

and 1392 to 1406, respectively. Theoretically, the resulting PCR products should be 354 bp, of which 314 bp are from the 16S rRNA genes of members of the domain *Bacteria*, including the highly variable V9 region (11). Each 100- μ l PCR mixture contained 50 ng of soil DNA extracts, 0.6 μ M (each) primer, 200 μ M PCR nucleotide mixture (Fisher Scientific Inc., Pittsburgh, Pa.), 1.75 mM MgCl₂, 1.5 μ g of bovine serum albumin, and 3.2 U of *Taq* DNA polymerase in PCR buffer A (Promega Chemicals, Madison, Wis.). PCR was performed by a modified procedure described by Ferris et al. (9). Briefly, it included an initial denaturation of 2 min at 94°C, followed by nine cycles consisting of 1 min of denaturation at 94°C, 1 min of annealing at 56°C with a touchdown of 1°C per cycle, and 2 min of extension at 72°C. Then, 22 additional cycles, each consisting of 40 s at 94°C, 40 s at 47°C, and 2 min at 72°C, were performed. The final extension at 72°C was 8 min. PCR products were checked for the expected size and quantified on 2.5% agarose gels.

DGGE analysis of rRNA genes. The PCR amplicons were separated with a DCode universal mutation detection system (Bio-Rad, Inc., Hercules, Calif.). A 6.5% acrylamide gel with a 30 to 55% parallel denaturing gradient was prepared with a Hoefer SG100 gradient maker (Amersham Pharmacia Biotech Inc., Piscataway, N.J.). Denaturant (100%) contained 7 M urea and 40% deionized formamide. The gel was polymerized for at least 4 h. Approximately 3 μ g of PCR products of the expected size was loaded in each well. The gel was run for 5 h at 200 mV in 1 \times Tris-acid-EDTA buffer at 60°C. The image of the gel was obtained with a Kodak (New Haven, Conn.) 1D scientific imaging system and a Kodak DC 290 zoom digital camera. DGGE banding patterns were analyzed with GeneProfiler (Scanalytics Inc., Fairfax, Va.).

Isolation and sequencing of dominant DGGE bands. Some dominant DGGE bands were excised and eluted by incubation in 20 μ l of sterilized distilled water overnight at 4°C. The following day, 10 μ l of liquid from each tube was used as the template to reamplify the band of interest with the primers previously described and a slightly different PCR mixture and conditions. Briefly, each 50- μ l PCR mixture contained 10 μ l of eluted DNA from the DGGE band, 0.2 μ M (each) primer, 200 μ M PCR nucleotide mixture (Fisher Scientific Inc.), 1.5 mM MgCl₂, 1 μ l of dimethyl sulfoxide, and 1.5 U *Taq* of DNA polymerase in PCR buffer A (Promega Chemicals). PCR included an initial denaturation of 3 min at 94°C, followed by 31 cycles consisting of 1 min of denaturation at 94°C, 30 s of annealing at 60°C, and 30 s of extension at 72°C, followed by a final extension of 8 min at 72°C. PCR products were checked for purity and expected size and quantified as described above.

The reamplified products were purified with the UltraClean PCR cleanup DNA purification kit (Mo Bio Laboratories, Inc.) to remove primers and short oligonucleotides and were cloned into plasmids with a TOPO TA cloning kit (Invitrogen Life Technologies). Plasmid DNAs were isolated from randomly selected clones and screened for inserts of the expected sizes. Following confirmation of their DGGE positions under the conditions previously stated, the plasmid DNAs were sequenced with primers (M13 reverse/forward) on an ABI PRISM 3700 DNA analyzer (Applied Biosystems, Foster City, Calif.).

