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Comparison of Parental and Transgenic Alfalfa Rhizosphere Bacterial Communities Using Biolog GN Metabolic Fingerprinting and Enterobacterial Repetitive Intergenic Consensus Sequence-PCR (ERIC-PCR)

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Abstract

Rhizosphere bacterial communities of parental and two transgenic alfalfa (*Medicago sativa* L.) of isogenic background were compared based on metabolic fingerprinting using Biolog GN microplates and DNA fingerprinting of bacterial communities present in Biolog GN substrate wells by enterobacterial repetitive intergenic consensus sequence-PCR (ERIC-PCR). The two transgenic alfalfa expressed either bacterial (*Bacillus licheniformis*) genes for alpha-amylase or fungal (*Phanerochaete chrysosporium*) genes for Mn-dependent lignin peroxidase (Austin S, Bingham ET, Matthews DE, Shahan MN, Will J, Burgess RR, *Euphytica* 85:381–393). Cluster analysis and principal components analysis (PCA) of the Biolog GN metabolic fingerprints indicated consistent differences in substrate utilization between the parental and lignin peroxidase transgenic alfalfa rhizosphere bacterial communities. Cluster analysis of ERIC-PCR fingerprints of the bacterial communities in Biolog GN substrate wells revealed consistent differences in the types of bacteria (substrate-specific populations) enriched from the rhizospheres of each alfalfa genotype. Comparison of ERIC-PCR fingerprints of bacterial strains obtained from substrate wells to substrate community ERIC-PCR fingerprints suggested that a limited number of populations were responsible for substrate oxidation in these wells. Results of this study suggest that transgenic plant genotype may affect rhizosphere microorganisms and that the methodology used in this study may prove a useful approach for the comparison of bacterial communities.

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Introduction

There is much interest in rhizosphere microbial communities due to their key roles in plant health and ecosystem function. The diversity of rhizosphere microorganisms is partially dependent on the plant species [6, 7, 26] and has been previously suggested to be due in part to plant-specific differences in the chemical composition of root exudates [7, 9]. Current advances in biotechnology have allowed the construction of genetically engineered plants possessing novel and useful characteristics for applications such as crop protection, phytoremediation of polluted soils, and production of specialty enzymes [31]. Many of these engineered plants have altered root exudates which potentially may affect rhizosphere communities. Commercial development and field testing of engineered plants is far exceeding the knowledge of the potential ecological effects of their large-scale introduction into the environment [21], and concerns regarding their effects on soil and rhizosphere microorganisms have been raised by the scientific community [11, 27, 31]. Much effort is currently being devoted to the development of methods to assess the affects of transgenic plants on microbial communities.

Approaches to the characterization of microbial communities include recently developed cultural and molecular techniques. Garland and Mills [14] reported a new cultural approach to the characterization of bacterial communities based on the inoculation of Biolog Gram negative (GN) microplates (Biolog, Inc., Hayward, CA) with environmental samples to generate sole carbon source utilization patterns (metabolic fingerprints) of bacterial communities. This approach has been applied in several recent plant-associated ecological studies, including the evaluation of agricultural practices on bacterial communities [5]; the classification of rhizosphere bacterial communities of hydroponically grown plants [13, 15]; and the evaluation of larch and spruce tree rhizosphere communities [16]. Another recent study utilized this approach to evaluate the influence of different plant species (ryegrass, bentgrass, wheat, and clover) on rhizosphere community structure [17]. It has been acknowledged that this is a cultural technique, and as such does not necessarily represent the activity of all members of the community; however, its simultaneous evaluation of 95 different carbon sources, automated data collection, and potential for rapid comparison of environmental samples represent several advantages over traditional culture-based methods [12, 18].

Despite the abundance of studies which used Biolog GN community metabolic fingerprinting, the microorganisms responsible for the observed metabolic fingerprints remained uncertain. Each well on a Biolog microplate may be considered a sole carbon source enrichment culture and the microorganisms enriched in each substrate well represent a community which may be unique for a given inoculum type. Recently, this was demonstrated using DNA fingerprint analysis of 16S rDNA fragments amplified from a potato rhizosphere sample and an activated sludge sample [28]. Another DNA fingerprinting technique involves the analysis of amplified genomic sequences located between interspersed repetitive elements (rep-PCR) [30]. This technique was developed primarily for the analysis of axenic bacterial cultures, but has also been used for the analysis of mixed bacterial populations obtained by substrate enrichment [25]. One type of rep-PCR relies on the amplification of genomic DNA located between enterobacterial repetitive intergenic consensus sequences (ERIC-PCR) [29]. ERIC elements are 126 bp in size and are distributed throughout extragenic regions of the genomes of many Gram negative bacterial genera [19, 29, 30], including many plant-associated bacteria [8, 10, 20, 24]. ERIC-PCR generates multiple distinct amplification products of sizes ranging from approximately 50 to 3,000 bp which collectively constitute a DNA fingerprint. The unique locations of ERIC elements in bacterial genomes allows discrimination at the genus, species, and even strain level based on the electrophoretic pattern of amplification products [10]. Our hypothesis was that Biolog GN microplate substrate bacterial communities may serve as another means of comparing inoculum types, such as different rhizosphere samples, and that ERIC-PCR DNA fingerprinting would be useful for this approach.

