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Diversity and Seasonal Fluctuations of the Dominant Members of the Bacterial Soil Community in a Wheat Field as Determined by Cultivation and Molecular Methods

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There is a paucity of knowledge on microbial community diversity and naturally occurring seasonal variations in agricultural soil. For this purpose the soil microbial community of a wheat field on an experimental farm in The Netherlands was studied by using both cultivation-based and molecule-based methods. Samples were taken in the different seasons over a 1-year period. Fatty acid-based typing of bacterial isolates obtained via plating revealed a diverse community of mainly gram-positive bacteria, and only a few isolates appeared to belong to the *Proteobacteria* and green sulfur bacteria. Some genera, such as *Micrococcus*, *Arthrobacter*, and *Corynebacterium* were detected throughout the year, while *Bacillus* was found only in July. Isolate diversity was lowest in July, and the most abundant species, *Arthrobacter oxydans*, and members of the genus *Pseudomonas* were found in reduced numbers in July. Analysis by molecular techniques showed that diversity of cloned 16S ribosomal DNA (rDNA) sequences was greater than the diversity among cultured isolates. Moreover, based on analysis of 16S rDNA sequences, there was a more even distribution among five main divisions, *Acidobacterium*, *Proteobacteria*, *Nitrospira*, cyanobacteria, and green sulfur bacteria. No clones were found belonging to the gram-positive bacteria, which dominated the cultured isolates. Seasonal fluctuations were assessed by denaturing gradient gel electrophoresis. Statistical analysis of the banding patterns revealed significant differences between samples taken in different seasons. Cluster analysis of the patterns revealed that the bacterial community in July clearly differed from those in the other months. Although the molecule- and cultivation-based methods allowed the detection of different parts of the bacterial community, results from both methods indicated that the community present in July showed the largest difference from the communities of the other months. Efforts were made to use the sequence data for providing insight into more general ecological relationships. Based on the distribution of 16S rDNA sequences among the bacterial divisions found in this work and in literature, it is suggested that the ratio between the number of *Proteobacteria* and *Acidobacterium* organisms might be indicative of the trophic level of the soil.

Agriculture is of prime importance for The Netherlands where traditionally high-input arable farming is being practiced. On high-input farms, microorganisms are generally thought to play a minor role in soil fertility because most nutrients in inorganic fertilizers are readily available for the plants and do not require degradation or mineralization. However, because the government aims to reduce tillage and the use of pesticides and inorganic fertilizer, it is generally thought that the role of soil microorganisms in the decomposition and mineralization of complex organic compounds and in the reduction of plant pathogens will increase (21, 25, 33). On the other hand, it is expected that the application of genetically modified crops (32a) and of genetically modified microorganisms will increase, which might change bacterial community composition and affect microbial processes (8, 9, 39). To date, only limited information exists on microbial diversity and dynamics in agricultural soil (3, 4, 41). In order to assess the magnitude of changes in the bacterial community as a result of anthropogenic activity, it is necessary to gain knowledge on bacterial diversity and seasonal changes in "healthy" agricultural soil (7, 13, 41).

The object of our study was a field plot on the Lovinkhoeve experimental farm, situated on reclaimed land in the Noordoostpolder (19, 21), on which summer and winter wheat has been grown alternately by using common agricultural practices since 1944. This field has never been treated with pesticides, and only organic manure has been applied. Studying the microbial community in this soil provides information on the bacterial diversity, species richness, and seasonal variation in bacterial composition of a nonpolluted agricultural soil with a known history of cultivation. Previously, microbial processes and food web interactions have been studied intensively on various plots of this farm, and it was found that decomposition processes were dominated by bacteria (4). Bloem and coworkers (5) found that microbial biomass and the ratio of dividing cells to divided cells in winter wheat fields showed peak levels in spring and autumn, which could be indicative of increased bacterial growth and activity.

