4-D were isolated from a variety of water samples and were phenotypically and genetically characterized (1). But this research provided only fragmentary information on the diversity of 2,4-D degrading bacteria and the isolation procedure using original environmental samples as inoculum for an enrichment culture with high amount of 2,4-D could select for only a certain type of 2,4-D degrading bacteria. Moreover, the inconsistency and heterogeneity of strain sources and identification make it difficult to get consistent results on diversity and on ecological significance of the isolates in an ecosystem.

In this work we studied the diversity of 2,4-D degrading bacteria isolated at different times from 1989 to 1992 from agriculture soils not treated or treated with 2,4-D at different rates by analyzing FAME profiles, REP PCR patterns obtained from the colonies, hybridization patterns with DNA probes, and capabilities to metabolize 2,4-D related compounds. The results were compared for analysis of relationships between the different grouping methods. Finally, we investigated the influences of different 2,4-D application rates and repeated treatments on the distribution of the hybridization groups of the isolates in field.

MATERIALS AND METHODS

Media and culture conditions. All isolates were maintained on MMO mineral medium (33) plus 500 ppm (μg/ml) of 2,4-D. Peptone-tryptone-yeast extract-glucose (PTYG) medium containing 0.25 g of peptone (Difco

Laboratories, Detroit, MI), 0.25 g of tryptone (Difco), 0.5 g of yeast extract (Difco), 0.5 g of glucose, 0.03 g of magnesium sulfate, and 0.003 g of calcium chloride was used for strain purification and colony production for REP PCR.

Isolation of bacterial strains. Agricultural soil samples from duplicate plots that have not been treated with 2,4-D or have been regularly treated with 2,4-D at different rates (1, 10, and 100 μg/g soil) from 1988 to 1992 (Table 1) were taken from the Long-Term Ecological Research (LTER) site of Kellogg Biological Station (KBS, Hickory Corners, MI), sifted through a 2-mm sieve, and kept at 4°C prior to use. A 10-g soil sample of each plot was homogenized with 95 ml of sterilized, cold 0.85 % saline by shaking at 200 rpm for 20 min in a New Brunswick G24 environmental incubator shaker (New Brunswick Scientific Co., New Brunswick, NJ). Samples (0.1 ml) of appropriate 10-fold dilutions were inoculated into MPN (most probable number) tubes containing 3 ml of 2,4-D mineral medium (MMO plus 500 ppm of 2,4-D). The tubes were incubated at 30°C for 3 weeks and then the degradation of 2,4-D was analyzed by using an Hewlett Packard series 1050 HPLC equipped with Lichrosorb RP-18 column (Anspec Co., Ann Arbor, MI). The culture of the highest dilution showing 2,4-D degradation was enriched through two additional transfers into fresh medium. The enriched culture was streaked onto 2,4-D agar medium (MMO + 500 ppm of 2,4-D + 0.1 % of Casamino acids + 1.5 % agar) and then single colonies were tested for 2,4-D degradation in a fresh 2,4-D mineral medium before it was further purified by streaking onto PTYG plates. Some cultures failed to produce single colonies able to degrade 2,4-D. Altogether 47 strains were isolated from the eight plots (duplicate control plots not treated with 2,4-D and duplicate plots treated with 1, 10, or 100 ppm 2,4-D) from 1989 to 1992 (Table 1). These isolates were preserved by freezing (-70°C) in sterile 15 % glycerol.

Chemicals. Analytical grade 2,4-D, phenoxyacetic acid (PA), and 2-methyl-4-chlorophenoxyacetic acid (MCPA) were obtained from Sigma Chemical Co. (St. Louis, MO) and 2-chlorophenoxyacetic acid (2-CPA), 4-chlorophenoxyacetic acid (4-CPA), 3-chlorobenzoic acid (3-CB), and 4-chlorobenzoic acid (4-CB) were obtained from Aldrich Chemical Co. (Milwaukee, WI).

FAME analysis. The isolates were cultured on tryptic soy agar medium (15) at 28°C for 48-72 h and then cells were harvested from the plate by scraping with a sterile glass loop and used for FAME analysis. Saponification, methylation and extraction were performed using the procedure described in MIDI (Microbial Identification, Inc.) manual (31). The extracts were assayed by using a Perkin Elmer 3920 gas-liquid chromatography equipped with a flame ionization detector. Cluster analysis was carried out using an inhouse cluster program and the MIDI software.

Colony REP PCR. This method was performed as described by deBruijin (7). Each isolate was grown on PTYG plate for 24-48 h and then small amount of cells was resuspended in 25 µl PCR mixture containing 50 pmol each of primers REP 1 and REP 2 (7), 1.25 mM deoxynucleoside triphosphates, 0.04 % bovine serum albumin, 10 % dimethylsulfoxide, and 2 U of Ampli*Taq* polymerase (Perkin-Elmer Cetus). The amplications were carried out with a DNA Thermal Cycler (Perkin-Elmer Cetus). After the reactions, 8 µl of REP PCR products were separated by electrophoresis on horizontal 1.5 % agarose gels.

Genetic diversity analysis. Individual isolates were cultured in 2,4-D broth medium, harvested, and lysed as described by Kado and Liu (21) with some modifications (19). DNA samples obtained from the cell lysate were subjected to gel electrophoresis to detect and separate plasmid DNA (if any)

from linear chromosomal DNA, Southern transferred to nitrocellulose hybridization membrane, and hybridized with ^{32}P-labelled DNA probes.

As preliminary experiments for the genetic diversity study, in 1989 we isolated two 2,4-D degrading bacteria (KI1 and 1443) from the plot treated with 2,4-D at 100 ppm and one strain (2811P) from the control plot not treated with 2,4-D. Strain KI1 was observed to have sequence homology with the *tfd* genes (*tfdA*, *tfdB*, *tfdC*, and *tfdD*) of the 2,4-D metabolic pathway of the well-known plasmid pJP4. However, strain 2811P had sequence homology only with the *tfdA* gene but not with the *tfdB*, *tfdC*, and, *tfdD* genes and moreover, strain 1443 did not have sequence homology with any of the *tfd* genes. Hence, the *tfd* genes (*tfdA*, *tfdB*, *tfdC*, and *tfdD*) (16) were selected as probes to examine the possible genetic diversification of the 2,4-D genes of other 2,4-D degrading isolates and we developed a new DNA probe, a 6.5 kb fragment, to distinguish strain 1443 types. The 6.5 kb fragment was subcloned as a *BamHI* fragment into the multiple cloning site of plasmid pUC19 (27) from the large plasmid of strain 1443 based on weak cross-hybridization under low stringency condition between the DNA of strain 1443 and the plasmid DNA of another 2,4-D degrading isolate, strain 712, which showed the same hybridization patterns with the *tfd* genes as strain 2811P. The basic idea for subcloning this fragment was that the 2,4-D degradative, transferable plasmid of strain 712, which does not have sequence homology with the *tfd*B, *tfd*C, and *tfd*D genes, might have some homology with the DNA of strain 1443, which does not have homology with all the *tfd* genes. The 6.5 kb fragment was found to be specific for strain 1443 types and did not show any detectable homology to plasmid DNA such as pJP4 and the plasmid of strain 712 under high stringency condition. The *tfd* genes were labelled with ^{32}P by using the Random Primed DNA Labeling kit (Boehringer

Mannheim, Indianapolis, IN) and the 6.5 kb fragment was labeled by using the Nick Translation kit (Boehringer Mannheim). Prehybridization, hybridization and post-hybridization washes were performed as described by Holben et. al. (17) with some modifications. The membranes were prehybridized for at least 24 h at 42°C and after hybridization, three washes were carried out at room temperature, followed by one wash at 65°C for 45 min. Hybridization signals were detected by autoradiography using Kodak X-omat AR film (Kodak, Rochester, NY) exposed at -70°C with a Quanta III (Sigma, St. Louis, MO) intensifying screen. If necessary, bound probe was stripped from the membranes prior to rehybridization by washing for 15 min, 3 times with boiled distilled water containing 0.1 % sodium dodesyl sulfate (w/v). Individual isolates were grouped based on their hybridization patterns with probes.

Degradation phenotype analysis. A representative strain selected from the isolates identified as the same species by FAME analysis was cultured in 2,4-D mineral medium. The same medium containing 250 ppm of sodium acetate instead of 2,4-D was used to produce cells not adapted to metabolize 2,4-D. Cultures were grown at 30°C and aerated by shaking at 200 rpm in an incubator shaker. Cells in the late log phase were harvested by centrifugation (10,000 x g, 10 min, 4°C), washed twice with an equal volume of 0.02 M phosphate buffer (pH 7.0), and resuspended in the same buffer. An aliquot of suspended cells was inoculated into culture tubes containing MMO mineral medium plus 250 ppm of one of the following seven compounds : 2,4-D, 2-chlorophenoxyacetic acid (2-CPA), 4-chlorophenoxyacetic acid (4-CPA), phenoxyacetic acid (PA), 2-methyl-4-chlorophenoxyacetic acid (MCPA), 3-chlorobenzoic acid (3-CB), and 4-chlorobenzoic acid (4-CB). The cultures were shaken at 150 rpm and 30°C for 2 weeks, after which optical density was read

at 550 mμ with a Hewlett Packard 8452A Diode Array spectrophotometer to measure the cell growth. To determine the degradation of phenoxyacetates, the cultures were centrifuged to remove the cellular material and the absorption of ultraviolet light at the wavelength of maximum absorption was determined by scanning the sample in Hewlett Packard spectrophotometer.

RESULTS

FAME analysis. FAME analysis of the isolates (Figure 1) revealed 12 FAME groups of strains showing a Euclidian distance of less than 10 and all isolates clustered at a Euclidian distance of 48.8. One dominant FAME group contained 34 % of the isolates (i. e., 16 strains, Table 2). Four FAME groups contained three to seven strains, and the rest (8 strains) were distributed among the remaining 7 FAME groups, each consisting of only one or two isolates. Among the isolates, only 55.3 % of the isolates was reasonably identified to the species level and the rest could not be identified due to their poor match to any profile within the MIDI (Microbial ID, Inc.) library and to lack of growth on laboratory medium (Table 3). The dominant species, *P. saccharophyla*, accounted for 25.5 % of the isolates (i. e., 12 strains, Table 3) and comprised the dominating FAME group with *P. paucimobilis*.

Colony REP PCR analysis. Colony REP PCR was performed to distinguish strains of the same genera and species determined by FAME analysis as well as to analyze the divergence of strains classified as different genera and species. Representative gel photographs are shown in Figure 2. REP PCR analysis of the isolates revealed 30 different DNA fingerprint patterns. Among the 16 strains belonging to the dominating FAME group, two isolates (1443 and 556) identified as *P. paucimobilis* by FAME analysis exhibited

identical REP PCR patterns, thus indicating that they are the same strains in a single species. Identical PCR patterns were also observed among strains 765, 1124, and 1136, among 9 isolates (936, 947, 957, 1146, 1165, 91462, 9174, 9247, and 9256) classified as *P. saccharophyla*, and between strains 1173 and 9157 identified as *Alcaligenes eutrophus*. Among the unidentifiable isolates, strains 583 and 773, strains 9136, 91461, and 9166, and strains 9236 and 9266 produced identical REP PCR patterns, respectively. Since these strains were isolated either at different times or from different plots, their detection frequencies reflect the extent of their dominance in the examined plots. From this point of view, the strain of *P. saccharophyla* which was detected from 1990 to 1992 and from five different plots seemed to be most widely distributed on the plots during this research. Among *Pseudomonas* species, the isolates of *P. paucimobilis* produced very similar PCR patterns to those of *P. saccharophyla*(Figures 2-A, lanes 1 - 6, 8 - 14, 2-B, lanes 18, 19, 2-C, lanes 40, 44, 45, 47), while both of their PCR patterns were quite distinct from other strains (Figures 2-A, lane 7, 2-B, lanes 22 - 24, 2-C, lane 46) classified as *Pseudomonas* species by FAME analysis. This observation is well correlated to FAME data in which the isolates of *P. paucimobilis* and *P. saccharophyla* were grouped together into one FAME group at a Euclidian distance of 6.4, whereas the other *Pseudomonas* strains were grouped into several more diverse FAME groups clustering at a Euclidian distance of 31.5. The latter groups, together with *Alcaligenes* species (Figures 2-B, lanes 21, 25, 26, 30, 2-C, lane 42) and most of the unidentifiable strains (Figures 2-A, lanes 15, 16, 2-B, lanes 20, 28, 29, 31, 2-C, lanes 33 - 39, 41, 43) exhibited patterns distinct from each other, which is also reflected in the FAME dendrogram (Figure 1).

Genetic diversity analysis. To investigate the sequence diversity of the genes involved in 2,4-D degradation and to examine the presence of 2,4-D degradative plasmid DNA, the isolates were subjected to the modified Kado's plasmid detection method. Chromosomal DNA and plasmid DNA (if any), after gel separation and Southern transfer, were hybridized to the *tfd* genes of the plasmid pJP4 and also to a 6.5 kb DNA probe which is specific for a certain group of 2,4-D degrading bacteria such as *P. paucimobilis* 1443 undetectable with the known *tfd* genes. Representative hybridization blots are shown in Figure 3. Based on their hybridization patterns, the 47 isolates were grouped into four groups (Table 4) : hybridization group I included 12 isolates having sequence homology with all of the four *tfd* genes used ; group II contained 3 isolates having homology only with the *tfdA* gene among the four genes ; group III contained 18 isolates showing homology only with the 6.5 kb probe ; group IV (all others) contained 14 isolates which did not hybridize with any of the probes used.

All of the isolates classified into the group I exhibited clear plasmid bands on the gel and most of the detected plasmids (75 %) had sequence homology with the *tfd* genes (Figures 3-A, lane 5, 3-B, C &D, lanes 9, 10). Although plasmids ranging from 2.6 to 350 Mdal were reported to be easily detected by the Kado's method (21), shearing of large plasmids seemed to occur during the procedure as shown by minor hybridization signals observed on the chromosomal and linear DNA band in addition to main signals on plasmid DNA. In some isolates such as 9136, 91461, and 9166, the plasmid DNA was clearly detected on the gel, but it did not hybridize to any of the *tfd* genes used ; instead the chromosomal DNA gave hybridization signals (Figure 3-A, lanes 2, 3, 6). It seemed that these isolates have 2,4-D degradative genes on the chromosome instead of on the plasmid, although it is in contrast to most

of the earlier reports (10, 13, 25) that all or most of the 2,4-D genes are contained on the independent plasmid DNA. However, this observation is not surprising since it has been recently observed that an entire 2,4-D degradative plasmid could integrate into (strain 2811C, Figure 3-B, lane 8) and excise from the chromosome in *Alcaligenes* sp. (19). Alternatively, they might have huge plasmids that behave like chromosomal DNA during the procedure. Another interesting strain in group I is 9112 (Figures 3-B, C & D, lane 14). Its plasmid DNA detected on the gel had sequence homology with the *tfdA* and *tfdB* genes but, with the *tfdC* and *tfdD* probes, the major hybridization signals were shifted to the chromosomal DNA band. It is likely that the genes corresponding to the *tfdA* and *tfdB* probes are contained on one plasmid, while the genes for the *tfdC* and *tfdD* are contained either on another large plasmid or on the chromosome. The 12 isolates belonging to group I clustered at a Euclidian distance of 31.5 in FAME analysis and most of their REP PCR patterns (Fig. 2-A, lane 7, 2-B, lanes 20, 23, 25 - 31) were quite different from each other, suggesting that they are not closely related.

The isolates classified into group II accounted for only 6.4 % of the total isolates, indicating that they are not the dominant species in the examined plots. Their hybridization patterns were more unique than that of isolate 9112 of group I in that they do not have sequence homology with the *tfdB*, *tfdC*, and *tfdD* genes, suggesting the diversification of the corresponding enzymes or pathways. These isolates were observed to have 2,4-D degradative, transferable plasmids (19). Although isolates 712 and 782 were identified as the same species, *P. pickettii*, by FAME analysis (Table 3) and observed to have the same plasmid (data not shown), their REP PCR patterns were quite distinct from each other (Figure 2-B, lanes 22, 24). This observation indicates that their 2,4-D degradative plasmids were obtained

through natural conjugation among the indigenous soil microorganisms. Isolate 2811P, identified as *Alcaligenes paradoxus*, was also observed to have nearly identical plasmid to those of isolates 712 and 782. The possible intergeneric gene transfer between isolates 2811P and 712 has been discussed in the previous paper (19). Strain 2811C (a derivative strain from isolate 2811P) having the entire plasmid integrated into the chromosome (19) is included in Fig. 3-B (lane 8).

The isolates classified to group III did not hybridize with any of the *tfd* genes but hybridized with the 6.5 kb probe (Figure 3-A & E, lanes 1, 4, 7), suggesting that the degradative enzymes (or pathways) of this group are different from those of groups I and II. Although plasmid DNA band was not detected from most of the isolates (83.3 %) of group III with the Kado's method, they seem to have huge plasmids which behave like chromosomal DNA since one isolate (strain 1443) was observed to have a huge plasmid (data not shown) with the direct lysis method (29). Hence, the hybridization signals detected on the chromosomal area from these isolates may have been derived from their broken large plasmids as was observed from the isolate 1443. The isolates of group III clustered at a Euclidian distance of 6.4 in FAME analysis and their REP PCR patterns were very similar or identical to each other (Figures 2-A, lanes 1 - 6, 8 - 16, 2-B, lanes 18, 19, 2-C, lanes 40, 44, 45, 47), suggesting that they are closely related to each other. FAME analysis identified them as either *P. paucimobilis* or *P. saccharophyla*, but REP PCR analysis revealed six different patterns among them.

The 14 isolates of group IV (all others) did not have sequence homology with the DNA probes used (Figures 3-B, C, D & E, lanes 11, 12, 13) and clustered at a Euclidian distance of 31.5 in FAME analysis (Figure 1). Only two isolates, 912 and 983, among them were identified as *Alcaligenes faecalis*

and *Pseudomonas pickettii*, respectively, but the rest could not be identified by FAME analysis. These isolates appeared not to be closely related to each other as indicated by their quite distinctive REP PCR patterns (Figure 2-C, lanes 33 - 39, 41 - 43, 46).

Degradative diversity analysis. Representative strains of the isolates identified as the same species by FAME analysis were grown on 2,4-D or acetate as the sole carbon source and then examined for their degradation capabilities of other 2,4-D related compounds (Table 5). The isolates selected from the hybridization group I were more versatile in substrate utilization ability than those from the other groups. Especially, isolate 965, identified as *P. solanacearum* by FAME analysis, was (Table 3) the most versatile strain among the isolates tested. Whether it was grown on 2,4-D or acetate, it vigorously utilized 2,4-D related compounds such as PA, 4-CPA, 3-CB, and MCPA as the sole carbon sources as indicated by the complete disappearance of the substrate absorption spectrum and by the substantial cell growth (Table 5). Isolates 1173 and 745 of the hybridization group I could degrade MCPA or 3-CB, respectively. On the other hand, the selected isolates belonging to the groups II, III, and IV were very stringent in substrate utilization ability. None of them could degrade any of the compounds examined when they were grown on acetate (Table 5). The degradation of 4-CPA by isolates 912, 1443, 1156, 91462, and 1173 only under 2,4-D adapted condition indicates that this compound was metabolized probably due to its structural similarity to 2,4-D or 2,4-D pathway intermediates. None of the selected isolates could degrade 2-CPA and 4-CB.

Distribution of 2,4-D degraders. The distribution of hybridization groups (group I, II, III, and IV) was influenced by 2,4-D application rates and repeated treatments of 2,4-D with time (Figure 4). The isolates of groups II

and IV were encountered more frequently from soils not treated with 2,4-D or treated at a low rate (1 ppm), whereas those of groups I and III preferred soils treated at high rates (10 and 100 ppm) (Figure 4). This is well indicated by the decreasing ratios of the populations of the former to the latter with the increasing rates of 2,4-D application : i. e., the ratios over the experiments were 6, 1, 0.33, and 0.15 in control soil and soils treated with 1, 10, and 100 ppm of 2,4-D, respectively.

At the early phase of the field experiments, the isolates belonging to the groups II and IV were detected as predominant 2,4-D degraders in most of the plots. For example, these isolates accounted for 87.5 % of the predominant species isolated from the soil samples of May, 1990. However, the frequency of their occurrence was decreased to 50 % and 37.5 % in July and September of 1990, respectively. Thereafter, with further repeated treatments of 2,4-D, they were rarely detected. In contrast, those of the groups I and III occurred at lower frequency at the early phase but were encountered more frequently with the increasing number of 2,4-D treatments and eventually they accounted for most or all of the predominant species detected in 1991 and 1992.

DISCUSSION

The diversity of predominant 2,4-D degrading bacteria isolated at different times from an agricultural soil not treated or treated with 2,4-D at different rates was analyzed by using different classification systems. The relationships between different grouping methods are summarized in Table 6. The 47 isolates of 2,4-D degrading bacteria exhibited high diversity in their FAME profiles (Figure 1), REP PCR patterns (Figure 2), hybridization

patterns (Figure 3), and phenotypic traits (Table 5). While a variety of 2,4-D degrading bacteria belonging to multiple genera were reported in 1950s and 1960s, species identification by FAME analysis placed 55.3 % (26 strains) of the isolates in the genus *Pseudomonas* or *Alcaligenes* in this study. The rest (21 strains) could not be identified by FAME analysis mainly due to their poor match to any profole of the MIDI library and to lack of growth on ordinary laboratory medium. The REP PCR analysis showed these unidentifiable isolates to be heterogenous, giving 16 distinctive patterns, 4 of which occurred more than once. It is also interesting that most of the isolates (85.7 %) belonging to the hybridization group IV, which included all isolates not showing any detectable homology to the DNA probes used, could not be identified.

The isolates of the groups II and IV degrade 2,4-D very slowly and are not versatile in capabilities to utilize 2,4-D related compounds as sole carbon sources (Table 5). They appeared not to be competitive in soils treated with a high rate of 2,4-D or repeatedly treated with 2,4-D over a year period, since their proportion among the isolates was low or decreased according to the respective conditions. This could influence the type of 2,4-D degrading bacteria to be isolated from environmental samples by using the enrichment culture technique. Since, in most of studies, 2,4-D degrading bacteria have been isolated by inoculating undiluted environmental samples to broth culture with a high concentration of 2,4-D and through repeated transfers to fresh medium, the type of obtained isolates could be biased. In this situation, generally, strains belonging to the hybridization groups I are preferably isolated due to their quick growth property, which has already been demonstrated and discussed in soil competition studies (20). This may be the main reason why most of the 2,4-D degradative plasmids independently

isolated from numerous *Alcaligenes* species showed a high degree of similarity to plasmid pJP4 in biophysical and genetic properties (10) and also why most of the 2,4-D degrading bacteria isolated from various natural water samples exhibited suprising uniformity in their phenotypes, metabolism, and cell structure (1). In this research, the possible bias during enrichment procedure has been avoided by using the highest dilution showing 2,4-D degradation as inoculum for enrichment culture. With this procedure, only the naturally predominant species would be enriched and isolated from the plots without depending on their growth property in laboratory 2,4-D medium. Some of the cultures failed to produce single colonies able to degrade 2,4-D, suggesting that either they were not culturable on laboratory plates or cometabolism was involved in the 2,4-D degradation.