A model system of parental and two transgenic alfalfa (*Medicago sativa* L.) is currently being used by the U.S. EPA to develop methods to assess the impact of transgenic plants on rhizosphere and soil microflora. The two transgenic alfalfa genotypes were developed for industrial enzyme production and expressed either bacterial (*Bacillus licheniformis*) genes for alpha-amylase or fungal (*Phanerochaete chrysosporium*) genes for Mn-dependent lignin peroxidase [1], respectively, and were otherwise isogenic. The expression cassette promoter used to construct these transgenic alfalfa was used in a previous study and resulted in the expression of an engineered protein in all parts of the plant, with units of soluble protein highest in the root [2]. In this study the rhizosphere bacterial communities of the parental and transgenic alfalfa were first compared based on community-level metabolic fingerprinting using Biolog GN microplates. Bio-

log GN substrates which were utilized differently by the rhizosphere communities were identified. Next, the types of bacteria present in these Biolog GN microplate substrate wells (substrate communities) of microplates inoculated with the different rhizosphere samples were compared using ERIC-PCR DNA fingerprinting. Finally, ERIC-PCR fingerprints of bacterial strains isolated from these substrate wells (substrate-specific populations) were compared to the ERIC-PCR fingerprints of the substrate communities and these bacterial strains were identified.

Methods

Plants and Propagation

Cuttings of isogenic parental, alpha-amylase- and manganese-dependent lignin peroxidase-expressing transgenic alfalfa (*Medicago sativa* L.) [1] were clonally propagated under greenhouse conditions (kindly provided by Sandra Austin-Phillips and Eric Triplett, University of Wisconsin—Madison). Greenhouse conditions included a 16-hour photoperiod and approximate temperatures of 24°C day, 18°C night. Plants used in this study came from internodal cuttings taken from clonally propagated mother plants. Six individual cuttings for each genotype were rooted directly in a 1:1 mix of nonpasteurized sand and Woodburn silty loam soil obtained from the U.S. EPA Willamette Research Station, Corvallis, OR, in 65 cm^3 capacity nursery pine cells (Stuewe & Sons, Inc., Kiger Island, OR) arranged randomly in a holder rack. Plants were harvested after 12 weeks of growth and replicate experiments (I and II) were performed 2 weeks apart.

Rhizosphere Extract Preparation and Community Biolog Metabolic Fingerprinting

Rhizosphere extracts were prepared from six individual plants of each genotype for each experiment. Growth containers were cut along their sides and plants and soil were placed onto clean sheets of plastic wrap. Roots with a diameter of 1 mm or less were cut from the root balls using ethanol-flamed scissors and forceps. Loosely adherent soil was removed by gently dipping the roots into sterile beakers containing sterile 0.85% NaCl. Washed roots were cut into pieces of approximately 1 cm in length using ethanol-flamed scissors and forceps. Samples of comparable weight (ca. 0.5 g) were placed in sterile test tubes containing 10 ml of extraction solution which contained 0.2% sodium hexametaphosphate (Pfaltz and Bauer, Waterbury, CT) and 6 μM Zwittergent detergent (Calbiochem Corp., La Jolla, CA) (Brendecke JW (1992) M.S. Thesis. University of Arizona). Rhizosphere microorganisms were extracted by vortexing samples for 2 min on high setting. The resulting slurries were transferred to new sterile test tubes and centrifuged at 1500 × *g* for 10 min. Supernatants were transferred to new tubes and diluted with sterile 0.85% NaCl to a final transmittance of 80–85% using a Biolog turbidimeter (Biolog, Inc., Hayward, CA).

Bacterial densities of the rhizosphere extracts were determined by spread-plating diluted samples on peptone yeast extract (PY) medium which contained peptone, 5.0 g; yeast extract, 3.0 g; $CaCl_2$, 1.1 g; and 15 g agar L^{-1} deionized water, supplemented with 100 μg ml^{-1} cycloheximide to inhibit fungal growth. Plates were incubated at 27°C for 1 week and viable plate counts (CFU ml^{-1}) were calculated.

Rhizosphere bacterial community fingerprints were generated using Biolog GN microplates. These 96-well microplates contained 95 different sole-carbon sources and a negative control well. Each well also contained the redox dye tetrazolium violet, which turns from colorless to purple in the presence of respiration. Microplates were inoculated with the six replicate rhizosphere extracts for each alfalfa genotype (each replicate derived from an individual plant), incubated at 27°C, and read with a microplate reader (Molecular Devices, Inc., Sunnyvale, CA) at 2-hour intervals from 36 to 48 hours of incubation. Absorbance data (A_{590}) was collected using Biolog ML3N software. After 48 hours of incubation samples were taken from the Biolog microplate wells for isolation of bacterial strains and substrate community DNA extraction.

Biolog GN Substrate Bacterial Communities

DNA of the bacterial communities enriched in Biolog GN microplate substrate wells was purified from microplates inoculated with different rhizosphere samples of each alfalfa genotype. Selection of Biolog GN microplate substrate communities for analysis was based on substrate utilization calculated by the Biolog ML3N software. ERIC-PCR was used to generate DNA fingerprints of the Biolog GN microplate substrate communities for comparison of the types of bacteria (substrate-specific populations) present in these wells. In addition, bacterial communities of a substrate utilized similarly by all of the alfalfa rhizosphere communities (functionally similar substrate communities) were analyzed.

To isolate members of the substrate-specific populations, aliquots from substrate wells were dilution-plated on PY medium. Colonies representing the dominant morphotypes were picked and purified. The ERIC-PCR fingerprint of each bacterial strain was compared to the ERIC-PCR fingerprints of the substrate communities, and the strains were identified using Biolog GN microplates and the Biolog ML3N software according to the manufacturer's instructions.