To obtain knowledge on the microbial diversity, in this work both cultivation- and molecule-based methods were applied to soil samples. The fraction of the bacterial community in soil that can be cultured is estimated to range from 0.1 to 1% of the total; using molecular methods, a substantially larger diversity

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of the bacterial community can be detected (11, 17, 38). Therefore, the use of molecular techniques for the detection and analysis of microorganisms in the environment is increasing steadily (6, 14, 16, 20, 22, 25, 28, 36). While typing of isolates and clones seems more suitable for studying bacterial diversity in detail, these methods might not be particularly suited to map seasonal changes. For this purpose the analysis of large numbers of isolates or clones is needed; therefore, denaturing gradient gel electrophoresis (DGGE) was performed to create banding patterns representing the bacterial community (27). DGGE banding patterns obtained using general prokaryotic primers have been shown to reveal only the predominant species and are expected to be more suitable for detecting significant changes in the microbial community (27). Gelsomino and coworkers (16) showed by DGGE analysis of soil samples taken from one plot over a 1-year period that seasonal fluctuations were small, suggesting that the soil bacterial community is dominated by a limited number of dominant and stable microorganisms. Previously, Felske and coworkers (14) also found little variation in DGGE patterns over time in grasslands. However, both studies lacked a statistical analysis of the variability of the banding patterns, which is of the utmost importance for determining the magnitude of the natural fluctuations.

In the present study, bacterial diversity and seasonal variability of the bacterial community in wheat field soil were investigated both by culturing techniques and by direct DNA analysis on four different dates throughout the year. The most abundant isolates were typed by fatty acid analysis, and the most abundant clones were identified by partial sequencing and phylogenetic analysis. Whole-community diversity in soil samples was visualized by DGGE of specific fragments of 16S DNA sequences (29). The aim of this study was to analyze bacterial diversity in Lovinkhoeve soil, to compare data obtained by cultivation-based methods with data found using molecular techniques, to investigate the magnitude of seasonal changes in the bacterial community, and to use the data to search for general ecological relationships (18, 41).

MATERIALS AND METHODS

Sampling site. To study the soil microbial community, samples were obtained from the De Lovinkhoeve experimental farm in the Noordoostpolder, The Netherlands. Detailed characteristics of the silt loam soil at this location have been described previously (19, 21). The field was studied from September 1995 to August 1996. On November 1, 1995, the field was ploughed and subsequently sown with winter wheat (*Triticum aestivum* var. Herzog). The field was manured on April 25, 1996 (500 kg/ha), and no pesticides were used. The wheat was harvested on September 9, 1996.

Soil samples (50 to 100 g; depth, 5 to 10 cm) were taken from a 12- by 70-m field plot on which wheat had been grown since 1944. Ten samples about 6 m apart in the plot were taken. Soil was sampled on September 29, 1995; January 29, 1996; May 10, 1996; and July 29, 1996, on approximately the same spots. These samples are referred to in this paper as September, January, May, and July. These separate samples were plated for enumeration of culturable cells and DNA extraction. To obtain cultured isolates for further characterization, the 10 samples taken at the same date were pooled before plating.

Plating of soil samples and analysis of cultured isolates. Aliquots of 5 g of soil were used for determining the number of culturable cells. Soil moisture content was determined by weighing fresh and dried soil (100°C for 24 h). Samples were plated onto 10-fold-diluted tryptic soy agar (Oxoid) containing 100 μg of cycloheximide ml^{-1} to inhibit fungal growth, as described by Smit and coworkers (36). Plates were incubated at 28°C, and the number of CFU was counted daily from day 2 to day 8 to include data on copiotrophs versus oligotrophs (10). The geometric means of the log number of CFU per gram of dry soil was calculated. The percentage of fast-growing bacteria was determined by dividing the number of CFU counted on day 2 by the number of CFU on day 8.

In order to obtain a representative subsample of bacterial isolates, all 10 soil samples were pooled. From this mix two pseudoreplicates of 5 g of soil were taken. These duplicate samples were plated as described above, and after 8 days of incubation, about 100 bacterial colonies were picked randomly from the plates of the highest dilutions with approximately 50 to 100 colonies. These isolates were cultured in 10-fold-diluted tryptic soy broth at 28°C in a Gyrotory shaker. Cultures were stored at −70°C.