2,4-D is known to be easily utilized by microorganisms able to degrade this compound (24). The presence of 2,4-D degradative genes on plasmids and the extensive use of this compound as a herbicide since the late 1940s have been assumed to stimulate the rapid dissemination of the 2,4-D genes among the indigenous microbial populations (28). Additionally, the studies on 2,4-D degrading bacteria such as *Arthrobacter* (4, 5, 11, 23, 25, 34) and *Alcaligenes* (9) species known to be among the predominant species revealed the details of pathways and genes involved in 2,4-D degradation. However, it is worth noticing that still 75 % of the 2,4-D degraders isolated as dominant species during this research do not have sequence homology with all or most of the known *tfd* genes. It is not likely that gene transfers have extensively occurred among the isolates in the environment, which is further supported by the observation that the isolates of each hybridization group showed a tendency to form a separate cluster in FAME dendrogram instead of being mixed with one another. Moreover, considering that the natural environment

usually would not be frequently exposed to high doses of 2,4-D such as 10 and 100 ppm, the commonly encountered dominant species in the environment would be the types of the hybridization groups II and IV.

The ability to metabolize 2,4-D related compounds was quite diverse among the isolates. The selected isolates of group I exhibited relatively versatile property in substrate utilization ability, but those of group II appeared unable to vigorously attack any of the compounds examined and those of group III and IV tended to incidentally attack 4-CPA. While it has been suggested that 2,4-D degrading bacteria isolated from environmental samples commonly degraded 2-CPA, 4-CPA, and MCPA (24), none of the selected isolates could degrade 2-CPA and only 30 % and 60 % of the selected isolates could degrade MCPA or 4-CPA, respectively.

It has been reported that soil microflora adapted to 2,4-D could rapidly degrade MCPA and *vice versa* (2). It was suggested that 2,4-D and MCPA each induced their own specific enzyme systems, either in two different microfloras or in the same microorganisms, and that each enzyme system could incidentally attack the other molecule with less efficiency (2). Torstensson et. al. concluded that two different microfloras were involved in this cross-adaptation, while Audus (2) attributed this to the same organism. However, our results revealed substantial diversification of MCPA metabolism among the isolates. While the 2,4-D-degrading enzyme system of isolate 712 appeared able to incidentally degrade MCPA with less efficiency, which is in agreement with the result of Audus, isolates 965 and 1173 seemed able to induce their own MCPA-degrading enzyme systems without depending on the previous growth conditions. Moreover, the rest of the selected isolates could not attack MCPA even when they were adapted to metabolize 2,4-D, a finding also observed by MacRae & Alexander (26).

The hybridization grouping of the isolates not only reveals the substantial genetic heterogeniety among them but also has usefulness in the interpretation of the ecological evaluation of highly diverse groups of 2,4-D degraders. The observed gradual shifting of the predominant 2,4-D degrading populations from the groups II and IV to the groups I and III in field studies demonstrates how the indigenous microbial populations respond to the environmental changes. Among the four hybridization groups, group III was the most homogenous in population structure, as indicated by the high similarity in FAME profiles, REP PCR patterns, and degradation phenotypes (Table 6).

The data presented in this study illustrate the ability of different classification methods to group the 2,4-D degrading isolates and provide new insights into their diversity and ecological dynamics.

ACKNOWLEDGMENTS

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Table 1. 2,4-D degrading bacteria and their source.

Site	Isolate	Rate of 2,4-D application[a]	Date of soil sample collection[b]
Plot 1	2811P	control	Aug., 1989
Plot 4	KI1, 1443	high	
Plot 1 & 8	512, 583	control	May, 1990
Plot 2 & 7	524, 573	low	
Plot 3 & 6	535, 565	intermediate	
Plot 4 & 5	546, 556	high	
Plot 1 & 8	712, 782	control	July, 1990
Plot 2 & 7	723, 773	low	
Plot 3 & 6	736, 765	intermediate	
Plot 4 & 5	745, 756	high	
Plot 1 & 8	912, 983	control	Sept., 1990
Plot 2 & 7	924, 974	low	
Plot 3 & 6	936, 965	intermediate	
Plot 4 & 5	947, 957	high	
Plot 2 & 7	1124, 1173	low	Nov., 1990
Plot 3 & 6	1136, 1165	intermediate	

Table 1 (con'd).

Plot 4 & 5	1146, 1156	high	
Plot 1 & 8	9112, 9182	control	Sept., 1991
Plot 7	9174	low	
Plot 3 & 6	9136, 9166	intermediate	
Plot 4 & 5	91461, 91462, 9157	high	
Plot 1	9212	control	May, 1992
Plot 2	9224	low	
Plot 3 & 6	9236, 9266	intermediate	
Plot 4 & 5	9247, 9256	high	

[a] Control : no 2,4-D treatment ; low, intermediate, and high : 2,4-D was applied at the rate of 1 (0.6), 10 (6), 100 (60) μg/g soil (kg/ha), respectively, at the following dates : one application each on October of 1988 and on May, August, October, and December of 1989 ; two applications on May, July, September, and November of 1990 ; on May, July, September, and November of 1991 ; and three applications on May of 1992.

[b] Soil samples for strain isolation were taken in one week following the last application.

Table 2. Distribution of isolates among FAME (fatty acid methyl ester) groups.

Number of isolates	Number of FAME groups[a]
16	1
7	1
5	1
4	1
3	1
2	1
1	6

[a] FAME groups include isolates with < 10 Euclidian distance.

Table 3. Identification of isolates to species level using FAME (fatty acid methyl ester) analyses.

Isolate	FAME identification
1443, 556, 9174, 9224	*Pseudomonas paucimobilis*
736, 756, 765, 936, 947, 957, 974, 1124, 1136, 1146, 1165, 91462	*Pseudomonas saccharophyla*
712, 782, 983, 9112	*Pseudomonas pickettii*
745	*Pseudomonas pseudomallei*
965	*Pseudomonas solanacearum*
1173, 9157	*Alcaligenes eutrophus*
912	*Alcaligenes faecalis*
2811P	*Alcaligenes paradoxus*
KI1, 512, 524, 535, 546, 565, 573, 583, 723, 773, 924, 1156, 9136, 91461, 9166, 9182, 9212, 9236, 9247, 9256, 9266	Unidentifiable[a]

[a] These isolates could not be identified due to their poor match to profiles of MIDI library and lack of growth on ordinary laboratory medium.

Table 4. Hybridization patterns of the isolates with DNA probes.

Isolate	DNA Probes[a,b]					Hybridization Group
	tfdA	*tfdB*	*tfdC*	*tfdD*	6.5 kb	
KI1, 745, 965, 1173, 9112, 9136, 91461, 9157, 9166,9212, 9236, 9266	+	+	+	+	-	I
2811P, 712, 782	+	-	-	-	-	II
1443, 556, 736, 756, 765, 936 947, 957, 974, 1124, 1136, 1146, 1165, 91462, 9174, 9224, 9247, 9256	-	-	-	-	+	III
512, 524, 535, 546, 565, 573 583, 723, 773, 912, 924, 983 1156, 9182	-	-	-	-	-	IV (all others)

[a] *tfdA*, *tfdB*, *tfdC*, *tfdD* gene probes were subcloned from plasmid pJP4 ; 6.5 kb probe was subcloned from the large plasmid of strain 1443.

[b] +, shows detectable hybridization signal ; -, does not show detectable hybridization signal.

Substrate[a]	Utilization by given isolate[b]																					
	I						II						III						IV			
	745		965		1173		2811P		712		1443		91462		565		912		1156	
	A	U	A	U	A	U	A	U	A	U	A	U	A	U	A	U	A	U	A	U	A	U
2,4-D	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
PA	-	-	++	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2-PA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4-PA	-	-	++	++	++	-	-	-	-	-	++	-	++	-	-	-	++	-	++	-	++	-
3-CB	+	+	++	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4-CB	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MCPA	-	-	++	++	++	++	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-

[a] PA, phenoxyacetic acid ; 2-PA, 2-chlorophenoxyacetic acid ; 4-PA, 4-chlorophenoxyacetic acid ; 3-CB, 3-chlorobenzoic acid ; 4-CB, 4-chlorobenzoic acid ; MCPA, 2-methyl-4-chlorophenoxyacetic acid.

[b] The selected isolates of the hybridization groups I, II, III, and IV were grown on 2,4-D (A) or on acetate (U) and then tested for substrate utilization capabilities. ++, >80 % reduction in peak height from UV scanning and substantial growth ($OD_{550} > 0.13$) ; +, 40 to 60 % reduction in peak and moderate growth ($OD_{550} > 0.08$) ; -, < 15 % reduction in peak and very scant growth ($OD_{550} < 0.01$).

Table 6. Comparison of similarities or differences among isolates of different groups using different methods.

	Hybridization group			
	I	II	III	IV
Number of isolates	12	3	18	14
FAME profile	diverse (31.5)[a]	moderate (11.8)	similar (6.4)	diverse (31.5)
REP PCR patterns	diverse	diverse	similar	diverse
Substrate utilization patterns	diverse (1 - 4)[b]	similar (0 - 1)	similar (1)	similar (0 - 1)

[a] Maximum Euclidian distance among isolates.

[b] Number of six related substrates utilized by isolates.

Figure legends

Fig. 1. Dendrogram of fatty acid profiles of the isolates. FAME groups were defined at less than 10 Euclidian Distance.

Fig. 2. REP PCR patterns of the isolates. *Hind*III digested Lambda DNA standard (1, 17, 32) is shown on the left. Lanes: strains 1443 (2), 556 (3), 936 (4), 947 (5), 957 (6), 965 (7), 1124 (8), 1136 (9), 1146 (10), 1165 (11), 91462 (12), 9174 (13), 9224 (14), 9247 (15), and 9256 (16) (A), strains 736 (18), 756 (19), KI1 (20), 2811P (21), 712 (22), 745 (23), 782 (24), 1173 (25), 1173 (26), 9112 (27), 9136 (28), 91461 (29), 9157 (30), and 9166 (31) (B), strains 512 (33), 524 (34), 535 (35), 547 (36), 565 (37), 573 (38), 583 (39), 765 (40), 773 (41), 912 (42), 924 (43), 957 (44), 974 (45), 983 (46), and 936 (47) (C).

Fig. 3. Autoradiogram of the gel after hybridization with the *tfdA* gene (A & B), *tfdB* gene (C), and *tfdC* gene (D) and the 6.5 kb probe (E). Lanes: strains 1124 (1), 9136 (2), 91461 (3), 91462 (4), 9157 (5), 9166 (6), 9174 (7), 2811C (8), 965 (9), 1173 (10), 912 (11), 924 (12), 983 (13), and 9112 (14). Positions of plasmid DNA (P) and chromosomal and linear DNA (chr) are shown.

Fig. 4. Distribution of hybridization groups of 2,4-D degraders in field.

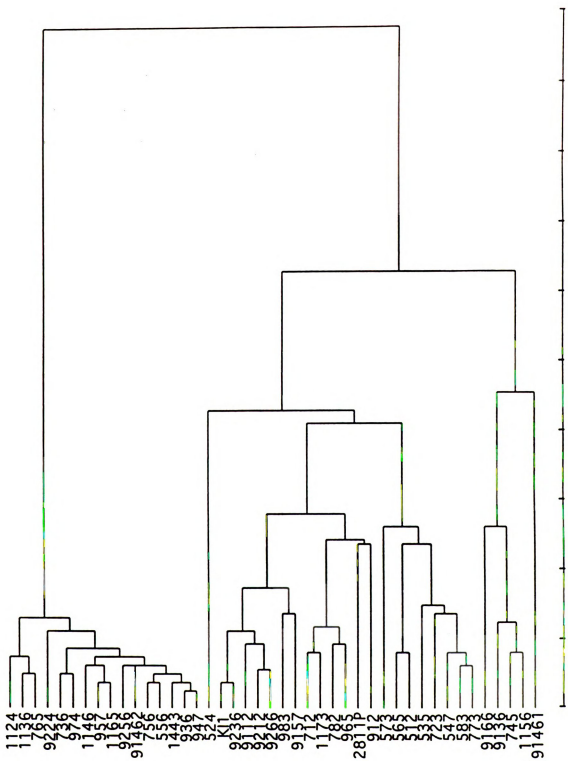
1124
1136
765
9244
696
1149
1165
9256
91462
756
555
1443
949
524
K11
9236
911
9212
9289
9157
717
113
783
965
2811p
912
573
593
516
733
547
583
773
9166
9136
745
1156
91461
0
10
20
30
40
50
Euclidian Distance

Figure 1.

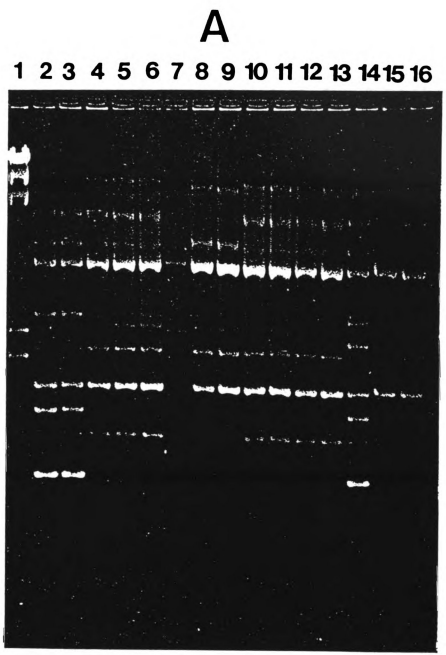

Fig. 2-A.

B

17 18 19 20 21 22 23 24 25 26 27 28 29 30 31

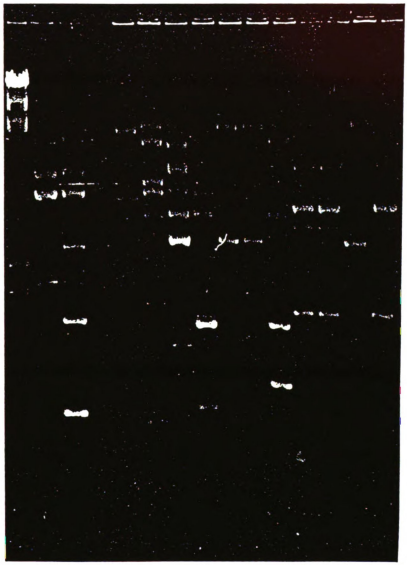

Fig. 2-B.

C

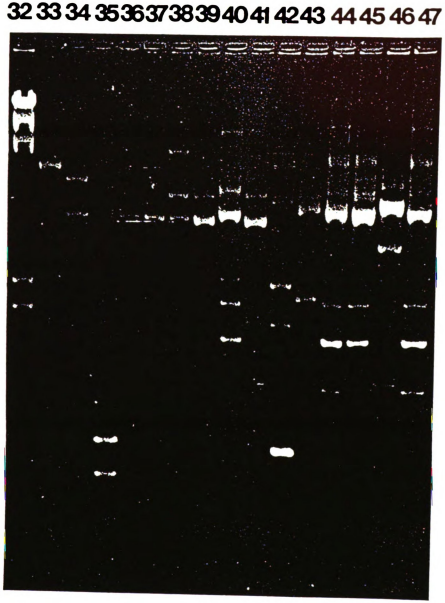

Fig. 2-C.

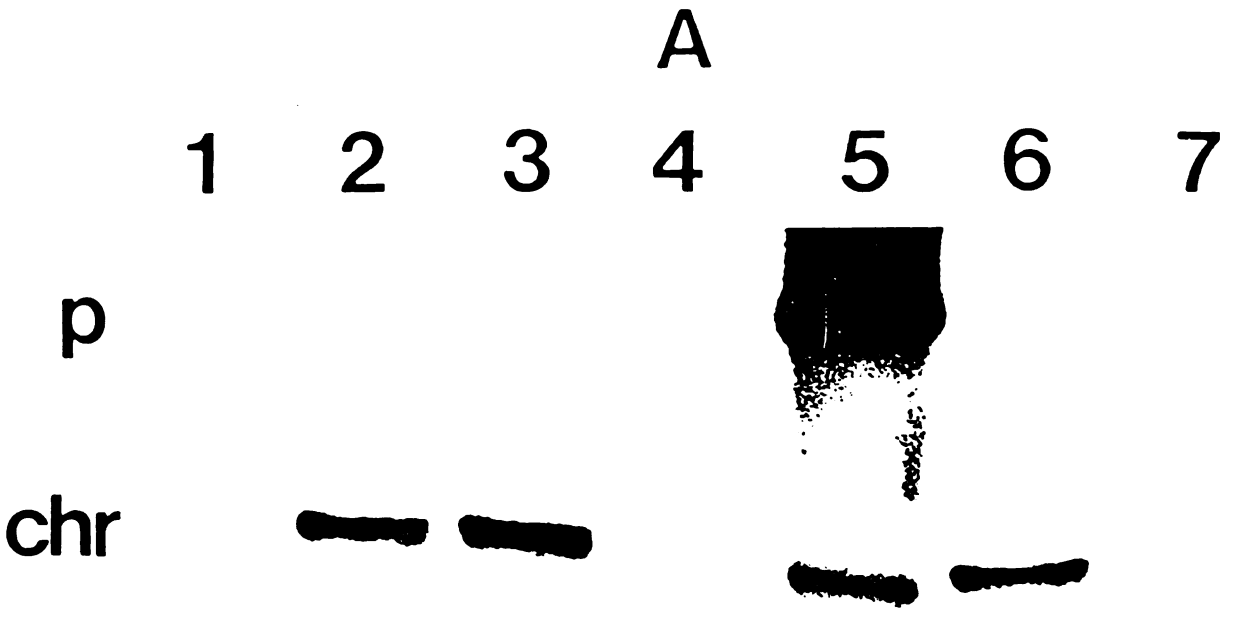

Figure 3-A.

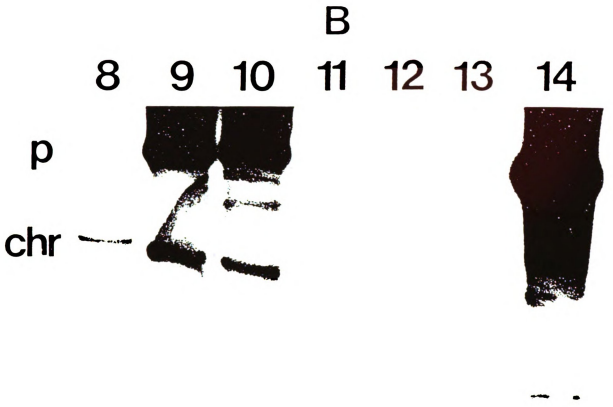

Figure 3-B.

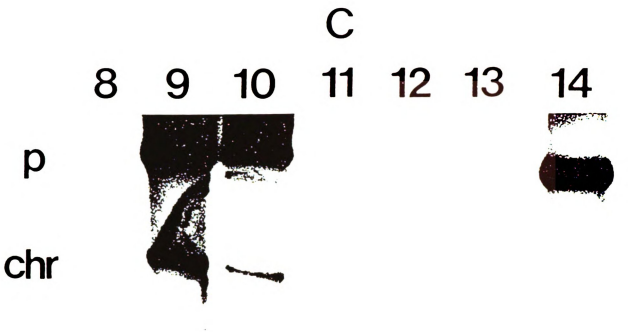

Figure 3-C.

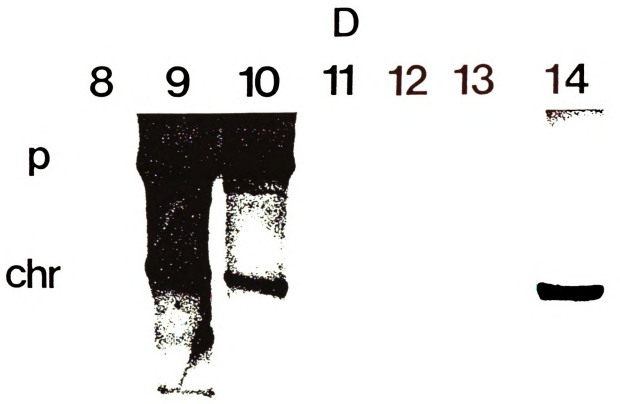

Figure 3-D.

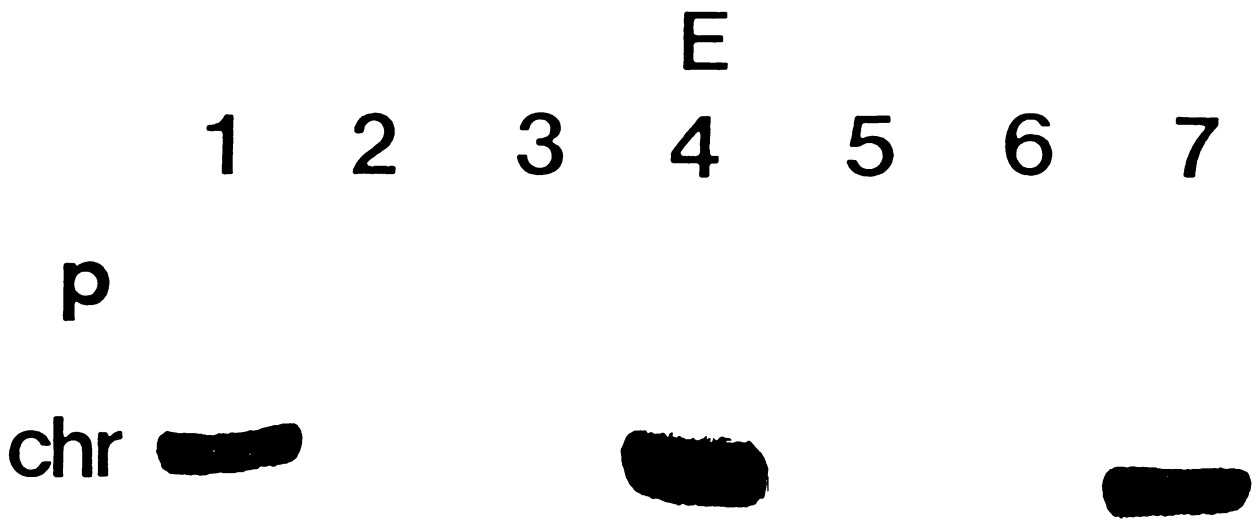

Figure 3-E.

