APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1997, p. 1326–1331 Vol. 63, No. 4
0099-2240/97/$04.00+0

Genetic Diversity through the Looking Glass: Effect of Enrichment Bias

JOHN DUNBAR,[1]† SCOTT WHITE,[2] AND LARRY FORNEY[1]*

NSF Center for Microbial Ecology and Department of Microbiology, Michigan State University, East Lansing, Michigan,[1] and Los Alamos National Laboratories, Los Alamos, New Mexico[2]

Received 26 August 1996/Accepted 16 January 1997

The effect of enrichment bias on the diversity of 2,4-dichlorophenoxyacetate (2,4-D)-degrading (2,4-D$^+$) bacteria recovered from soil was evaluated by comparing the diversity of isolates obtained by direct plating to the diversity of isolates obtained from 85 liquid batch cultures. By the two methods, a total of 159 isolates were purified from 1 g of soil and divided into populations based on repeated extragenic palindromic sequence PCR (rep-PCR) genomic fingerprints. Approximately 42% of the direct-plating isolates hybridized with the *tfdA* and *tfdB* genes from *Alcaligenes eutrophus* JMP134(pJP4), 27% hybridized with the *tfdA* and *tfdB* genes from *Burkholderia* sp. strain RASC, and 30% hybridized with none of the probes. In contrast, the enrichment isolates not only represented fewer populations than the isolates obtained by direct plating but also exhibited, almost exclusively, a single hybridization pattern with 2,4-D catabolic gene probes. Approximately 98% of the enrichment isolates possessed pJP4-type *tfdA* and *tfdB* genes, whereas isolates containing RASC-type *tfdA* and *tfdB* genes were obtained from only 2 of the 85 enrichment cultures. The skewed occurrence of the pJP4-type genes among the isolates obtained by enrichment suggests that the competitive fitness of 2,4-D$^+$ populations during growth with 2,4-D may be influenced either by specific *tfd* alleles or by genetic factors linked to these alleles. Moreover, the results indicate that evaluation of the diversity and distribution of catabolic pathways in nature can be highly distorted by the use of enrichment culture techniques.

Our understanding of the genetic diversity underlying specific bacterial phenotypes in nature is shaped by the organisms used for study. The use of cultured instead of uncultured bacteria is a well-known source of potential bias. Culturable bacteria represent only 0.1 to 5% of the bacteria observed in microbial communities (16, 29). Since culturable bacterial strains are the ones used for biochemical and genetic studies, it is possible that only a small portion of the biochemical and genetic diversity underlying particular phenotypes has been documented. Even within the culturable fraction of bacteria, obtaining organisms which are representative of the diversity in nature can be difficult as a result of inherent biases in the methods used for isolation. Batch culture enrichment is the most widely used method for isolation of bacteria expressing specific phenotypes. It has long been recognized that enrichment limits the number and relative growth rates of organisms obtained from the environment (13). These limitations may also affect the diversity of catabolic pathways obtained from the environment.

By design, enrichment in liquid batch culture yields only one or a few strains possessing a specific phenotype. This limitation is potentially overcome by establishing numerous enrichment cultures, but to our knowledge this has not been systematically evaluated. A more serious limitation is the fact that liquid batch culture enrichments typically select for fast-growing organisms (13). A number of researchers have demonstrated that both fast- and slow-growing organisms possessing the same phenotype are found simultaneously in natural communities and can be isolated from the same inoculum source by use of chemostats with different dilution rates (17, 32) or by direct plating (11). However, as a result of the high substrate concentrations ($\gg K_s$) commonly used in batch culture enrichments, slower-growing organisms are typically outcompeted by organisms with a higher maximum specific growth rate, μ_{max}. The genetic factors responsible for differences in growth rates and competitive fitness are poorly understood. Thus, the bias which enrichment might impose on the diversity of a particular catabolic pathway detected in environmental samples is unknown.

To evaluate the potential for bias due to the use of enrichment cultures, we compared the genetic diversity of 2,4-dichlorophenoxyacetate (2,4-D) catabolic pathways among 2,4-D-degrading bacteria obtained by enrichment and by direct plating. All of the isolates originated from just 1 g of soil. Gene probes representing different alleles for the first two genes in the 2,4-D catabolic pathway were used in Southern hybridization analyses to evaluate the diversity among the isolates obtained.

MATERIALS AND METHODS

Media and reagents. Defined aerobic basal (DAB) medium amended with 2,4-D at 250 μg ml^{-1} was used for specific cultivation of 2,4-D-degrading bacteria. DAB medium consists of a mineral salts base supplemented with amino acids and vitamins at low concentrations and has been described previously (11). Reagent grade 2,4-D and [UL-^{14}C]2,4-D were purchased from Sigma Chemical Co., St. Louis, Mo.

Soil. Soil was obtained from an experimental field plot at Kellogg Biological Station (Hickory Corners, Mich.) which had been treated with 2,4-D (100 μg/g of soil) from 1989 to 1992 (19). A 1-kg sample of soil was collected in October 1993 and stored at 4°C until used. For isolation of 2,4-D-degrading bacteria, 1 g of soil was mixed with 9 ml of 10 mM phosphate buffer (pH 7.0) containing 6.1 mM Na_2HPO_4 and 3.9 mM KH_2PO_4 and shaken for 20 min at 250 rpm on a rotary shaker, and aliquots were used for enrichment or direct plating.

