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Influence of enhanced malate dehydrogenase expression by alfalfa on diversity of rhizobacteria and soil nutrient availability

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Abstract

Transgenic alfalfa over-expressing a nodule-enhanced malate dehydrogenase (neMDH) cDNA and untransformed alfalfa plants were grown at the same field site and rhizosphere soils collected after 53 weeks of plant growth. These alfalfa lines differ in the amount and composition of root organic acids produced and exuded into the rhizosphere. Nucleotide sequencing of PCR-based 16S ribosomal DNA (rDNA) clone libraries and Biolog™ GN microtiter plates were employed to assess the activity of naturally occurring rhizobacteria in the two alfalfa rhizospheres. Selected macro- and micro-elements in the two alfalfa rhizosphere soils were also measured. Analysis of 240 16S rDNA clone sequences indicated the existence of about 11 bacterial phyla and their major subdivisions in the two alfalfa rhizosphere samples. There were qualitative changes in the abundance of bacterial phylogenetic groups between rhizosphere soils of transgenic and untransformed alfalfa. Carbon substrate utilization profiles suggested that rhizosphere samples from transgenic alfalfa had significantly greater microbial functional diversity compared with rhizosphere samples from untransformed alfalfa. The concentrations of nitric acid extractable P, K, Mn, Zn and Cu increased significantly in the transgenic alfalfa rhizosphere compared with the untransformed alfalfa rhizosphere. These observations indicate that organic acids produced by plant roots significantly influence rhizosphere microbial diversity and availability of macro- and micro-nutrients and demonstrate the utility of such trangenic plants as tools for studying the potential impact of plant root exudates on soil microbial ecosystems.
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Keywords: Alfalfa; Lucerne; Medicago; Malate dehydrogenase; Bacterial diversity; rDNA; Transgenic plants; Organic acids; Nutrient uptake

1. Introduction

Plant roots release as much as 20% of their assimilates as root exudates in the form of organic acids, amino compounds, sugars and phosphate esters (Uren, 2001; Whipps, 1990). Root exudates are known to enhance growth rates of bacteria (Hartwig et al., 1991), to act as chemoattractants to attract bacteria towards the root (Dharmatilake and Bauer, 1992), and to act as transcriptional signals in the communication between soil bacteria and host plants during nodule formation and biological nitrogen fixation by plants (Phillips and Streit, 1996; Redmond et al., 1986). Different plant species release different organic compounds that vary in their ability to induce or inhibit effective signaling by different soil bacteria (Phillips and Streit, 1996). Root exudation may create a niche that influences which microorganisms colonize the rhizosphere, thereby altering the composition and diversity of rhizomicrobial communities in a plant-specific manner (Grayston et al., 1998; Marilley et al., 1998). Although the mechanisms by which plants regulate root exudation are not fully understood, enhanced rates of exudation of organic acids have been associated with plant responses to toxic levels of aluminum in acid soils (Kochian, 1995; Ryan et al., 2001), as well enhanced

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acquisition of sparingly soluble plant nutrients such as P, K or Fe (Dinkekaker et al., 1989; Jones, 1998; Johnson et al., 1994; Kraffczyk et al., 1984). Increased exudation of citrate and malate by plant roots has been shown to occur under conditions of P deficiency and Al toxicity (Johnson et al., 1994; Kochian, 1995). Differences among plant species in the ability to solubilize soil nutrients and to chelate toxic elements influence the ability of plants to cope with adverse soil conditions.

We recently reported the production of transgenic alfalfa plants over-expressing a full-length nodule-enhanced malate dehydrogenase (neMDH) cDNA under the control of the cauliflower mosaic virus (CaMV) 35S promoter (Tesfaye et al., 2001). One of the transgenic alfalfa lines (M16-27) displayed a 4.2-fold increase in the accumulation of neMDH mRNA transcript and a 1.6-fold increase in the activity of malate dehydrogenase compared with the untransformed alfalfa cultivar Regen-SY (Tesfaye et al., 2001). The enzyme, malate dehydrogenase (MDH: EC 1.1.1.82), catalyzes the reversible reduction of oxaloacetate to malate (Vance, 1997). In neutral pH medium, enhanced amounts of malate and other organic acids were exuded into the rhizosphere by roots of the transgenic line (Tesfaye et al., 2001). Other research groups have previously generated transgenic plants that displayed enhanced amounts of organic acid with subsequent increased capacity for P uptake (Koyama et al., 2000; Lopez-Bucio et al., 2000) and tolerance of aluminum (de la Fuente et al., 1997; Koyama et al., 2000). Although numerous studies have focused on the effect of organic acids on amelioration of Al toxicity or P uptake, there have been few investigations on the influence of these transgenic plants on microbial populations or on the availability of other mineral nutrients. It was shown previously that microbial population density and diversity can be influenced by introducing genes into plants for the production of novel compounds (Oger et al., 1997; Savaka and Farrand, 1997).

Only about 1% of soil organisms can be cultivated on routine laboratory media (Amann et al., 1995). The immense range of rhizophere soil microbes, together with technical limitations associated with cultivation-based bacterial enumeration techniques, compel researchers to employ alternative approaches to study microbial diversity (Amann et al., 1995; Pace, 1997). The availability of 16S rDNA nucleotide sequences from diverse bacterial groups in the public domain is making soil microbial studies easier by providing valuable information for identifying both culturable and non-culturable organisms (Pace, 1997). The Biolog™ system was developed to use carbon source utilization patterns of individual bacterial isolates for taxonomic identification (Bochner, 1989). Substrate utilization patterns derived from inoculating soil suspensions directly into Biolog™ GN microtiter plates have also been shown to provide information relevant to soil microbial functional diversity (Garland, 1996; Zak et al., 1994). For example, relatively subtle shifts in microbial functional diversity due to plant age were discernible with this method (Garland, 1996). The present study employed both 16S rDNA sequencing and Biolog GN plates to assess potential changes in diversity and structure of naturally occurring rhizosphere bacterial communities associated with a transgenic alfalfa line and its untransformed parental alfalfa line grown in the field.