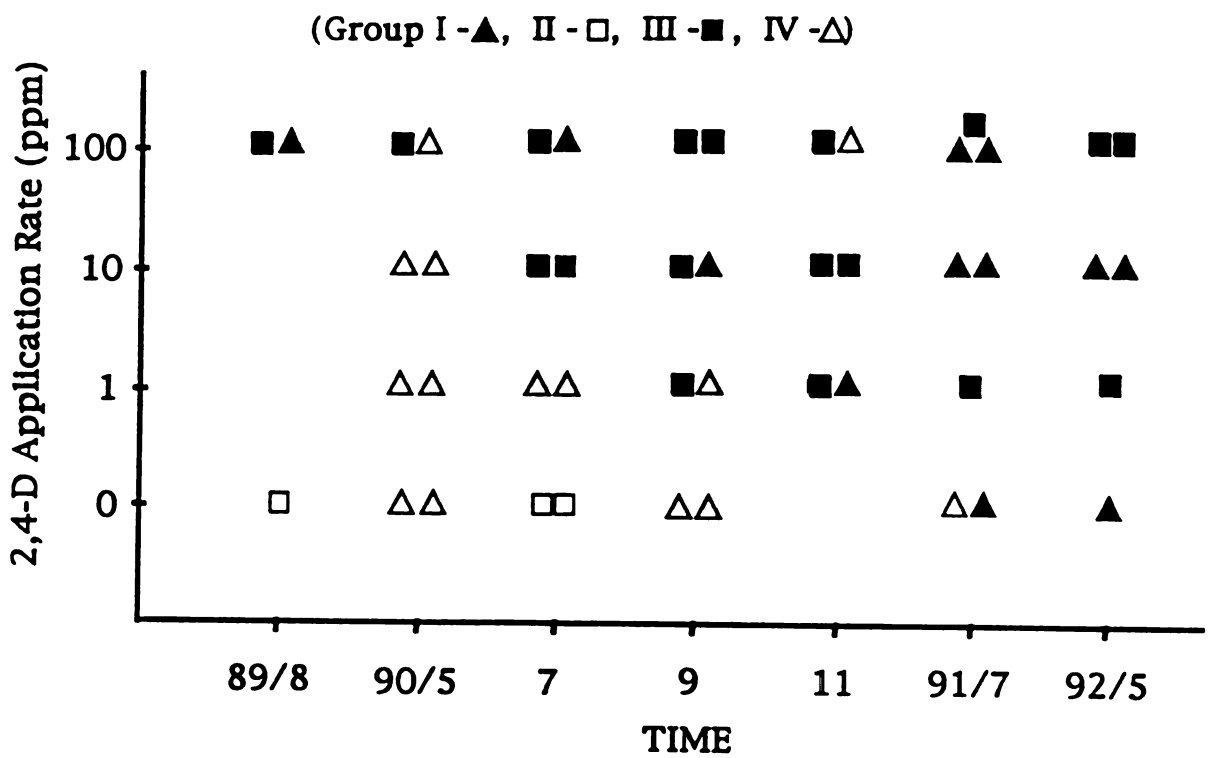
(Group I -▲, II - □, III -■, IV -△)
2,4-D Application Rate (ppm)
100
10
1
0
89/8
90/5
7
9
11
91/7
92/5
TIME

Figure 4.

LIST OF REFERENCES

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Chapter Three

Use of Gene Probes to Detect 2,4-D Degrading Populations in Soil Maintained under Selective Pressure.

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ABSTRACT

2,4-D was applied to soils in microcosms and degradation was monitored for each of 5 repeated additions. Total DNA was isolated from the soil bacterial communities after each 2,4-D treatment. The DNA samples were digested with restriction enzymes, and analyzed on Southern blots, using a *tfdA* gene probe subcloned from plasmid pJP4 and also a 6.5 kb probe derived from the large plasmid of a new 2,4-D degrading isolate (*Pseudomonas paucimobilis*) which has no detectable cross-homology with the *tfdA* gene probe. 2,4-D applied to soil with no prior history of 2,4-D treatment was quickly degraded by indigenous soil microbial populations. There was good correlation between 2,4-D degradation and banding patterns in hybridization analyses performed after each 2,4-D treatment using both the *tfdA* gene probe and the 6.5 kb probe. In one microcosm, where soil water content was adjusted to 10% initially, changes in banding patterns over time were observed with the *tfdA* gene probe in Southern blot analyses, suggesting that population changes or possibly genetic rearrangement in 2,4-D degrading microbial populations occurred in this soil. In another microcosm adjusted to 25% water content, 2,4-D degrading populations were maintained stably throughout the 5 additions. Isolates responsible for hybridization patterns observed in the total community DNA were identified. The data demonstrate that gene probe analyses were capable of detecting and discriminating indigenous 2,4-D degrading populations in soil amended with 2,4-D.

INTRODUCTION

Large amounts of man-made chlorinated organic chemicals have been used extensively in agriculture as herbicides and pesticides. Among these, 2,4-dichlorophenoxyacetic acid (2,4-D) has received widespread use as a herbicide for more than 40 years. Unlike many of the recalcitrant synthetic compounds that have been released into the environment over the past several decades, 2,4-D is rapidly degraded by soil bacteria (4, 13, 29, 39, 40) and its fate in soil is critically affected by microbial degradation (13, 25). Populations of microorganisms able to degrade 2,4-D have been estimated by a most probable number (MPN) method in soils (7, 19, 26, 29, 35). Organisms capable of degrading 2,4-D have been identified in a number of genera such as : *Achromobacter, Alcaligenes, Arthrobacter, Corynebacterum, Flavobacterium, Pseudomonas*, and *Streptomyces* (2, 8, 11, 23, 28, 34, 41). Furthermore, the pathway for 2,4-D degradation on plasmid pJP4 originally identified in *Alcaligenes eutrophus* was studied and described (8, 9, 38).

However, past research describing herbicide biodegradation by microbial populations has provided virtually no information regarding population or genetic changes of microbial communities in natural environments in response to the herbicide. Traditionally, the isolation of pure cultures (6) and the fluorescent antibody techniques (5,12) have been used to confirm the presence of specific microorganisms in environmental samples, each of which is useful but limited in some aspects. The cultural method is selective for certain microorganisms which can be cultivated on or in particular laboratory media. The fluorescent antibody technique has high specificity even to the strain level, but is labor-intensive, relatively expensive and is not well-suited to routine screening for the presence of organisms. As a new methodological

approach, gene probes were developed to detect specific microorganisms in environmental samples by using colony hybridization (18, 36). But this method requires that microorganisms be cultivated in laboratory media prior to analysis. Since more than 90% of soil microorganisms are non-culturable in laboratory media, the colony hybridization method would have limited utility for detecting these non-culturable microorganisms in soils.

It has been suggested that a DNA probe method for analyzing total soil bacterial DNA might make it possible to detect multiple organisms and populations or genetic changes in natural environmental samples (21, 22). However, in those reports, particular gene probes were used to detect microorganisms inoculated into soils in large numbers under laboratory conditions rather than the indigenous soil microfloras. Moreover, little attention has been given to the population dynamics and genetic changes of the soil microfloras in response to environmental changes.

It is likely that when the involved genes of the indigenous microbial populations are diversified, it is difficult to detect and assess all of the responsive, diverse populations with a single gene probe. The diversity of 2,4-D degradative genes was described in the previous study (24) where numerous indigenous 2,4-D degrading microorganisms did not show any cross-homology with the *tfdA* gene which is specific in the 2,4-D degradative pathway. Among the four hybridization groups described in the study, the dominating group III accounted for about 38 % of the total 47 2,4-D degrading bacteria isolated from Kellogg Biological Station (KBS) soils. Since the dominating populations did not have any sequence homology with the *tfdA* gene (as well as with the *tfdBCD* genes), a new probe, a 6.5 kb fragment, has already been developed to discriminate them from others undetectable with the *tfdA* probe (24). These two DNA probes, the *tfdA* gene probe and the 6.5

kb probe, are able to account for about 70 % of the indigenous 2,4-D degrading populations in KBS soil and the isolates of the hybridization groups I and III, which can be detected with the *tfdA* gene probe or the 6.5 kb probe, respectively, dominated in KBS soil repeatedly treated with high amount of 2,4-D (24). Hence, the two DNA probes appear to make it possible to monitor the population dynamics and genetic changes of the indigenous, dominant 2,4-D degrading populations in KBS soil under 2,4-D selective condition.

In this research, the objectives were to detect indigenous 2,4-D degrading microbial populations in soil using DNA probes to understand the genetic response of the microbial community to 2,4-D selective conditions, to identify specific 2,4-D degrading isolates responsible for hybridization patterns observed in the total soil community DNA, and to demonstrate that a single gene is not enough to monitor all of the 2,4-D degrading microbial populations in soil due to the diversity of the 2,4-D degradative genes.

MATERIALS AND METHODS

Bacterial strains and media. The strains of *Pseudomonas pickettii* and *Pseudomonas paucimobilis* capable of utilizing 2,4-D as the sole source of carbon were isolated from agricultural soil samples in Long-Term Ecological Research (LTER) site of Kellogg Biological Station (KBS, Hickory Corners, MI) (24). *Escherichia coli* JM83 is described elsewhere (3). Strains *P. paucimobilis* and *P. pickettii* were cultivated in MMO mineral media (37) containing 500 ppm of 2,4-D at 30°C. *E. coli* JM83 was cultivated in Luria broth (30) at 37°C. Plasmid DNA was isolated by using the method of Hirsch et. al. for large plasmids (19) or Maniatis et. al. for small plasmids (30).

2,4-D amendment of soil. Soil (loam) with no history of 2,4-D treatment was taken from the LTER site of KBS, on which a plot has been subjected to microbial ecological research in response to 2,4-D treatment as one of the LTER projects. Soil was stored at field moisture levels at 4°C until used. Soil samples (500g) which had been sifted through a 2-mm sieve were transferred to sterile polyethylene wide-mouth bottles. Soil water content was adjusted to 10% for two of four bottles and 25% for the other two by adding sterile, distilled water. 2,4-D was added to one of the 10% moisture soils and one of the 25% moisture soils to a concentration of 250 ppm and thoroughly mixed. Each control soil received only phosphate buffer. The disappearance of 2,4-D in soil was monitored as described above and the soil respiked for each of 5 cycles of degradation. At the end of each cycle of degradation, a 50 g subsamples were taken from both the 2,4-D-treated and control soils and the total soil bacterial DNA was extracted with cell extraction method which involves the separation of the bacterial cells from the soil particles by differential centrifugation followed by lysis of the recovered cells (21).

Analysis of 2,4-D concentration in soil. Stock solutions (20 mg/ml) of analytical grade 2,4-D (Sigma, St. Louis, MO) were prepared by dissolving in 0.1M NaH_2PO_4 buffer (pH 7.0) and stored at 4°C until use. For analysis of the concentration of 2,4-D in soil, 1 g soil subsamples were combined with 1ml of sterilized distilled water in an eppendorf tube and vortexed for 1 min. The soil was pelleted by centrifugation in a microfuge (14,000 rpm, 5 min) and the supernatant was transferred into a clean eppendorf tube. This sample was filtered through a Millipore Millex-GS syringe filter and analyzed for 2,4-D with an Hewlett Packard series 1050 HPLC equipped with

Lichrosorb RP-18 column (Anspec Co., Ann Arbor, MI) and a UV detector set at 230nm, using methanol/0.1% phosphoric acid (60:40) as eluant.

Probe construction. To detect 2,4-D degrading microbial populations, the *tfdA* gene was chosen as a probe among the six structural genes (*tfdA, B, C, D, E* and *F*) for 2,4-D metabolism of the well-known plasmid pJP4 (1, 10), because it would be apparently more specific to the 2,4-D degradation pathway of plasmid pJP4 than the other genes (17, 19, 24). The *tfdA* gene probe was cloned as a 556 bp *EcoR*1-*Bam*H1 fragment into the multiple cloning site of plasmid pUC19 (30). A 6.5 kb fragment, which was cloned as a *Bam*HI fragment from the large plasmid of *P. paucimobilis* into plasmid pUC19, was used as a new probe to detect *P. paucimobilis* and similar 2,4-D degrading bacteria that do not hybridize to the *tfdA* gene probe due to the diversity of 2,4-D degradative genes (24). All restriction enzymes and T4 DNA ligase were purchased commercially and used according to the manufacturer's specifications. The cloned *tfdA* gene probe and the 6.5 kb probe were isolated as restricted fragment from the corresponding cloned plasmid pUC19 on 0.7% agarose gel, purified with Geneclean Kit (Bio 101 Inc, La Jolla, CA), and labeled with ^{32}P by using the Random Primed DNA Labeling Kit (Boehringer Mannheim, Indianapolis, IN) or the Nick Translation Kit (Boehringer Mannheim) according to manufacturer's specifications. Labeled probes were separated from unincorporated nucleotides prior to use with spun column (30).

Restriction analysis and Southern hybridization. Total soil bacterial DNA was digested with appropriate restriction endonucleases according to manufacturer's specifications. Digested DNA was size fractionated by

electrophoresis through horizontal 0.7% agarose gels and transfered to nitrocellulose hybridization membrane.

Prehybridization, hybridization and post-hybridization washes were performed as described by Holben et. al. (21) with some modifications. The membranes were prehybridized for at least 24 h at 42°C and after hybridization, three washes were carried at room temperature, followed by one wash at 65°C. Hybridization signals were detected using the Betascope radioactive blot analyzer (Betagen Corp., Waltham, MA) or by autoradiography using Kodak X-Omat AR film (Kodak, Rochester, NY) exposed at -70°C with a Quanta III (Sigma, St. Louis, MO) intensifying screen. Exposure times were 1 to 7 days depending on the intensity of the radioactive signal.

RESULTS

Degradation of 2,4-D in soil. Exposure of 2,4-D degrading populations of soil to 2,4-D for the first time resulted in slow degradation for first treatment of 2,4-D in both 10% and 25% moisture microcosm soils (Figure. 1), requiring about 3 weeks for the added 2,4-D to be degraded completely. The rate of degradation for subsequent additions of 2,4-D was greatly increased, requiring 1 week or less for the complete degradation for each of four more 2,4-D additions.

Gene probe hybridization results. The results of Southern hybridization of the *tfdA* gene probe are presented in Figure 2. For both the 10% and the 25% moisture soils, a single band of hybridization was detected from the second through the fifth addition. No detectable hybridization was observed after the first treatment. In the 10% soil moisture microcosm, the location of

the band of hybridization shifted between the second and third treatments and thereafter remained constant. A possible interpretation of this data is that the dominant 2,4-D degrading population present after two treatments was displaced by another 2,4-D degrading population that then stably dominated throughout the course of the experiment. Alternatively, it is possible that the sequence encoding the *tfdA* homology was somehow rearranged (e.g. by deletion) resulting in a shift in the band of hybridization and that this derivative strain displaced the original population possibly because of its increased fitness. In the 25% soil-moisture sample the band of hybridization was first detected after 2 treatments with 2,4-D and its location remained constant throughout the experiment. Note that the apparent size (8.8kb, Figure 2-B) of this band is similar to the size of the band observed after the shift observed in the 10% soil-moisture samples.

A total of 47 2,4-D degrading microorganisms were isolated from KBS soil by serial dilution of soil suspensions and selection for growth on 2,4-D (24). All isolates were screened for homology with the *tfdA* gene probe. Among them, fifteen isolates including strain *P. pickettii* hybridized to this probe and, through another prescreening hybridization experiment, *P. pickettii* was expected to correspond to the hybridization bands of microcosm soil bacterial DNA. Plasmid DNA obtained from this strain was compared to total soil bacterial DNA of the fifth treatment of 2,4-D in Southern analyses using the *tfdA* gene probe (Figure 3). Matching band patterns were obtained between these two DNA samples when digested with three different restriction enzymes, suggesting that the dominant 2,4-D degrading organism responsible for the hybridization signals observed in soil bacterial DNA from the third to the fifth treatment of this microcosm experiment could be *P. pickettii*. Moreover, its population level was increasing with repeated additions of 2,4-

D through the fifth treatment, as indicated by increasing band intensities on Southern blot (Figure 2-A). Since each lane contained the same amount of total DNA (1.5μg), the intensity of the hybridization for each time point is proportional to the amount of target DNA in each DNA sample. Quantitative hybridization analysis using the Betascope radioactive blot analyzer indicated that the hybridization signal exhibited a 5-fold increase when the third 2,4-D treatment is compared to the fifth treatment, suggesting a five fold increase of the target sequence in the degrading population detected (presumably strain *P. pickettii*). In the 25% moisture soil the same strain *P. pickettii* population seemed to have been dominant from first detection (after 2 treatments with 2,4-D) through the fifth treatment, since a single band of hybridization that is same size as in the 10% moisture sample persists (Figure 2-B). For both the 10% and the 25% moisture control soils not treated with 2,4-D, the total bacterial DNA had no detectable hybridization to the *tfdA* gene probe (Figures 2-A & B).

Strain *P. paucimobilis* is one of the 2,4-D degrading isolates from KBS soil. The 6.5 kb fragment was developed as a new probe to detect *P. paucimobilis* in KBS soil amended with 2,4-D. This was necessary because this strain, which was one of the dominant 2,4-D degrading bacteria in both these experiments and in field experiments, has no detectable homology to the *tfdA* gene probe. The 6.5 kb fragment was also observed to hybridize to other similar 2,4-D degrading populations that are not detectable with the *tfdA* probe (24). Total soil bacterial DNA from the 25% moisture soil was digested with *Hind*III, then hybridized with the 6.5 kb fragment (Figure 4). Throughout the 5 treatments of soil with 2,4-D, two bands (5.0 kb and 11.3 kb in size) of hybridization were detected with the 6.5 kb probe. The position of the hybridization bands remained constant throughout the experiment,

indicating that they represent a single, stably maintained population. When total soil bacterial DNA from the fifth treatment with 2,4-D was compared to DNA isolated from *P. paucimobilis*, the observed hybridization band patterns between these two DNA samples were identical in Southern analyses using the 6.5 kb probe (Figure 5). This suggests that *P. paucimobilis* corresponded to the detected hybridization bands from soil bacterial DNA and that this strain was one of the dominant 2,4-D degraders throughout this experiment. Quantitative hybridization analysis indicates that this population was increased about two-fold in size throughout the course of 5 treatments with 2,4-D. Again, there was no hybridization signal to the total DNA of control soil not treated with 2,4-D.

DISCUSSION

2,4-D applied to soil with no prior history of 2,4-D treatment was quickly degraded by indigenous 2,4-D degrading microbial populations. Lag phase was observed in the first treatment of 2,4-D, indicating that during which 2,4-D degrading microbial populations of soil were adapting to a new selective conditions and inducing their 2,4-D degradative enzymes as suggested by Loos(27).

The hybridization results presented in this report show that both the *tfdA* gene probe and the 6.5 kb probe were useful for selective detection of 2,4-D degrading microorganisms in natural soil amended with 2,4-D. Good correlation was observed between 2,4-D degradation activities and hybridization signals with these two probes and no hybridization signal was observed in control soil not treated with 2,4-D. It is known that the fate of 2,4-D applied to soil is critically affected by microbial degradation (13, 25, 27)

and that 2,4-D treatment of soil significantly increases the number of 2,4-D degrading organisms (14,15). The enrichment effect of 2,4-D on the number of degrading microorganisms made it possible to detect these organisms using DNA probe method which has a lower sensitivity limit of about 10^4 cells per g (21) without requiring amplification of target sequences or probe signal.

Traditionally, numbers of 2,4-D degrading microorganisms in soils have been estimated by the most probable number enumeration method (MPN) (20, 26, 29, 31). This MPN method has restricted usage because it requires successful cultivation of indigenous 2,4-D degraders and only indicates the presence or absence of 2,4-D degraders which can be cultivated. On the other hand, the DNA probe method allows us to study which species is predominant and whether or not population and genetic changes occur among 2,4-D degrading microorganisms, in addition to the relative population levels, in relation to their environment without cultivation of the recovered microorganisms. The use of this DNA probe method in conjuntion with Southern transfer is analytically powerful, being able to detect multiple organisms, population changes, deletion of sequences, and genetic rearrangements (21, 22). The observed changes in band patterns with *tfdA* gene probe (Figure 2-A) and the co-existence of the apparently dissimilar 2,4-D degrading strains *P. pickettii* and *P. paucimobilis* in the same microbial community under 2,4-D selective conditions (Figures 2 & 4) indicates that there may be population fluctuations or dynamic interactions between populations that would be undetectable by the MPN method.

Strain *P. paucimobilis*, which appears to be one of the dominant 2,4-D degraders in both experiments, has no detectable homology to the *tfdA* gene probe, suggesting that the 2,4-D degradative genes in this strain are substantially different at the DNA sequence level and possibly at the

functional level. In contrast to the *tfdA* gene probe, *tfdC* and *tfdD* gene probes subcloned from the plasmid pJP4 did not hybridize to the plasmid DNA of *P. pickettii*(24). In fact, in both microcosms experiments, no hybridization signal was observed with the *tfdC* and *tfdD* gene probes on Southern analyses. This observed diversity of the 2,4-D degradative genes and the results presented here demonstrate that all of the indigenous 2,4-D degrading microbial populations can not be monitored with a single probe in soil.

Among the 2,4-D degrading isolates, some strains (*P. pickettii* and *P. paucimobilis*) were found to account for bands of hybridization obtained on Southern blots probed with the *tfdA* gene probe and the 6.5 kb probe (Figures 3 & 5). Digestion with three different restriction enzymes and hybridization to specific probes gave good evidence that hybridization bands detected from total bacterial community DNA correspond to a particular soil isolate. It is desirable to relate environmental soil DNA samples to laboratory pure DNA of specific isolate in microbial ecological studies and thus it is a good demonstration that DNA probe method can discriminate a strain among a variety of indigenous microorganisms in soil.

While it is possible that there were additional 2,4-D degrading populations present that were not detected by either probe, these data demonstrate that DNA probe method with total soil bacterial DNA was capable of detecting and discriminating the indigenous 2,4-D degrading microbial populations in soil under selective conditions and that it could represent a means to study microbial ecology both autecologically and synecologically at the DNA level.