Enrichment cultures. Enrichment cultures were established by transferring 0.1 ml of soil slurry to each of 74 test tubes containing 3 ml of DAB medium amended with 250 μg of 2,4-D ml^{-1}. After 1 week of incubation at 30°C on a rotary shaker set at 250 rpm, the disappearance of 2,4-D in each enrichment

* Corresponding author. Mailing address: Department of Microbiology, Michigan State University, East Lansing, MI 48824. Phone: (517) 432-1365. Fax: (517) 432-3770. E-mail: forney@pilot.msu.edu.

† Present address: Life Sciences Division M888, Los Alamos National Labs, Los Alamos, NM 87544.

culture was confirmed spectrophotometrically with a Hewlett-Packard 8452A diode array spectrophotometer. A 0.03-ml aliquot of each culture was then transferred to 3 ml of fresh DAB–2,4-D medium, incubated for 1 week, and monitored spectrophotometrically for 2,4-D disappearance. After three serial transfers, each culture was streaked on one-quarter-strength Trypticase soy agar (TSA). 2,4-D-degrading bacteria on each plate were identified by transferring colonies representing different morphologies into 3 ml of DAB-2,4-D medium and monitoring the disappearance of 2,4-D. Cultures which tested positive were streaked on one-quarter-strength TSA to check for purity. Pure cultures were fingerprinted by rep-PCR (see below), and representatives of different rep-PCR patterns were regrown in DAB–2,4-D medium to establish 15% glycerol stocks, which were stored at −80°C.

Direct plating. To isolate 2,4-D-degrading bacteria by direct plating, the soil slurry was serially diluted and appropriate dilutions were spread directly on HATF nitrocellulose filters (Millipore, Bedford, Mass.) atop R2A agar medium (Difco, Detroit, Mich.). After 1 week of incubation at 30°C, colonies able to degrade 2,4-D were identified by autoradiography based on their ability to incorporate ^{14}C from [U-^{14}C]2,4-D. This procedure has been described previously with $^{35}SO_4^{2-}$ instead of a ^{14}C-labeled carbon source (11). The procedure was modified for [U-^{14}C]2,4-D as follows. Each master filter was replicated once, and each replica filter was incubated on DAB–2,4-D solid medium containing 1.5% agar and 0.1 μCi of [U-^{14}C]2,4-D ml^{-1}. After 3 days of incubation, the replica filters were transferred to 1% agar medium containing 10 mM phosphate buffer and 750 μg of 2,4-D ml^{-1} and incubated for 30 min to remove unincorporated [U-^{14}C]2,4-D. This washing step was repeated once. The replica filters were dried, mounted on paper, covered with a layer of Saran, and exposed to Kodak X-OMAT XAR (Eastman Kodak, Rochester, N.Y.) film overnight at −80°C. The film was developed as recommended by the manufacturer. The autoradiography films were aligned with appropriate master filters, and colonies corresponding to film signals were considered putative 2,4-D$^+$ colonies. Since the filters used for isolation of 2,4-D$^+$ bacteria were crowded with bacterial colonies unable to degrade 2,4-D (2,4-D$^-$), putative 2,4-D$^+$ colonies were transferred to 3 ml of DAB-2,4-D medium to reduce the relative abundance of 2,4-D$^-$ bacterial contaminants. Cultures were transferred two or three times and purified as described above for enrichment cultures.

MPN estimation. The most probable number (MPN) of 2,4-D-degrading bacteria in the soil was determined by standard methods (6) with five tubes per dilution. For each 10-fold dilution of the soil slurry, 1 ml was transferred to each of five tubes containing 3 ml of DAB–2,4-D medium. The MPN tubes were incubated for 2 weeks at 30°C. The disappearance of 2,4-D in the tubes was determined spectrophotometrically, and MPN estimates of the 2,4-D degrading bacteria were obtained from statistical tables (6). 2,4-D-degrading bacteria were isolated from MPN tubes as described above for enrichment cultures.

rep-PCR. Genomic fingerprints of 2,4-D-degrading isolates were obtained with consensus primers for the repeated extragenic palindromic (REP) sequence (33). PCRs were performed by the method of de Bruijn (8) with colonies of pure cultures grown on one-quarter-strength TSA as the template. Approximately 1 μl of cells was added to each 24 μl of PCR mixture. PCR products were electrophoresed through 1.5% agarose gels containing one-half-strength TAE (26) and 0.25 μg of ethidium bromide ml^{-1} and then photographed. After fingerprints of all isolates were obtained, the reproducibility of the rep-PCR patterns was confirmed by retesting representative isolates regrown in DAB–2,4-D medium.

16S rDNA restriction analysis. 16S rDNA was amplified with primers 8-27 and 1541-1522 (4) under the reaction conditions described by Laguerre et al. (22). PCRs were performed in a Perkin-Elmer 9600 thermal cycler. Cells from broth cultures subjected to four freeze-thaw cycles were used as templates for PCR amplifications. For each 2,4-D$^+$ strain, 10 μl of amplified 16S rDNA was digested separately with *Cfo*I, *Hae*III, *Msp*I, *Rsa*I, and *Hin*fI (Gibco). After a 3-h incubation, digested 16S rDNA was electrophoresed through 3% Metaphor agarose gels (FMC Bioproducts, Rockland, Maine) in TAE buffer. Following electrophoresis, the gels were stained with ethidium bromide and photographed.