DNA Purification and PCR Conditions

DNA was purified from Biolog microplate substrate communities and bacterial cultures using a modification (which excluded the spin column steps) of our previously reported protocol [32]. Briefly, DNA was extracted from 100-μl samples of substrate communities from Biolog microplate wells or from resuspended cells of isolated bacterial colonies by applying SDS, EDTA, and guanidine isothiocyanate at 68°C with mild sonication. DNA fingerprints were obtained using the ERIC1R and ERIC2 primers [29]. Negative

and positive controls were included in PCR runs and duplicate PCR were routinely run for samples to verify reproducibility of fingerprints. The following was added to each 50 µl reaction mixture: 5.0 µl of 10× amplification buffer with Mg (Boehringer Mannheim, Indianapolis, IN); 1.0 µl of a 10 mM mix of each dATP, dTTP, dCTP, and dGTP (Promega, Madison, WI); 31.25 pmol of each ERIC1R and ERIC2 oligonucleotide primer (Center for Gene Research, Oregon State University, Corvallis, OR); 2.5 µl of 30 mg ml^{-1} bovine serum albumin (Sigma Chemical Co., St. Louis, MO); and ca. 25 ng of purified template DNA. PCR mixtures were overlaid with two drops of mineral oil and the PCR was performed using a PTC-100 Programmable Thermal Controller with heated lid (MJ Research Inc., Watertown, MA). Amplification conditions were as follows: initial denaturation at 95°C for 7 min; samples held at 75°C while 2.0 U *Taq* DNA polymerase (Boehringer Mannheim) was added; followed by 35 cycles of denaturation at 94°C for 10 sec, 92°C for 40 sec; annealing at 49°C for 8 sec, 51°C for 1 min; extension at 74°C for 10 sec, 72°C for 5 min; and a single final extension at 72°C for 10 min. The samples were maintained at 4°C until analysis by electrophoresis. PCR products were separated by electrophoresis on a 2.0% agarose gel (Life Technologies, Inc., Menlo Park, CA) containing 0.5 µg ml^{-1} ethidium bromide and photographed under UV light using Polaroid type 667 film (Polaroid Co., Cambridge, MA). The ERIC-PCR protocol in this study differed from the protocol of de Bruijn [10]. Notable differences included addition of *Taq* DNA polymerase, reduced in concentration by 50%, after the initial denaturation step (''hot start''). These modifications were aimed at reducing the complexity of banding patterns, obtaining highly reproducible fingerprints, and reducing nonspecific products. Preliminary studies revealed that increasing the primer annealing temperature resulted in poor amplification.

Statistical Analysis

Cluster analysis and principal components analysis (PCA) of the rhizosphere bacterial community metabolic fingerprints were performed using SYSTAT V. 5.2.1 software (SPSS, Inc., Chicago, IL). To normalize for minor differences in inoculum density, the microplates were compared at a standardized reference point in color development termed the average well color development (AWCD) value [12, 14]. AWCD values for the microplates were calculated and absorbance data from microplates having AWCD values of 0.60 ± 5% were used for statistical analyses. This AWCD value corresponded to color development in approximately half of the substrate wells. Cluster analysis by the average linkage method was used to produce Euclidean distance dendrograms of the metabolic fingerprints. The results of PCA were displayed using principal components 1 and 2 with points and error bars representing the mean and standard deviation of PC scores for the alfalfa rhizosphere bacterial community metabolic fingerprints.

Cluster analysis of Biolog GN substrate community ERIC-PCR fingerprints was performed by converting the fingerprints to binary patterns. Starting from the top of a gel each substrate community ERIC-PCR fingerprint was scored in comparison to adjacent lanes for the presence (1) or absence (0) of identifiable bands. Cluster analysis of binary patterns and dendrogram construction using the average linkage method and Euclidian distance was performed using SYSTAT V. 7.0 software (SPSS, Inc., Chicago, IL).

Results

Differences in Rhizosphere Bacterial Community Metabolic Fingerprints

Bacterial densities of the rhizosphere extracts varied little among replicates, genotypes, or experiments and had a mean of $5.9 \times 10^5 \pm 1.6 \times 10^5$ CFU ml^{-1}. In addition, all microplates reached the target AWCD value after 40–46 hours of incubation, indicating inocula of similar activity. It is therefore considered unlikely that minor differences in inoculum densities contributed to the observed differences in metabolic fingerprints.

Cluster analysis and PCA of replicate experiments I and II data revealed plant genotype-specific differences in the rhizosphere bacterial community metabolic fingerprints. Cluster analysis of the experiment I data indicated some overlap in cluster composition between the rhizosphere bacterial community metabolic fingerprints of the parental genotype and those of the alpha-amylase transgenic plants, whereas rhizosphere bacterial community metabolic fingerprints of the lignin peroxidase transgenic plants clustered distinctly (Fig. 1A). Cluster analysis of the experiment II data gave similar results in that some overlap between the rhizosphere bacterial community metabolic fingerprints of the parental genotype and of the alpha-amylase transgenic plants occurred, but to a lesser degree than in experiment I (Fig. 1B). Similarly to experiment I, the experiment II rhizosphere bacterial community metabolic fingerprints of the lignin peroxidase transgenic plants formed a distinct cluster. Cluster analysis of combined experiments I and II data sets gave similar trends in clustering: the rhizosphere bacterial community metabolic fingerprints of the lignin peroxidase transgenic plants clustered tightly, while the rhizosphere bacterial community metabolic fingerprints of the parental genotype and of the alpha-amylase transgenic plants overlapped (Fig. 1C).

PCA of the rhizosphere bacterial community metabolic fingerprints strongly agreed with the results of cluster analyses. For experiment I, PCA indicated overlap between the rhizosphere bacterial community metabolic fingerprints of the parental genotype and those of the alpha-amylase transgenic plants, while the rhizosphere bacterial community metabolic fingerprints of the lignin peroxidase plants were differentiated along the first principal component axis (Fig. 2A).