ACKNOWLEDGMENTS

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Figure legends

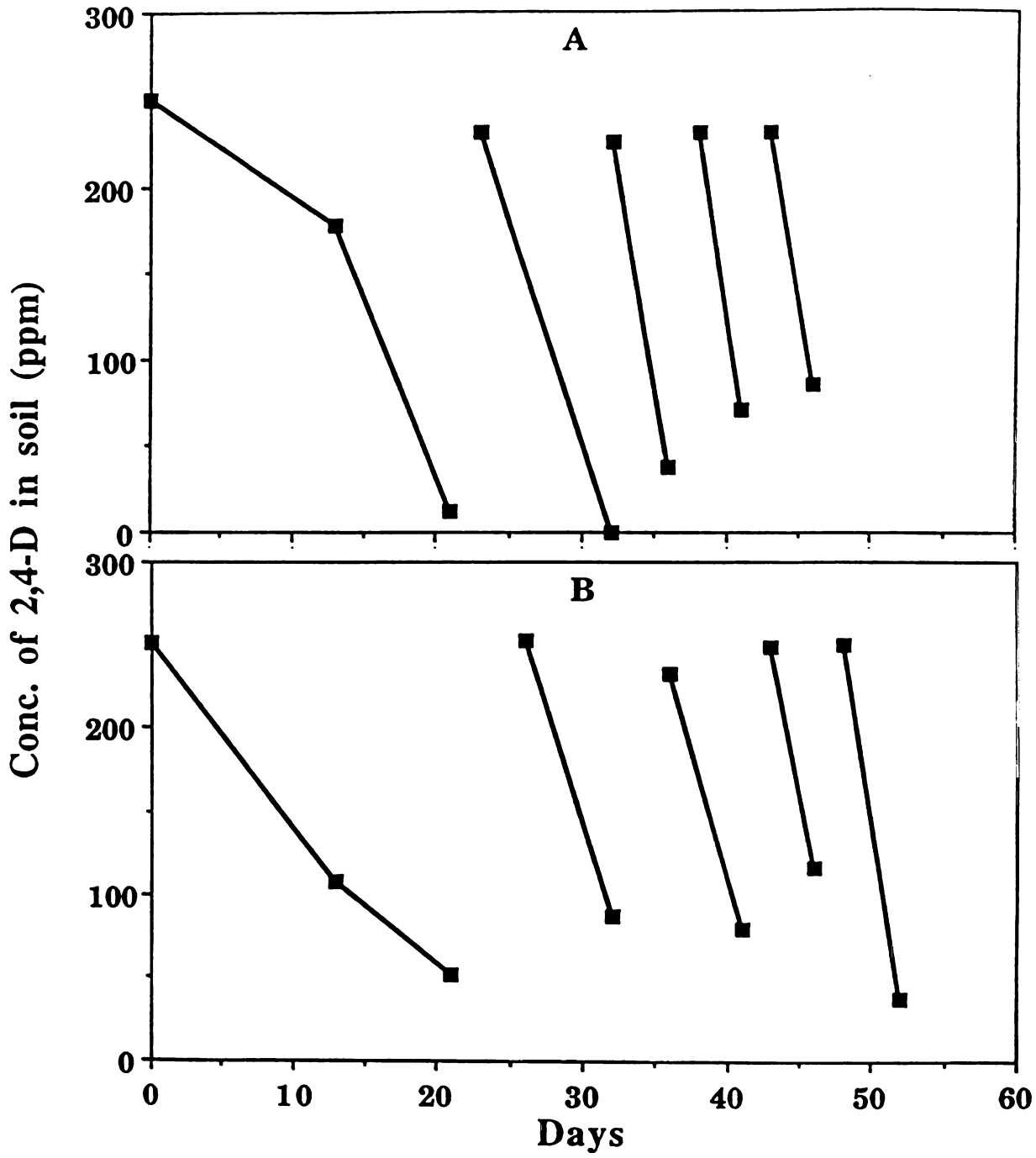
Fig. 1. Degradation of 2,4-D in soil. 2,4-D was applied to microcosm soils at a concentration of 250 ppm for each of the 5 repeated treatments. Degradation of 2,4-D was monitored by HPLC. Initial soil water content was adjusted to (A) 10% and (B) 25%.

Fig. 2. Detection of indigenous 2,4-D degrading populations in soil by Southern blot analysis. Total soil bacterial DNA was isolated at time zero and after 1, 2, 3, 4 and 5 cycles of 2,4-D treatment (+ : 2,4-D treated, – : no 2,4-D controls). 1.5μg DNA samples were digested with *Bam*HI, transferred to nitrocellulose hybridization membrane, and hybridized with the *tfdA* gene probe. Initial soil water content was (A) 10% and (B) 25%.

Fig. 3. Comparison by DNA probe hybridization of soil bacterial DNA and plasmid DNA digested with three different restriction enzymes. Soil bacterial DNA isolated after 5 cycles of 2,4-D treatment at 10% moisture soil microcosm was used. Plasmid DNA was extracted from *P. pickettii* which was isolated from the same agricultural soil. The *tfdA* gene was used as probe.

Fig. 4. Detection of indigenous 2,4-D degrading soil bacteria not detected with the *tfdA* gene probe. Soil DNA samples (1.5μg) from 25% moisture soil microcosm were digested with *Hind*III, size-fractionated by agarose gel electrophoresis, then transferred to nitrocellulose hybridization membrane. A 6.5kb fragment derived from the large plasmid of *P. paucimobilis* was used as probe. 0 : soil DNA sample of time zero. 1, 2, 3, 4 and 5 : soil DNA samples after 1, 2, 3, 4 and 5 treatments of 2,4-D (+ : 2,4-D treated, – : no 2,4-D controls), respectively.

Fig. 5. Comparison by DNA probe hybridization of soil bacterial DNA and *P. paucimobilis* DNA digested with three different restriction enzymes. Soil bacterial DNA isolated after 5 cycles of 2,4-D treatment at 25% moisture soil microcosm was used. A 6.5kb fragment derived from the large plasmid of *P. paucimobilis* was used as the probe.



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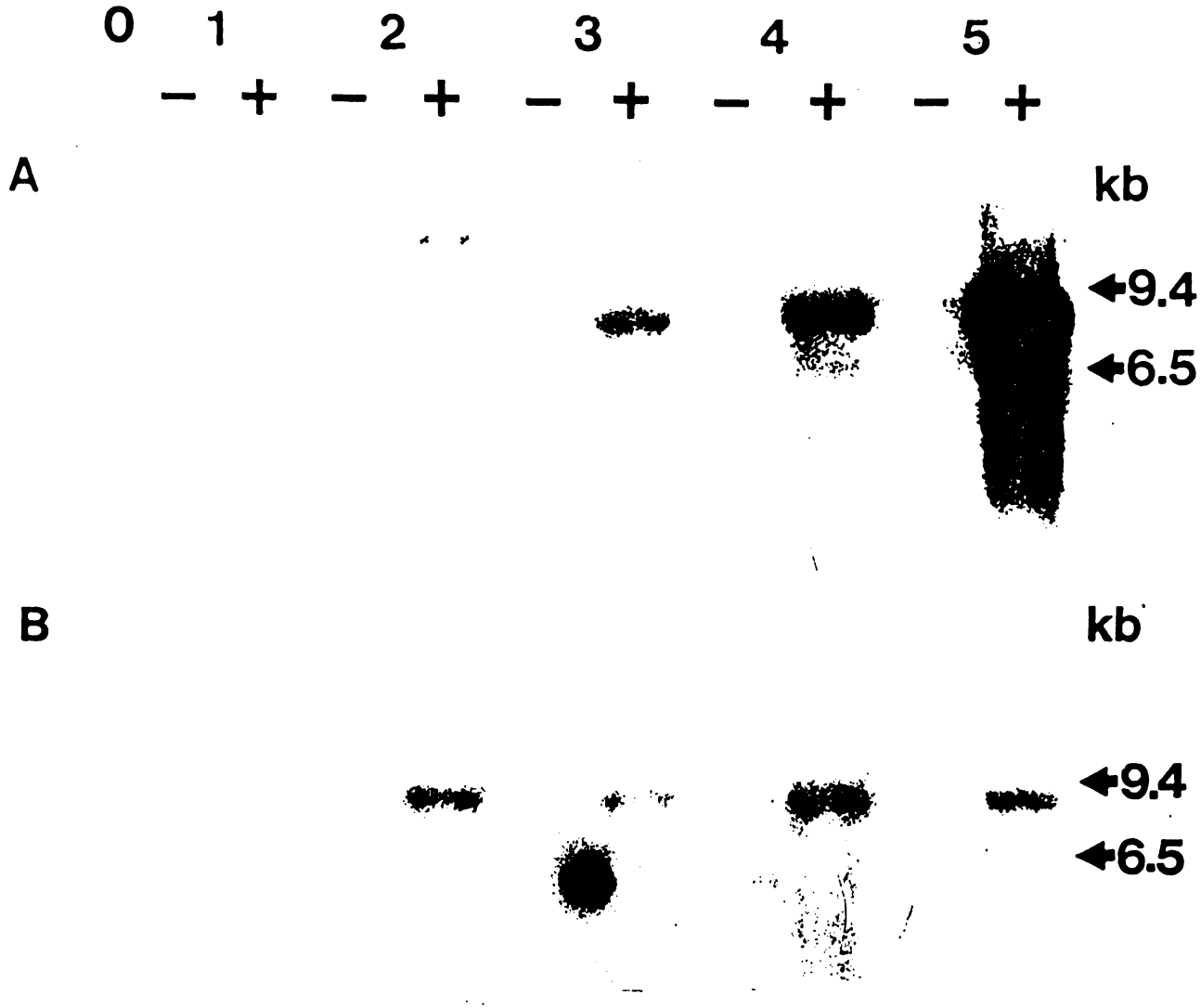

Figure 2.

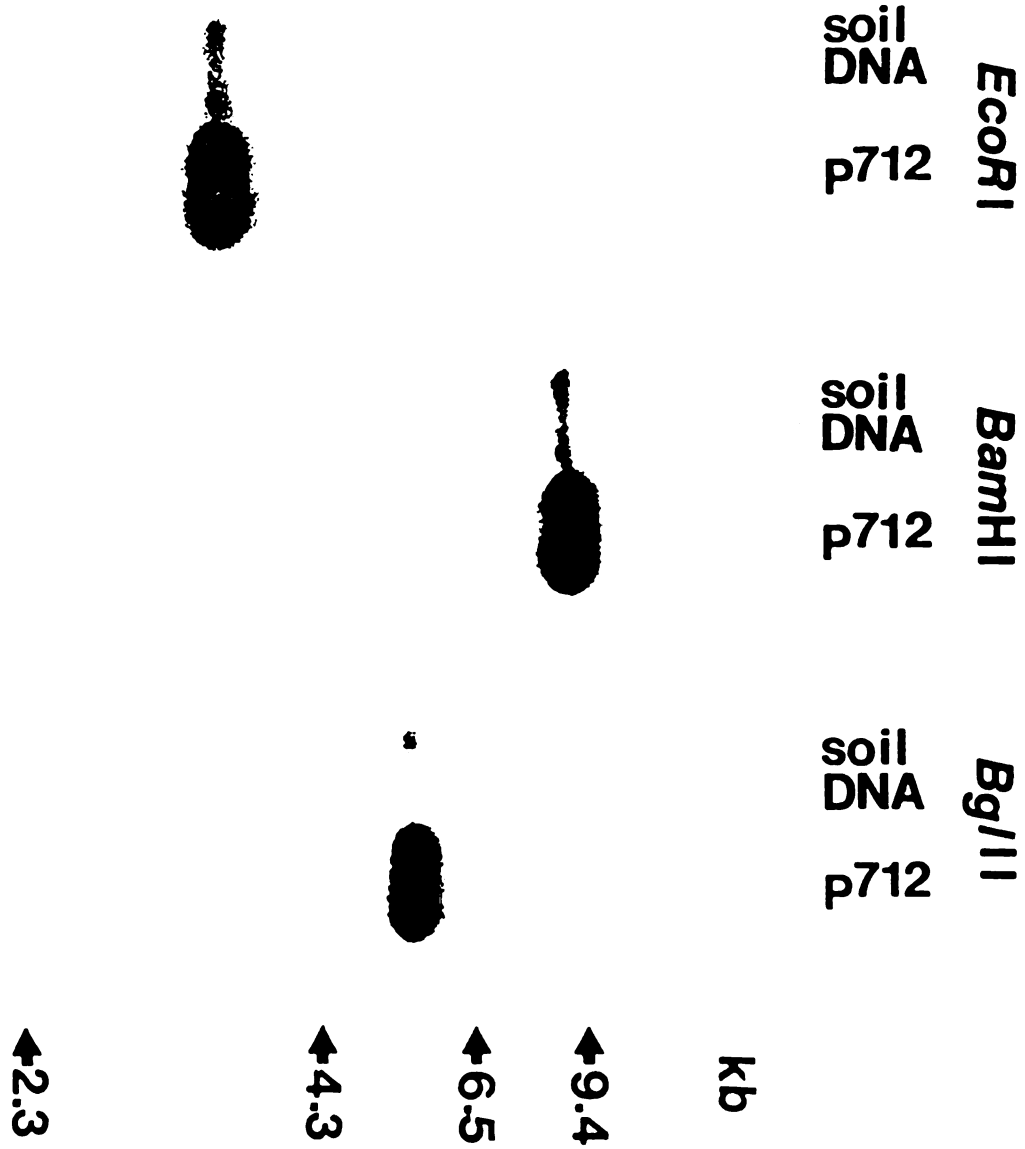
EcoRI
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Figure 3.

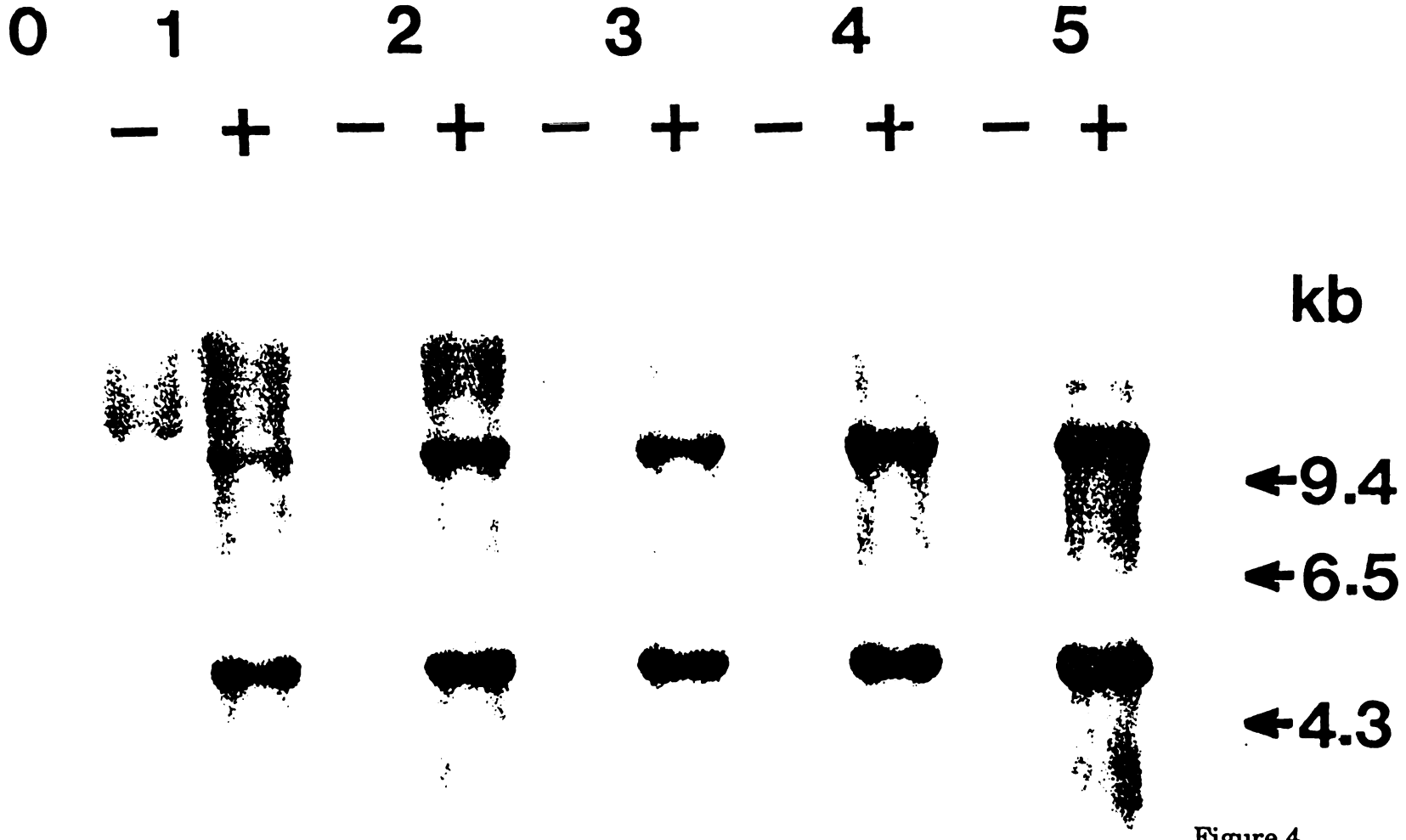
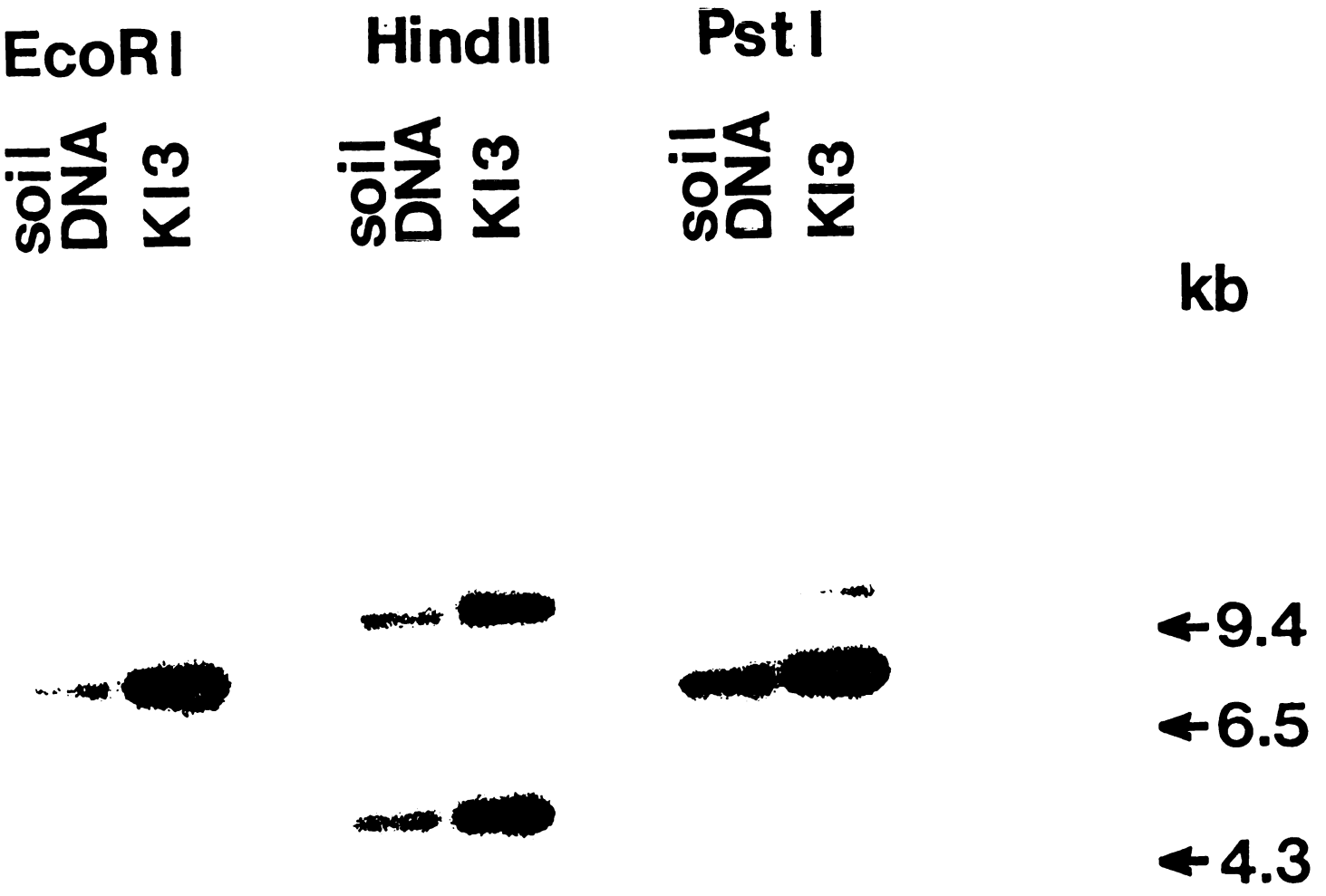


Figure 4.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HindIII
PstI
soil DNA
KI3
soil DNA
KI3
soil DNA
KI3
kb
9.4
6.5
4.3

Figure 5.

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Chapter Four

Gene Probe Analysis of Competition among 2,4-D-degrading Bacteria in Soil under Selective Conditions.

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ABSTRACT

Competition among indigenous and inoculated 2,4-D degrading bacteria was studied in a native Kansas prairie soil following 2,4-D additions. The inoculated soil received four different 2,4-D degrading strains at densities of ~10^3 cells/g soil : *Pseudomonas cepacia* strain DBO1/pJP4 ; and three Michigan soil isolates identified as *Pseudomonas pseudomallei*, *Pseudomonas paucimobilis* and *Pseudomonas pickettii*. The soil community DNA samples were analyzed on Southern blots using a *tfdA* gene probe and also a 6.5 kb probe derived from *P. paucimobilis* since this strain has no homology to *tfd* genes. *P. cepacia* DBO1/pJP4, a constructed strain, outcompeted both of the other added strains and the indigenous 2,4-D degrading bacteria. *P. paucimobilis* was detected as a secondary dominant and *P. pseudomallei* and *P. pickettii* were not detected. In contrast to the outcome of competition experiments in soil, relative fitness coefficients determined in axenic broth cultures indicated that *P. pseudomallei* along with *P. cepacia* DBO1/pJP4 should have been the best competitors. These parameters predicted the outcome of competition in soil for some but not all strains. Growth in mixed broth culture enhanced the growth of the slower growing strains principally by reducing the lag time over that found in axenic culture. Plasmids containing the 2,4-D pathway were important determinants of competitiveness since pKA4 in *P. cepacia* DBO1 had the slower growth characteristics of its original host, *P. pickettii*, rather than the more rapid growth found when this strain harbors pJP4.

INTRODUCTION

Microorganisms seldom exist in complete segregation from others but interact both positively and negatively in their habitats. One of the major goals of microbial ecology is to understand how interactions between microbial species influence their survival and abundance in nature. Among the various types of interactions between microbial populations (18), competition for carbon is often the major determinant of the relative abundance of the indigenous organisms in the soil environment (3, 20). Pesticides and other xenobiotic compounds are new carbon compounds that have been introduced into the environment during past several decades. Some of these compounds are good carbon sources for soil microorganisms capable of their degradation. 2,4-D, for example, is known to be a growth substrate for a number of different soil microorganisms (4, 14, 16, 17). Since these compounds are usually not present in nature and their supply is under human control, they make good models to study microbial resource competition in soil.

Batch liquid culture and chemostats have provided the basic background for understanding the principles of microbial competition for common carbon sources (9, 10), but it is not clear that we can easily extrapolate this information to predict the outcome of competition in the poorly mixed soil habitat. Furthermore, competition studies in soil have been difficult to perform due to limited techniques for enumeration of different competiting organisms. DNA probe methods offer the potential advantage of being able to distinguish and identify a number of competiting organisms in the inoculated as well as native communities without the requirement for prior selective culture.

In this study, DNA gene probes were used to study competition for the 2,4-D growth substrate among inoculated and indigenous 2,4-D degrading bacteria in a native prairie soil which has no history of cultivation or agricultural chemical use. In addition, batch liquid cultures were used to study the growth patterns of 2,4-D degrading bacteria in axenic and mixed cultures and the result was compared with that from the soil competition experiment. Finally, we evaluated the effects of different 2,4-D degradative plasmids on both the growth patterns and the competitive advantage of the host bacteria.