Phylogenetic analysis. The restriction fragment profiles of all strains were compared and the presence or absence of fragments was recorded, resulting in 96 binary characters for each strain. The data were analyzed with PAUP version 3.1.1 (31). Excluding redundant taxa (i.e., strains with identical 16S restriction fragment length polymorphism data), the data matrix contained 45 phylogenetically informative characters. All characters were treated as Fitch (unordered) characters, and searches were unrooted. A bootstrap analysis with 1,000 replicates was performed by a heuristic search procedure with global branch swapping and 10 randomized taxon additions for each replicate. A 50% majority rule consensus tree (31) was computed from all minimal trees recovered in the bootstrap analysis. All nodes supported by bootstrap values of less than 50 were collapsed into polytomies.

2,4-D gene probe analysis. Genomic DNA was obtained by standard methods (2). Bacterial strains were grown overnight in 5 ml of 1/10-strength Trypticase soy broth (TSB), and 1.5 ml was transferred to an Eppendorf tube for DNA extraction. For each strain, approximately 5 μg of genomic DNA was digested with 10 U of *Eco*RI for 4 h and then electrophoresed in ethidium bromide-stained 0.8% agarose gels. Digested DNA was transferred to Hybond-N nylon membranes (Amersham, Arlington Heights, Ill.) by capillary blotting (28) and cross-linked with a UV Stratalinker 1800 (Stratagene, La Jolla, Calif.) for subsequent hybridization with gene probes.

Gene probes were derived from 2,4-D catabolic genes encoded on plasmid pJP4 from *Alcaligenes eutrophus* JMP134 and from the chromosomally encoded 2,4-D genes in *Burkholderia* sp. strain RASC. Four probes that consisted of internal fragments of *tfdA* and *tfdB* from pJP4 (14) and of *tfdA* (30) and *tfdB* from strain RASC were used. The RASC *tfdB* probe consisted of a 1-kb *Sac*I-*Kpn*I fragment from a 1.2-kb *Sac*I-*Sac*I fragment which had been cloned into pUC9 and was kindly provided by Yuichi Suwa. The RASC *tfdA* and *tfdB* genes have 73 and 65% DNA sequence similarity, respectively, to the corresponding pJP4 alleles (30). Gel-purified probes were labeled with digoxigenin-dUTP by using the DIG DNA labeling and detection kit (Boehringer Mannheim, Indianapolis, Ind.). Prehybridization and hybridization solutions contained 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (26), 0.1% *N*-lauroylsarcosine, 0.02% sodium dodecyl sulfate, 5% blocking reagent, and 50% formamide. Hybridizations were conducted at 62°C to achieve high-stringency conditions (90 to 100% similarity). Following hybridization, membranes were washed twice in 2× SSC and processed for probe detection.

RESULTS

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

The diversity of isolates obtained by direct plating was greater than the diversity of isolates obtained by enrichment (Fig. 2). Among the 74 isolates obtained by direct plating, 25 distinct populations were detected, while only 7 were identified among the 74 enrichment isolates. Similar results were obtained in previous experiments in which 2,4-D-degrading isolates were obtained from 1 g of soil either by direct plating or by enrichment (Table 1). The estimated population diversity, $E(S)$, for a standardized sample size of 50 isolates was calculated by rarefaction (15, 27) and used to compare the diversity of isolates obtained in different experiments since the actual number of isolates in each experiment varied (Table 1). The average population diversity, $E(S)$, among isolates obtained by direct plating from each of three separate 1-g samples of soil was 19 populations (standard deviation = 1.026). In contrast, the average value of $E(S)$ for isolates obtained from two separate 1-g samples of soil by enrichment was 8.6 populations (standard deviation = 3.182). These results demonstrate that numerous 2,4-D$^+$ populations can be obtained by establishing multiple enrichment cultures, but the population diversity of isolates from enrichment cultures is still significantly lower than that obtained by direct plating.

Catabolic diversity. Significant differences were also observed in the diversity of 2,4-D catabolic genes obtained by enrichment and by direct plating (Fig. 3). Hybridization of 2,4-D gene probes with genomic DNA from each of the 25 populations obtained by direct plating identified three groups of catabolic genes. Nine populations, representing approximately 42% of the isolates, belonged to catabolic group A_1B_1, since these populations hybridized with the *tfdA* and *tfdB* genes from *A. eutrophus* JMP134(pJP4). Nine populations representing 27% of the isolates belonged to group A_2B_2 based on hybridization with the *tfdA* and *tfdB* genes from *Burkholderia* sp. strain RASC, and seven populations representing 30% of the isolates belonged to group A_XB_X, since these populations hybridized with none of the probes. In contrast, six populations representing 98% of the isolates obtained by enrichment belonged to group A_1B_1. A population exhibiting the A_2B_2 hybridization pattern was obtained from only 1 of 74 enrichment