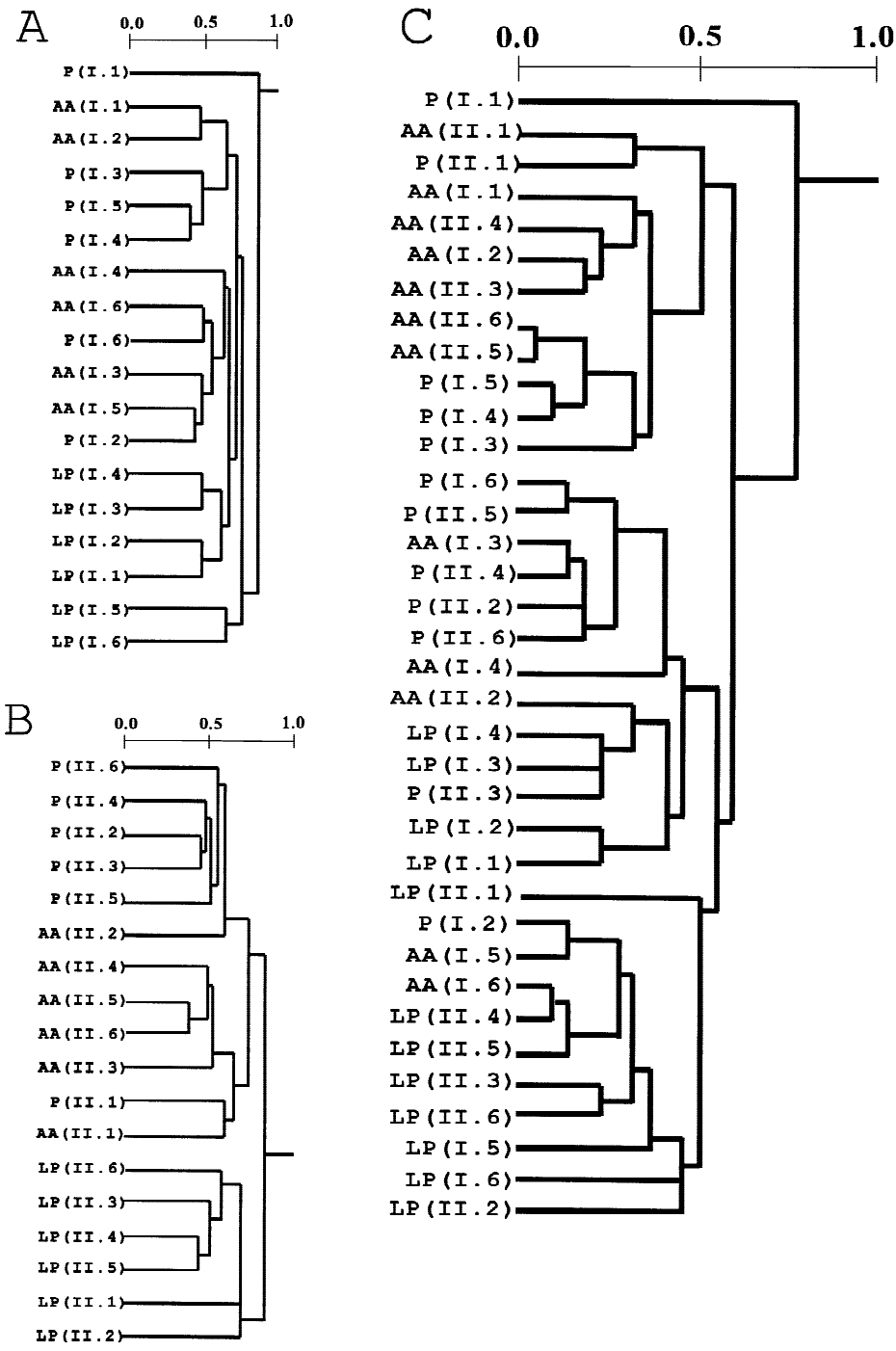

Fig. 1. Cluster analysis dendrograms of alfalfa rhizosphere bacterial community metabolic fingerprints for experiments I (A), II (B), and combined experiment I and II data sets (C). The metabolic fingerprints were generated using Biolog GN microplates (Biolog, Inc., Hayward, CA) and the microplates were compared at a standardized reference point in color development (average well color development (AWCD) value [14] of 0.60 ± 5%). Each Biolog GN microplate was inoculated with a rhizosphere extract obtained from one of six individual 12-week-old plants per alfalfa genotype (experiments I and II; P = parental, AA = alpha-amylase, and LP = lignin peroxidase; replicates 1 through 6). The average linkage method with Euclidian distance was used to produce the dendrograms.

PCA of experiment II data also indicated some overlap between the rhizosphere bacterial community metabolic fingerprints of the parental genotype and those of the alpha-amylase transgenic plants, but to a lesser degree than in experiment I (Fig. 2B). Again, the rhizosphere bacterial community metabolic fingerprints of the lignin peroxidase transgenic plants were clearly separated along the first principal component axis (Fig. 2B). Figure 2C represents PCA results of combined experiments I and II data sets which corresponded well with the results from the individual experiments. There was a significant amount of overlap between the rhizosphere bacterial community metabolic fingerprints of the parental genotype and those of the alpha-amylase transgenic plants, while rhizosphere bacterial community metabolic fingerprints of the lignin peroxidase transgenic plants were well separated.