MATERIALS AND METHODS

Bacteria and soil. The bacterial strains used or isolated in this work and any sequence homology to *tfd* genes from plasmid pJP4 are described in Table 1. *P. cepacia* DBO1/pKA4 was obtained through filter mating between *P. cepacia* DBO1 and *P. pickettii* 712/pKA4. All *tfdA* hybridizing strains were distinguishable by unique restriction fragments.

The soil used in this study was from the Konza Prairie near Manhattan, Kansas. This is the largest native prairie area remaining in the U. S. (3,400 hectare tract), and has never been cultivated or treated with agricultural chemicals. MPN experiments indicated a low background of native 2,4-D degraders in this soil, ~30 cells/g. This soil was also selected because it should provide a microbial environment dissimilar to the one from which the inoculated strains were isolated, and therefore competition between indigenous strains and invaders (perhaps less fit) could be evaluated. The inoculated strains are from the KBS soil which was formed from glacial till in a humid region and under cultivated agriculture, while the Konza soil is

derived from layered limestone and shale in a semi-arid region and under native grasses.

Media and culture conditions. All strains were maintained on MMO mineral medium (19) plus 500 ppm of 2,4-D. Strains to be inoculated in soils and flasks were cultured to full growth at 30°C in Luria broth and then reinoculated in fresh Luria broth (1:50) for an overnight culture. Enumeration of total viable cells was accomplished by plating appropriate dilutions of soil suspensions onto PTYG agar, which contained 0.25 g of Bacto-peptone, 0.25 g of Bacto-tryptone, 0.5 g of yeast extract, 0.5 g of glucose, 0.03 g of $MgSO_4$, 0.003 g of $CaCl_2$ and 15 g of agar per liter. Strains for plasmid isolation were cultured in MMO mineral medium with 500 ppm of 2,4-D. Plasmids were isolated by the method of Hirsch et. al.(5).

Inoculation and sampling of bacteria in soil. The above overnight cultures of four different 2,4-D degrading bacteria, *P. cepacia* str DBO1/pJP4, *P. pseudomallei* 745, *P. paucimobilis* 1443 and *P. pickettii* 712, grown at 30°C were harvested by centrifugation (10,000 x g, 10 min., 4°C), washed twice with an equal volume of sodium phosphate buffer (15 mM, pH 7.0), and again collected by centrifugation. The cells were resuspended in 1/10 volume of sodium phosphate buffer and left at 4°C for 5 h.

Konza Prairie soil was sifted through a 2-mm (square aperture) sieve, adjusted to a water content of ~35%, and inoculated with each of four different species to a final concentration of ~1.5 x 10^3 cells/g soil. The soil was thoroughly mixed and 500 g was transferred to each of three replicate polyethylene wide-mouth bottles (microcosms I - III). Three additional replicates were not inoculated with 2,4-D degrading bacteria. Inoculated and uninoculated soils were treated with 2,4-D dissolved in 0.1 M NaH_2PO_4 buffer (pH 7.0) to a concentration of 250 ppm (mg/g) and thoroughly mixed.

The disappearance of 2,4-D in soil was monitored by HPLC (8) and the soils were respiked with 2,4-D (to 250 ppm) after its removal for each of 10 cycles of degradation.

Stresses of cold temperature and of starvation were imposed on two sets of microcosms at a later stage of treatment to evaluate the robustness of the community to other conditions. One inoculated and uninoculated soil microcosm was incubated at 4°C for 14 days immediately after the eighth addition of 2,4-D and then returned back to room temperature (microcosm II). In another microcosm from each group, the 2,4-D addition was delayed for 14 days after the end of the seventh degradation cycle before these two microcosms were re-subjected to the normal 2,4-D addition (microcosm III). At time zero and at the end of the second, the fifth and the tenth treatment of 2,4-D, a 10 g soil subsample from each microcosm was used for both MPN determination (6) and total viable counts on PTYG plates, and a 50 g soil subsample was used for isolation of total bacterial DNA by using the cell extraction method (7).

Batch culture experiments. The strains were cultured, harvested and prepared in sodium phosphate buffer as described above. Liquid culture studies were conducted in 250 ml-flasks containing 100 ml of 2,4-D minimal medium (500 ppm). Axenic culture studies were in duplicate and mixed culture studies in triplicate. Mixed cultures were inoculated at a ratio of 1 : 1 : 1 among these strains ; the initial population densities on PTYG plates were ~7 $x10^5$ cells/ml and ~1 x 10^6 cells/ml in the two experiments. Individual strains were discriminated and counted based on distinctive colony morphology. All cultures were incubated at 30°C and aerated by shaking at 200 rpm in a New Brunswick G24 environmental incubator shaker (New Brunswick Scientific Co., New Brunswick, NJ).

Probe preparation. The *tfdA* gene and a 6.5 kb fragment of *P. paucimobilis* were used as probes to detect and discriminate the inoculated and the indigenous 2,4-D degrading bacteria by Southern blot. The *tfdA* gene probe was cloned as a 556 bp *EcoR*I-*Bam*HI fragment from the well-known 2,4-D degradative plasmid pJP4 into the multiple cloning site of plasmid pUC19 (15). The 6.5 kb probe, which is specific for *P. paucimobilis* 1443, was cloned into pUC19 as a *Bam*HI restriction fragment from the large plasmid of this strain. Cloned plasmids were amplified and isolated by the method of Maniatis et. al. (15). These probes were isolated as restricted fragments from the corresponding cloned plasmid pUC19 on 0.7% agarose gel, purified with Geneclean kit (Bio 101 Inc, La Jolla, CA), and labeled with ^{32}P by using the Random Primed DNA Labeling kit (Boehringer Mannheim, Indianapolis, IN) or the Nick Translation kit (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's specifications. Labeled probes were separated from uninoculated nucleotides by spun column prior to use (15). The probe was used at approximately 10^7 cpm/10 ml of hybridization fluid.

Southern hybridization. Total soil bacterial DNA was digested with appropriate restriction endonucleases according to the manufacturer's specifications. Digested DNA was size-fractionated by electrophoresis through horizontal 0.7% agarose gel and transferred to nitrocellulose hybridization membranes (15). Prehybridization, hybridization and post-hybridization washes were performed as described by Holben et. al. (7) with some modifications. The membranes were prehybridized for at least 24 h at 42°C and after hybridization, three washes were carried out at room temperature, followed by one wash at 65°C. Hybridization signals were detected by autoradiography using Kodak X-omat AR film (Kodak,

Rochester, NY) exposed at -70°C with a Quanta III (Sigma, St. Louis, MO) intensifying screen. Exposure times were 1 to 4 days depending on the intensity of the radioactive signal.

RESULTS

Degradation of 2,4-D in soil. The degradation patterns of 2,4-D in Konza Prairie soil with and without the added 2,4-D degrading bacteria are shown in Figure 1. 2,4-D was quickly degraded without a lag period in inoculated soil maintained at a soil water content of ~35% and incubated at room temperature(Figure 1-A). Under these conditions, it took about 1 week for each addition of 250 ppm 2,4-D to be degraded completely throughout the 10 treatments. In microcosm II kept at 4°C for two weeks during the eighth treatment, the 2,4-D degradation rate was significantly retarded during this period, but it was restored to the preceding rate when microcosm was transferred to room temperature (data not shown). In microcosm III, where the addition of 2,4-D was delayed for two weeks after the seventh cycle, the 2,4-D degradation rate in subsequent treatments was not affected (data not shown).

Without added 2,4-D degrading bacteria, the exposure of indigenous 2,4-D degrading populations of Konza Prairie soil to 2,4-D for the first time resulted in a lag period of about two weeks prior to complete degradation in 3 weeks (Figure 1-B). The lag period was not observed following the second addition and the 2,4-D degradation rate was similar to that found for inoculated soil. In uninoculated microcosm soils incubated at 4°C for two weeks (or delayed in 2,4-D treatment), the 2,4-D degradation patterns in subsequent treatments were similar to those found for inoculated soils.

In soil inoculated with 2,4-D degrading bacteria, the initial population of 2,4-D degraders was 7.5 x 10^3/g soil (Figure 2). After the second treatment of 2,4-D, the population markedly increased to 1.8 x 10^8/g soil which was stably maintained throughout the subsequent 2,4-D treatments. In the uninoculated soil, the initial population detected by MPN was 3.1 x 10^1/g soil which increased to 3.1 x 10^7/g soil with two additions of 2,4-D and then was maintained at this level through subsequent additions of 2,4-D (Figure 2). Throughout the 10 treatments of 2,4-D, the population of 2,4-D degraders detected by MPN was higher by a factor of 6 in inoculated soil than in uninoculated soil.

Relationship of 2,4-D degrading populations to total viable counts.

Addition of 2,4-D as a specific carbon resource had a marked effect on the community structure (Table 2). In Konza Prairie soil, where the initial total viable counts were 7.8 x 10^7 and 4.8 x 10^7 cells/g soil in inoculated soil and uninoculated soil, respectively, the total viable count increased three to five-fold with repeated 2,4-D treatments in both soils (Table 2). By contrast, 2,4-D degrading populations increased 2.4 x 10^4-fold and 10^6-fold with repeated 2,4-D treatments in inoculated soil and uninoculated soil, respectively (Figure 2). Under this selective condition, the rapidly growing populations of 2,4-D degrading bacteria became dominant members of the total bacterial community, as indicated by the decreasing ratio of total viable count to 2,4-D degrading bacteria and the absolute increase in viable counts (Table 2).

In inoculated soil, the typical colonies of *P. cepacia* DBO1/pJP4 (irregular, white and large colonies)were always detected in greatest numbers on PTYG plates (Figure 2), suggesting that this strain was predominant throughout this experiment. The ratio of 2,4-D degrading bacteria to *P. cepacia* DBO1/pJP4 decreased from 5.0 to 0.9-1.4 indicating that this strain

dominated the 2,4-D degrading community as well as the total community (Table 2).

Probing total soil bacterial DNA. Total bacterial DNA isolated from the three replicate microcosm soils at the indicated times was hybridized to the ^{32}P-labeled *tfdA* gene probe after restriction digestion, gel separation and Southern transfer. In microcosm soils inoculated with the four 2,4-D degrading bacteria, a single DNA hybridization band was detected in the DNA isolated after the second, the fifth and the tenth treatment of 2,4-D, and no band was observed in the DNA sample from time zero (Figure 3-A). The size of the hybridized band obtained from total soil bacterial DNA digested with *EcoR*I was about 8.3 kb, which corresponds to a fragment containing the *tfdA* gene in plasmid pJP4 digested with the same enzyme. To confirm the origin of the band from soil, plasmid DNA of pJP4 and total soil bacterial DNA of the second treatment were digested separately with three different restriction enzymes and the size of bands that hybridized to the *tfdA* probe were compared (Figure 4-A). Matching band patterns were obtained between these two DNA samples, indicating that these hybridized bands corresponded to *P. cepacia* DBO1/pJP4 and that this strain predominated in this experiment. This result from hybridization experiments is consistent with that from plate counts on PTYG which indicated that *P. cepacia* DBO1/pJP4 was the predominant colony type among the added and indigenous 2,4-D degraders throughout the experiment.

When total soil bacterial DNA was digested with *Hind*III, subjected to Southern transfer and hybridized to the 6.5 kb probe, two hybridized DNA bands (5.0 kb and 11.3 kb in size) were obtained from DNA samples isolated after the second, the fifth and the tenth treatment of 2,4-D, and no band

was observed in the DNA sample from time zero (Figure 3-B). These two hybridized bands were also detected on Southern blots when *P. paucimobilis* 1443 DNA was digested with the same enzyme and hybridized to the same probe (8), suggesting that this strain produced the hybridization bands in the microcosm DNA. Since each lane contained the same amount of total DNA (1.5 μg), the weaker intensities (1/180 - 1/35) of these latter bands indicate that *P. paucimobilis* 1443 was maintained at a lower density than *P. cepacia* str DBO1/pJP4. This interpretation is further supported by the fact that the number of *P. paucimobilis* 1443 colonies on PTYG plates were not obvious.

In uninoculated soils, bands hybridizing to the*tfdA* probe began to appear only after five treatments of 2,4-D (Figure 5). This population was perhaps becoming prominant at this time since the bands were weak from two of the three microcosms. The intensities of bands from the microcosm with weak hybridization increased and became similar to that of the strong band of uninoculated microcosm I after the tenth treatment of 2,4-D. Total soil bacterial DNA from the tenth treatment and plasmid DNA of a new 2,4-D degrading bacterium, isolated from the same soil and designated strain K17, were digested separately with three different restriction enzymes, and compared (Figure 4-B). Matching band patterns were observed between these two DNA samples, suggesting that one of the indigenous 2,4-D degrading bacteria, K17, had become predominant in the uninoculated soil. No band was detected when the soil bacterial DNA was hybridized to the 6.5 kb probe.

Axenic growth characteristics of 2,4-D degrading bacteria in broth culture. To understand axenic growth patterns of 2,4-D degraders, each strain was inoculated into 2,4-D minimal medium under uninduced

conditions. The lag period, specific growth rate (2) and relative fitness coefficients (11) were measured (Table 3). *P. pseudomallei* 745, *P. cepacia* DBO1/pJP4 and K17 showed short lag periods (<15 h) and began to grow exponentially at about 20 h of incubation (Figure 6). By contrast, *P. paucimobilis* 1443 showed a longer lag period (~35 h) and *P. pickettii* 712 showed the longest lag period (>60 h). The specific growth rates of these bacteria during exponential growth were similar except for *P. pickettii* which was markedly lower.

The role of the host cell versus the plasmid in determining growth characteristics was evaluated by comparing growth of the same host with two different plasmids, and the same plasmid in two different hosts. *P. cepacia* DBO1 carrying plasmid pKA4 instead of plasmid pJP4, showed the lower growth rate of the plasmid in its original host (Table 3) and a lag time intermediate between the original host, *P. pickettii*, and the new host, *P. cepacia* (Figure 6). *P. pseudomallei* 745 showed the largest number of doublings during the first 30 h of incubation and had the highest fitness coefficient among these 2,4-D degraders, suggesting that it might be the best competitor.

Competition experiments in broth culture. The behavior of each 2,4-D degrading species in three member broth cultures was monitored by plate counts. When LB-grown *P. pseudomallei* 745, *P. cepacia* str DBO1/pJP4 and K17 were inoculated together into 2,4-D minimal medium at a ratio of 1 : 1 : 1, these 2,4-D degraders exhibited similar growth patterns (Figure 7-A). This result is consistent with that of the axenic growth experiment in which all of these strains showed a short lag period and rapid growth. In mixed cultures of *P. pseudomallei* 745, *P. paucimobilis* 1443 and *P. pickettii* 712 at a ratio of 1 : 1 : 1 (Figure 7-B), *P. pseudomallei* 745 multiplied quickly and

slightly outgrew the other two strains in the initial phase, but after 33 h of incubation its growth rate began to decline even though 65 % of the substrate remained. By contrast, *P. paucimobilis* 1443 initiated growth earlier in mixed culture than in axenic culture, thereby overcoming *P. pseudomallei* 745 in the last phase. This result was unexpected because, from the axenic growth experiment, *P. pseudomallei* 745 should have reached stationary phase and depleted the substrate before *P. paucimobilis* 1443 and *P. pickettii* 712 would have begun to grow. *P. pickettii* 712 also showed significant growth during the first 40 h of incubation in mixed culture, whereas it did not show any growth during 80 h of incubation in axenic culture.

To further analyze the stimulatory effect of co-culture on the strains that grew slowly in axenic culture, we grew the slowest growing strain, *P. pickettii* 712, under several cultural conditions (Figure 8). Since the above experiments included a nutritional shiftdown from LB medium to 2,4-D mineral medium, we evaluated whether strain 712 could shift to a 2,4-D pathway intermediate, succinate, in the same mineral medium. This occurred without significant lag (Figure 8) and produced a rapid doubling time (1.4 h). Growth in 2,4-D and transfer to 2,4-D resulted in continuous growth. Growth of inoculum on succinate and LB produced significant lag periods when shifted to 2,4-D, suggesting that induction of the 2,4-D pathway may be delayed. When culture filtrate from 2,4-D grown *P. pseudomallei* 745, a member of the stimulatory triculture, was added to the 2,4-D mineral medium, the lag time of *P. pickettii* 712 was reduced by one-third. While this does not achieve the shorter lag time found for the triculture, it does suggest that products of *P. pseudomallei* 745 do benefit this slow growing and otherwise non-competitive strain.

DISCUSSION

The study evaluated competition in three different environments : non-sterile soil, mixed broth culture and axenic culture, and among strains with different habitats of origin ; a laboratory host strain containing a plasmid isolated in Australia (1), three strains from Michigan agricultural soils, and one strain indigenous to the native Kansas prairie soil used in the study. The key features of these strains and their competitive performance are summarized in Table 4. Kinetic parameters such as μ_{max} and Ks, that underlie growth rate, are well-documented factors important to competition ; this has recently been illustrated for 2,4-D degrading strains (12). But, other factors such as induction period, adaptation to the environment, and crossfeeding of growth factors can also contribute to competitive outcome and were the focus of this study.

When four 2,4-D degrading organisms were added at equal densities to a soil from a different climatic region than their origin, primary and secondary dominant strains emerged, an outcome which was not entirely predicted from their fitness coefficients measured in axenic broth culture. *P. pseudomallei* 745, *P. cepacia* DBO1/pJP4 and K17 belong to the fast-growing group and thus were anticipated to outgrow both of the slow-growing bacteria, *P. pickettii* 712, and *P. paucimobilis* 1443 in soil. Although *P. pseudomallei* 745 had the highest fitness coefficient among these strains, it was not detected with the DNA probing method but instead the constructed strain, *P. cepacia* DBO1/pJP4, was observed as the predominant strain in this experiment based on both the plate counts and the hybridization results. K17, the indigenous 2,4-D degrading bacterium in Konza Prairie soil, was not detected on Southern blot throughout 10

treatments of 2,4-D in inoculated soil, whereas this strain was predominant in the uninoculated soil from the fifth treatment of 2,4-D. Considering that this strain has never been exposed to 2,4-D in the past and that its initial population density was lower by a factor of 50, it apparently had no chance for detectable growth because *P. cepacia* DBO1/pJP4 and *P. paucimobilis* 1443 likely consumed most of the 2,4-D. This outcome was not necessarily expected since it did have the highest specific growth rate on 2,4-D and should have been well adapted to the other conditions of its native habitat.

P. paucimobilis 1443 was detected as a secondary dominant on Southern blots throughout this experiment in inoculated soil. This strain showed an intermediate lag time in broth culture which was followed by a specific growth rate similar to those of *P. pseudomallei* 745, *P. cepacia* DBO1/pJP4 and K17. This suggests that *P. paucimobilis* 1443 may grow slowly initially but has the potential to catch up with other fast-growing bacteria. This potential was demonstrated in mixed broth culture where *P. paucimobilis* 1443 outgrew *P. pseudomallei* 745 in the later phase, and may have also led *P. paucimobilis* 1443 to a secondary dominance in the soil competition experiment. *P. pickettii* 712, which showed a long lag time followed by slow growth in axenic culture, was not detected on Southern blot throughout this experiment in inoculated soil. It is also noteworthy that *P. paucimobilis* 1443 shares no DNA sequence homology with the *tfd* genes that were presumably important to the competitive growth of *P. cepacia* DBO1, as well as the lesser growth of the other strains.

In uninoculated soil after the second 2,4-D treatment the population density of indigenous 2,4-D degrading bacteria reached levels which should be detected by DNA probes. However, no hybridization bands were detected on Southern blot after the second treatment, and after the fifth treatment

bands of weak intensity were seen in two of three microcosms while a strong band was seen in the third (Figure 5). This suggests that another 2,4-D degrading microbial population not detected with the available gene probes dominated in the initial phase and was replaced (or co-established) with K17 after the fifth treatment of 2,4-D.

The importance of interactions among competiting organisms on the outcome of competition is apparent when comparing patterns from the axenic broth culture with the results from the mixed broth cultures (Table 4). *P. paucimobilis* 1443 and *P. pickettii* 712 began to grow much earlier in mixed culture with *P. pseudomallei* 745 than in axenic culture, suggesting that some intermediates produced from *P. pseudomallei* 745 stimulated the growth of these two strains in mixed culture. One explanation is that pathway intermediates induced the 2,4-D pathway of these two less fit strains, thereby substantially reducing the long lag times. Another is that nutritional factors provided by crossfeeding aided growth on this less favorable substrate. Lag periods before 2,4-D degradation in soil are typical ; Loos (13) suggested this could be due to enzyme induction while Miwa and Kuwatsuka (16) felt it was related to the initial populations of 2,4-D degraders. An interesting result in these studies is how dominant lag time can be in determining fitness, but also how this can be compensated for by population interactions. The difference in outcome of competition in the broth vs soil experiments may be due to the fact that the rapid exchange of cell products in the mixed broth environment reduced the fitness difference while this did not occur in the unmixed, physically isolated niches in the soil environment.

Although coldness and starvation stresses were applied to replicate inoculated and uninoculated microcosms, these stresses were not enough to

change the composition of the 2,4-D degradative microbial communities. Soil microbial communities may be rather stable to typical environmental perturbations.

The impact of a different 2,4-D degradative plasmids on 2,4-D growth pattern of the host microorganism was noticeable. For example, *P. cepacia* DBO1 carrying pJP4 exhibited a short lag period followed by rapid growth, but the same strain carrying pKA4 instead of pJP4 showed a relatively long lag period followed by slow growth (Figure 6). The specific growth rate of *P. cepacia* DBO1/pKA4 (0.077) was similar to that of *P. pickettii* 712/pKA4 (0.078) and not that of *P. cepacia* DBO1/pJP4 (0.179). However, the lag time of *P. cepacia* DBO1/pJP4 was intermediate between that of *P. cepacia* DBO1 with pJP4 and *P. pickettii* with pKA4. Thus plasmid and plasmid-host interactions determined growth rate and lag time, respectively, key determinants of competitive outcome.

This research demonstrates that a xenobiotic compound is a useful model to study competition in soil as well as provide population information important to understanding biodegradation. The most interesting findings were that species interactions appear to affect competition to a different degree in soil vs broth perhaps making data derived from broth culture less predictive for the soil habitat, that superior competitiveness can be determined by a plasmid, and that non-native soil strains, and in fact a constructed strain not even originating from soil was the most successful competitor in soil.

ACKNOWLEDGMENTS

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Table 1. Bacterial strains and plasmids.

Strain	Plasmid	*tfd* genes which hybridize to plasmid	Original habitat of isolate
Pseudomonas cepacia DBO1/pJP4	pJP4	*A, B, C, D*	Laboratory[a]
Pseudomonas cepacia DBO1/pKA4	pKA4	*A*	Laboratory
Pseudomonas pseudomallei 745	pBS5	*A, B, C, D*	KBS soil, MI[b]
K17	pKO51	*A, B, C, D*	Konza Prairie soil, KS
Pseudomonas paucimobilis 1443	pBS3	None	KBS soil, MI[b]
Pseudomonas pickettii 712/pKA4	pKA4	*A*	KBS soil, MI[b]

[a] Provided by R. Olsen, Univ. of Michigan ; plasmid was isolated in Australia from an *Alcaligenes* strain (1).