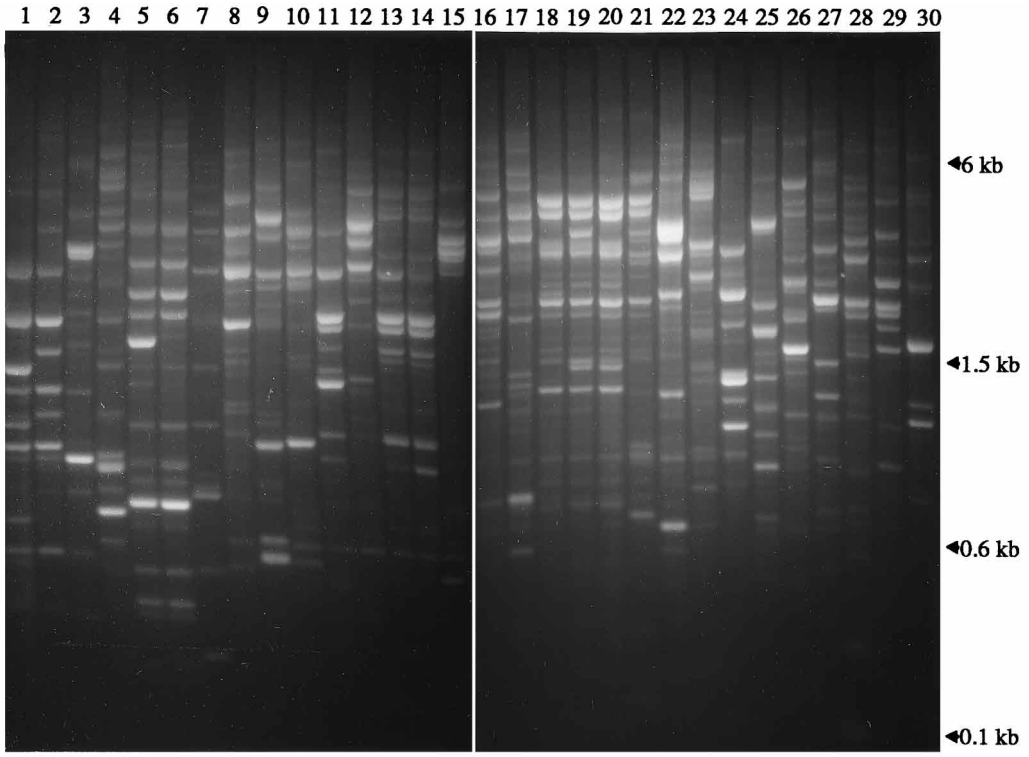

FIG. 1. Unique rep-PCR patterns of 2,4-D$^+$ strains obtained by direct plating and by enrichment. Strains 1 to 16, 18, and 20 to 27 were obtained by direct plating, whereas strains 9, 17 to 19, 21 to 23, and 28 to 30 were obtained by enrichment.

cultures. Similarly, a population representing group A_2B_2 was obtained from only 1 of 11 MPN tubes; the remaining tubes yielded populations exhibiting the A_1B_1 hybridization pattern. These data demonstrate a marked bias in the catabolic genes obtained from soil by use of enrichment.

Phylogenetic analysis. To determine if the skewed enrichment of the A_1B_1 group was due to outgrowth of populations representing a single species, an estimate of the phylogeny of the populations was constructed based on restriction analysis of 16S rDNA amplified from each population. The most parsimonious reconstruction (Fig. 4) had a rescaled consistency index of 0.531 (21). Each operational taxonomic unit (OTU) represents a unique, five-enzyme 16S restriction fragment length polymorphism pattern. While such patterns identify coherent phylogenetic units, the resolution of this approach is not always sufficient to delineate individual species (25). Nonetheless, the diversity of OTUs revealed by this approach provides an estimate of the minimum number of species present. The 25 populations obtained by direct plating represented 16 different OTUs, which were distributed among three catabolic groups (Fig. 4). The frequency distribution of the OTUs obtained from enrichment cultures and MPN tubes was highly skewed. The enrichment and MPN isolates (combined) represented seven OTUs, and one OTU (no. 5) accounted for 72%

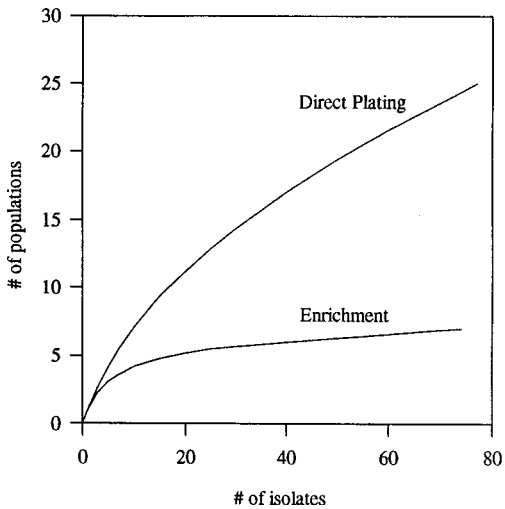

FIG. 2. Cumulative diversity curves for isolates obtained by direct plating and by enrichment. Rarefaction (15, 27) was used to estimate population diversity for numerous samples sizes between 0 and 74. The estimates were then plotted to construct the curves shown.

TABLE 1. Population diversity of 2,4-D$^+$ isolates obtained by enrichment and by direct plating

Expt.[a]	Enrichment			Direct plating		
	No. of isolates[b]	$E(S)$[c]	Variance[d]	No. of isolates	$E(S)$	Variance
1				50	18	0.745
2	50	10.8	0.146	50	20	0.000
3	50	6.3	0.440	50	19.4	2.871

[a] For each experiment, 1 g of soil was used.
[b] The actual number of isolates collected in each experiment varied from 51 to 74. To standardize the diversity measure, $E(S)$, between experiments, $E(S)$ was calculated for a standardized sample size of 50 isolates for each experiment.
[c] The standardized population diversity, $E(S)$, was obtained by rarefaction (15, 27).
[d] The variance arises from inherent statistical uncertainty in the estimation of population diversity, $E(S)$, for sample sizes smaller than the actual sample size.