2. Materials and methods

2.1. Plant culture, soil sample collection and elemental analysis

The experiment was done at the University of Minnesota Experiment Station, St Paul, Minnesota, USA. This study was part of a larger field experiment designed to evaluate the performance and productivity of several transgenic alfalfa lines that included the untransformed alfalfa cultivar Regen-SY and its derivative transgenic alfalfa line (M16-27).

The experimental field was plowed, harrowed and rolled during April and May of 1999. The site had been planted with alfalfa in the previous three seasons and had composted animal waste applied in the 1997 season. Alfalfa plants for this experiment were clonally propagated as described by Tesfaye et al. (2001) and were transplanted by hand in June 1999. A randomized complete block experimental design with three replicates was used. Each plot comprised a single row of seven alfalfa plants, with plants being 0.3 m apart and a distance between plots of 0.6 m. The plants did not receive fertilizer or rhizobial inoculant. Pre-emergent herbicides were applied before transplanting, and plots were hand-weeded during the experimental period. Potato leaf hoppers were controlled in both years by regular foliar application of Ambush™ according to the manufacturer's recommendations. To determine aerial biomass accumulation, plants were clipped to ground level on four occasions: 8/27/1999, 9/28/1999, 6/14/2000 and 7/14/2000. Dry matter (DM) yield was determined after drying shoot tissue in a 70 °C oven.

Fifty three weeks after transplanting plants in the field, two plants from each plot were excavated randomly, and loose soil was shaken off the roots and discarded. The soil that adhered to the roots was brushed off, and soil from the two plants in each plot was pooled. The rhizosphere soil was immediately transported to the laboratory and screened through a 1 mm sieve. Half of the soil was stored at −80 °C and the other half was stored at 4 °C.

For macro- and micro-element analyses, soil samples were air-dried, screened through a 2-mm sieve, and 1N HNO_3 extracts were analyzed using inductively coupled

plasma-atomic emission spectroscopy (ICP-AES) at the Research Analytical Laboratory, University of Minnesota.

2.2. Soil DNA extraction and 16S rDNA library construction

Soil samples stored at − 80 °C were used for total DNA extraction. Soil DNA was extracted from 0.5 g soil sample using the UltraClean™ Soil DNA Isolation Kit (MO BIO Laboratories, Inc., Solana Beach, CA) following the manufacturer's directions. Approximately a 1.5 kb 16S rDNA fragment was amplified in a PCR reaction using extracted soil DNA as the template. Primers used were: 27f (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492r (5′-TACGGYTACCTTGTTACGACTT-3′), corresponding to positions 8–27 and 1492–1513, respectively, of the *Escherichia coli* 16S rDNA (Lane, 1991). PCR reactions (100 μl) consisted of 2.5 U of *Taq* DNA polymerase, 10 mM Tris-HCl (pH 8.3), 50 mM KCl and 1.5 mM $MgCl_2$ (Roche Chemicals, Inc.), dNTPS at 250 μM each, 62 pmol each of 27f and 1492r primers, and 300 pg soil DNA as the template. Amplification conditions were 30 cycles of 94 °C for 1 min, 55 °C for 30 s and 72 °C for 1 min, followed by 72 °C for 5 min. Amplified PCR products were purified using mini-spin columns (Qiagen USA, Madison, WI) following the manufacturer's instructions. The 16S rDNA products were ligated into pGEM-T Easy vector and transformed into competent *E. coli* JM109 cells (Promega, Inc., Madison, WI). A total of 320 plasmid DNA clones containing the expected insert size were submitted for automated DNA sequencing by the Advanced Genetic Analysis Center, University of Minnesota, St Paul, Minnesota, USA, using 27f as a sequencing primer.

2.3. Phylogenetic analysis

The resulting 16S rDNA partial sequences were edited and were compared with sequences in the Ribosomal Database Project (RDP) using the SEQUENCE_SIMILARITY option (Maidak et al., 2001). Nucleotide sequences were checked for chimeric properties using the CHECK_CHIMER option of the Ribosomal Database Project (Maidak et al., 2001). On the basis of database searches and similarity indexes, nucleotide sequences resulting from this study were aligned pair-wise with published bacterial sequences from RDP and GenBank databases using the CLUSTAL W option (Thompson et al., 1994). The accession numbers of bacterial 16S rDNA sequences used for sequence alignment and/or phylogenetic analyses are listed in Table 1. Aligned nucleotide sequences corresponded to positions 110 and 357 of the *E. coli* 16S rDNA sequence numbering (Lane, 1991). A series of 100 bootstrap data sets of the same size as the original nucleotide sequence data were re-sampled using the SEQBOOT option of the PHYLIP software (Felsenstein, 1995). As provided in the PHYLIP software (Felsenstein, 1995), a matrix of Jukes–Cantor distances (Jukes and Cantor, 1969) was used to construct unrooted phylogenetic trees using the neighbor-joining method of Saito and Nei (1987). A consensus phylogenetic tree was constructed using the CONSENSE option of the PHYLIP software (Felsenstein, 1995).