[b] From LTER gene flow plot at the Kellogg Biological Station, Hickory Corners, MI.

Table 2. Comparison of total viable counts, 2,4-D degrading populations and numbers of *P. cepacia* DBO1/pJP4 in Konza Prairie soil.

Soil	Number of 2,4-D treatments	Total viable counts ($x10^7$/g)	Total viable counts per 2,4-D degrading bacterium	2,4-D degrading bacteria per *P. cepacia* DBO1/ pJP4
Inoculated soil	0	7.8	1.0×10^4	5.0
	2	41.4	2.3	0.9
	5	37.9	1.1	1.0
	10	21.2	1.0	1.4
Uninoculated soil	0	4.8	1.5×10^5	-
	2	25.6	8.8	-
	5	17.9	3.0	-
	10	12.3	2.7	-

Table 3. Growth characteristics of 2,4-D degrading bacteria in axenic broth culture[a].

Strain	Lag period	Specific growth rate	Relative fitness coefficient[b]
P. pseudomallei 745	<15h	0.173	1.0
P. cepacia DBO1/pJP4	<15h	0.179	0.95
K17	<15h	0.185	0.74
P. paucimobilis 1443	25 - 40h	0.181	0.03
P. cepacia DBO1/pKA4	25 - 40h	0.077	0.05
P. pickettii 712/pKA4	>60h	0.078	~0

[a] All values represent means from two independent broth cultures.

[b] Relative fitness coefficient determined in axenic broth culture according to reference (8), i.e., ratio of the number of doublings of each strain to that of *P. pseudomallei* under same conditions. The first 30 h of incubation was chosen at the time to evaluate relative fitness since the most rapidly growing strain, *P. pseudomallei,* stopped growing at this time due to depletion of substrate.

Table 4. Summary of key features of 2,4-D degrading strains and the outcome of competition in different environments.

Strain	Source	Hybridization to *tfd* genes	Competitive rank[a]		
			Axenic broth	Mixed broth	Soil
P. cepacia DBO1/pJP4	Constructed	A - D	1	-	1
P. pseudomallei 745/pBS5	Mich. soil	A - D	1	2	0
Strain K17 /pKO51	Native soil	A - D	1	-	0
P. paucimobilis 1443/pBS3	Mich. soil	none	2	1	2
P. pickettii 712/pKA4	Mich. soil	A	3	3	0

[a] 0 = present but not detected ; - = not included in this triculture competition.

Figure legends

Fig. 1. Degradation of 2,4-D in soil during 10 repeated additions in microcosms inoculated with 2,4-D degrading bacteria (A) and with only indigenous 2,4-D degrading bacteria (B).

Fig. 2. Change in number of soil microorganisms in response to repeated additions of 2,4-D. MPNs of 2,4-D degraders in inoculated soil (—Δ—) and in uninoculated soil (—▲—). Viable counts of *P. cepacia* DBO1/pJP4 on PTYG (···✱···). Total viable counts on PTYG in inoculated soil (—o—) and in uninoculated soil (—●—).

Fig. 3. Hybridization of labeled *tfdA* gene probe (A) and 6.5 kb fragment (B) with total soil bacterial DNA from three replicate inoculated soils. Lanes : Total soil bacterial DNA of time zero (0) and after the second (2), the fifth (5), and the tenth (10) treatment of 2,4-D. Each lane contains 1.5 µg of total soil bacterial DNA digested with *EcoR*I (A) and *Hind*III (B).

Fig. 4. Comparison by DNA probe hybridization of total soil bacterial DNA and plasmid DNA digested with three different restriction enzymes. Lanes : Total soil bacterial DNA of the second treatment of inoculated microcosm I (1, 3 and 5) and plasmid pJP4 DNA of *P. cepacia* DBO1/pJP4 (2, 4 and 6), total soil bacterial DNA of the tenth treatment of uninoculated microcosm I (7, 9 and 11) and plasmid DNA of strain K17 (8, 10 and 12). DNA was digested with *EcoR*I (1, 2, 7 and 8), *Hind*III (3, 4, 9

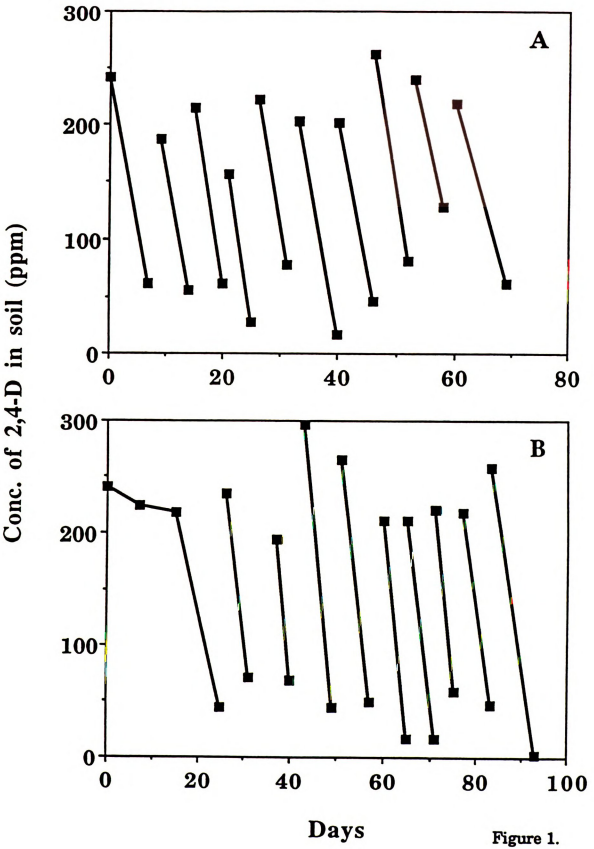
and 10), and *BglII* (5, 6, 11 and 12).

Fig. 5. Hybridization of labeled *tfdA* gene probe with total soil bacterial DNA from three replicate uninoculated soil microcosms. Lanes : Total soil bacterial DNA of time zero (0) and after the second (2), the fifth (5), and the tenth (10) treatment of 2,4-D. Each lane contains 1.5 μg of total soil bacterial DNA digested with *HindIII*.

Fig. 6. Growth patterns of 2,4-D degrading bacteria in axenic culture. *P. pseudomallei* 745 (—■—), *P. cepacia* DBO1/pJP4 (—▲—), K17 (—●—), *P. paucimobilis* 1443 (—○—), *P. cepacia* DBO1/pKA4 (—△—), and *P. pickettii* 712/pKA4 (—□—). Each point represents an average value of replicate flask cultures.

Fig. 7. Competition for 2,4-D (A) among *P. pseudomallei* 745 (—■—), *P. cepacia* DBO1/pJP4 (—□—) and K17 (—△—), and (B) among *P. pseudomallei* 745 (—■—), *P. paucimobilis* 1443 (—○—) and *P. pickettii* 712 (—▲—) in mixed liquid culture. Each point represents an average value of three replicate broth cultures.

Fig. 8. Effect of culture conditions on enhancing the growth of *P. pickettii* 712. Lines are labeled with medium on which the inoculum was grown followed by the growth medium. *P. cepacia* DBO1/pJP4 is shown for comparison.



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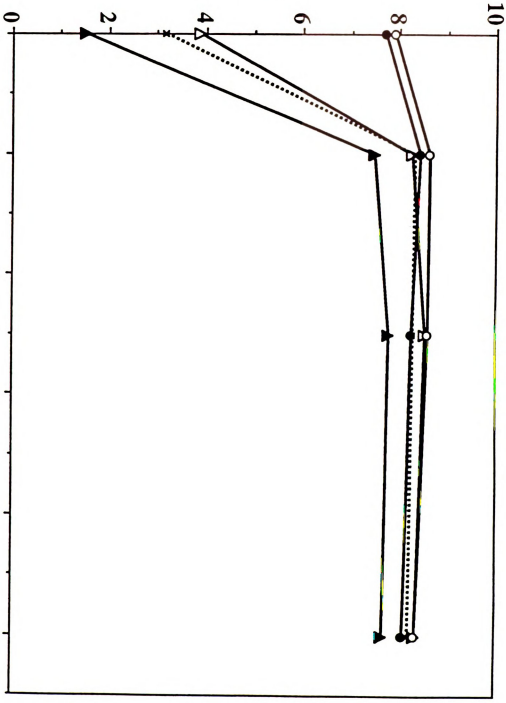

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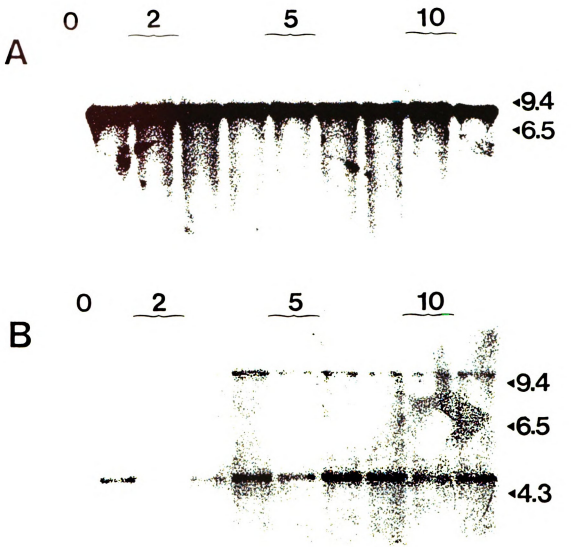

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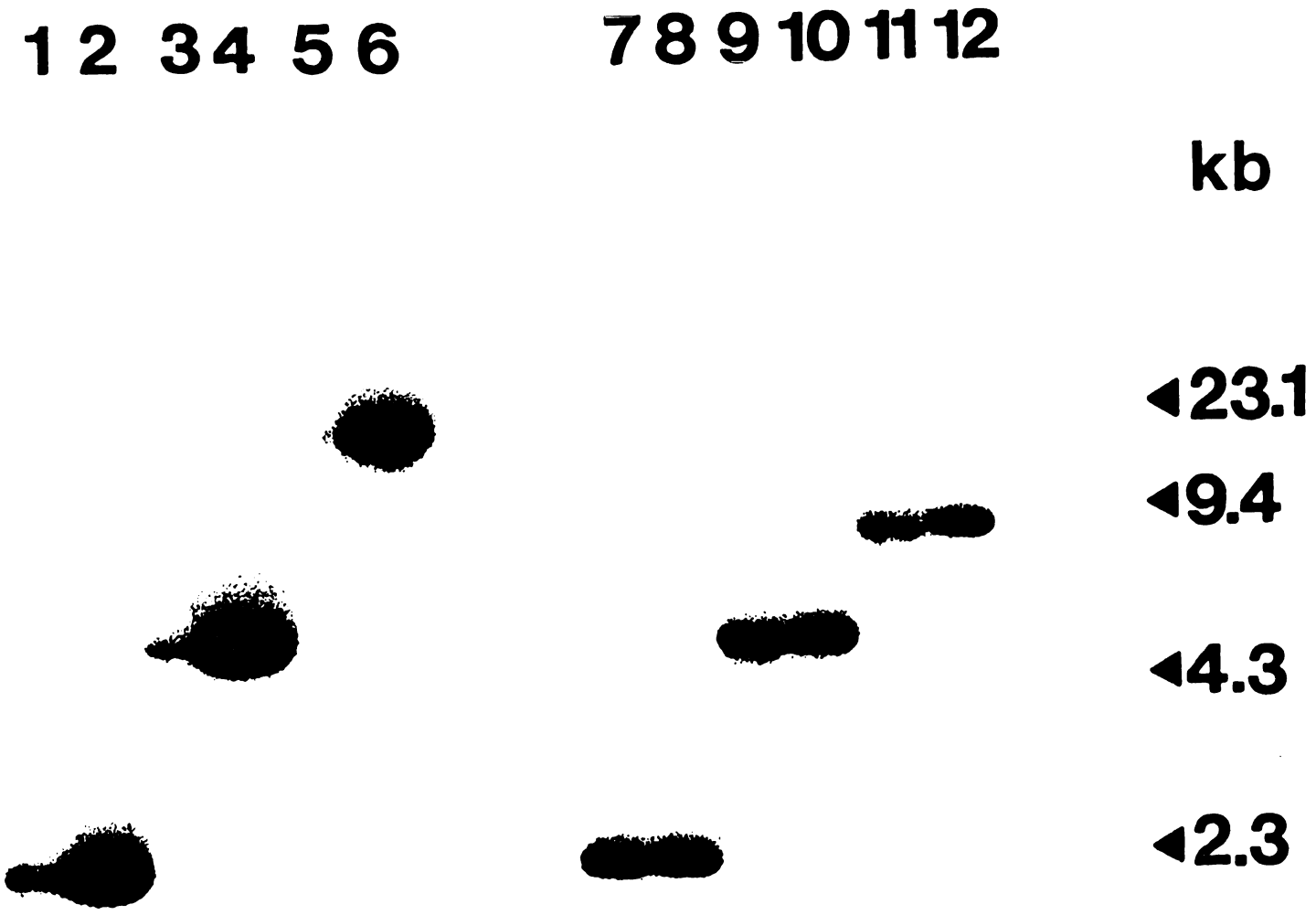

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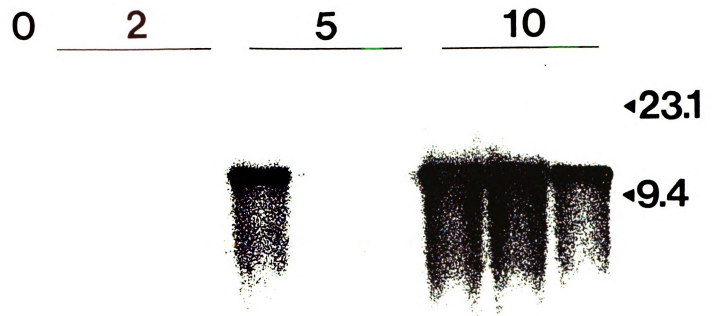

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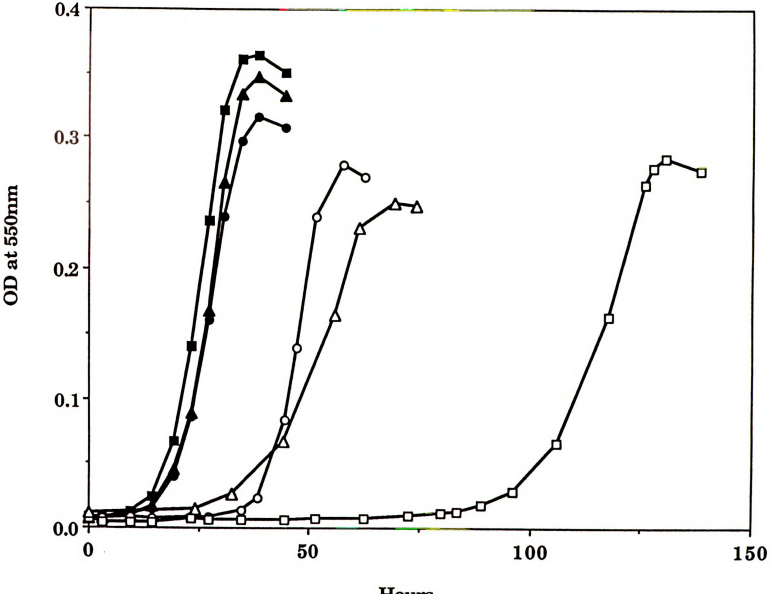

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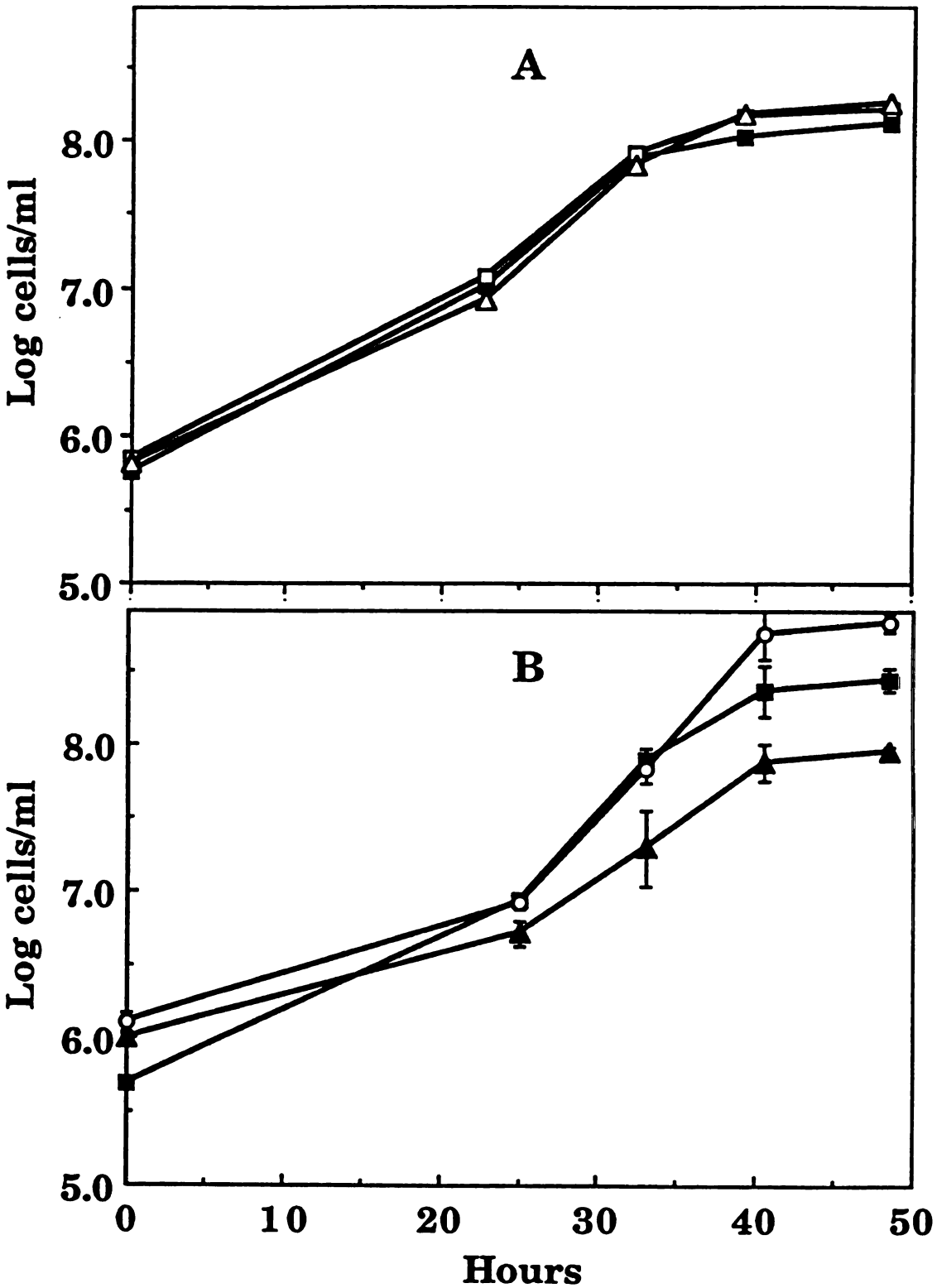

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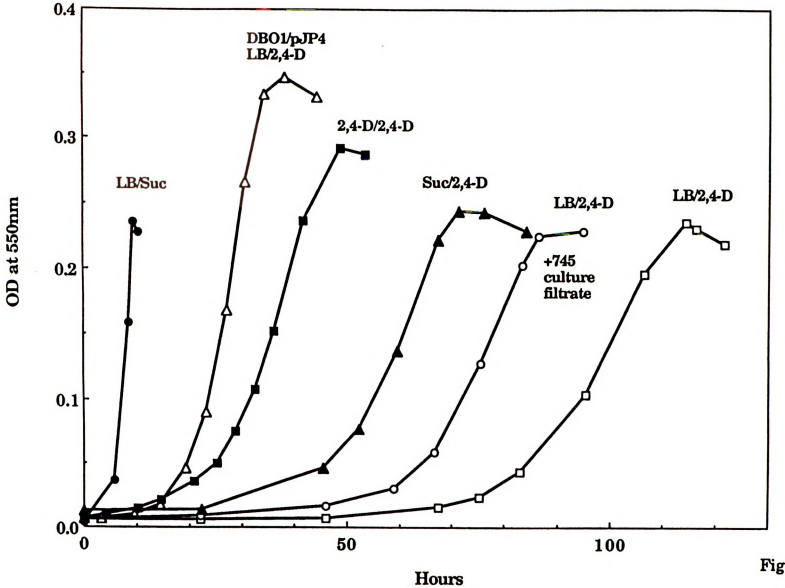
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culture
filtrate
LB/2,4-D

Figure 8.

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Chapter Five

Integration and Excision of a 2,4-D Degradative Plasmid in *Alcaligenes paradoxus* and Evidence of Its Natural Intergeneric Transfer

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ASTRACT

A self-transmissible 2,4-D degradative plasmid, pKA2, has been identified in a new 2,4-D degrading strain, *Alcaligenes paradoxus* 2811P, isolated from agricultural soil. pKA2 occurred as a 42.9 kb plasmid in strain 2811P. However, a derivative strain 2811C was isolated from glycerol stock culture of 2811P kept at -20°C for two years. In *A. paradoxus* 2811C, the whole plasmid pKA2 was integrated into the host chromosome without loss of 2,4-D$^+$ phenotype, as suggested by disappearance of a free plasmid DNA band, shifting of the hybridization signal with the *tfdA* gene probe to the chromosome, nontransmissibility, and Southern hybridization results with plasmid and whole cell DNA. Furthermore, we demonstrated that the integrated plasmid could be excised either precisely or in a different way. Another new 2,4-D degrading strain, *Pseudomonas pickettii* 712, which was isolated from the same plot but at a different time, was found to carry a nearly identical plasmid to pKA2. The plasmid of this strain, pKA4, is 40.9 kb and shares common features with pKA2 such as high self-transmissibility and hybridization only to the *tfdA* gene among 2,4-D metabolic genes of the 2,4-D degradative plasmid pJP4. The similar profiles of restriction endonuclease-generated fragments and the genetic homology shown by Southern hybridization between two plasmids indicate that these two 2,4-D degradative plasmids are closely related to each other, thus suggesting the occurrence of intergeneric gene transfer in natural environments.

INTRODUCTION

A number of catabolic plasmids have been isolated and described which enable host microorganisms to utilize xenobiotic compounds as their carbon and energy source. Most catabolic plasmids exist as extrachromosomal genetic elements that replicate autonomously. However, there have been some reports that catabolic gene recombination occurs between the chromosome and plasmids under selective conditions. This is well documented with chloroaromatic degradative genes on the *Pseudomonas* TOL plasmid ; these are excised occasionally by reciprocal recombination between the direct repeats (15) and integrated into the host chromosome (2, 10, 21). The integrated genes are shown to be rescued by plasmids R2 and pMG18 to form novel recombinants (10).