Analysis of the DGGE community profiles. Banding patterns of three replicated experiments were analyzed by a GeneProfiler database manager with TreeCon software (LI-COR, Inc.). The lower limit for band detection was set by the band peak height threshold of 0.1% of the total detected optical density in the lane. The total number of different bands was determined for the samples being compared, and then each sample was scored based on the presence or absence of each band in its profile compared with the profile of each of the other samples. This provided a comparison of detected microbial community structures among soils, but not the relative dominance of each detected ribotype in that community. The Shannon diversity indices were calculated to reflect species richness and evenness of the bacterial community (29). Species evenness of the community was further estimated with Pielou's diversity (*J*) (26). Similarity among treatments was determined by pairwise matching to determine matching bands by comparing two treatments at a time. The band sharing coefficient (*S*; also termed the Dice similarity coefficient) (7) was used as a measure of similarity between two given treatments; it was determined from the equation $S = 2N_s/NT$, where N_s is the number of shared bands in samples A and B and NT is the total number of detected bands in samples A and B. Relatedness of bacterial communities was determined by determining similarity coefficients for bands common to two samples. Two bands were common if they migrated the same distance on a gel. Based on the number, intensity values, and positions of detected bands, cluster analysis and statistical analysis were performed. Comparisons of relative band intensity within lanes were made. Significant difference among treatment means was determined by the least significant difference test at a *P* value of <0.05. Percentage data were normalized by arc sine transformation before analysis (10). Bacterial species richness in a community was indicated by the total number of bands detected, and species distribution evenness was evaluated by

determining the intensity, mobility, and distribution of bands in the rRNA gene fingerprint.

rRNA gene cloning and sequencing, and phylogenetic analysis. The PCR products obtained for DGGE analysis from the manure-treated soil were also directly cloned into PCR 2.1-TOPO plasmids by the procedure recommended by the manufacturer. Ninety-four clones were randomly selected and sequenced.

Sequence similarity searches were conducted using the BLAST network service (1) of the GenBank database to identify the nearest relatives of the partially sequenced 16S rRNA genes and the excised dominant bands. Sequences were screened for chimeras by the CHECK-CHIMERA program of the Ribosome Database Project (<http://rdp.cme.msu.edu/cgi/chimera.cgi?su=SSU>) and by BLAST alignment. Sequences yielding a bell-shaped histogram, matching <93% with sequences in the database, and showing different closest relatives from two sides of a breakpoint were considered chimeras and were removed from the data set. Phylogenetic analyses were conducted with Biology WorkBench, version 3.2 (San Diego Supercomputer Center; <http://workbench.sdsc.edu>). The phylogenetic tree of the aligned sequences was constructed with a maximum-likelihood tree algorithm.

Nucleotide sequence accession numbers. A total of 105 nucleotide sequences obtained in this study have been sent to the National Center for Biotechnology Information database. The accession numbers for the 11 excised band sequences are AY391554 to AY391564, and the 94 randomly selected clone sequences are assigned AY391617 to AY391710.

RESULTS

DGGE analysis of bacterial community. Bacterial species richness and evenness are demonstrated by DGGE banding patterns of 16S rRNA genes amplified by PCR with the bacterium-specific primer and a universal primer containing a GC clamp (Fig. 1). The DGGE patterns from the six soils were distinctly different and were reproducible according to DGGE banding patterns generated in three repeated experiments. Numerous discrete DGGE bands, resulting from differences between the 16S rRNA gene sequences of different bacterial species, were apparent (Fig. 1). Each band represents at least one unique ribotype. A significantly higher number of ribotypes was detected in the manure-treated soil, while the least number of ribotypes was detected in the control soil (Table 1). The different banding patterns among the soils tested suggest that each soil contained different bacterial community structure and diversity.

When the band sharing coefficients between treatments were compared, bacterial community structures in P- and NP-treated soils showed the highest similarity, with an average band sharing coefficient of 0.68, followed by NPK- and NPKL-treated soils, with an average band sharing coefficient of 0.64 (Table 2). The bacterial community structure in the manure-treated soil was most similar to that of the control soil (0.56) and least similar to that of the NPKL-treated soils (0.41) (Table 2).

The Shannon diversity index for the bacterial community in the manure-treated soil (1.19 ± 0.07) was significantly higher than that for the bacterial community in the untreated control (0.97 ± 0.12). Compared to that for the manure-treated soil, lower Shannon diversity indices were obtained for soils treated with P (1.10 ± 0.10), NP (1.11 ± 0.05), NPK (1.16 ± 0.12), and NPKL (1.11 ± 0.04), though the differences were not statistically significant. However, the indices for evenness of the bacterial community were not significantly different among control soil (0.81 ± 0.078) and soil given manure (0.84 ± 0.039), P (0.83 ± 0.058), NP (0.85 ± 0.032), NPK (0.89 ± 0.033), and NPKL (0.87 ± 0.025) treatments.