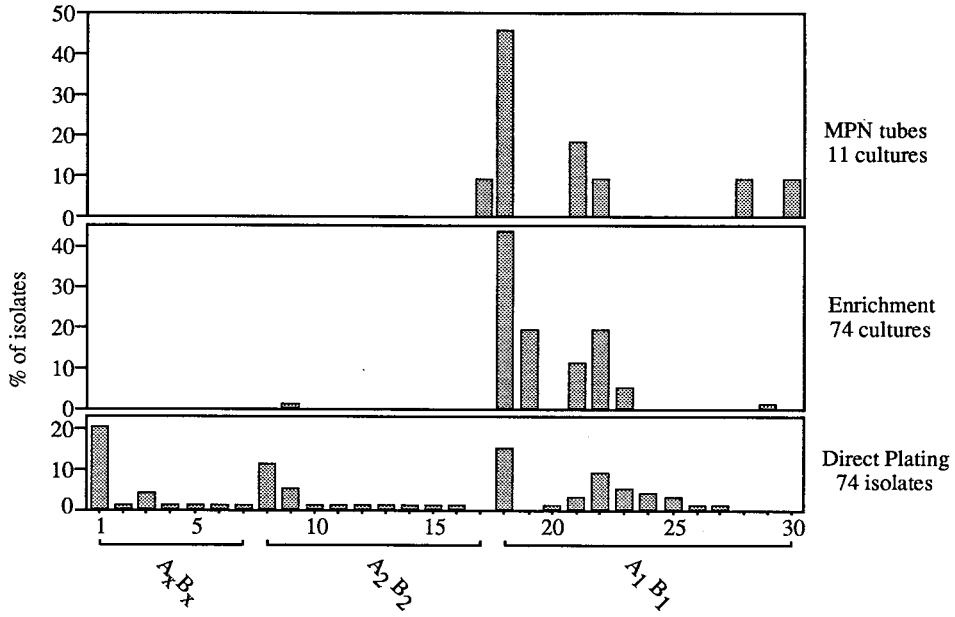

FIG. 3. Frequency distribution of populations obtained by direct plating and by enrichment. The numbers below the *x* axis correspond to lane numbers in Fig. 1. The hybridization pattern of each population with 2,4-D catabolic gene probes is indicated by the brackets below the *x* axis. Genotype A_1B_1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

of the isolates. Populations belonging to the A_1B_1 group represented six OTUs (Fig. 4). The enrichment of multiple species possessing predominantly the pJP4-type *tfd* genes suggests that the competitive fitness of these species during competition for 2,4-D may be enhanced by pJP4-type *tfd* alleles or genetic factors linked to these alleles.

DISCUSSION

Bacteria expressing specific phenotypes are most commonly obtained from natural samples by enrichment. Competition among populations during the enrichment process typically results in dominance of one or a few populations with the highest growth rates. This phenotypic bias of enrichment culture techniques is well known. We have demonstrated the genetic consequences of this phenotypic bias among 2,4-D-degrading bacteria. Enrichment of 2,4-D-degrading bacteria from 1 g of soil resulted in the almost exclusive isolation of species possessing *tfdA* and *tfdB* genes that were highly similar, if not identical, to the genes from *A. eutrophus* JMP134(pJP4). In contrast, the species obtained by direct plating represented three different groups of 2,4-D genes in roughly equal abundance. These results suggest that there is an association between specific alleles of 2,4-D catabolic genes and the competitive fitness of the species obtained by enrichment. Furthermore, the data demonstrate that the direct-plating method facilitates the acquisition of strains with previously uncharacterized and genetically distinct catabolic genes.

It is not clear whether the association between specific 2,4-D alleles and competitive fitness in enrichment cultures is coincidental or causal. Preferential selection of specific *tfd* alleles may simply be a coincidental function of host range. It is conceivable that the pJP4-type *tfdA* and *tfdB* alleles are distributed among species which, in general, are better adapted to rapid exploitation of new resources than are species harboring other *tfd* alleles. In previous studies, the pJP4-type alleles have been identified primarily in species of *Alcaligenes* and *Burkholderia* (1, 3, 5, 9, 10, 12). However, the RASC-type alleles also occur primarily in *Burkholderia* species (12, 24, 30). Although the phylogenetic identities of the OTUs obtained in the present study were undetermined, restriction analysis of amplified 16S rDNA identified three cases in which the host range of pJP4-type alleles and RASC-type alleles overlapped (OTUs 10, 15, and 17). In these cases, the pJP4-type alleles and RASC-type alleles appeared in different populations belonging to the same (or very closely related) species (Fig. 4). Thus, host range seems unlikely to account for the differential abundance of pJP4-type and RASC-type alleles following enrichment. The fact that these two sets of alleles occur in the same or very similar organisms would suggest that both sets have a similar chance of being obtained by enrichment if, in fact, competitiveness during enrichment were primarily a result of host-specific factors. However, the overabundance of the pJP4-type alleles among the enrichment isolates in the present study suggests that host-specific factors alone are insufficient to account for differences in competitive fitness.