Table 1
GenBank accession numbers of 16S rDNA sequences used in the analysis of 16S rDNA clone libraries in this study

Bacterial group	GenBank accession number
Proteobacteria	AB009013, AB021128, AB025572, AJ227797, AJ295071, D12795, D86605, U29388, U31074, U89831, AB008801, AB004790, AB00800, AF078756, AB003627, AF078767, AF288737, AY036898, AJ012108, AB021389, AB021423, X97652, Y12639, Y17661, AF177916, AF159581, X72771, X80728, D89798, AJ233936, AJ233915, Y17712, Y11560, AF354664, X99236, U96917, AJ233940, AJ233915, AY038046, AF097690, L42994, U51874
CFB green sulfur group, including bacteroides	AB015265, M58766, AB023660, AB050106, AB021165, AB03390, AF260710, AF162266, M58794, AF137381, M62798, AJ224415, Y18838, AF070444, U85888, Y07837, M58468
High and low G + C content gram-positive	U75647, AB045884, Z68094, D87974, AF116559, AB015539, AJ308603, X92708, AJ408875, AY043395, AF062535, AJ278870, D78478, AF071857, X60644, AJ311893, AY030338, X64465, D82064, U49247, X73436, X76739, X68415, AJ229251, AJ243423, X68461, AJ240976
Fibrobacter/Acidobacteria	D26171
Nitrospira	Y14644
Green non-sulfur	AJ009480
Planctomycetales	AB015527
Cyanobacteria	AF132783, AF137631
Thermotogales/Thermus/Deinococcus	AB020888, AJ22911, AB039769
Aquificales	AJ001049

2.4. Bacterial density and patterns of substrate utilization

Ten grams of each replicate soil sample stored at 4 °C was weighed and the soil samples pooled (30 g of soil in total for each treatment). Soil suspensions were prepared by mixing 6 g of the pooled soil with 54 ml of sterile quarter-strength Ringer's solution (EM Industries, Inc., Gibbston, NJ) and shaken for 1 min. The suspensions were allowed to settle for 1 h at room temperature, and the supernatant was 10-fold serially diluted in sterile quarter-strength Ringer's solution. Samples from 10^{-2} to 10^{-6} dilution series were used for estimating bacterial population densities and evaluating patterns of substrate utilization.

For bacterial counts, 100 μl of the suspensions were plated onto R2A medium (DIFCO, Becton Dickson and Co., Sparks, MD) containing 150 μg/ml cycloheximide. Plates were incubated at 28 °C for 24–48 h and colony-forming units (cfu) were counted.

The remaining soil suspensions from each dilution series were added to the Biolog™ Gram-negative (GN) Microtiter plates (Biolog, Inc., Hayworth, CA). Each well was inoculated with a 150-μl soil dilution sample using an eight-channel repeating pipettor. The plates were inoculated with four replicates of soil extracts and incubated at 30 °C under static conditions in the dark. The reduction of tetrazolium violet present in each well to colored formazan in respiring cells was used to estimate the degree of substrate oxidation by measuring absorbance at 590 nm using a Multiskan® EX microtiter reader (Labsystems, Helsinki, Finland), after 24 and 48 h of incubation. The absorbance value of the control well was subtracted from that of each well containing a carbon substrate. Substrate wells with resulting absorbance values below 0.05 were assigned an absorbance value of '0' in subsequent data analysis.

The 95 substrate-wells on each Biolog™ GN plate were categorized into six groups following the classification of Zak et al. (1994). Catabolic response profiles were evaluated by determining catabolic richness and evenness (Degens, 1998; Degens et al., 2000). Average well color development (AWCD) was calculated for each substrate group according to Garland and Mills (1991). A threshold value was calculated as the total plate absorbance for each substrate group divided by the total number of substrate wells that showed positive absorbance values in each substrate group (Vahjen et al., 1995). Substrate wells utilized were substrate wells with absorbance values above the threshold and represent catabolic richness. Catabolic evenness was determined by calculating the Shannon–Weaver index (Degens, 1998; Degens et al., 2000; Magurran, 1988), and is said to be an indicator of a component of microbial diversity that can be applied across all soil types (Degens et al., 2000).

3. Results

3.1. Relative abundance and phylogenetic analysis of 16S rDNA clone sequences

A total of 240 clones from the 320 plasmid clones submitted for sequencing from the transgenic and untransformed alfalfa rhizosphere 16S rDNA libraries produced usable nucleotide sequence information and were further analyzed. Several of the 16S rDNA clone sequences were identical in the region analyzed, including 10 pairs of duplicate 16S rDNA clones, three sets of triplicate 16S rDNA clones and one set of five 16S rDNA clones from the untransformed alfalfa rhizosphere, together with 12 pairs of duplicate 16S rDNA clones, three sets of triplicate 16S rDNA clones and one set of seven identical 16S rDNA clone sequences from the transgenic alfalfa rhizosphere. Eight 16S rDNA clones from the untransformed alfalfa rhizosphere showed identical nucleotide sequences to 16S rDNA sequences in the GenBank database: *Pseudomonas* sp. accession AF177916 (one clone), *Variovorax paradoxus* accession AF288737 (one clone), *Bacillus globisporus* acession X68415 (one clone) and *E. coli* accession X80728 (five clones). In contrast, there was only one 16S rDNA clone from the transgenic alfalfa rhizosphere whose nucleotide sequence was identical to the GenBank nucleotide sequences from *E. coli* acession X80728.