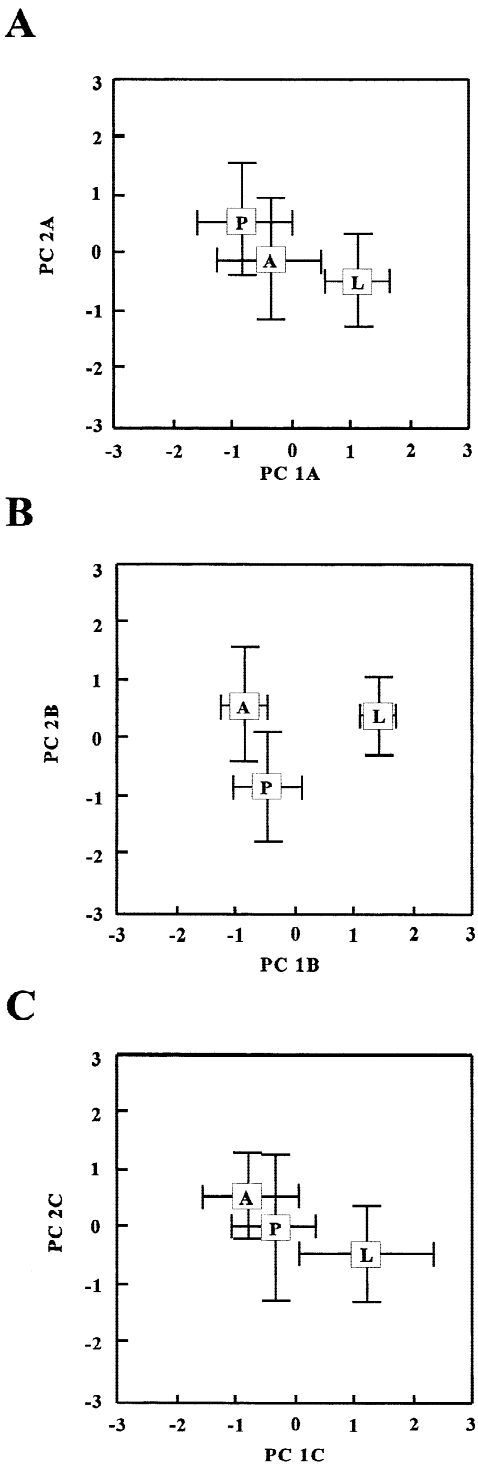

Fig. 2. Principal components analyses (PCA) of alfalfa rhizosphere bacterial community metabolic fingerprints for experiments I (A), II (B) and combined experiment I and II data sets (C). The results of PCA are displayed using principal components 1 and 2 with points and error bars representing the mean and standard deviation of PC scores for the alfalfa rhizosphere bacterial community metabolic fingerprints. (P = parental, A = alpha-amylase, L = lignin peroxidase.)

Comparison of Biolog GN Microplate Substrate Bacterial Communities

Analysis of the rhizosphere bacterial community metabolic fingerprints using the Biolog ML3N software revealed differences in the utilization of α-cyclodextrin (Biolog GN microplate well A2), D-alanine (well F5), and L-ornithine (well G4) by the rhizosphere bacterial communities of the three alfalfa genotypes. In addition, the substrate dextrin (well A3) was utilized similarly by the rhizosphere bacterial communities of all three alfalfa genotypes. These substrate communities were selected for ERIC-PCR fingerprinting and isolation of bacterial populations. Comparison of these experiment I and II substrate community ERIC-PCR fingerprints revealed consistent plant genotype-specific differences in these populations of rhizosphere bacteria (Figs. 3A and 3B, respectively; Fig. 4). ERIC-PCR fingerprints of replicate substrate communities obtained from different Biology GN microplates were highly comparable for most substrates and plant genotypes, with the exception of the D-alanine substrate communities (Figs. 3A and 3B, lanes 14–19; Fig. 4C). ERIC-PCR fingerprints of the α-cyclodextrin substrate communities were the most comparable between the two experiments (Figs. 3A and 3B, lanes 2–7; Fig. 4A). Surprisingly, the ERIC-PCR fingerprints of the functionally similar dextrin substrate communities were unique for the different alfalfa genotypes (Figs. 3A and 3B, lanes 8–13; Fig. 4B). The occurrence of several ERIC-PCR products of analogous mobility suggested that the substrate bacterial communities contained one or more common or closely related substrate-specific populations. Of particular interest was the unique low molecular weight amplification product (ca. 100 bp in size) associated exclusively with the lignin peroxidase transgenic alfalfa dextrin, D-alanine, and L-ornithine substrate bacterial communities in both experiments (Fig. 3A, lanes 12–13, 18–19, 24–25; Fig. 3B, lanes 12–13, 18, 24–25).

Biolog GN Microplate Substrate-Specific Bacterial Populations

A total of 45 bacterial strains isolated from the Biolog substrate communities were fingerprinted by ERIC-PCR. These individual strains were fingerprinted by ERIC-PCR and all but one gave amplification products. Based on their ERIC-PCR fingerprints, 41 of the 44 strains could be divided into 4 dominant groups (A–D) which contained 16, 9, 8, and 4 members each, respectively, with the remainder having dif-