DNA extraction from soil, PCR amplification and cloning of 16S ribosomal DNA (rDNA). Total DNA was extracted from 10-g aliquots of soil by using a bead-beater as described by Smalla et al. (35), and the DNA was purified as described by Smit et al. (36). To date it is unknown which percentage of the soil bacteria is lysed; however, bead-beating has been shown to lyse spores of *Bacillus*, which are generally difficult to break (26).

To obtain a collection of 16S rDNA clones, total DNA extracts from the 10 samples taken at the same date were pooled before PCR. Amplification was performed with a primer set for eubacteria: 338 to 355, 5′-ACTCCTACGGG[A/G][G/C]GCAGC-3′ (12), and 1491 to 1473, 5′-GGTTACCTTGTTACGACTT-3′ (36).

PCR mixtures of 50 μl contained 5 μl of PCR buffer 2 (10-fold concentrated; Boehringer); 1.7 mM $MgCl_2$; 200 μM concentrations of each deoxynucleoside triphosphate, 300 nM primer, and 2.6 U of Expand Long Template enzyme mix (Boehringer); 1 μl of template DNA; and sterile Millipore water up to 50 μl. For incubation of the PCR mixtures, a temperature touchdown program was used which consisted of incubation at 94°C for 1 min, 64°C for 1 min, and 72°C for 3 min for two cycles. Then the annealing temperature was lowered in 2°C steps with each two cycles until 54°C was reached, and at this annealing temperature, 30 more cycles were performed. The incubation was finished with a 72°C, 10-min incubation step. PCR-amplified 16S rDNA sequences from the soil microbial community were separated on a 1% agarose gel in Tris-borate-EDTA buffer (89 nM, 89 mM, and 2 mM). Bands were excised, and the DNA was purified by centrifuging the agar pieces for 15 min at 16,000 × *g* in a Wizard column without resin. The flowthrough containing the DNA was collected and used without further purification. DNA fragments were ligated into a pGEM-T vector, which has 3′-T overhangs to facilitate cloning of PCR products (Promega, Madison, Wis.). Ligation mixes were transformed into ultracompetent *Escherichia coli* XL1-Blue cells (Stratagene, Cambridge, United Kingdom) according to the manufacturer's instructions. White colonies on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) medium were picked, and the insert size was determined by performing direct PCR on the cell material using M13 forward and reverse primers. Clones containing an insert with the expected size were cultured in 2 ml of Luria broth and then stored at −70°C.

For DGGE analysis, PCR primers F-968 and R-1401, which were described by Nübel et al. (29), were used and amplification was performed in a Hybaid PCR Express thermocycler. Samples were first denatured at 94°C for 5 min, followed by 40 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. Amplification was finished by a final extension step at 72°C for 10 min.

ARDRA to select the most abundant isolates and clones. In order to select the dominant members from both the clone and isolate collection, amplified rDNA restriction analysis (ARDRA) was performed. DNA was liberated from bacterial isolates using a method which was shown to be effective for both gram-negative and gram-positive bacteria (M. Vaneechoutte, State University Ghent, Ghent, Belgium, personal communication). Briefly, bacterial colonies were grown on 10-fold-diluted tryptic soy agar at 28°C for 2 to 3 days. Cells were suspended in 20 μl of lysis buffer (0.05 M NaOH, 0.25% sodium dodecyl sulfate) and heated for 15 min at 95°C. The resulting lysate was diluted with 200 μl of distilled water and centrifuged for 5 min at 16,000 × *g*. One microliter of the cleared supernatant was used for PCR amplification (see above). The amplified DNA was digested with 20 U of *Taq* I for 2 h at 65°C to generate ARDRA profiles. Digests were separated on 2% agarose gels. Both clones and isolates were grouped based on their digestion patterns using the Biogene (V96.15) software package (Vilber Lourmat, Marne-la-Vallee, France).