Table 2 shows the broad phylogenetic distribution of all of the 16S rDNA clones analyzed from the two alfalfa rhizospheres. Many of the 16S rDNA clone sequences aligned phylogenetically with previously determined bacterial groups, such as proteobacteria, cytophaga-flexibacter-bacteriodes (CFB) group including flavobacteria, gram positive bacterial groups (*Bacillus* and *Actinomyces*), fibrobacter/acidobacterium, green non-sulfur, nitrospira, cyanobacteria, planctomycetales, and green sulfur bacterial groups. Approximately 8% of the total 16S rDNA clones did not cluster with any of the major bacterial groups included in the construction of the phylogenetic trees and may be regarded as unclassified and/or uncultured bacterial groups (Table 2). Below we describe the comparative phylogenetic analysis of the 16S rDNA sequence data from the rhizosphere soils of the transgenic alfalfa line M16-27 and its parent, untransformed alfalfa cultivar Regen-SY.

3.1.1. Proteobacteria group

Clone sequences belonging to members of the proteobacteria groups were most abundant and comprised up to 36% of the total 16S rDNA clone libraries of the transgenic and untransformed alfalfa rhizospheres (Table 2). All but ε-proteobacterial groups were recovered in this study. The β-proteobacterial group dominated the proteobacterial clones and comprised over half of the total proteobacterial 16S rDNA clones from both alfalfa rhizospheres (Table 2). Major β-proteobacterial genera recovered from both alfalfa rhizospheres included several species of *Burkholderia*,

Table 2
Relative abundance (%) of partial 16S rDNA clone sequences from untransformed and transgenic alfalfa rhizospheres

Bacterial group	Untransformed alfalfa	Transgenic alfalfa	Pooled data
Proteobacteria			35.8
α-proteobacteria	5.9	9.1	
β-proteobacteria	19.3	19.0	
γ-proteobacteria	6.7	2.5	
δ-proteobacteria	3.4	5.8	
CFB group			27.9
Cytophaga	11.8	15.7	
Flexibacter*	9.2	4.1	
Flavobacter + others	9.2	5.9	
High G + C grampositive*			7.5
Actinobacterial sp.	5.0	6.6	
Uncultured/unclassified	0.8	2.5	
Low G + C gram-positive			3.3
Bacillus sp.	5.0	1.7	
Fibrobacter/Acidobacterium*	4.2	9.1	6.7
Nitrospira*	1.7	6.6	4.2
Green Sulfur	NR	1.7	1.7
Green non-sulfur	4.2	4.1	4.2
Planctomycetales	1.7	NR	1.7
Cyanobacteria	0.8	NR	0.8
Unclassified/unknown*	10.9	5.8	8.3

Relative abundance (%) was calculated as the proportion of 16S rDNA clones representing a bacterial group to the total 16S rDNA clones in each rhizosphere soil. Total numbers of 16S rDNA clones were 121 from the transgenic alfalfa rhizosphere and 119 from the untransformed alfalfa rhizosphere; NR–not recovered; Bacterial groups followed by * were generally significant at $P < 0.1$ as determined by the *t*-test procedure of SAS for windows (SAS Institute, 1989).

Variovorax, *Acidovorax* and *Denitrobacter*. Bacterial genera that aligned with γ-proteobacterial 16S rDNA clone sequences included *E. coli*, *Pseudomonas* and *Xanthomonas*. There were more γ-proteobacterial clone sequences in the untransformed alfalfa rhizosphere than the transgenic alfalfa rhizosphere. In contrast, a greater proportion of the α- and δ-proteobacterial 16S rDNA clones were recovered from the transgenic alfalfa rhizosphere than from the untransformed alfalfa rhizosphere. Major α-proteobacterial genera included *Sphingomonas*, *Agrobacterium* and *Methylobacterium*, while *Desulfovibrio*, *Stigmatella* and *Geobacter* dominated the δ-proteobacterial 16S rDNA clone sequences.

3.1.2. CFB group

The CFB bacterial group comprised 26 and 30% of the transgenic and untransformed alfalfa rhizosphere 16S rDNA clone libraries, respectively (Table 2). The cytophaga group represented the bulk of the clone sequences in this category; there were more cytophaga 16S rDNA clones recovered in the transgenic alfalfa rhizosphere than in the untransformed alfalfa rhizosphere (Table 2). In contrast, significantly more 16S rDNA clone sequences representing flexibacter and flavobacter groups were recovered from the untransformed alfalfa rhizosphere than from the transgenic alfalfa rhizosphere 16S rDNA clone libraries (Table 2).

3.1.3. Gram positive bacterial group

Approximately 11% of the total 16S rDNA clone sequences were of the gram positive bacterial group, which were recovered in equal proportions from both alfalfa rhizospheres (Table 2). However, analysis of the major sub-divisions with this group revealed that bacterial groups with high G + C content comprised 6–9% of the total 16S rDNA clone libraries of the transgenic and untransformed alfalfa rhizospheres (Table 2). Representative genera that dominated this bacterial group were of the actinobacterial species that included actinomycetes, *Frankia*, *Arthrobacter* and *Acidomicrobia*. Approximately 3.3% of the total 16S rDNA clones aligned with nucleotide sequences from uncultured and unidentified actinomycete clones from published domains (Table 2).

Gram positive bacterial groups with low G + C content were also recovered and comprised about 2–5% of the 16S rDNA clone libraries from both alfalfa rhizospheres (Table 2). All of the 16S rDNA clones were of the *Bacillus* group, including *B. gobisporus*, *B. megaterium*, *B. methanolicus* and *B. subtilis*. Considerably more *Bacillus* 16S rDNA clones were recovered from untransformed alfalfa rhizospheres than from transgenic alfalfa rhizospheres (Table 2).