The predominance of the pJP4-type *tfdA* and *tfdB* alleles among the seven OTUs obtained by enrichment suggests that the competitive fitness of 2,4-D-degrading species may be strongly influenced by one or more of the pJP4-type alleles or by other genetic factors linked to these alleles. Plasmids encoding different sets of *tfd* alleles have been shown to confer different growth rates on *B. cepacia* DBO1 transconjugants (18). The growth rate of a transconjugant harboring pJP4 was twofold greater than the growth rate of a transconjugant harboring a different plasmid, which contained a pJP4-type *tfdA* allele and alleles for *tfdB* and *tfdC* with less than 90% DNA sequence similarity to the pJP4 alleles (18). At maximum growth rates, TfdB, the second enzyme in the pJP4 pathway, may limit the rate of 2,4-D metabolism based on the observed accumulation of 2,4-dichlorophenol in a chemostat culture (7). The fact that 2,4-dichlorophenol is toxic to bacterial cells would tend to magnify the effects of catalytic differences be-

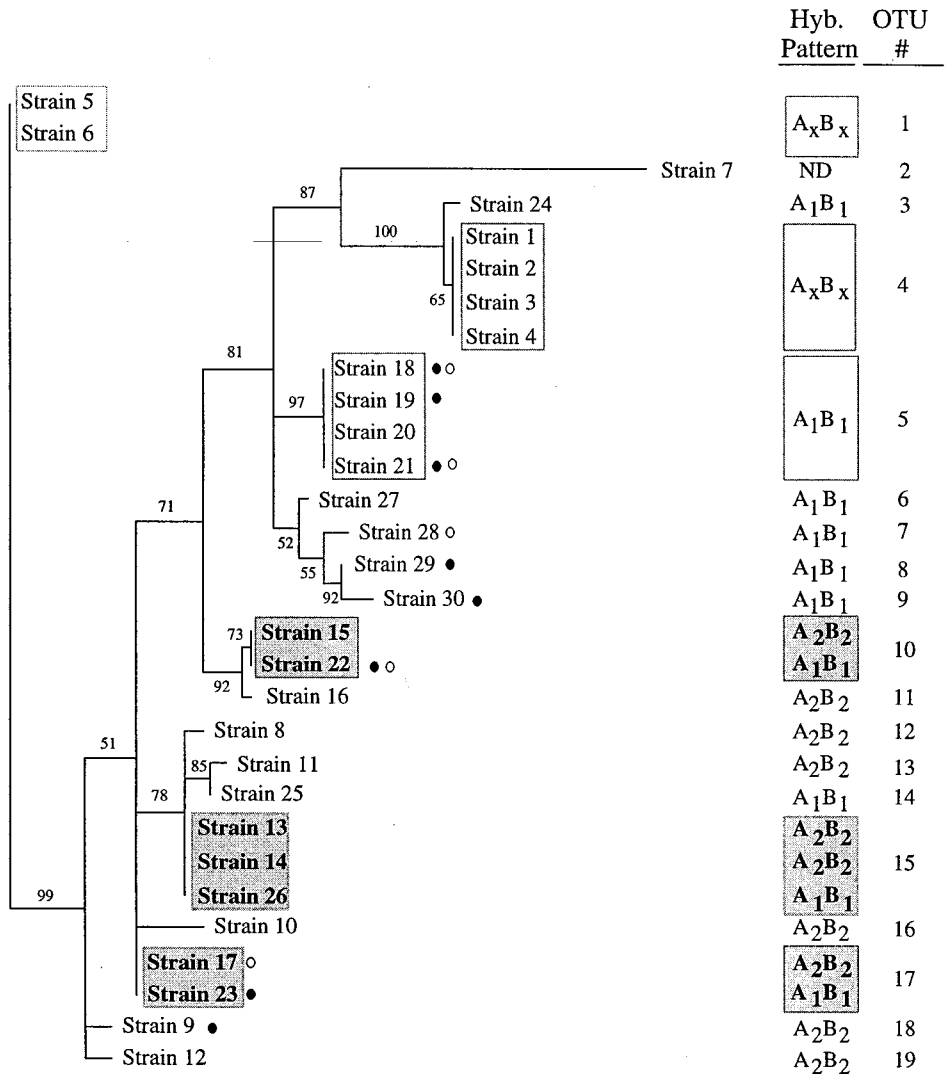

FIG. 4. Phylogenetic reconstruction of 2,4-D$^+$ strains. The number above each node indicates the bootstrap value for that node. Branch lengths are proportional to numbers of derived characters. Solid circles indicate strains obtained from standard enrichments. Open circles indicate strains obtained by enrichment in MPN tubes. All but five strains (strains 17, 19, 28, 29, and 30) were obtained by direct plating; these five strains were isolated only by standard enrichment or enrichment in MPN tubes. Strains enclosed by boxes belong to the same OTU. Boxes that are shaded include strains of the same OTU that have dissimilar 2,4-D catabolic genes.

tween TfdB isozymes. Although the kinetics of TfdB isozymes have not been measured, the deduced amino acid sequences of TfdB (pJP4) and TfdB (RASC) are 80.5% identical (29a). The isozymes encoded by different *tfdB* alleles may thus differ in catalytic efficiency and cause differences in the growth rates of 2,4-D-degrading populations. If the pJP4 *tfdB* allele is in fact optimal for rapid growth, genetic linkage with other pJP4 2,4-D genes could account for the frequent appearance of the genetic pathway encoded on pJP4 following selection for maximum growth rates. Alternatively, other genetically linked factors, such as *tfd* gene copy number per cell or elements involved in the regulation of transcription or translation of *tfd* genes, may affect the fitness of strains with different *tfd* genotypes.