Partial sequencing and phylogenetic analysis of the most abundant soil clones. Clones obtained from Lovinkhoeve soil samples were partially sequenced using the primers F-968 and R-1401 previously described by Nübel et al. (29). Fragments for sequence analysis were obtained by cycle sequencing using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit. Samples of both forward and reverse primers were analyzed on an ABI 373 DNA sequencer. Consensus sequences were obtained using the DNAsis software package (version 2.5; Hitachi Ltd., San Francisco, Calif.). The partial 16S rDNA sequences were screened against those in GenBank/EMBL by using Blast (1). The most homologous sequences were used to construct a multiple alignment

TABLE 1. Isolates used[a]

Isolate or index	Division	No. of isolates collected or diversity index score taken in			
		September	January	May	July
Isolates					
Micrococcus roseus	High GC	4	5	3	3
Micrococcus kristinae	High GC	2		1	1
Micrococcus luteus	High GC	1	1		
A. oxydans	High GC	11	12	8	6
Arthrobacter globiformis	High GC	2			
Arthrobacter atrocyaneus	High GC	1	1	1	2
Arthrobacter viscosus	High GC				1
Arthrobacter mysorens	High GC			1	
Arthrobacter pascens	High GC	1			1
Corynebacterium aquaticum	High GC	1	1	1	1
Cellulomonas flavigena	High GC	2			1
Oerskovia xanthineolytica	High GC	1			
Clavibacter michiganensis	High GC		6		2
Rhodococcus rhodochrous	High GC		1		
Acinetobacter johnsonii	High GC		4		
Brevibacterium casei	High GC			1	
Streptoverticillium reticulum	High GC			1	
Nocardia asteroides	High GC			1	
Aureobacterium barkeri	High GC				1
Aureobacterium liquefaciens	High GC				2
Bacillus panthothenticus	Low GC				1
Bacillus atrophaeus	Low GC		1		2
Bacillus brevis	Low GC				1
Paenibacillus macerans	Low GC	3			
Staphylococcus haemolyticus	Low GC		1		
Staphylococcus cohnii	Low GC	1			
Staphylococcus hominis	Low GC			1	
Methylobacterium extorquens	α-Prot	1		1	
Agrobacterium radiobacter	α-Prot			1	
Agrobacterium rubi	α-Prot			1	
Hydrogenophaga pseudoflava	β-Prot			1	
Pseudomonas putida	γ-Prot	1			
Pseudomonas fluorescens	γ-Prot	1	2	3	
Pseudomonas chlororaphis	γ-Prot	2	1	3	
Flavobacterium resinovorum	Green sulfur			1	
Sphingobacterium heparinum	Green sulfur	1			
Deinococcus erythromyxa	Therm/Dein[b]			1	
Diversity Indices					
D		10.3	7.1	11.4	9.4
H		0.901	0.881	1.12	1.054
E		0.73	0.83	0.89	0.92

[a] Numbers and identity of the most abundant bacterial isolates obtained from Lovinkhoeve soil samples taken on September, January, May, and July. The bacterial division to which they belong is given. At the bottom the diversity (D and H) and evenness (E) indices based on these data are presented (see Materials and Methods).

[b] Therm/Dein, *Thermococcus*/*Deinococcus*.

using ClustalW (an online program at the Institute Pasteur website [http://www.pasteur.fr]). A phylogenetic tree was made from these aligned sequences by neighbor joining using the Treecon program (version 1.3b; Yves van de Peer). The clones sequenced in this study are coded LC (Lovinkhoeve clone), followed by the month of sampling and a number. The tree was rooted using *Verrumicrobium* as the outgroup (see Fig. 2).

Fatty acid analysis to identify the most abundant isolates. Isolates were streaked on Trypticase soy broth agar in four quadrants, and the plates were incubated at 28°C for 24 h. A loopful of cell material of late-log-phase cells was harvested. Fatty acids were extracted and methylated according to the procedure described by the manufacturer (Microbial ID, Inc.). Samples were analyzed using the Microbial Identification System on a Hewlett-Packard 5898A gas chromatograph (Palo Alto, Calif.). Chromatograms were compared to a large database of well-known reference cultures previously grown on Trypticase soy broth agar. Species names of the organisms with the most similar chromatograms are given (Table 1).