3.1.4. Other bacterial groups

Only about 3% of the 16S rDNA clone sequences representing the cyanobacteria and planctomycetales bacterial groups were recovered from the untransformed alfalfa

rhizosphere (Table 2). In contrast, 16S rDNA clone sequences representing the green sulfur bacterial groups were only recovered from the transgenic alfalfa rhizosphere (Table 2). The proportions of 16S rDNA clone sequences representing the green non-sulfur bacterial groups were the same in the transgenic and untransformed alfalfa rhizospheres (Table 2). The fibrobacter–acidobacterium group was recovered from both the transgenic and the untransformed alfalfa rhizosphere and comprised 4–9% of the 16S rDNA clone libraries (Table 2). Significantly more nitrospira 16S rDNA clone sequences were recovered from the transgenic alfalfa rhizosphere (Table 2).

3.1.5. Unclassified bacterial groups

About 8% of the 16S rDNA clone sequences in the alfalfa rhizospheres did not show significant similarity with Genbank nucleotide sequences and are, thus, regarded as unknown or unclassified bacteria (Table 2). In the phylogenetic tree presented in Fig. 1, the unknown 16S rDNA clones recovered appear to form two major clusters that did not appear to align phylogenetically to known bacterial groups included in the construction of the phylogenetic tree. There were twice as many unclassified 16S rDNA clone sequences recovered in the untransformed alfalfa rhizosphere than the transgenic alfalfa rhizosphere (Table 2; Fig. 1).

3.2. Carbon source utilization profiles

Colony forming units (log cfu) of cultivable bacteria were not significantly different between soil suspensions from the transgenic and untransformed alfalfa rhizospheres. Approximately 3.35×10^7–3.39×10^7 bacterial cfu/g of air-dry soil were cultivated on R2A agar plates.

The Biolog data using the 10^{-3}–10^{-5} soil dilutions were subjected to Principal Component Analysis (PCA) (Fig. 2). The first principal component which explained 59.4% of the variance, showed clear separation based on rhizosphere soil as well as on soil dilution (inoculum density). The second principal component explained 7.0% of the total variance, although there was no meaningful separation along this axis. Data from each soil dilution were evaluated separately by PCA, and results showed a clear separation of treatments according to rhizosphere soil (data not shown).

Data from the 10^{-4} soil dilutions were further evaluated using other measures of diversity. Comparisons of the mean AWCD, catabolic richness and evenness at the 10^{-4} soil dilutions showed that bacteria in the rhizosphere of transgenic alfalfa utilized significantly more substrates and showed significantly greater functional diversity than bacteria in the rhizosphere of untransformed alfalfa (Fig. 3a–c). The significant contributions of carboxylic acids and amino acids between the two rhizosphere soils were consistently evident for the AWCD, number of substrates utilized and the Shannon–Weaver diversity indexes (Fig. 3a–c).

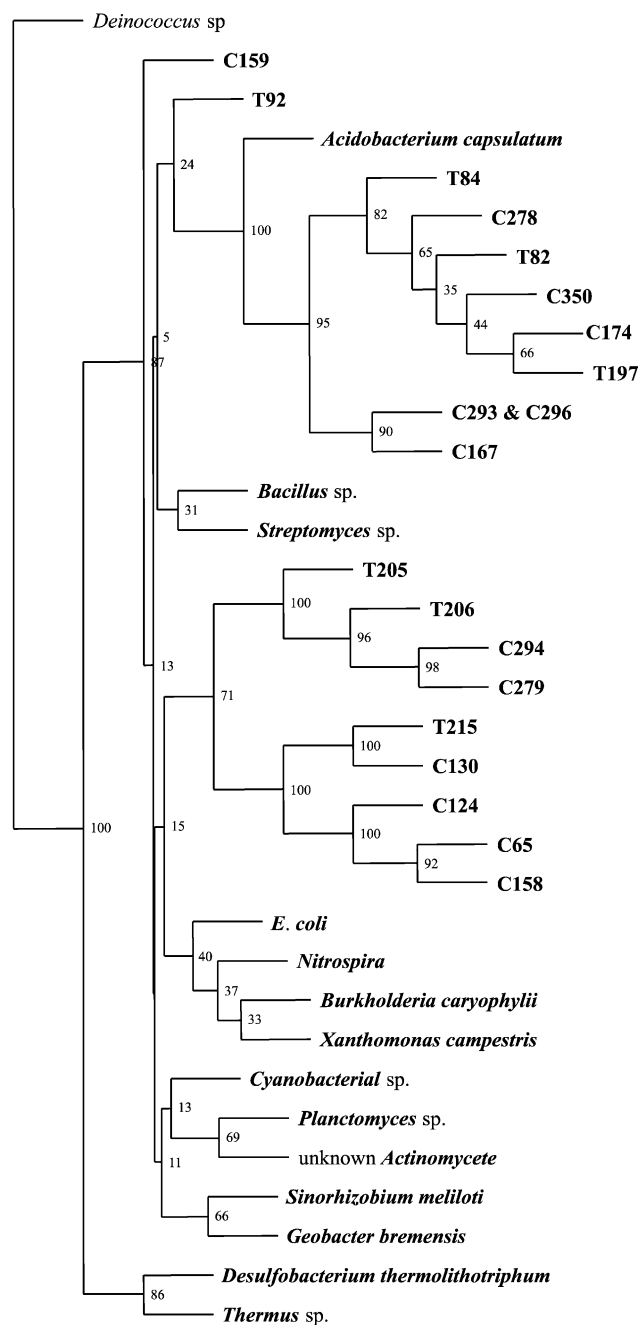

Fig. 1. Neighbor-joining tree showing the relationships of partial 16S rDNA clone sequences representing the unclassified bacterial groups recovered from the rhizosphere soils of transgenic (T) and untransformed (C) alfalfa. The tree was constructed from a Jukes–Cantor distance matrix derived from aligned partial 16S rDNA sequences in relation with reference 16S rDNA sequences from the GenBank databases. The numbers on the branches are bootstrap values based on 100 bootstrap resamplings.