For the herbicide 2,4-D, all or most of the genes coding for the degradative enzymes have been reported to be contained on plasmids (3, 4, 17, 18). Unlike the aromatic hydrocarbon degradative genes on TOL (2, 10, 21, 24) and in *Alcaligenes* BR60 (27), 2,4-D degradative genes have not been shown to move either between chromosomal DNA and plasmid DNA or between plasmids in microorganisms which degrade 2,4-D. Furthermore, there have been no reports that the entire catabolic plasmid can be integrated into the host chromosome and then be excised to form a free plasmid depending on the culture conditions. This phenomenon could play an important role in persistence and efficient gene expression of plasmid DNA in environments where resources fluctuate as well as in the evolution of plasmids and bacterial chromosomes.

The increasing use of xenobiotic compounds may be stimulating a more widespread dissemination of the corresponding degradative plasmids among

microbial populations in nature. The transfer of catabolic plasmids, such as the 2,4-D degradative plasmid pJP4 (5), on agar media is well known. However, naturally occurring horizontal gene transfer between bacterial populations is difficult to detect due to the absence of the appropriate means for investigating such events in open environments. The horizontal transfer of genes responsibile for 3-chlorobenzoate degradation in a natural aquatic community was suggested in a microcosm study, where the conditions in a shallow bay were simulated (6). Physically and genetically indistinguishable 2,4-D degradative plasmids were detected in different species of *Alcaligenes* isolated independently from soil (3), which provides indirect evidence of natural gene transfer. Nonetheless, there have been few reports that supported degradative gene exchange between different genera in open environments.

In this paper we present the evidence of chromosomal integration of an entire 2,4-D degradative plasmid and demonstrate that it can be excised either precisely or in a different way. In addition, we present evidence of natural intergeneric transfer of this plasmid between *A. paradoxus* and *P. pickettii* strains that were isolated from the same soil at two different times.

MATERIALS AND METHODS

Bacterial strains. Strains 2811P and 712 were isolated from the Long-Term Ecological Research (LTER) site of Kellogg Biological Station (KBS, Hickory Corners, MI) by enrichment for growth on 2,4-D as the sole source of carbon and identified as *Alcaligenes paradoxus* and *Pseudomonas pickettii*, respectively, by FAME analysis (11). Strain 2811C was isolated from a glycerol stock culture of strain 2811P kept at -20°C for two years and was

reconfirmed as *Alcaligenes paradoxus* by FAME. *Pseudomonas cepacia* DBO1, which was obtained from Dr. R. Olsen (Univ. of Michigan), has Tn 5 transposon and is resistant to kanamycin (75 mg/ml), bacitracin (50 mg/ml) and carbenicillin (50 mg/ml).

Media and growth conditions. Peptone-tryptone-yeast extract-glucose (PTYG) broth consisted of 0.25 g of peptone (Difco Laboratories, Detroit, MI), 0.25 g of tryptone (Difco), 0.5 g of yeast-extract (Difco), 0.5 g of glucose, 0.03 g of magnesium sulfate, and 0.003 g of calcium chloride. Solid medium containing 1.5 % (wt/vol) agar was used for strain purification. MMO mineral medium (22) plus 500 ppm of 2,4-D was used to cultivate 2,4-D degrading strains to detect and isolate plasmid DNA. All cultures were incubated at 30°C. Broth cultures were aerated by shaking at 200 rpm in a New Brunswick G24 environmental incubator shaker (New Brunswick Scientific Co., New Brunswick, NJ).

DNA isolation. Plasmid DNA was isolated by using the procedure of Hirsch et. al. (7). For the detection of plasmid DNA, cells were lysed as described by Kado and Liu (12) with some modifications. 2,4-D degrading bacteria were cultivated in 5 ml of MMO mineral broth containing 500 ppm of 2,4-D at 30°C up to full growth. Cells were harvested by microcentrifugation (14,000 rpm for 1 min), washed with 1 ml of sterilized distilled water and repelleted in a 1.5 ml Eppendorf microcentrifuge tube. The pellet was resuspended in 30 ul of distilled water and lysed by adding 120 ul of lysing solution (50 mM Tris base, 3 % sodium dodecyl sulfate, pH 12.6). The solution was incubated at room temperature for 15 min, then heated at 80°C for 1 min in a water bath, and extracted with one volume of phenol-chloroform solution (1:1 vol/vol) saturated with TE buffer (10 mM Tris base, 1 mM EDTA, pH 8.0). The lysate was incubated overnight at room temperature. After microcentrifugation

(14,000 for 15 min), the aqueous phase was transferred into a new Eppendorf tube and mixed with 1/5 volume of 5x loading dye (14). Samples of 110 ul were subjected to electrophoresis in 0.7 % (wt/vol) agarose in Tris-acetate buffer (40mM Tris-acetate, 1mM Na_2-EDTA). Gels were photographed on a model Foto/Prep I DNA Transilluminator (Fotodyne Inc.) with type 55 positive-negative film (Polaroid Corp.). Chromosomal DNA was isolated by the procedure of Watson et. al. (20) and subsequently purified by cesium chloride-ethidium bromide ultracentrifugation (14).

Southern hybridization. To study hybridization patterns with 2,4-D metabolic genes (*tfdA*, *tfdB*, *tfdC*, and *tfdD*), plasmid and chromosomal DNA were prepared in crude lysate as described above, separated on horizontal 0.7 % agarose gel, transferred to nitrocellulose hybridization membranes (14), and hybridized with *tfd* gene probes. To analyse the restriction fragment profile, purified plasmid DNA and chromosomal DNA were digested with appropriate restriction endonucleases according to the manufacturer's specifications. Digested DNA was size fractionated by electrophoresis through horizontal 0.7 % (wt/vol) agarose gel and transferred to nitrocellulose hybridization membranes (14). As probes, internal segments of the *tfd* metabolic genes (8) and 23S rRNA gene (19) were labeled with ^{32}P by using the Random Primed DNA Labeling kit (Boehringer Mannheim, Indianapolis, IN). The plasmid DNA probe was labeled with ^{32}P by using the Nick Translation kit (Boehringer Mannheim). Prehybridization, hybridization and post-hybridization washes have been previously described (9). Hybridization signals were detected by autoradiography using Kodak X-omat AR film (Kodak, Rochester, NY) exposed at -70°C with a Quanta III (Sigma, St. Louis, MO) intensifying screen. If necessary, bound probe was stripped from the

membranes prior to rehybridization by washing for 15 min, 3 times with boiled distilled water containing 0.1 % sodium dodesyl sulfate (w/v).

Conjugation. Matings were performed on membrane filters as described by Willetts (26). Cells were grown in PTYG broth. An exponential broth culture (0.5 ml) of the donor strain was mixed with 1.0 ml of the recipient culture (*P. cepacia* DBO1, exponential phase) in an Eppendorf tube. Cells were pelleted, resuspended in 50 ml of PTYG media and spread onto a filter membrane (0.45 um pore-size) which was placed on PTYG agar. The filter was incubated overnight at 30°C and immersed in 2 ml of saline (0.85 % NaCl). Cells were resuspended by vortexing, diluted appropriately, and plated on 2,4-D minimum selective agar containing MMO mineral medum, 500 ppm of 2,4-D, kanamycin (75 mg/ml), bacitracin (50 mg/ml), carbenicillin (50 mg/ml), and 1.5 % Noble agar. Transconjugants were purified and their plasmid content was analyzed by agarose gel electrophoresis of crude lysates. The donor viable count was measured by plating dilutions on PTYG plates and counting the distinctive colonies of the donor. The frequency of transfer was calculated as the number of exconjugants per donor cell.

RESULTS

Properties of 2811P, 2811C and 712. Strains 2811P and 712 were isolated from the same plot at different times ; they contained a 2,4-D degradative plasmid pKA2 and pKA4 (Figure 1-A and Table 1), respectively, and grew with 2,4-D as the sole carbon source. Strain 2811C, a derivative of strain 2811P, appeared not to have any free plasmid (lane 3, Figure 1-A), but had the whole pKA2 plasmid DNA integrated into chromosome. The presence of a free plasmid in 2811C could not be demonstrated even by pulse field

electrophoresis (23). Southern blot hybridization with 23S rRNA gene probe to the total DNA of strains 2811P and 2811C confirmed that these were identical strains as indicated by matching band patterns (Figure 2) except for the different location of a 2,4-D degradative plasmid. Southern blot hybridization with 2,4-D metabolic genes (*tfdA*, *tfdB*, *tfdC*, and *tfdD*) to the plasmid DNA and chromosomal DNA of these three strains revealed that these strains had sequence homology only with the *tfdA* gene (Table 1) and that the homologous sequence was located on plasmid DNA in strains 2811P and 712, whereas it was located on the chromosomal DNA in strain 2811C (Figure 1-B). This indicated that the plasmid DNA not detected in the agarose gel was integrated into the host chromosome in strain 2811C.

These three strains showed a relatively long lag time (>50 h) and slow growth in 2,4-D minimal medium under uninduced condition (Figure 3). Strain 2811C had relatively longer lag time than strain 2811P in 2,4-D medium, presumably due to the chromosomal location (i. e., lower copy number) of a 2,4-D degradative plasmid. On the other hand, strains 2811P and 712, which were identified as different genera but had a nearly identical 2,4-D degradative plasmid, showed similar growth patterns in 2,4-D medium.

Plasmid transfers. The possibility of transfer of the 2,4-D degradative genes was tested by filter mating. In three independent experiments, transfer of pKA2 from strain 2811P to *P. cepacia* DBO1 was obtained at an average frequency of 7.7×10^{-3} per donor colony formed after the mating period (Table 2). Under similar conditions, the 2,4-D$^+$ phenotype was not transferred at a detectable frequency ($<10^{-9}$) with strain 2811C, supporting the interpretation that the 2,4-D genes were not contained on a free conjugative plasmid in this strain. Plasmid pKA4 of strain 712 was transferred at an average frequency of 1.0×10^{-2} per donor cell (Table 2).

Plasmid bands exhibiting identical electrophoretic mobilities were observed in agarose gels of the transconjugants and their respective donors (arrowheads, Figure 4). A cryptic plasmid DNA band of recipient (*P. cepacia* DBO1) was also observed in agarose gels of the transconjugants and recipients.

Physical evidence for the integration of plasmid into chromosomal DNA. To investigate the fate of pKA2 in strain 2811C, plasmid pKA2 and total DNA of strain 2811P and strain 2811C were digested with two different restriction enzymes separately, and analyzed on Southern blots hybridized with ^{32}P-labeled pKA2 plasmid DNA (Figure 5). As expected, all the seven hybridized fragments of pKA2 (Figure 5) were also observed in the *EcoR*I-digested total DNA of strain 2811P that contained the same plasmid, in addition to an unexpected novel fragment (CA, Figure 5-A). The novel fragment was not observed in the digested plasmid DNA but observed in the digested total DNA of both 2811P and 2811C. For this reason, it was believed to be derived from the digested host chromosomal DNA. This was further supported by the fact that its radioactive signal was much weaker than the signal with the plasmid DNA fragment of similar size due to its lower copy number in total DNA. On the other hand, in *EcoR*I-digested total DNA of strain 2811C, one fragment (ED) disappeared with concomitant appearance of three extra hybridized fragments (arrowheads, Figure 5-A). This observation indicates that the whole pKA2 plasmid was integrated into the host chromosomal DNA without the loss in 2,4-D$^+$ phenotype, although normally the appearance of only two additional bands is expected with the loss of one band with chromosome integrations. The loss of one hybridized fragment (HB) with concomitant appearance of three novel fragments (arrowheads) was also observed in *Hind*III digests (Figure 5).

Excision of pKA2 plasmid. The existence of pKA2 plasmid as both an independent plasmid in 2811P and a chromosomally integrated plasmid in 2811C, together with the observed difference in their growth patterns on 2,4-D medium, suggested that the population containing a free plasmid might be derived from strain 2811C and that it could be preferably amplified under selection imposed by growth on 2,4-D. To investigate this hypothesis, strain 2811C was cultivated in 2,4-D broth with repeated transfers into fresh medium. After the seventh transfer, a weak plasmid band was detected on the agarose gel. The intensity of this band was increased with increasing number of transfers. Southern hybridization of the gel with *tfdA* exhibited a clear hybridized plasmid band from the seventh transfer and the radioactive intensity was much increased after the nineth and the eleventh transfer (Figure 6). After longer film exposure, weak hybridized bands were observed in chromosomal DNA band on Southern blots of the original culture and in cultures after the seventh, the nineth, and the eleventh transfer (data not shown). With further transfers on 2,4-D medium, two different plasmid bands were detected on agarose gel from the twentieth transfer culture, suggesting that the integrated plasmid could excise in several different ways during the continuous growth on 2,4-D. These results indicated that initial pure culture of strain 2811C became a mixed culture of 2811C and at least two other plasmid-containing strains that are probably more fit for 2,4-D growth.

To estimate the ratio of population 2811C to the population of the excised plasmid-containing strain, colonies from cultures after the eighth and the fourteenth transfer were randomly selected, purified on PTYG plates, and subjected to Kado's procedure. Whereas no plasmid DNA band was detected from the 30 colonies examined from the eighth transfer, clear plasmid DNA

bands were observed in five colonies out of the 28 colonies examined from the fourteenth transfer. The result suggested that the mixed population was mainly composed of strain 2811C even at the eighth transfer, while plasmid-containing strains have gradually become more predominant and comprised approximately 18 % of the population after the fourteenth transfer. Plasmid DNA was isolated from three colonies selected randomly from the five colonies to analyze the fragment profiles of restriction endonucleases digests. Two of three plasmids showed identical restriction profiles to plasmid pKA2 in *EcoR*I and *Hind*III digests (Figure 7), while the third plasmid revealed deletion of four fragments as well as appearance of an extra novel fragment (arrowhead) in *EcoR*I digests (Figure 7). The derivative plasmid could result from either an alternative excision event of the integrated pKA2 in strain 2811C or rearrangement of the precisely excised plasmid pKA2 during continuous selection on 2,4-D.

Comparison of pKA2 of *A. paradoxus* with pKA4 of *P. pickettii*. As stated above, plasmid pKA2 of *A. paradoxus* has similar properties to plasmid pKA4 of *P. pickettii* in regard to size, high transmissibility and hybridization pattern with *tfd* probes. In addition to that, restriction enzyme analysis with *EcoR*I and *Hind*III revealed the restriction pattern similarity between these two plasmids (Figure 8-A). In *EcoR*I digests, only two (EB and EC) of the seven fragments showed altered mobilities in the agarose gel electrophoresis, while one (HB) of the three fragments revealed different mobility in *Hind*III digests (Figure 8-A). When fragments EA, ED and EE of both plasmids (pKA2 and pKA4) were isolated from the agarose gel and digested with *Bgl*II, the new fragments were identical for each plasmid (data not shown). To determine whether all the DNA fragments of plasmids pKA2 and pKA4 generated by *EcoR*I restriction endonuclease are homologous to one another,

the fragments were analyzed on Southern blots hybridized with pKA4 DNA as the probe (Figure 8-B). All the fragments of pKA2 were shown to be hybridizable with pKA4. The identical restriction profiles and hybridization patterns demonstrate that pKA2 of *A. paradoxus* and pKA4 of *P. pickettii* are genetically homologous, suggesting that intergeneric gene transfer between these two strains has occurred in natures.

DISCUSSION

The data presented here demonstrated that the 2,4-D degradative plasmid pKA2 can exist as both an integrated plasmid into the host chromosome and a free plasmid in a natural isolate, *A. paradoxus*. Since, for the herbicide 2,4-D, all or most of the degradative genes have been reported to be carried on a free plasmid in a number of independent natural isolates (3, 4, 17, 18), the observation of its chromosomal location was surprising. The integrated plasmid could be precisely excised, thus forming the same plasmid pKA2. The selective advantage of strain 2811P over strain 2811C in 2,4-D minimal medium (Figure 3) might make it possible to detect the 2811P population which may normally arise from strain 2811C at a low frequency. In any case, the detection of strain 2811C in glycerol stock culture of 2811P and the reproduction of 2811P from 2811C on 2,4-D medium strongly suggest that the entire plasmid pKA2 can move into and out of the host chromosome depending on its environment. However, we have been unable to demonstrate the reproducibility of plasmid integration into chromosome due to the absence of any selection for this variant.

It has been reported that TOL genes could be integrated into and rescued from the bacterial chromosome (2, 10, 21). In those cases, part of the TOL

plasmid was located in the chromosome and the integrated DNA was rescued only in the presence of other plasmids to form novel recombinants, suggesting that the TOL genes may be carried on a transposon-like element(1, 10, 24). It has also been postulated that repeated sequences were present and involved in gene expression in several 2,4-D degradative plasmids (28) and that repeated sequences were involved in the complex interaction between chromosomal DNA and plasmid DNA in *Acetobacter xylinum* (25). It is important to note that part of the chromosomal DNA of strains 2811P and 2811C has sequence homology with plasmid pKA2 (Figure 5). Although it is not known whether the process is dependent of a host *recA* product, the hybridization results and the occurrence of the precise excision of pKA2 suggest that homologous repeated sequences may be involved in the interaction between host chromosome and plasmid pKA2.

While chromosomally located plasmid pKA2 has lost the freedom of conjugal transfer possessed by the free plasmid, there may be some compensations which come from the chromosomal location. First, it would allow the stable maintenance of plasmid DNA in any circumstance, while the stability of a free plasmid is influenced by cultural conditions, presence of other incompatible plasmids, the efficiency of partitioning into daughter cells, and the environment. Second, the chromosomally located plasmid pKA2 could be available to other populations of bacteria in good times because it still retains a means of transferability by virtue of its ability to excise followed by conjugation. Third, it may play an important role in the exchange of bacterial genome because an alternative excision could allow for conjugal transfer of chromosomal DNA as well as plasmid DNA to other microbial populations. In fact, the observation that some extra fragments of the digested chromosomal DNA were hybridizable with pKA2 plasmid DNA

would indicate that DNA exchange between chromosome and plasmid occurred in the past.

It is also interesting to see how chromosomally located pKA2 excises during continuous growth on 2,4-D medium. In the smaller derivative plasmid, a 19.6-kb region corresponding to four fragments of *EcoR*I digests has been deleted with concomittant appearance of a novel fragment (Figure 7). The identical profile of the other fragments generated by *EcoR*I digestion between pKA2 and the smaller derivative affords a striking illustration of two different plasmids with the same origin and demonstrate that such rearrangement of DNA plays an important role in the evolution of plasmids.

We isolated two different 2,4-D degrading bacteria belonging to the genera of *Alcaligenes* and *Pseudomonas* from the same soil at two different times. The observation that plasmid pKA2 of *A. paradoxus* isolated in 1989 showed nearly identical restriction profile to that of pKA4 of *P. pickettii* isolated in 1990, together with their similar physical properties and genetic homology, strongly suggests that intergeneric gene transfer followed by plasmid modification, or vice versa, has occurred between these two species in nature.

While it is not clear why and how pKA2 is integrated into and excised from the chromosome, our results demonstrated that a conjugative plasmid can integrate in its entirety into the host chromosome, and that the chromosomally located plasmid can be excised either precisely or in a different way. Additionally, we presented the evidence that intergeneric gene transfer occurred between *Alcaligenes* and *Pseudomonas* species in nature. Future research on the different physiology of strains 2811P and 2811C on 2,4-D medium, the integration site on chromosome of pKA2, and the mechanism of integration and excision would reveal the mechanism and strategy for this genetic flexibility.

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Table 1. Sequence homology of plasmid and chromosome with 2,4-D metabolic genes.

Strain	2,4-D degradative plasmid (size)	[a]Homology to *tfdA*	*tfdB*	*tfdC*	*tfdD*	Location of hybridization signal
P. pickettii 712	pKA4 (40.9 kb)	+	-	-	-	plasmid
A. paradoxus 2811P	pKA2 (42.9 kb)	+	-	-	-	plasmid
A. paradoxus 2811C	none	+	-	-	-	chromosome

[a] +, has sequence homology ; -, does not have sequence homology.

Table 2. Transferability of 2,4-D$^+$ phenotype from donor to *P. cepacia* DBO1.

Donor	Plasmid transferred	Antibiotic selection[a] (ug/ml)	Transfer frequency[b]
P. pickettii 712	pKA4	Km, 75 ; Cb, 50 ; Bc, 50	1.0×10^{-2}
A. paradoxus 2811P	pKA2	Km, 75 ; Cb, 50 ; Bc, 50	7.7×10^{-3}
A. paradoxus 2811C	-	Km, 75 ; Cb, 50 ; Bc, 50	$< 10^{-9}$

[a] Km, kanamycin ; Cb, carbenicillin ; Bc, bacitracin.
[b] Values are means of three independent matings.

Figure legends

Fig. 1. Resolution of plasmids of 2,4-D degrading strains. (A) Negative photograph of agarose gel and (B) autoradiogram of the gel after hybridization with *tfdA* gene as a probe. Lanes : *P. pickettii* 712 containing plasmid pKA2 (1), *A. paradoxus* 2811P containing plasmid pKA4 (2), *A. paradoxus* 2811C not containing plasmid DNA (3). Positions of plasmid DNA (P) and chromosomal and linear DNA (Chr) are shown. Films were exposed for (a) 20 min (b) 2 days.

Fig. 2. Southern blot hybridization with the 16S rRNA gene to the total DNA of strains 2811P and 2811C. Lanes : Total DNA of 2811C (1 and 3) and 2811P (2 and 4) digested with *Hind*III (1 and 2) and *Eco*RI (3 and 4).

Fig. 3. Growth characteristics of 2,4-D degrading strains in 2,4-D mineral medium under uninduced condition. *P. pickettii* 712 (—Δ—), *A. paradoxus* 2811P (—□—), and *A. paradoxus* 2811C (—■—).

Fig. 4. Resolution of plasmids in donors, transconjugants, and recipient. Lanes : recipient *P. cepacia* DBO1 (1), transconjugant from 712 x DBO1 (2), donor *P. pickettii* 712 (3), transconjugant from 2811P x DBO1 (4), donor *A. paradoxus* 2811P (5).

Fig. 5. (A) Line drawing and (B) autoradiogram of a Southern blot hybridization with labeled plasmid pKA2 of *Eco*RI (left) and *Hind*III (right) restriction enzyme digests of pKA2 plasmid DNA (1), total DNA of strain 2811P (2), and total DNA of strain 2811C (3). Novel extra bands presumably resulting from intergation process are indicated by arrowheads in panel (A).

Fig. 6. Excision of plasmid DNA in strain 2811C during continuous growth on 2,4-D medium. Each 5 ml of culture grown initially (1) and after the seventh (2), nineth (3), and eleventh (4) transfer was subjected to Kado's procedure and then plasmid and chromosomal DNA in lysate were gel-separated, and analyzed on Southern blots hybridized with labeled *tfdA* probe. Film was exposed for 4 h.