The distribution evenness of a community is also indicated

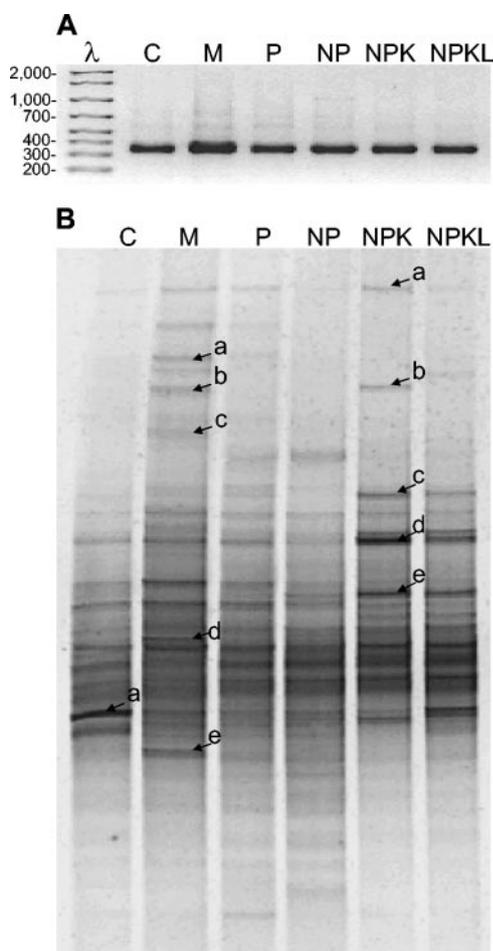


FIG. 1. (A) A 2.5% agarose gel electrophoresis of PCR-amplified 16S rRNA gene fragments. (B) DGGE analysis of PCR-amplified 16S rRNA genes. Each lane was loaded with 3 μ g of DNA. Bands marked were excised and sequenced. C, control; M, manure treatment.

by dominance of individual ribotypes in a community, as reflected by the relative band intensities on a DGGE gel. The higher the intensity, the more dominant is the bacterial ribotype corresponding to that band. According to the intensity of the three most intense bands in each community, expressed as percentages of total DNA intensity in the lane, the most even distribution of bacterial ribotypes occurred in the manure-

TABLE 1. Total bands detected on a DGGE gel with 6.5% acrylamide and a 30 to 55% parallel denaturing gradient

Treatment	No. of bands detected in:			Avg ^a
	Rep I	Rep II	Rep III	
Control	14	16	16	15.3 \pm 0.7c
Manure	29	23	29	27.0 \pm 2.0a
P	21	19	22	20.7 \pm 0.9b
NP	20	21	21	20.7 \pm 0.3b
NPK	23	18	20	20.3 \pm 1.5b
NPKL	21	18	18	19.0 \pm 1.0b

^a Means of three replicates (Rep) \pm standard errors. Different letters indicate significantly different means ($P < 0.05$) according to the least significant difference test.

TABLE 2. Average band sharing coefficient of DGGE bands detected in three repeated experiments between the paired treatments

Treatment	Mean avg band sharing coefficient \pm SE for:				
	Manure	P	NP	NPK	NPKL
Control	0.56 \pm 0.10	0.50 \pm 0.04	0.50 \pm 0.07	0.47 \pm 0.06	0.46 \pm 0.09
Manure		0.51 \pm 0.03	0.43 \pm 0.05	0.45 \pm 0.05	0.41 \pm 0.11
P			0.68 \pm 0.06	0.47 \pm 0.05	0.44 \pm 0.06
NP				0.49 \pm 0.05	0.44 \pm 0.07
NPK					0.64 \pm 0.03

treated soil, with the top three ribotypes comprising about 40% of the total bacterial community detected (data not shown). The top three dominant ribotypes in the control soil comprised, by average, 56% of the total bacterial community detected, a value significantly higher than that for the manure-treated soil. In fact, one ribotype distinctly dominated the bacterial community of the control soil (Fig. 1), comprising, by average, 28% of the total community (data not shown). The top three dominant ribotypes in P-, NP-, NPK-, and NPKL-treated soils comprised, by average, 47, 45, 42, and 43% of the total bacterial community detected, respectively.