3.3. Plant biomass and soil elemental analysis

Soil pH_{H_2O} in the transgenic alfalfa rhizosphere was 7.15 compared to 7.25 in the untransformed alfalfa rhizosphere. Significantly increased levels of nitric acid-extractable P, K, Mn, Cu and Zn were found in the transgenic alfalfa

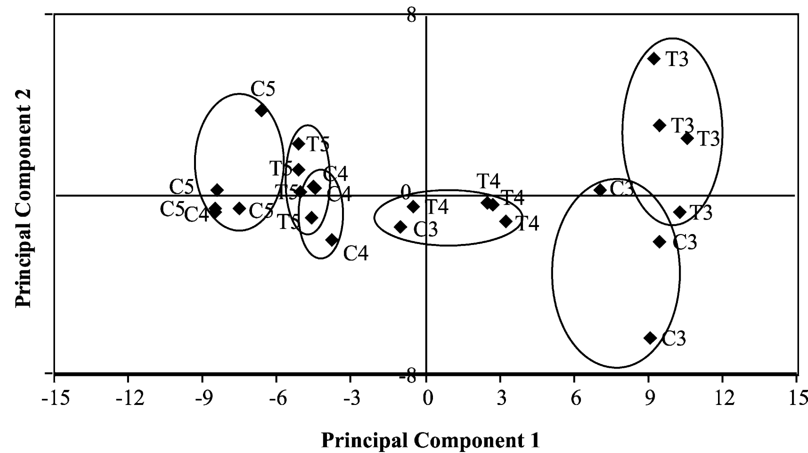

Fig. 2. Principal component analysis of absorbance data from 95 substrate wells of Biolog™ GN microtiter plates. The first and second principal components explained 59.4 and 7.0% of the total variance, respectively. Micotiter plates with greater catabolic response profiles are located to the right of the plot along the first principal axis. Rhizosphere soils from transgenic (T) and untransformed (C) alfalfa were resuspended in quarter-strength Ringers solution and soil dilutions made; soil dilutions are $10^{-3} = 3$; $10^{-4} = 4$ and $10^{-5} = 5$.

rhizosphere soil than in the untransformed alfalfa rhizosphere soil (Table 3). In contrast, nitric acid-extractable Mg concentration in untransformed rhizosphere soil was significantly higher than the transgenic alfalfa rhizosphere soil.

In 1999, the transgenic alfalfa plants accumulated significantly less shoot DM than did the untransformed alfalfa plants, indicating that the transgenic plants may be slower growing and slower to establish (Tesfaye et al., 2001). However, DM yield pooled from four consecutive harvests over the 1999–2000 growing period showed no statistically significant differences in shoot DM yield between the transgenic and untransformed alfalfa plants (data not shown).

4. Discussion

The composition and metabolic activity of naturally occurring rhizosphere bacterial populations differed between field-grown transgenic and untransformed alfalfa plants. The 16S rDNA clone libraries obtained by PCR amplification of soil DNA from the two alfalfa rhizospheres represented a wide range of bacterial phylogenetic groups and suggested that alfalfa plants with enhanced production of organic acids induced changes in the distribution and relative abundance of the different bacterial phylogenetic groups, which resulted in qualitative changes in bacterial community structure between the rhizospheres of transgenic and untransformed alfalfa.

Differences in root exudation patterns between plant species and among cultivars of the same species have been well documented (Cieslinski et al., 1997). Several lines of evidence indicate that differences in the activities of rhizosphere bacteria are related to both the types and amounts of root exudates. Increased associative N_2-fixation by *Azospirillum brasilence* and roots of three wheat cultivars differing in organic acid exudation correlated with exudation of higher amounts of carboxylic acids, such as succinic acid, malic acid and oxalic acid by the host plant (Christensen-Weniger et al., 1992). The intense microbial activity near plant roots is assumed to be in response to increased nutritional substrates in root exudates. Total numbers of bacteria capable of utilizing different classes of opines were significantly higher in the rhizospheres of transgenic tobacco and *Lotus corniculatus* plants producing these compounds (Oger et al., 1997; Savaka and Farrand, 1997), although the total populations of culturable bacteria around the rhizospheres of wild-type and opine-producing transgenic *L. corinculatus* were not significantly different (Oger et al., 1997). We also found no significant differences in the populations of total culturable bacteria between transgenic and untransformed alfalfa rhizospheres. The similar total bacterial numbers in the two alfalfa rhizospheres in our study indicated that increased exudation of organic acids by the roots of the transgenic line may not have been sufficient to produce a measurable increase in total bacterial numbers. It may also be that increases in total bacterial numbers may be confined to sites of organic acid exudation, such as root tips and lateral root emergence sites, although such localized increases in bacterial populations, especially of uncultured bacteria, would not necessarily have been detected by the viable plate counts used in the present study. Moreover, enhanced exudation of organic acids may not necessarily increase the rhizobacterial-carrying capacity of plant roots. Nevertheless, the ability to utilize specific organic compounds present in the rhizosphere was shown previously to provide a selective advantage to the bacterial strain that uses the substrate(s) and, perhaps, may lead to the dominance of a few bacterial species (Marilley et al., 1998; Oger et al., 1997; Savaka and Farrand, 1997). When opine-catabolizing