Fig. 7. Negative photograph of agarose gel of restriction enzyme-digested plasmid DNA. Lanes : Lambda DNA digested with *Hind*III as size marker (1), plasmid pKA2 (2), plasmids isolated from three independent colonies recovered on 2,4-D selection from strain 2811C (3, 4, and 5).

Fig. 8. (A) Negative photograph of agarose gel of plasmids digested with restriction endonucleases. Lanes : Plasmid pKA2 (1) and plasmid pKA4 (2). (B) Autoradiogram of a Southern blot hybridized with labeled plasmid pKA4 of *Eco*RI-digested pKA2 (1) and pKA4 (2).

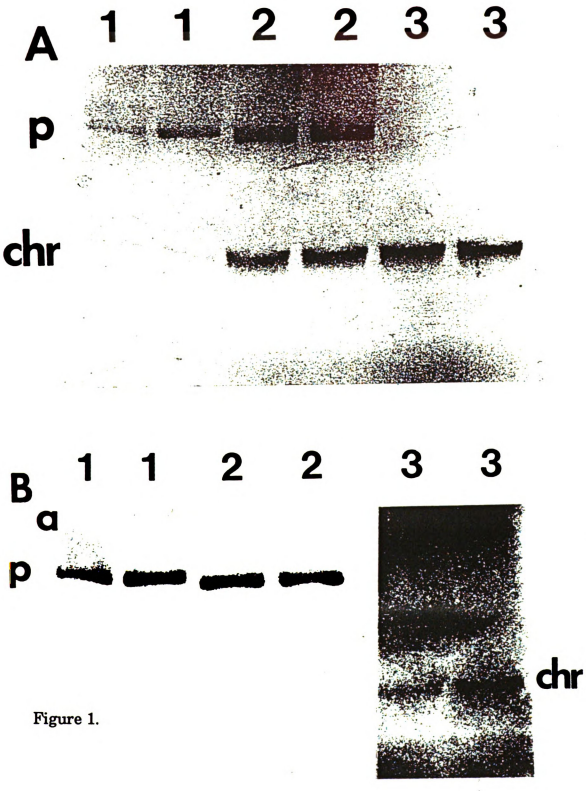
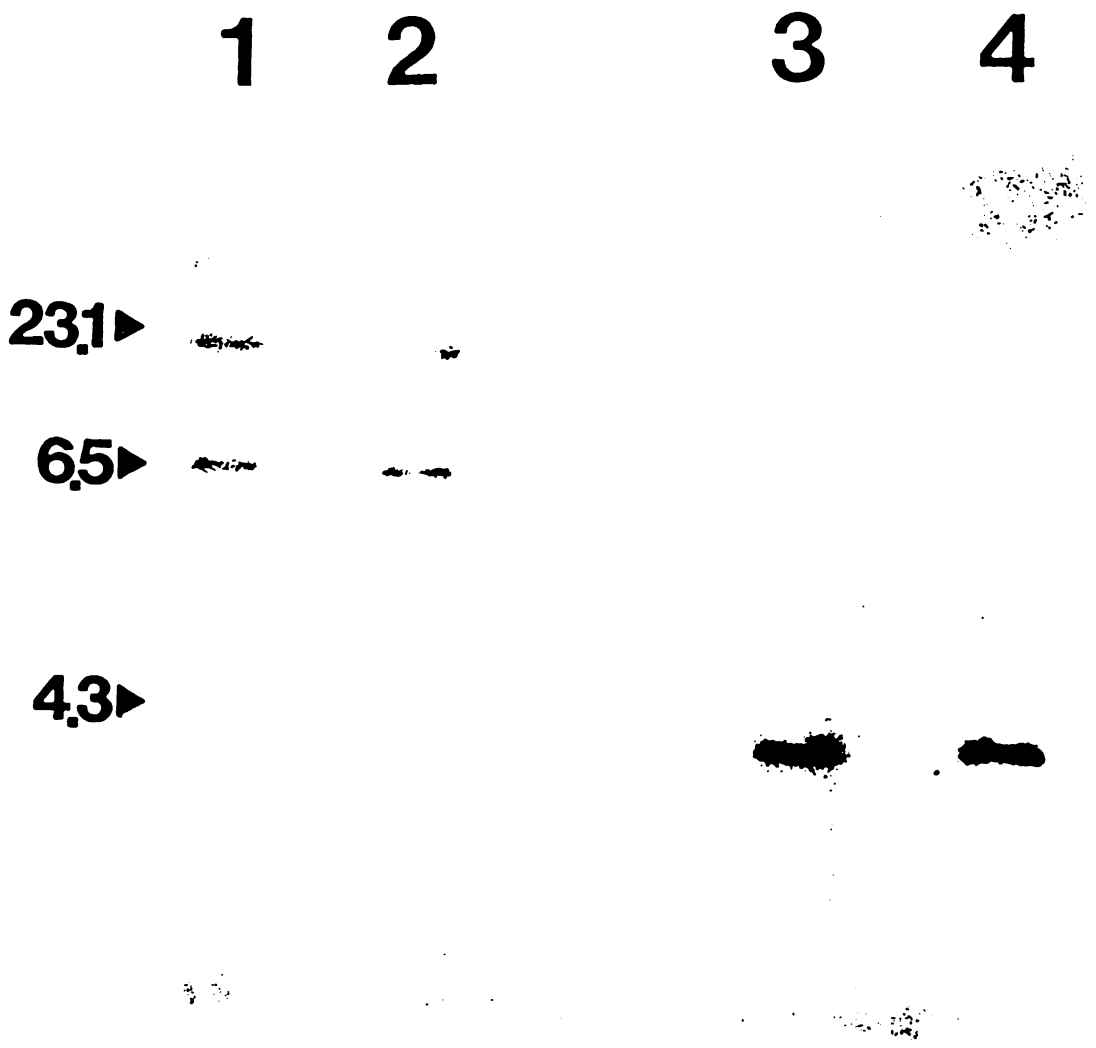


Figure 1.



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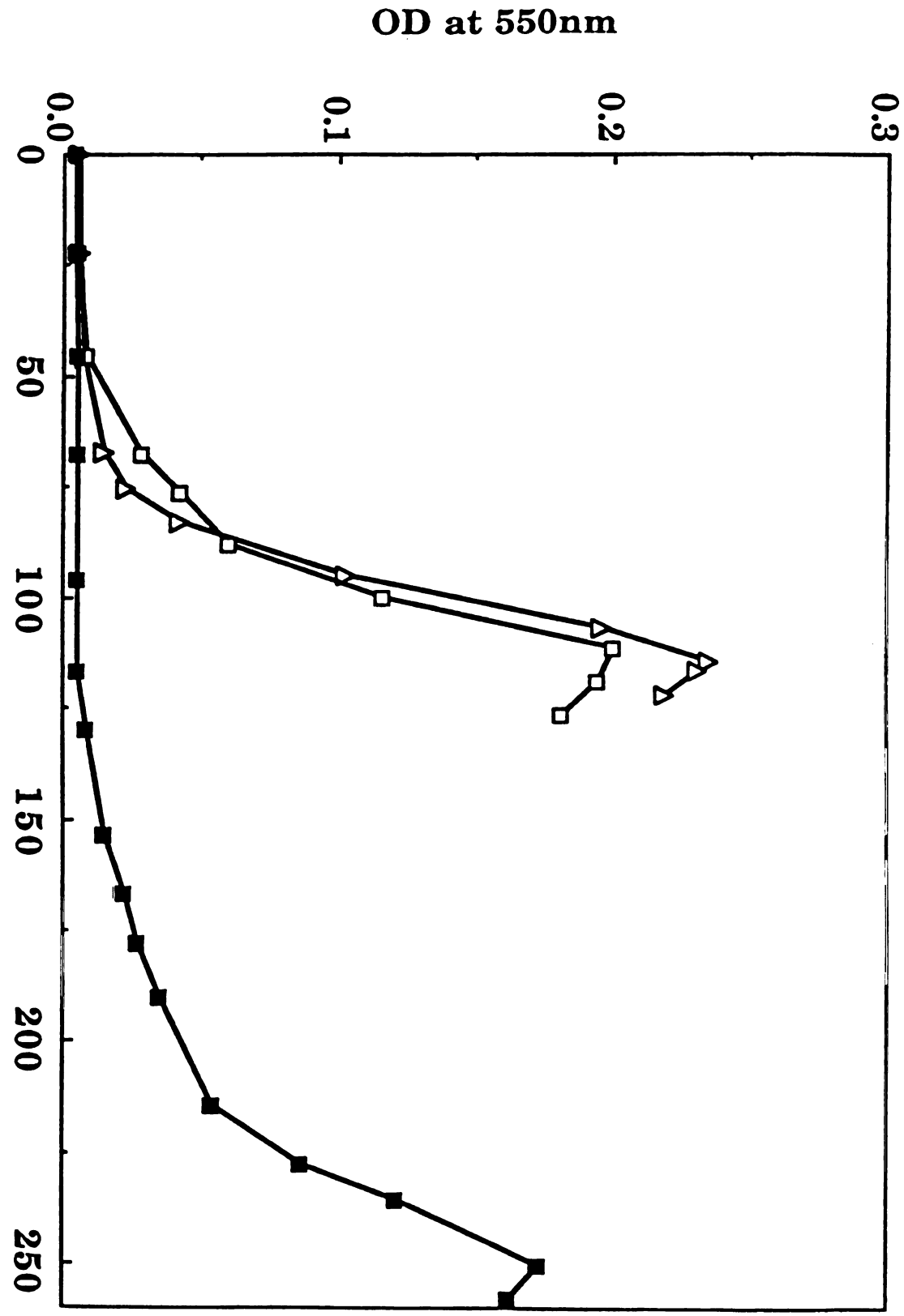

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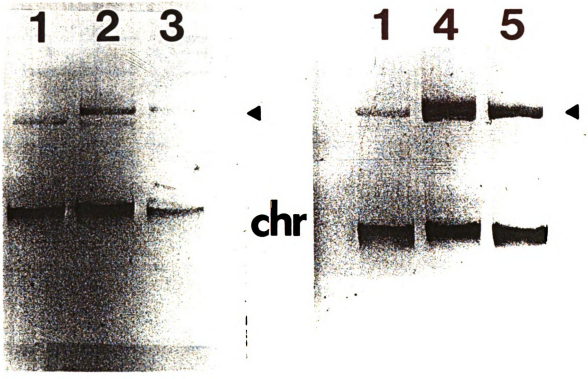

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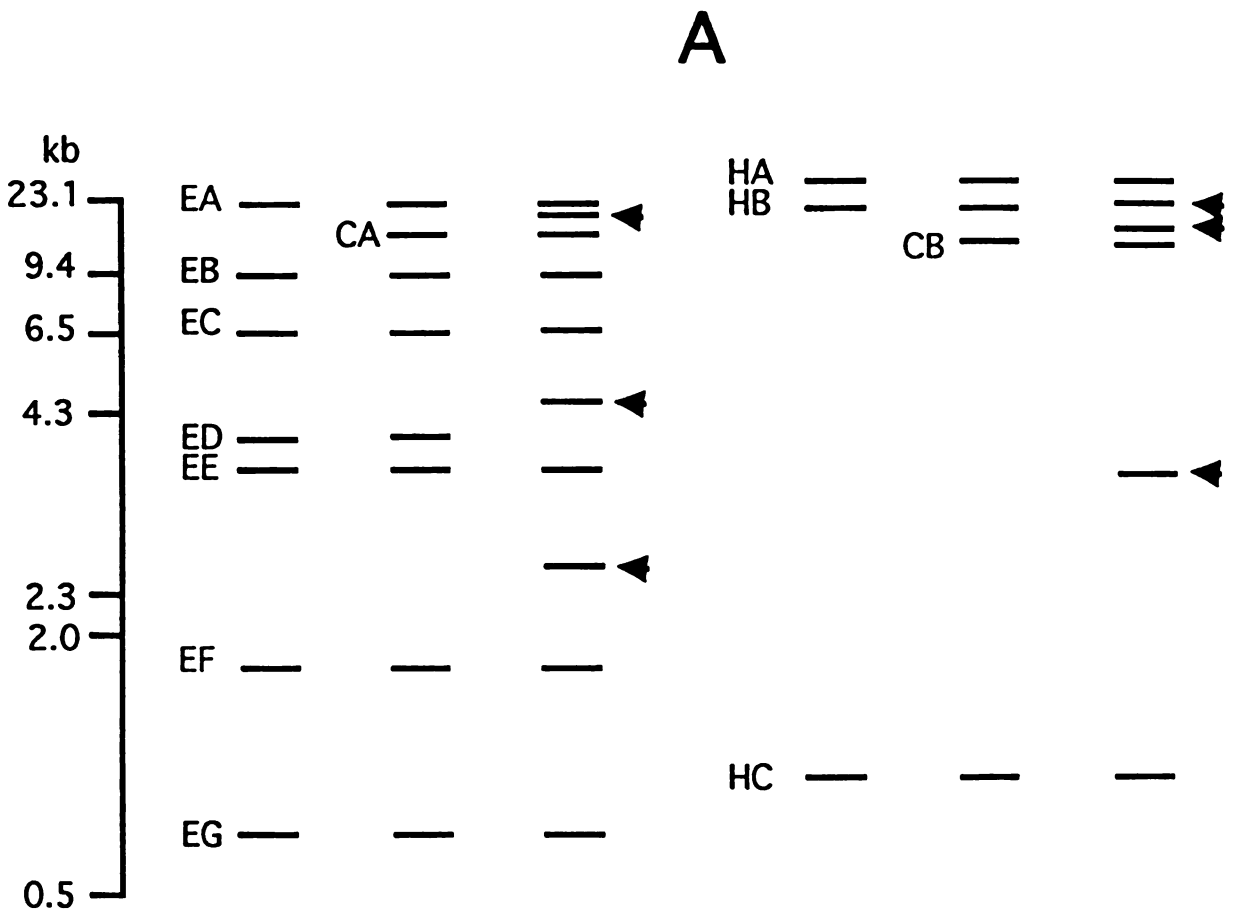
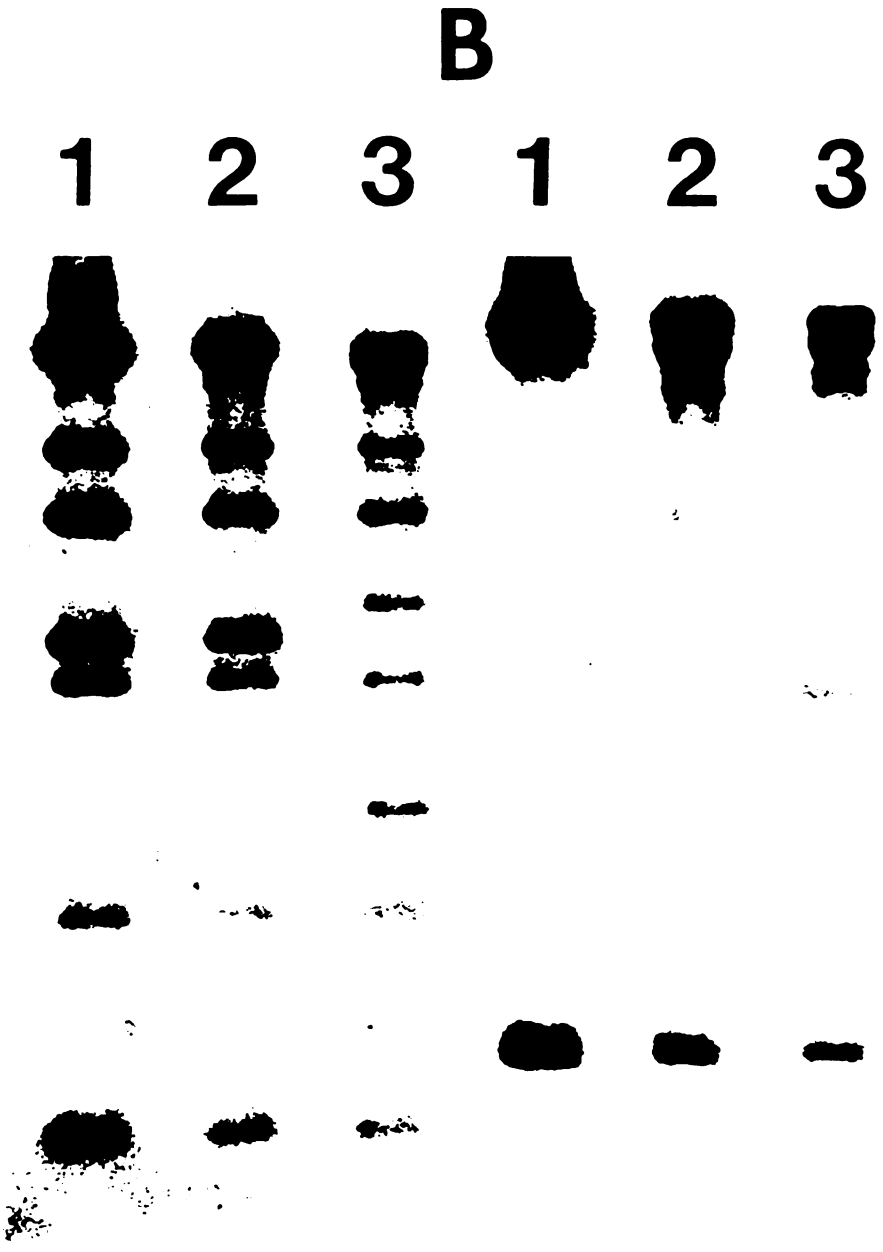


Figure 5-A.



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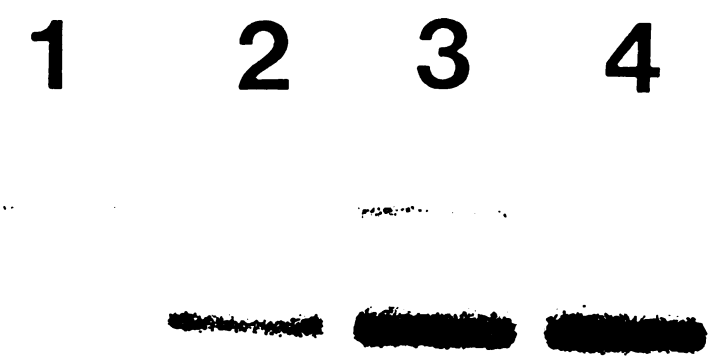

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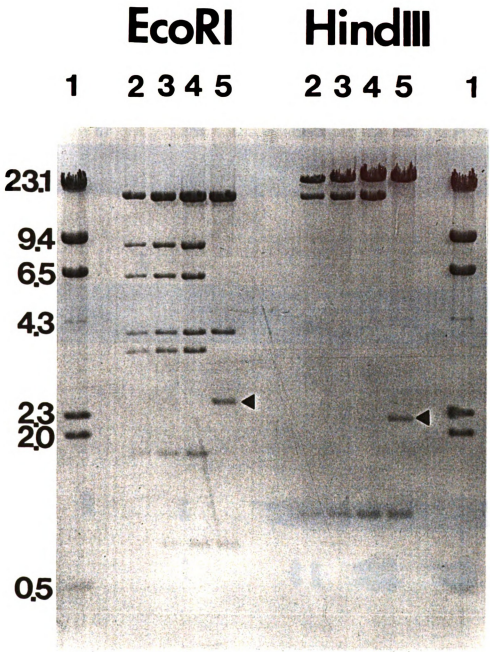

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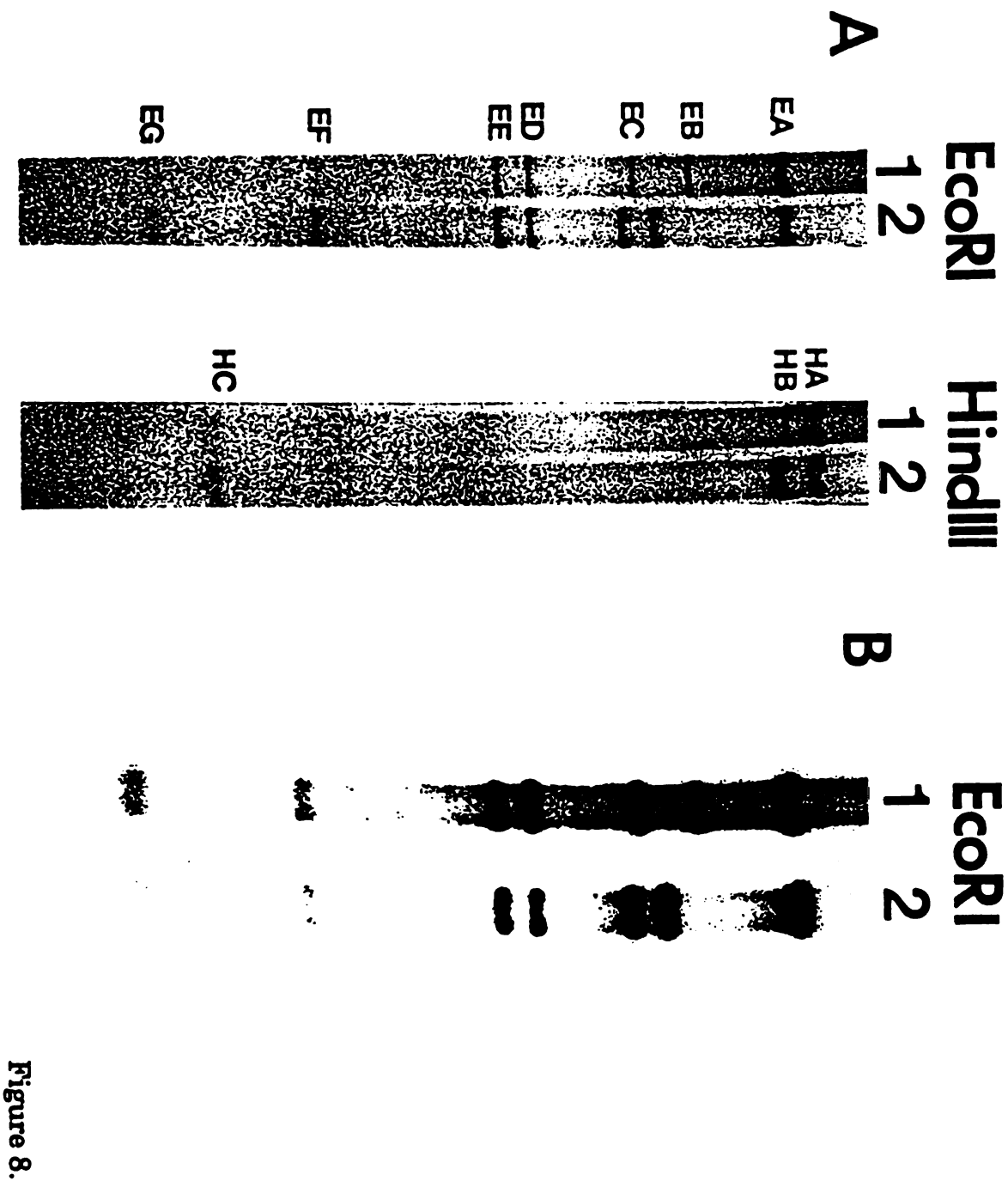

Figure 8.

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