Several indices were used to calculate bacterial diversity, richness, and evenness (31, 34). To describe the abundance of species distribution or species richness, the following equation was used: $D = S-1/logN$, in which N represents the total number of isolates and S is the number of different species. To calculate diversity in relation to the sampling size, the Shannon index was used: $H = -\Sigma (n_i/N) (log\ n_i/N)$ (34). The evenness of the species distribution was calculated using the equation $E = H/logS$ (31).

DGGE analysis of the bacterial soil community. Partial 16S rDNA sequences were amplified from soil extracts using the primers F-968 and R-1401 described by Nübel et al. (29). DGGE gels were made using the Bio-Rad Gradient Delivery System establishing a gradient from 30% to 60% denaturant. Two 6% (wt/vol) polyacrylamide (acrylamide-*N, N′*-methylenebisacrylamide 37; 5:1) stock solutions were made, one with 30% denaturant containing 1× TAE (40 mM Tris, 20 mM acetic acid, 1 mM EDTA [pH 8.3]), 12% (vol/vol) formamide, and 2 M urea and one with 60% denaturant containing 24% formamide and 4.2 M urea. Polymerization was achieved by adding 0.26% (vol/vol) ammonium persulfate (10% solution) and 0.15% (vol/vol) *N,N,N′,N′*-tetramethylethylenediamine (TEMED). On top of this gradient gel a 1-cm stacking gel was poured, consisting of 6% polyacrylamide in 1× TAE without denaturant.

An approximately 6-μl PCR sample was applied on the gel, and gels were run at 60°C at a constant voltage of 100 V for 16 h in a DCcode Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, Calif.). Gels were stained in 25 ml of 1:10,000 diluted Sybr Gold (Molecular Probes, Eugene, Oreg.) in 1× TAE for 15 min and were destained in ultrapure water for 45 min. Banding patterns were visualized on a Dark Reader (Clare Chemical Research, Denver, Colo.), and pictures were digitized using a charge-coupled device camera and the Biocapt software program (Vilber Lourmat).

Analysis of the DGGE community profiles. Banding profiles of duplicate samples from September, January, May, and July from Lovinkhoeve soil were analyzed using the Bionumerics program (Applied Maths, Kortrijk, Belgium). Normalized intensity values and positions of detected bands of all lanes were used for cluster analysis and statistical analysis. Similarity coefficients calculated based on detected bands according to Dice were used to construct a complete linkage dendrogram (see Fig. 4). Band positions and intensity values were also used for statistical analysis. The Euclidean distance was calculated and visualized by multidimensional scaling (not shown). In order to test if the banding profiles of the repeat samples (i.e., samples taken simultaneously) were more similar than those of samples taken at different months of the year, a permutation approach was used (1a). Data consisting of density values and band positions of the eight samples were grouped in all four possible groups of two. The average distance of the four pairs was calculated for all possible 40,320 combinations. The null hypothesis is that the duplicates behave like a random grouping of 4×2 profiles. We then considered the distances between the four matched pairs and calculated the probability that the average distance is less than or equal to the observed distance. When the duplicates behave randomly, the average distance would lie in the middle of this distribution, whereas if the duplicate profiles were more similar, the average distance would be smaller.

RESULTS

Analysis of cultured bacteria. The log number of CFU per gram of dry soil was similar on all sampling dates. The log number of CFU was 7.7 (0.23) in September, 7.8 (0.15) in January, 7.7 (0.19) in May, and 7.7 (0.20) in July. The portion of fast-growing cells was low in January (17%) and relatively high in July (35%). The mean air temperature during the sampling months was September, 14°C; January, −2°C; May, 8°C; and July, 17°C; and the soil moisture content was September, 22%; January, 26%; May, 15%; and July, 17%.

From each of the four sampling dates, 100 randomly picked colonies were subjected to ARDRA analysis by digesting the amplified 16S rDNA fragment with *Taq* I. By using restriction patterns from this one enzyme, isolates with similar patterns were grouped. Isolates from groups with three or more members were further characterized by fatty acid analysis using the Microbial Identification System. Similarity index values ranged from 0.154 to 0.915 with a mean of 0.5, which is quite good for typing environmental strains. Identifications with similarities below 0.3 were not used in this analysis. Results revealed a broad diversity of mainly gram-positive bacteria (Table 1).