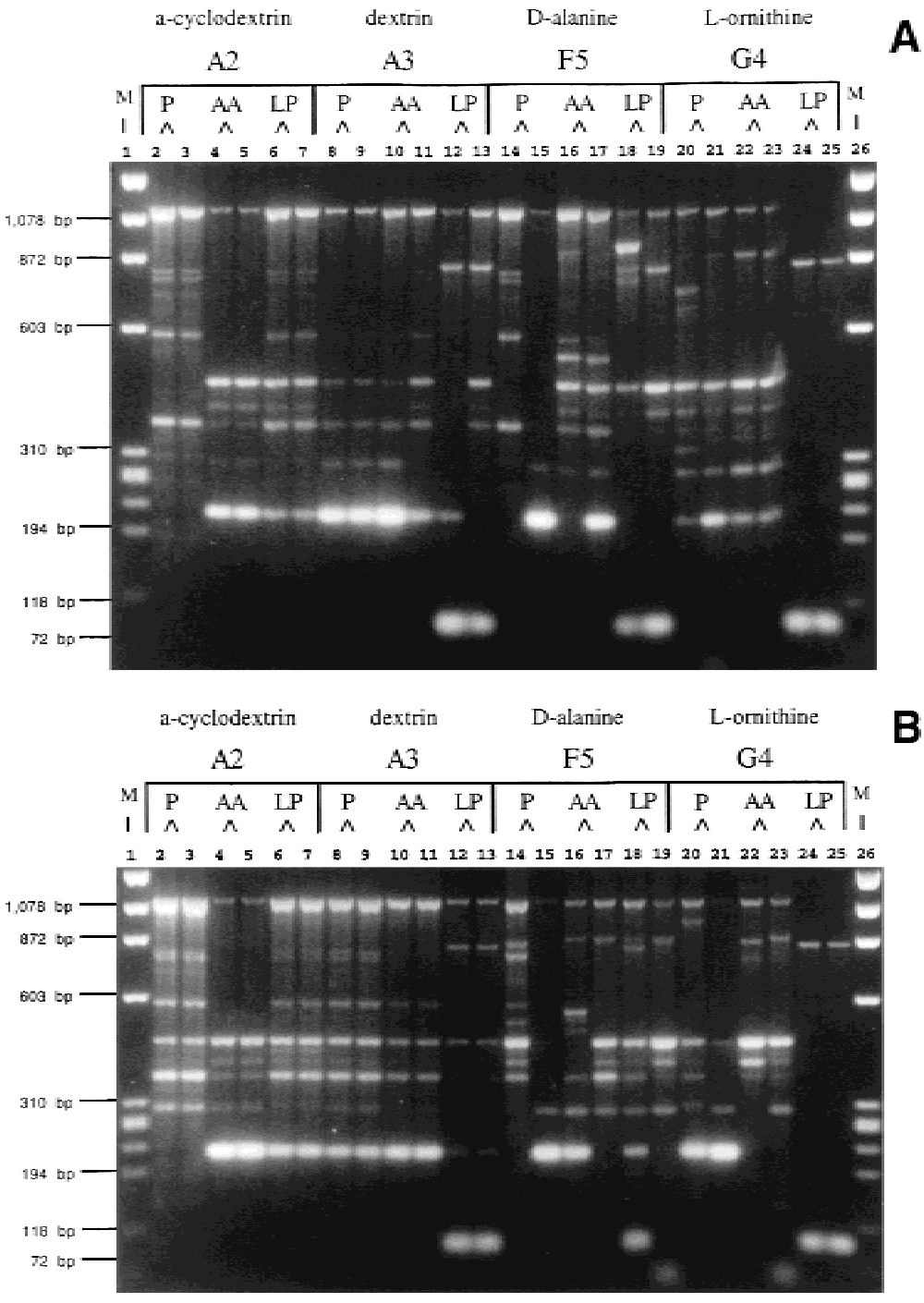

Fig. 3. ERIC-PCR fingerprints of experiment I (A) and experiment II (B) alfalfa rhizosphere Biolog GN substrate communities. ERIC-PCR was used to compare the types of rhizosphere bacteria present in the following Biolog GN substrate wells of two different Biolog microplates inoculated with individual rhizosphere samples of each genotype (P = parental, AA = alpha-amylase, LP = lignin peroxidase). A2 (α-cyclodextrin), A3 (dextrin), F5 (D-alanine), and G4 (L-ornithine). Lanes 1 and 26, ϕX174/*Hae* III molecular weight markers (Stratagene, La Jolla, CA).

ferent individual fingerprints. Only members of the A, B, C, and D groups gave bands of analogous mobility to those present in the Biolog GN substrate community fingerprints. ERIC-PCR fingerprints of these four groups are presented in Fig. 5. Representative isolates of these groups had the following Biolog identifications (tested in duplicate): group A, *Enterobacter cloacae* (sim 0.888); group B, *Pseudomonas fluorescens* Type G (sim 0.750); group C, *Pseudomonas fluorescens* Type G (sim 0.599); and group D, *Pseudomonas putida* Type A1 (sim 0.826). The group D strains (*Pseudomonas putida* Type A1; Fig. 5, lane 9) gave a unique low molecular weight ERIC-PCR amplification product analogous in mobility to that associated exclusively with the lignin peroxidase transgenic alfalfa Biolog GN substrate communities (Fig. 3A, lanes 12–13, 18–19, 24–25; Fig. 3B, lanes 12–13, 18, 24–25; Fig. 5, lane 4).