Seven of 11 excised dominant DGGE bands (marked in Fig. 1) had sequences most similar to unknown uncultured bacteria in the GenBank database (Table 3). Three of these 11 excised band sequences belong to *Proteobacteria*, and one belongs to *Acidobacteria*. The sequence of the most dominant bacterial community in the control soil exhibited 99% similarity to sequences of *Sinorhizobium* spp. and *Agrobacterium* spp. In general, these 11 bacterial ribotypes belong to a diverse group of bacteria with distant phylogenetic relationships, but with little soil specificity. Among them, NPKd and NPKe were closely related to one another, as were Ma and Mb.

Bacterial diversity in soil treated for a century with manure. Phylogenetic analysis of 99 sequences, including 94 randomly selected clones and 5 excised bands, from a DGGE gel from the manure-treated soil is shown in Fig. 2. Based on similarity matching ($\geq 98\%$) of these sequences with those in the GenBank database, 17 clones were tentatively identified as members of *Proteobacteria*, *Bacteroidetes-Sphingobacteria*, *Nitrospirae*, and *Firmicutes* (Table 4). Two of the clones (M2 and M19) are possible chimeras. These two clones showed not only typical bell-shaped histograms from the CHECK-CHIMERA program but also low percentages of matching with sequences in the database. Several other clones also showed typical bell-shaped histograms but matched over 95% with sequences in the database, including several sequences of culturable bacteria. For example, the sequence of M50 (AY391666) matched 96% with the *Nitrosomonas* sp. strain Nm86 16S rRNA gene (AY123798). These clones are less likely to be chimeras. The remaining 80 clones were most similar to unclassified or uncultured bacterial 16S rRNA genes or showed less than 98% matching with sequences in the GenBank database.

Cluster analysis of clone sequences revealed six major clusters with numerous diverse groups (Fig. 2). One of the major clusters was not closely related to any sequences in the database, indicating a potential novel bacterial group. Among the 99 clones analyzed, 56 (58%) were assigned taxon affiliations and 41 were unclassified (Fig. 2). The *Acidobacteria* were the

TABLE 3. Tentative identification of dominant DGGE bands by sequencing the excised bands and BLAST analysis^a

Band name	Sequence size (bp)	Accession no.	Closest relative (accession no.)	Alignment, % similarity	Score	Taxonomic affiliation
Ca	314	AY391554	<i>Sinorhizobium</i> sp. and <i>Agrobacterium</i> sp. (e.g., AJ300187 and L39882)	313/314, 99	615	α - <i>Proteobacteria</i>
Ma	300	AY391555	Uncultured soil bacterium (AF507686)	290/292, 99	563	Unknown
Mb	301	AY391556	Uncultured soil bacterium (AF507686)	289/292, 98	555	Unknown
Mc	317	AY391557	Uncultured soil bacterium (AF507740)	312/317, 98	589	Unknown
Md	315	AY391558	Uncultured acidobacterium (AY177759)	310/315, 98	585	<i>Acidobacteria</i>
Me	302	AY391559	Uncultured soil bacterium (e.g., AF423305 and AF423295)	294/294, 100	583	Unknown
NPKa	313	AY391560	Uncultured bacterium (AB075101)	306/313, 97	565	Unknown
NPKb	301	AY391561	Uncultured bacterium (AJ318200)	282/292, 96	500	Unknown
NPKc	314	AY391562	Uncultured soil bacterium (AY326617)	304/314, 96	543	Unknown
NPKd	309	AY391563	<i>Massilia timonae</i> (e.g., U54470 and AY157761)	306/309, 99	589	β - <i>Proteobacteria</i>
NPKe	309	AY391564	β - <i>Proteobacterium</i> (AY278883)	306/309, 99	589	β - <i>Proteobacteria</i>

^a Sequences were aligned with the closest relatives (highest score) in the GenBank database with BLAST. Bands correspond to those excised from the DGGE gel (Fig. 1). The percentage of similarity was calculated without taking gaps into account. The part of the total sequence used for alignment is indicated by the alignment data. Nucleotide sequences can be accessed by using the accession numbers at <http://www.ncbi.nlm.nih.gov>.

second-most-dominant group (16.5%). The sequences in this group matched $\geq 97\%$ with sequences of uncultured acidobacteria in the database. When the 16S rRNA gene sequence of *Acidobacterium capsulatum* was included in the analysis, it was clustered closely with Md (data not shown). The third major group belonged to *Bacteroidetes*. Two clones (M1 and M81) in this group exhibited 99% similarity to the culturable bacteria *Pedobacter* sp. strain LMG 10342 (AF329966) and *Pedobacter* sp. strain LMG9525 (AF329962). In addition, two closely related clones (M13 and M56) also exhibited similarity to sequences of *Bacteroidetes*, but at lower percentages (92 and 89%, respectively). Among the 94 randomly selected clones, three ribotypes belonging to γ -*Proteobacteria* and *Acidobacteria* were selected three or four times (Fig. 2).