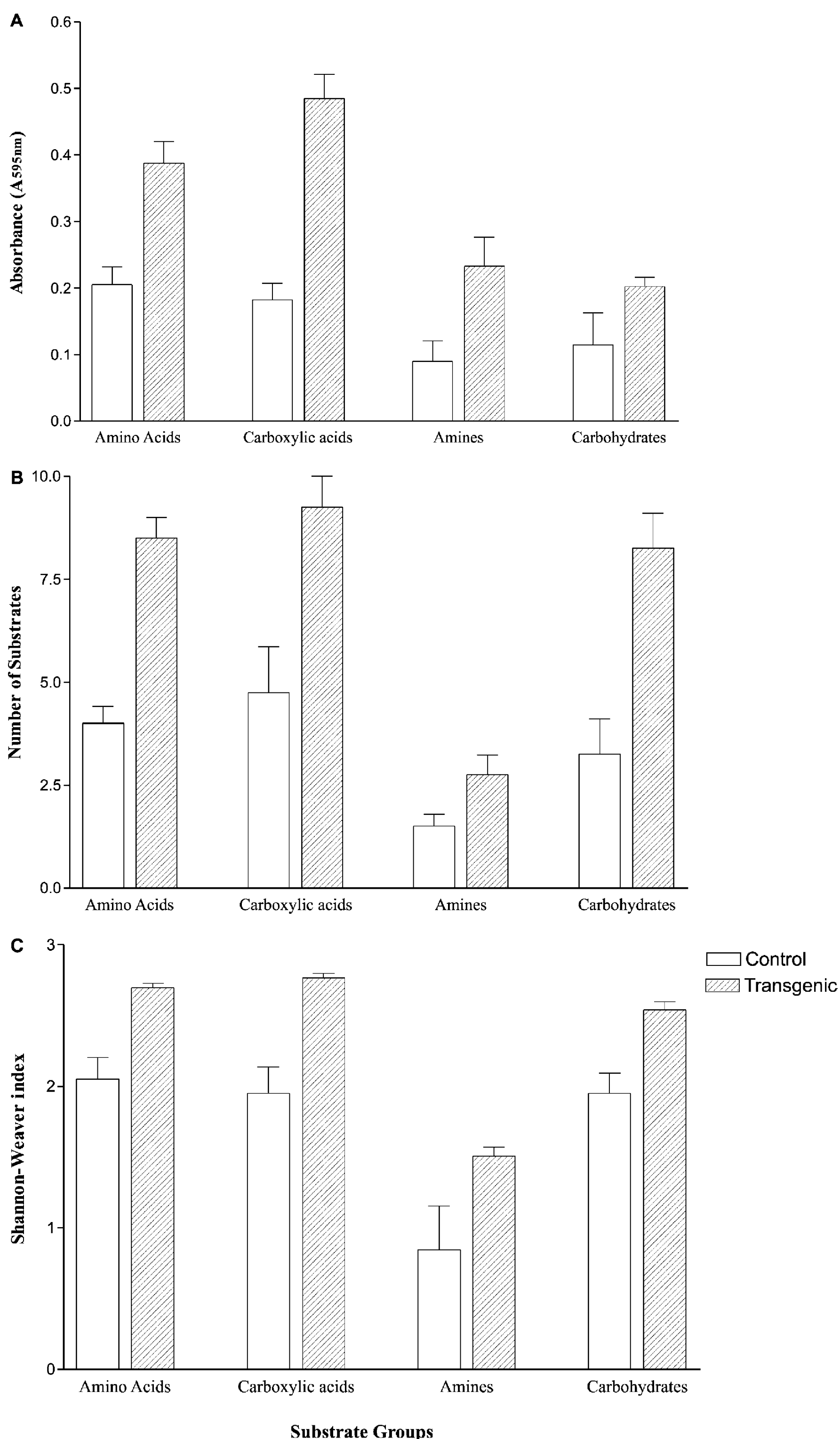

Fig. 3. Overall activity (A), number of utilized substrates (B) and Shannon–Weaver diversity index (C) of samples from the transgenic and untransformed alfalfa rhizosphere. Mean + SE of the 10^{-4} soil dilution data using the Biolog™ GN plates are shown for amino acids, carboxylic acid, amine and carbohydrate substrate groups. Data presented are after 48 h of incubation from 4 replicate Biolog™ plates for each treatment.

and non-catabolizing strains of *Pseudomonas fluorescens* were coinoculated on a transgenic tobacco plant that produces opines, the opine-catabolizing *Pseudomonas* strain had a significantly higher population than the non-catabolizing strain (Savaka and Farrand, 1997). Microbial diversity was considerably reduced in the rhizospheres of *Lolium perenne* and *Trifolium repens* compared with the microbial diversity in the bulk soil, suggesting that

Table 3
Macro- and micro-nutrient analysis of rhizosphere soils from untransformed and transgenic alfalfa plants grown for 53 weeks in field plots

Rhizosphere soil	P mg/g	K mg/g	Mg mg/g	Ca mg/g	Mn μg/g	Fe μg/g	Cu μg/g	Zn μg/g
Untransformed alfalfa	0.72	0.67	1.05	5.84	355.0	1701.5	37.3	42.0
Transgenic alfalfa	0.77	0.78	0.92	5.36	368.4	1757.6	56.4	49.9
Significance	**	**	**	NS	**	NS	**	**

**, means different at $P < 0.01$; NS, not significantly different.

proximity to root exudates reduces microbial diversity (Marilley et al., 1998). Substantially higher levels of 16S rDNA clones of the nitrogen-fixing soil bacterium, *Rhizobium* sp. were recovered from the rhizosphere of the host, *T. repens*, compared with the bulk soil, indicating that the host plant can select for specific bacteria (Marilley et al., 1998).