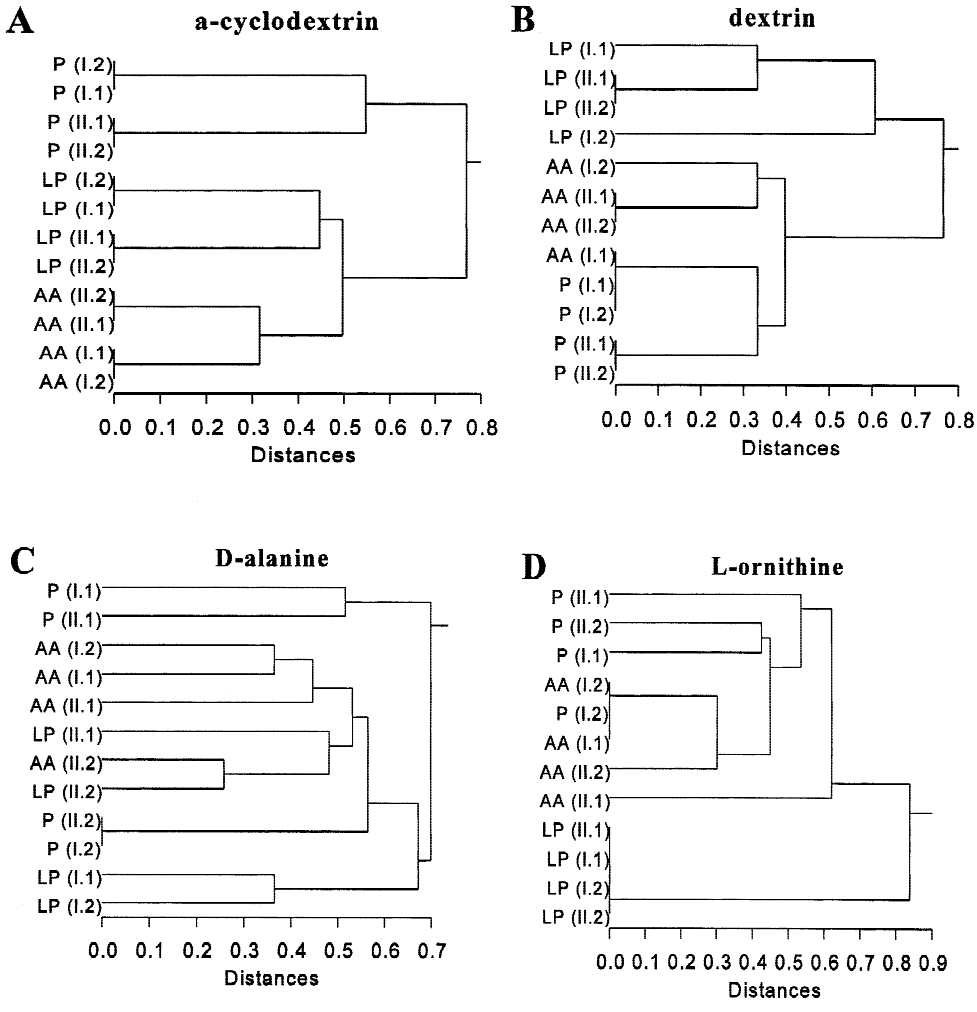

Fig. 4. Cluster analysis dendrograms of the ERIC-PCR fingerprints of experiment I and experiment II alfalfa rhizosphere Biolog GN substrate communities presented in Fig. 3. Samples are labeled as follows: Biolog GN substrate communities α-cyclodextrin (A), dextrin (B), D-alanine (C), and L-ornithine (D); each genotype (P = parental, AA = alpha-amylase, LP = lignin peroxidase); experiments I and II; and replicate samples 1 and 2. The average linkage method with Euclidian distance was used to produce the dendrograms.

Discussion

The cultural and molecular approach used in this study consistently detected differences between the rhizosphere bacterial communities of the parental genotype and the alpha-amylase and lignin peroxidase transgenic alfalfa. These findings support and extend earlier studies which suggested that plant genotype may affect rhizosphere microbial populations [3, 4, 13, 16, 17, 22, 23]. In a field performance trial the alpha-amylase transgenic alfalfa plants were phenotypically identical to the parental plants, while the lignin peroxidase transgenic alfalfa plants eventually became stunted and had reduced dry mass and height [1]. In this study, comparison of the rhizosphere bacterial communities of these alfalfa genotypes using Biolog GN metabolic fingerprinting agreed with these observations. In both experiments, cluster analysis and PCA revealed overlap between the Biolog GN rhizosphere bacterial community metabolic fingerprints of the parental genotype and the alpha-amylase transgenic alfalfa, while the rhizosphere bacterial community metabolic fingerprints of the lignin peroxidase transgenic alfalfa were the most distinctive. Results of cluster analysis and PCA of combined experiment I and II data sets indicated comparability of the two experiments (Figs. 1C and 2C, respectively) and confirmed the overlap between the rhizosphere community metabolic fingerprints of the parental genotype and alpha-amylase transgenic alfalfa.

Some variability or "noise" is inevitable when analyzing environmental microbial communities, particularly when using culture-based assays. Since Biolog GN metabolic fingerprinting is a selective culture assay, it is also prone to this noise. As mentioned earlier, each well on a Biolog microplate may be considered a sole substrate enrichment culture. As such, results of the assay may be affected by inoculum homogeneity and density. In addition, substrate utilization likely reflects the activity of only a fraction of the entire