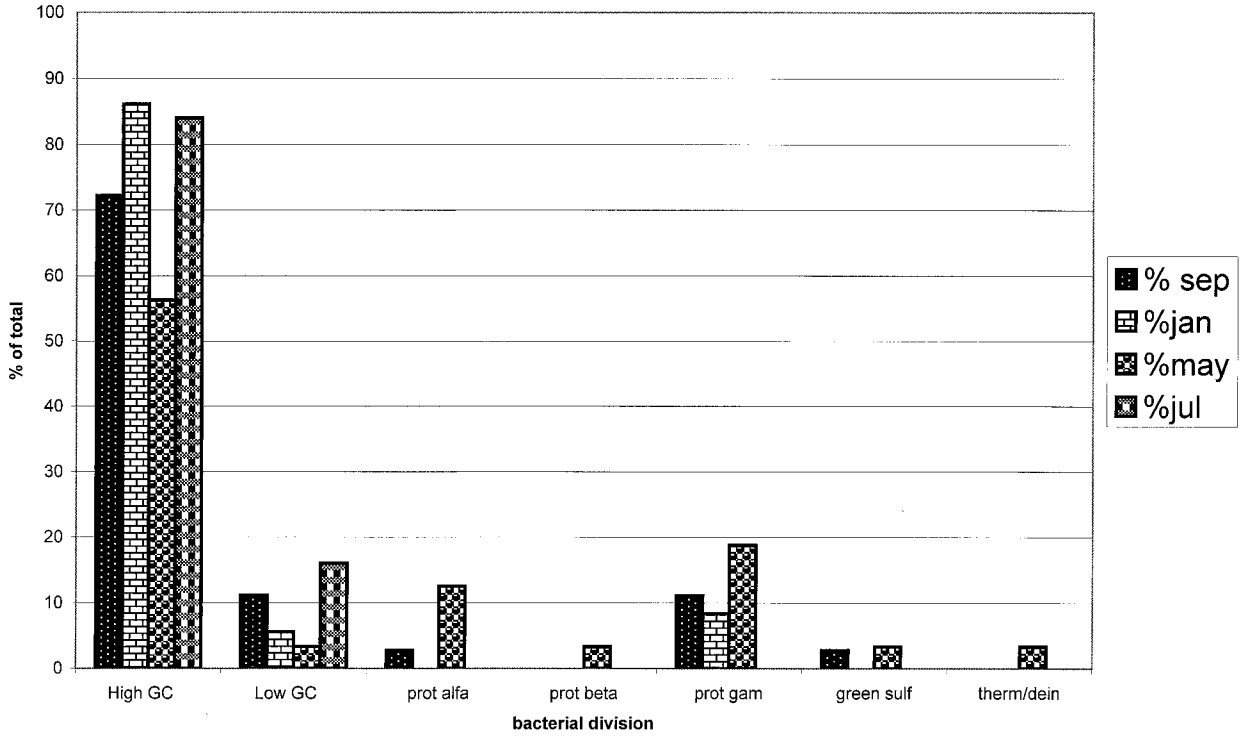

FIG. 1. Distribution of the isolates obtained from Lovinkhoeve soil samples by cultivation-based techniques taken at the different months of the year (Table 1) over various bacterial divisions. prot alfa, α-*Proteobacteria*; green sulf, green sulfur bacteria; therm/dein, *Thermococcus*/*Deinococcus*.

From a total of 128 isolates, 38 different species and 21 different genera were found. Only a few species, mainly *Micrococcus* sp. and *Arthrobacter* sp., which belong to the high-guanine-plus-cytosine-content (high-GC) gram-positive bacteria, were detected on all sampling dates. The data show a reduction in the number of the most dominant microorganism, *Arthrobacter oxydans*, in July. Similarly, members of the genus *Pseudomonas* also appear to be reduced in summer, as they are found only in September, May, and January. Quite remarkable is the relatively high number of *Clavibacter* and *Acinetobacter* bacteria in the January sample.

Several indices of community diversity, richness, and evenness were used to calculate bacterial diversity in the samples (Table 1). Both the Shannon-Weaver index (H) and richness index (D) indicate relatively little diversity in the January sample.