Five excised dominant bands from the DGGE gel in the manure-treated soil (Fig. 1) were distributed in three groups, the *Acidobacteria* (Md) group, an unclassified group that is closely related to *Bacteroidetes* (Mc), and an unclassified group that is a major cluster in the phylogenetic tree (Ma, Mb, and Me) (Fig. 2).

DISCUSSION

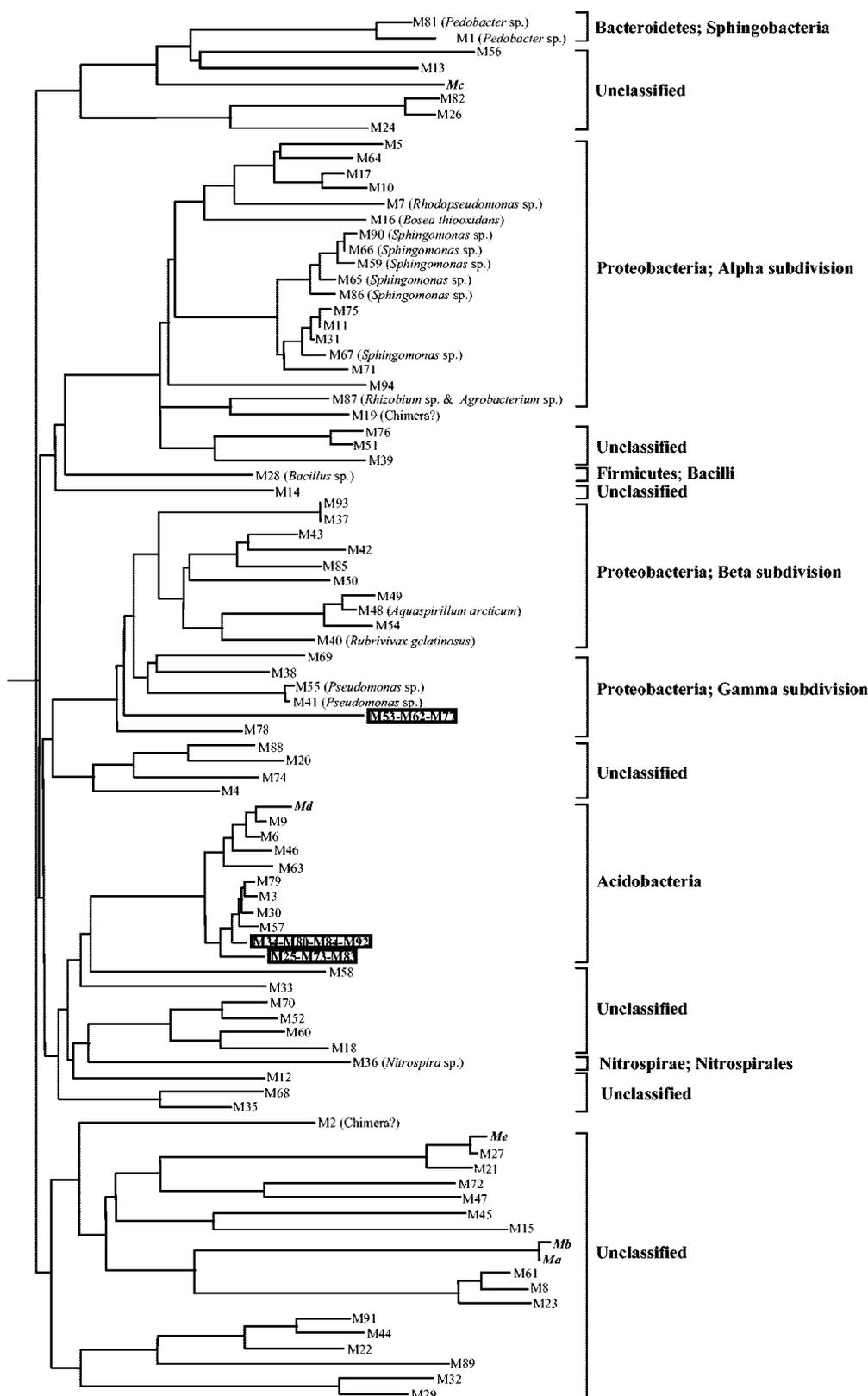
DGGE analysis demonstrated different banding patterns of bacterial community structures as well as species richness and evenness in the agroecosystems evaluated. Some of the discrete DGGE bands were further purified and sequenced to reveal specific differences in the bacterial community. Among the numerous recently emerged molecular methods for microbial community analysis, DGGE is one of few that holds this potential and has also been shown to be a sensitive method for microbial community analyses in compost-amended soils (13) and bacterial biofilms (17). Compared to amplified ribosomal DNA restriction analysis (ARDRA), the patterns of DGGE are more useful for a direct comparison of structural diversity among different microbial communities from different natural sites or environments. In ARDRA, each potential ribotype may be represented by multiple bands, and the patterns are affected by restriction enzymes used. In addition, ARDRA is not as sensitive as DGGE in detecting differences in microbial community structure (23).