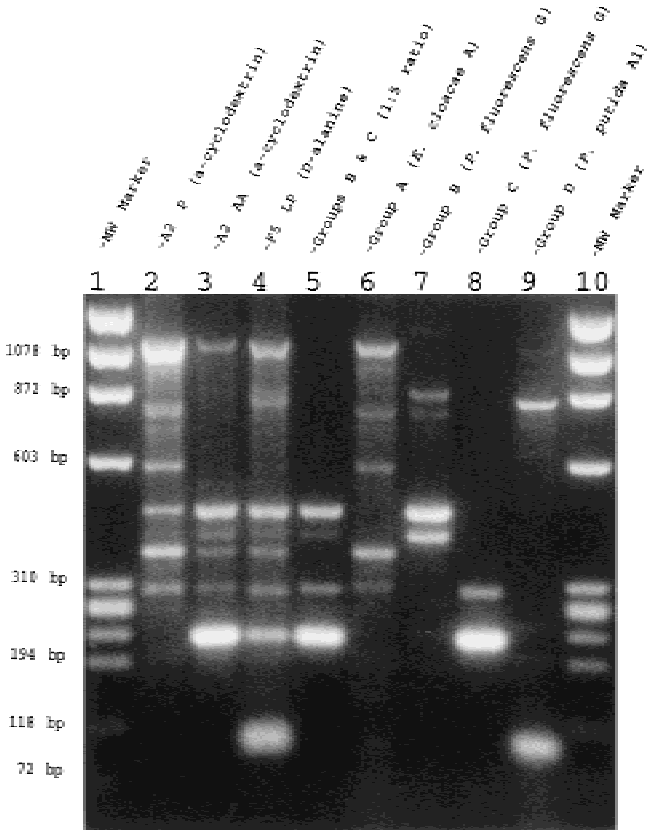

Fig. 5. ERIC-PCR fingerprints of bacterial strains (groups A–D) isolated from Biolog GN microplate substrate wells. Lanes: 2–4, ERIC-PCR fingerprints of Biolog GN substrate communities [experiment II, wells A2 (P), A2 (AA), and F5 (LP)] for amplification product size reference; 5, fingerprint of a 1:5 mix of purified DNA from the group B (*Pseudomonas fluorescens* Type G) and group C (*Pseudomonas fluorescens* Type G) strains; 6, group A (*Enterobacter cloacae*); 7, group B (*Pseudomonas fluorescens* Type G); 8, group C (*Pseudomonas fluorescens* Type G); 9, group D (*Pseudomonas putida* Type A1); 1 and 10, ϕX174/*Hae* III molecular weight markers (Stratagene, La Jolla, CA).

microbial community. Given the selective nature of the assay, changes in community metabolic fingerprints likely represent changes in community structure (species evenness) and not necessarily community diversity (species richness). Similarly, since it is an *in vitro* assay, correlations to *in situ* community function should be considered with caution. However, it should be reinforced that valuable information may be obtained by studying portions of microbial communities.

Despite the similarity of the parental genotype and alpha-amylase transgenic alfalfa Biolog GN rhizosphere community metabolic fingerprints, the ERIC-PCR fingerprints of Biolog GN substrate communities revealed plant genotype-specific differences between these populations of rhizosphere bacteria for each of the alfalfa genotypes. Cluster analysis revealed that ERIC-PCR fingerprints of replicate substrate communities, obtained from different Biolog GN microplates inoculated with rhizosphere samples derived from individual plants, were highly comparable for most substrates and plant genotypes and reproducible between experiments I and II. Cluster analysis also revealed some variability or noise in the ERIC-PCR fingerprints of some Biolog GN substrate communities, notably the D-alanine substrate communities (Fig. 4C). This likely reflects variations in the selective enrichment of different bacterial populations in these substrate wells.

An unexpected finding was that the ERIC-PCR fingerprints of the dextrin substrate communities revealed consistent plant genotype-specific differences between these bacterial populations, despite their comparable utilization of this substrate. These data suggest that in some cases functionally similar Biolog GN substrate communities may differ structurally.

Comparison of ERIC-PCR fingerprints of substrate communities and bacterial strains obtained from the same substrate wells suggested that the substrate communities were made up of relatively few types of bacteria, and that the strains isolated accounted for all major ERIC-PCR amplification products of the substrate communities. We evaluated this possibility by attempting to recreate the ERIC-PCR fingerprint of a substrate community by mixing purified DNA from the group B (*Pseudomonas fluorescens* Type G) and group C (*Pseudomonas fluorescens* Type G) substrate-specific bacterial populations in different ratios. The finding that these ERIC-PCR fingerprints were similar (Fig. 5, lanes 3 and 5) supported our interpretation. It is noteworthy that these two substrate-specific populations, representing two different strains of *Pseudomonas fluorescens* Type G, were isolated from the same substrate well on several occasions during the study. These results provide evidence that differences in Biolog GN substrate bacterial communities can be due not only to species-level, but also to strain-level differences in bacterial populations.

While it appears that the Biolog GN substrate communities analyzed contained a limited number of populations, it is possible that other populations which contributed to substrate oxidation were not detected. One possibility is that populations which may have initiated substrate oxidation in the Biolog GN wells were superseded during incubation by populations which were eventually isolated from the wells. A

recent study evaluated how Biolog GN substrate communities change during incubation [28] and found that in some cases populations detected in the inoculum were dominant following incubation, while in other cases populations which were undetectable in the inoculum were enriched and became dominant after incubation. It may therefore prove useful when comparing inoculum types to analyze Biolog GN substrate communities of interest over the course of incubation, which could allow for a more comprehensive evaluation of the populations contributing to substrate oxidation.

Regardless of the mechanisms involved, dominant Biolog GN substrate-specific populations repeatedly obtained from specific types of inocula may represent bacterial indicators of niche conditions and may be useful in future investigations. An example from this study is the *Pseudomonas putida* strain isolated only from substrate communities of Biolog GN plates inoculated with lignin peroxidase transgenic alfalfa rhizosphere samples. Characterization of this *Pseudomonas putida* strain, including the evaluation of its competitiveness in the rhizospheres of the different alfalfa genotypes, may provide insight into the effects of niche conditions on rhizosphere populations.

To clarify the relevance of our findings to the environmental risk assessment of these transgenic plants requires further investigation, since the alfalfa used in this study were grown under greenhouse conditions. This approach is currently being used to determine if the field-grown parental and transgenic alfalfa have unique rhizosphere bacterial populations associated with them. Note that while this study revealed that transgenic plant genotype may affect rhizosphere bacterial communities, additional studies are needed to determine whether such changes may or may not have adverse effects on downstream biological processes or the success of future rotational crops.

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