In Fig. 1 the percentages of the isolates belonging to the various bacterial divisions in September, January, May, and July are given. This figure shows little division diversity in the July sample, since only high- and low-GC gram-positive bacteria were found, while in the other months *Proteobacteria* and green sulfur bacteria were also detected.

Phylogenetic analysis of the dominant 16S rDNA clone sequences. Clones belonging to the most abundant ARDRA groups were selected for sequence analysis, since they were thought to represent bacteria which were present in Lovinkhoeve soil in relatively high numbers. Phylogenetic analysis of these 16S rDNA sequences revealed that they displayed close relationships to a wide range of clones or bacterial species of various divisions (Fig. 2). There is no correlation between sampling date and clone types found, and apart from the *Acidobacterium* division, no distinct, closely related clone groups can be recognized. The great diversity and low number of clones analyzed can be responsible for this phenomenon. The majority of clones appeared to cluster in bacterial taxonomic groups which are generally found in soil. In most analyses of 16S rDNA sequences from soil, members belonging to the *Acidobacterium* division dominated the clone collection (11, 20). It was remarkable that a number of clones, e.g., those belonging to the α- and γ-*Proteobacteria,* resemble clones previously found in freshwater or sediment samples. However, others have also detected sequences affiliated with isolates or clones from water and more extreme environments, such as hydrothermal vents and hot springs (25). In order to compare the diversity of the isolates with the diversity of the 16S rRNA clones, an overall diversity of the 16S rRNA clones was calculated, based on the assumption that sequences with three or more different base pairs belong to different operational taxonomic units representing certain species. Using this criterion, bacterial diversity calculated using the Shannon-Weaver index (H) was found to be 1.34 and the evenness was 0.985. In the July sample, the number of clones belonging to the *Proteobacteria* was also relatively low, although considering the small sample size, conclusions based on these statistics cannot be made. To compare the results from the culture-based analysis and the sequence analysis, the percentage of isolates or clones belonging to the different bacterial divisions was determined (Fig. 3). The large difference between the results obtained by both methods is clear. The majority of the bacteria detected by cultivation-based methods belong to the high- and low-GC

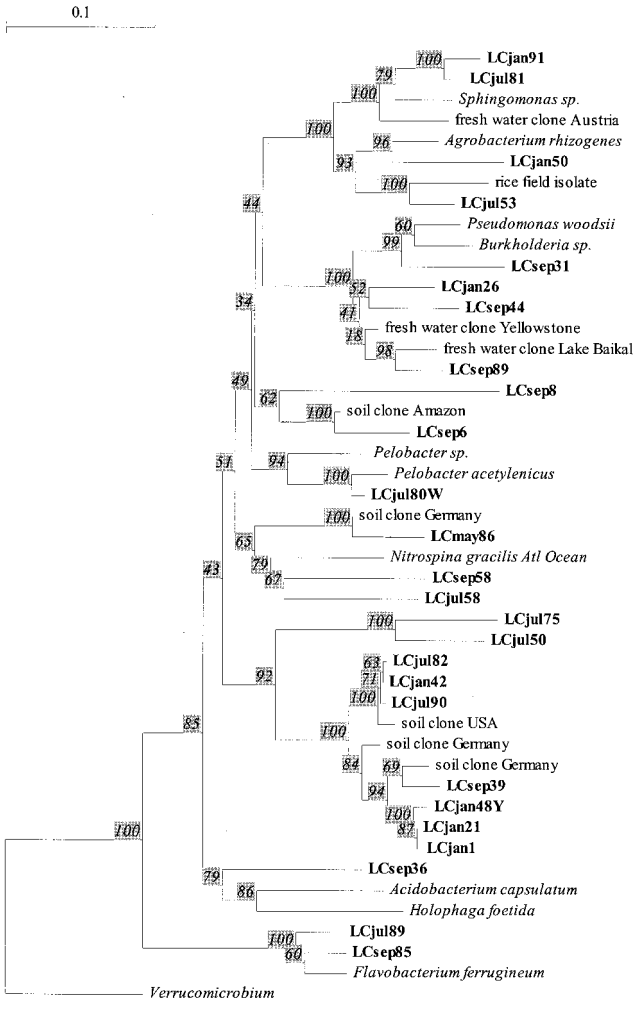

FIG. 2. Neighbor-joining tree representing the phylogenetic relationship of the most abundant 16S rDNA sequences from Lovinkhoeve soil samples taken in September, January, May, and July to various closely related clone and isolate sequences obtained from Blast searches (clones detected in this study are given in boldface). The scale indicates genetic distance.