DGGE banding patterns from soils of different agroecosys-

tems obtained in this study indicated that bacterial community structure was affected by soil management practices. Shifts in bacterial community structure following adaptation of soil management practices have been reported by other studies (5, 6, 25, 30). The observed impact was greater for long-term soil management practices than for short-term land use or plant community composition (6). In this study, the similarity in community structures in NPK- and NPKL-treated soils, as demonstrated by DGGE banding patterns and by band sharing coefficients, suggests that recognizable bacterial community structures exist in relation to field management following over 70 years of management practices. This finding is consistent with the conclusions reported by Buckley and Schmidt (6).

While the significance of the microbial community shifts following soil management practices remains to be recognized, it is evident that organic amendment, in general, enriches the soil bacterial community and promotes diversity and a more even distribution of bacterial species within the community. Among treatments evaluated in this study, the number of bands (ribotypes) detected and the Shannon diversity index were highest for the manure-treated soil, in which the sum of the three most dominant bacterial ribotypes comprised the lowest percentage of the total community, indicating the most even distribution. These results are in agreement with those obtained by evaluating the culturable bacterial populations (23). They also support the studies of Øvreås and Torsvik (22), who reported that the bacterial community in an organic soil was more diverse and more evenly distributed than that in a sandy soil. Enrichment of the bacterial community following manure treatment has also been shown by enhanced microbial biomass C contents (12, 24), soil enzyme activities (24), N flush (27), and gram-negative bacterial populations (25).

The bacterial community in the soil treated with manure for a century was further evaluated by sequencing 94 randomly selected clones and 11 excised dominant bands from a DGGE gel. Five major taxa, α -, β -, and γ -*Proteobacteria*, *Acidobacteria*, *Bacteroidetes*, *Nitrospirae*, and *Firmicutes*, were identified. These results deviate from those reported by Smit et al. (30), who found that *Acidobacteria*, *Proteobacteria*, *Nitrospira*, *Cyanobacteria*, and green sulfur bacteria were the main bacterial



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FIG. 2. Phylogenetic analyses of 99 sequences obtained from the soil treated for a century with manure, including 94 randomly selected clones and 5 excised bands (boldface and italics) from a DGGE gel (Fig. 1). Sequences were designated by the prefixes M (indicating manure treatment), followed by replicate numbers (randomly selected clones) or letters (excised bands). Three ribotypes were repeated three or four times (boxed) in the randomly selected clones. Clones exhibiting $\geq 98\%$ sequence similarity were used as references for the corresponding taxonomic-unit assignments.

TABLE 4. Summary of the phylogenetic assignments of 99 rDNA clones from soil treated for a century with manure

Major taxon	Phylogenetic assignment based on:	
	BLAST search ($\geq 98\%$ matching)	BioWorkBench cluster analysis
<i>Proteobacteria</i>		
α - <i>Proteobacteria</i>	9	18
β - <i>Proteobacteria</i>	2	10
γ - <i>Proteobacteria</i>	2	8
<i>Bacteroidetes-Sphingobacteria</i>	2	2
<i>Acidobacteria</i>	0	16
<i>Firmicutes</i>	1	1
<i>Nitrospirae</i>	1	1
Unclassified groups (possible chimeras, 2)	80	41

divisions in a manure-treated winter wheat field in The Netherlands. Three of the five divisions, *Proteobacteria*, *Acidobacteria*, and *Nitrospira*, were dominant in this study.