Only a few studies have investigated the influence of transgenic plants on their soil environment and on microorganisms that associate with plant roots. The insecticidal toxin, encoded by the *cry1Ab* gene from *B. thuringiensis* in transgenic corn (Bt corn), was released into the rhizosphere (Saxena and Stotzky, 2000; Saxena et al., 1999; 2002). Although the toxin remained active in the soil, it did not appear to influence soil microbiota as there were no significant differences in the total number of culturable bacteria and fungi or in the numbers of protozoa and nematodes between rhizosphere soil of Bt corn and untransformed corn (Saxena and Stotzky, 2001). However, evidence from other studies showed that plant species and cultivars strongly impact the composition and diversity of rhizosphere bacterial communities in agricultural soils. Based on Fatty Acid Methyl Ester (FAME) analysis of field-grown plants, fewer *Arthrobacter* and *Bacillus* isolates were recovered in the rhizosphere of a transgenic canola (*Brassica napus* cv. Quest) than in the rhizospheres of untransformed canola cultivars (*B. rapa* cv. Parkland and *B. napus* cv. Excel) (Siciliano and Germida, 1999; Siciliano et al., 1998). The reasons for the difference in bacterial community structures between the canola rhizospheres were not clear. Similarly, rhizosphere bacterial communities of wheat and untransformed canola (*B. napus* cv. Westar) grown at the same field site differed: *Bacillus* sp. dominated the wheat rhizosphere, whereas the canola rhizosphere had a uniform distribution of other bacterial types besides *Bacillus* sp. (Germida et al., 1998). In the present study, the structure and evenness of 16S rDNA clones representing the proteobacterial sub-groups differed between the two alfalfa rhizospheres. Furthermore, there was an increased incidence of 16S rDNA clones representing the high G + C content, nitrospira and acidobacterium bacterial groups in the transgenic alfalfa rhizosphere. The reasons for the shift in microbial structure between the two alfalfa rhizospheres were not clear. The transgenic alfalfa line M16-27 produced 7.1-fold increased organic acid exudation over untransformed alfalfa plants (Tesfaye et al., 2001). Assuming that these changes in patterns of organic acid exudation in transgenic alfalfa plants occurred in the field, they may be responsible for the differences observed in bacterial community structure and composition between rhizosphere soil samples from field grown transgenic alfalfa line M16-27 and untransformed alfalfa cultivar Regen-SY. Utilization of organic acids in root exudates has been shown to be the basis of root colonization by *Pseudomonas* sp. (Lugtenberg et al., 2001).

Metabolic response profiles of rhizosphere soils from the same plant species tend to be similar regardless of soil type, while catabolic response profiles of rhizosphere soils from different plant species were quite distinct, suggesting the presence of dissimilar microbial communities associated with roots of different plants (Garland, 1996; Grayston et al., 1998). Based on Biolog™ GN microtiter plates, there was significantly greater catabolic response profiles for rhizosphere soil samples from transgenic alfalfa than from untransformed alfalfa. Although it may be difficult to determine the direct effects of different types of organic substrates on functional diversity (Degens, 1998), the short assay time has been shown to provide a measure of catabolic functional diversity in soil (Degens et al., 2000).

Many soils contain large amounts of aluminum oxides that adsorb substantial amount of P (Fox et al., 1990). Exogenous application of organic acids to such soils and organic acid exudation from plant roots have been shown to improve P availability (Fox et al., 1990; Lopez-Bucio et al., 2000; Koyama et al., 2001). Mobilization of other soil nutrients, such as Fe, Mn, or Zn, has been related to complexation with organic acids contained in root exudates (Dinkekaker et al., 1989; Marschner and Römheld, 2001). Citrate and oxalate appear to be the most efficient components of root exudates with respect to mobilization of P, Al, Fe or Mn from soils low in readily available nutrients (Jones, 1998). Increased mineral availability in the transgenic alfalfa rhizosphere was consistent with increased exudation of citrate, oxalate, malate, succinate and acetate by transgenic alfalfa expressing neMDH (Tesfaye et al., 2001). Although it may be difficult to separate the contribution of root exudates from those of soil bacteria capable of solubilizing soil nutrients, the ability of the transgenic alfalfa line to liberate soil nutrients should be valuable in field soils.

The many roles of plant root exudates in plant-microbe interactions, nutrient mobilization, and crop adaptation to adverse soil conditions are not only of research interest, but also have practical implications for the engineering of plants suited for diverse agricultural systems. It is less clear how plants influence rhizosphere microbiota as a whole. Transgenic plants differing from wild-type plants in the composition of root exudates can be valuable tools for assessing the ecological significance of components of root exudates in microbial ecology. We should also be aware of the possibility that there may be transgenes that affect root exudation as a pleiotropic effect of the primary transformation event. With the increasing use of transgenic plants in agriculture, the ecological effects of genetically engineered plants in the soil environment needs to be addressed experimentally.

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