In previous studies on the genetic basis of 2,4-D metabolism, the pJP4 alleles have been the most frequently encountered. The prototypical strain, *A. eutrophus* JMP134 containing the broad-host-range 2,4-D-degradative plasmid pJP4, was obtained in 1980 from an enrichment culture in Australia (10). Genes highly similar, if not identical, to the pJP4 alleles were subsequently identified in isolates obtained from India (3), Estonia (23), Florida (5), and Oregon (1) by standard enrichment techniques. While these studies established the widespread distribution of pJP4-type *tfd* alleles, enrichment bias may have obscured the presence of other 2,4-D catabolic genes in the environment. The existence of different alleles for 2,4-D catabolic genes was established only recently. Hybridization of the pJP4 alleles with DNA from pure cultures and from entire microbial communities known to contain 2,4-D-degrading populations has indicated that numerous other alleles occur in nature for each of the 2,4-D catabolic genes and may be widespread (12, 18, 19, 34). Recently, the RASC *tfdA* and *tfdB* alleles were cloned and sequenced (20, 24, 30). These alleles have 77.2 and 78.5% DNA sequence similarity, respectively, to the pJP4 alleles. Other alleles with even lower similarities have also been identified (12). In the present study, the use of direct plating instead of enrichment demonstrated that at least three groups of 2,4-D catabolic genes occurred in roughly equal abundance in a single 1-g sample of soil. Thus, isolation techniques which separate competing populations improve the as-

sessment of the diversity and redundancy of catabolic genes and hosts in the environment.

It is conceivable that some strains are detected by enrichment and not by direct plating. Only the strains able to form colonies on a specific medium will be detected by direct plating. Furthermore, only the most abundant organisms expressing a specific phenotype within a community will be detected. By using enrichment culture techniques, strains may be obtained which are rare relative to other organisms expressing the same phenotype, provided that they are highly competitive in liquid batch cultures under the selective conditions used. In this study, strains 17, 19, 28, 29, and 30 were obtained only from enrichments (Fig. 3). However, three of these strains were obtained from MPN tubes representing 10^{-4} dilutions of the original sample. Based on MPN and autoradiographic plate counts, there were 10^4 to 10^5 2,4-D^+ organisms per g of soil (data not shown). The rarefaction curves (Fig. 2) suggest that additional isolates obtained by direct plating would reveal additional diversity. Thus, it is likely that the strains obtained from MPN enrichments would have been detected by direct plating if a larger collection of isolates had been examined.

Although populations with different 2,4-D catabolic alleles have occasionally been obtained by enrichment, this method severely distorts the apparent diversity of 2,4-D-degrading populations in individual samples from the environment. The limited number of populations obtained in a single enrichment culture can be partially overcome by establishing multiple enrichment cultures. However, although a larger number of populations may be obtained, the population diversity is still substantially lower than that obtained by other methods such as direct plating. Furthermore, specific alleles of catabolic genes may be exclusively obtained as a result of the enrichment bias for organisms with relatively high growth rates. Future use of alternative methods to obtain isolates exhibiting a specific phenotype may identify novel biochemical pathways or, at least, a broader array of isozymes. With a better understanding of the diversity and distribution of particular catabolic pathways, new insights into the evolution of catabolic pathways may be obtained.

ACKNOWLEDGMENT

This research was funded by a grant from the National Science Foundation (BIR 9120006).