The most dominant bacterial group was *Proteobacteria*, which is consistent with reports by several other studies related to agricultural agroecosystems (4, 30, 33). In this study, *Proteobacteria* comprised 37% of the 99 sequences obtained, a percentage that closely resembled that reported by Smit et al. (30), who showed that *Proteobacteria* comprised 35% of the bacterial community in an agroecosystem in The Netherlands. This may reflect the similarities of the two agroecosystems; both were winter wheat fields treated with manure. In contrast, only 16% of 122 bacterial clones from a clover-grass pasture in southern Wisconsin (4) and only 18% of 51 clones from the clay fraction of an animal manure-treated soil (28) were *Proteobacteria*.

The *Acidobacteria* were the second-most-dominant group based on cluster analysis, a result which was further evidenced by sequence analysis of excised DGGE bands and by the fact that two of the three repeatedly selected clones belonged to *Acidobacteria*. The dominance of *Acidobacteria* in a bacterial community is not surprising. Following a survey of 43 environmental samples, Barns et al. (3) concluded that members of the division *Acidobacteria* are as genetically and metabolically diverse, environmentally widespread, and ecologically important as the division *Proteobacteria*.

Dominance of *Firmicutes-Bacillus* in agroecosystems has been reported in California soils (13, 33). Among the five major taxa revealed in this study, *Bacteroidetes-Sphingobacteria* are well-known rumen bacteria that are commonly found in the gastrointestinal tracts of warm-blooded animals. The presence of this major group of bacteria in soil from a clover-grass pasture also was reported (4). With increasing concerns about adaptation of fecal bacteria in the environment following manure application (14), these findings merit additional research effort.

Results from this study suggested that bacterial diversity in agricultural soils was substantially different from that in uncultivated soils in a geothermal heating environment. Phylogenetic analysis of 39 clones from a soil in Yellowstone National Park suggested that the majority belonged to *Acidobacteria* (51%) and *Planctomyces* (18%) (20). *Acidobacteria* also dominated Arizona woodland soils (8).

Ratios between the ribotype numbers of *Proteobacteria* and

Acidobacteria in an ecosystem might reflect the nutrient status in the soils tested (30). The reported ratios ranged from 0.16 in an oligotrophic soil (8) to 0.87 in a high-input agricultural soil (16). In a soil in The Netherlands, this ratio was 0.46 (30), and in the manure-treated soil evaluated in this study, it was 0.44, demonstrating another consistency of the data obtained from these two studies.

Although rRNA gene sequence-based analysis of microbial communities has been widely used to reveal bacterial diversity in the soil environment (4, 19, 30, 35), a limited number of sequences can be obtained and analyzed. The randomly selected clones represent dominant ribotypes in the bacterial community tested. Few of the DGGE-excised band sequences were similar to those of the randomly selected clone sequences, indicating that a vast diversity of bacterial ribotypes existed in the community. This discrepancy may also suggest that limited information on bacterial community structure and diversity can be derived from analysis of a limited number of randomly selected clones. Following analysis of 99 rRNA gene sequences in the manure-treated soil, only two matched 100% and a small percentage (about 17.5%) of the clones matched $\geq 98\%$ with sequences in the GenBank database, indicating limitations of our knowledge of environmental microbial ecology as a whole. Similarly, 7 out of 11 excised bands from a DGGE gel remain unknown, further suggesting that a considerable portion of the bacterial community is largely unknown. It has been estimated that 80 to 99% of the microorganisms in soil are unidentified due to limitations in culture techniques (4). Less than 1% of the cells observed by direct counting can be recovered using standard cultivation methods (2, 15, 31, 32).

In summary, the soil bacterial community was impacted considerably by management practices of the agroecosystems studied. The distinct bacterial community structures in different agroecosystems reflected soil management practices conducted for over 70 years. Manure application increased soil bacterial diversity and resulted in a more even distribution than that in soil given inorganic fertilizer treatments and in an untreated control soil.

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