REFERENCES

1. **Amy, P. S., J. W. Schulke, L. M. Frazier, and R. J. Seidler.** 1985. Characterization of aquatic bacteria and cloning of genes specifying partial degradation of 2,4-dichlorophenoxyacetic acid. Appl. Environ. Microbiol. **49:** 1237–1245.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. A. Smith, J. G. Seidman, and K. Struhl.** 1987. Current protocols in molecular biology. John Wiley & Sons, Inc., New York, N.Y.
3. **Bhat, M. A., M. Tsuda, K. Horiike, M. Nozaki, C. S. Vaidyanathan, and T. Nakazawa.** 1994. Identification and characterization of a new plasmid carrying genes for degradation of 2,4-dichlorophenoxyacetate from *Pseudomonas cepacia* CSV90. Appl. Environ. Microbiol. **60:**307–312.
4. **Bruce, K. D., W. D. Hiorns, J. L. Hobman, A. M. Osborn, P. Strike, and D. A. Ritchie.** 1992. Amplification of DNA from native populations of soil bacteria by using the polymerase chain reaction. Appl. Environ. Microbiol. **58:**3413–3416.
5. **Chaudry, G. R., and G. H. Huang.** 1988. Isolation and characterization of a new plasmid from a *Flavobacterium* sp. which carries the genes for degradation of 2,4-dichlorophenoxyacetate. J. Bacteriol. **170:**3897–3902.
6. **Cochran, W. G.** 1950. Estimation of bacterial densities by means of the "most probable number." Biometrics **6:**105–116.
7. **Daugherty, D. D., and S. Karel.** 1994. Degradation of 2,4-dichlorophenoxyacetic acid by *Pseudomonas cepacia* DBO1(pRO101) in a dual-substrate chemostat. Appl. Environ. Microbiol. **60:**3261–3267.
8. **de Bruijn, F. J.** 1992. Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergenic consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. Appl. Environ. Microbiol. **58:**2180–2187.
9. **Ditzelmuller, G., M. Loidl, and F. Streichsbier.** 1989. Isolation and characterization of a 2,4-dichlorophenoxyacetic acid-degrading soil bacterium. Appl. Microbiol. Biotechnol. **31:**93–96.
10. **Don, R. H., and J. M. Pemberton.** 1981. Properties of six pesticide degradation plasmids from *Alcaligenes paradoxus* and *Alcaligenes eutrophus*. J. Bacteriol. **145:**681–686.
11. **Dunbar, J., D. C. L. Wong, M. J. Yarus, and L. J. Forney.** 1996. Autoradiography method for isolation of diverse bacterial species with unique catabolic traits. Appl. Environ. Microbiol. **62:**4180–4185.
12. **Fulthorpe, R. R., C. McGowan, O. V. Maltseva, W. H. Holben, and J. M. Tiedje.** 1995. 2,4-Dichlorophenoxyacetic acid-degrading bacteria are mosaics of catabolic genes. Appl. Environ. Microbiol. **61:**3274–3281.
13. **Harder, W., and L. Dijkhuizen.** 1982. Strategies of mixed substrate utilization in microorganisms. Philos. Trans. R. Soc. London Ser. B **297:**459–480.
14. **Holben, W. E., B. M. Schroeter, V. G. M. Calabrese, R. H. Olsen, J. K. Kukor, V. O. Biederbeck, A. E. Smith, and J. M. Tiedje.** 1992. Gene probe analysis of soil microbial populations selected by amendment with 2,4-dichlorophenoxyacetic acid (2,4-D)-degrading bacteria isolated from 2,4-D-treated field soils. Appl. Environ. Microbiol. **58:**3941–3948.
15. **Hurlbert, S. H.** 1971. The non-concept of species diversity: a critique and alternative parameters. Ecology **52:**577–586.
16. **Jannasch, H. W., and G. E. Jones.** 1959. Bacterial populations in seawater as determined by different methods of enumeration. Limnol. Oceanogr. **4:**128–139.
17. **Jannasch, H. W.** 1967. Enrichments of aquatic bacteria in continuous culture. Arch. Mikrobiol. **59:**165–173.
18. **Ka, J. O., W. E. Holben, and J. M. Tiedje.** 1994. Analysis of competition in soil among 2,4-dichlorophenoxyacetic acid-degrading bacteria. Appl. Environ. Microbiol. **60:**1121–1128.
19. **Ka, J. O., W. E. Holben, and J. M. Tiedje.** 1994. Genetic and phenotypic diversity of 2,4-dichlorophenoxyacetic acid (2,4-D)-degrading bacteria isolated from 2,4-D-treated field soils. Appl. Environ. Microbiol. **60:**1106–1115.
20. **Kamagata, Y., Y. Suwa, A. Wright, J. Tiedje, and L. Forney.** 1996. Unpublished data.
21. **Kluge, A. G., and J. S. Farris.** 1969. Quantitative phyletics and the evolution of anurans. Syst. Zool. **18:**1–32.
22. **Laguerre, G., M. Allard, F. Revoy, and N. Amarger.** 1994. Rapid identification of rhizobia by restriction fragment length polymorphism analysis of PCR-amplified 16S rRNA genes. Appl. Environ. Microbiol. **60:**56–63.
23. **Mae, A. A., R. O. Marits, N. R. Ausmees, V. M. Koiv, and A. L. Heinaru.** 1993. Characterization of a new 2,4-dichlorophenoxyacetic acid degrading plasmid pEST4011: physical map and localization of catabolic genes. J. Gen. Microbiol. **139:**3165–3170.
24. **Matheson, V. G., L. J. Forney, Y. Suwa, C. H. Nakatsu, A. J. Sexstone, and W. E. Holben.** 1996. Evidence for acquisition in nature of a chromosomal 2,4-dichlorophenoxyacetic acid/α-ketoglutarate dioxygenase gene by different *Burkholderia* spp. Appl. Environ. Microbiol. **62:**2457–2463.
25. **Moyer, C. L., J. M. Tiedje, F. C. Dobbs, and D. M. Karl.** 1996. A computer-simulated restriction fragment length polymorphism analysis of bacterial small-subunit rRNA genes: efficacy of selected tetrameric restriction enzymes for studies of microbial diversity in nature. Appl. Environ. Microbiol. **62:**2501–2507.
26. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
27. **Simberloff, D.** 1978. Use of rarefaction and related methods in ecology, p. 150–165. *In* K. L. Dickson, J. Cairns, Jr., and R. J. Livingston (ed.), Biological data in water pollution assessment: quantitative and statistical analyses. American Society for Testing and Materials, Washington, D.C.
28. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:**503–517.
29. **Staley, J. T., and A. Konopka.** 1985. Measurement of *in situ* activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats. Annu. Rev. Microbiol. **39:**321–346.

29a. **Suwa, Y.** Personal communication.

30. **Suwa, Y., A. D. Wright, F. Fukimori, K. A. Nummy, R. P. Hausinger, W. E. Holben, and L. J. Forney.** 1996. Characterization of a chromosomally encoded 2,4-dichlorophenoxyacetic acid (2,4-D)/α-ketoglutarate dioxygenase from *Burkholderia* sp. strain RASC. Appl. Environ. Microbiol. **62:**2464–2469.
31. **Swofford, D. L.** 1993. PAUP: phylogenetic analysis using parsimony, 3.1.1 ed. Smithsonian Institution, Washington, D.C.
32. **Veldkamp, H., and J. G. Kuenen.** 1973. The chemostat as a model system for ecological studies. Bull. Ecol. Res. Comm. NFR **17:**347–355.
33. **Versalovic, J., T. Koeuth, and J. R. Lupski.** 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res. **19:**6823–6831.
34. **Xia, X., J. Bollinger, and A. Ogram.** 1995. Molecular genetic analysis of the response of three soil microbial communities to the application of 2,4-D. Microb. Ecol. **4:**17–28.