gram-positive bacteria and, to a lesser extent, to the *Proteobacteria*. The 16S rDNA clones detected with molecularly based methods are more evenly distributed among the *Proteobacteria*, the *Acidobacterium* division, and the *Nitrospira*, cyanobacteria, and green sulfur bacteria.

DGGE profiles of Lovinkhoeve soil samples and seasonal fluctuations. At first glance the DGGE patterns from the different months look similar because the most intensely stained bands appear in all lanes (Fig. 4). This indicates that there is probably a relatively large, stable population of microorganisms detectable by molecular techniques. High similarities between DGGE banding patterns of soil samples taken on different months in the year are indicative of the existence of a stable, dominant community; previously, similar results were also found in other soils (14, 16). Nevertheless, a more thorough analysis showed quite a number of low-intensity bands which differed in the various samples. These low-intensity bands are responsible for the differences between the samples, which can be analyzed using the Bionumerics software package. Cluster analysis revealed that the patterns generated from duplicate samples are more similar to each other than to those from other months. The September and May profiles are relatively similar and cluster together with the January one, while the July profile differs substantially from all other samples (Fig. 4). Multidimensional scaling confirmed that the July sample differs from those of the other months (not shown). In order to test if the profiles of duplicate samples were more similar than the profiles from different data, a permutation approach was used. The null hypothesis was that the duplicates would behave like a random grouping of 4×2 profiles. Data from all eight profiles were grouped in all 40,320 possible combinations, and the average distance between the pairs was calculated. The average distance of 1,726 random pairings was smaller than the observed one. The one-sided P value is then 1,726/40,320 (0.04), which is <0.05 and indicates that the null hypothesis should be rejected. This indicates that duplicate profiles are significantly more similar to each other than to the profiles of the other dates.

DISCUSSION

Bacterial diversity and dynamics in Lovinkhoeve soil samples were assessed by fatty acid-mediated typing of isolates. While the log number of CFU appeared to be stable around 7.7 to 7.8 per g of dry soil on all sampling dates, both the percentage of fast-growing cells and the diversity varied (Table 1). The data suggest that the culturable bacterial population in January differed from the population on the other dates, although the differences might also have been caused by differences in cell physiology in certain microorganisms. Certain bacterial species or genera isolated from soil in a cold period could experience a shock when plated and incubated at relatively high temperatures, which could prevent them from growing. The same species might grow very well on plates when isolated from soil in a warm period. Such drawbacks are intrinsic to the use of culture-based methods for the analysis of microbial communities. The percentage of fast-growing cells reached the highest value in the July sample, and diversity was greater than in the January one, although below the values of September and May. This coincides with the appearance of *Aureobacterium* and *Bacillus* species. The most dominant bacterial genera detected by plating appeared to be *Micrococcus* and *Arthrobacter*. These genera are often found in various soils, such as those of wheat fields, deciduous woodlands, grasslands, and sand dunes (23, 37). These organisms seem to be typical inhabitants of bulk soil; data from a study by Olssen and Persson (30) showed that gram-positive bacteria were reduced in the rhizosphere, as opposed to the bulk soil. In another study, plant roots were shown to have a selective effect towards γ-*Proteobacteria* (pseudomonads) to the detriment of gram-positive bacteria and those of the *Acidobacterium* division (24). Nevertheless, a number of *Pseudomonas* species were detected throughout the year except for the July sample. Many *Pseudomonas* species are R strategists and have copiotrophic characteristics, and many are associated with plant roots which provide them with nutrients (40). Nutrient-limited and relatively warm and dry conditions in bulk soil are thought to be unfavorable to pseudomonads and might be responsible for this

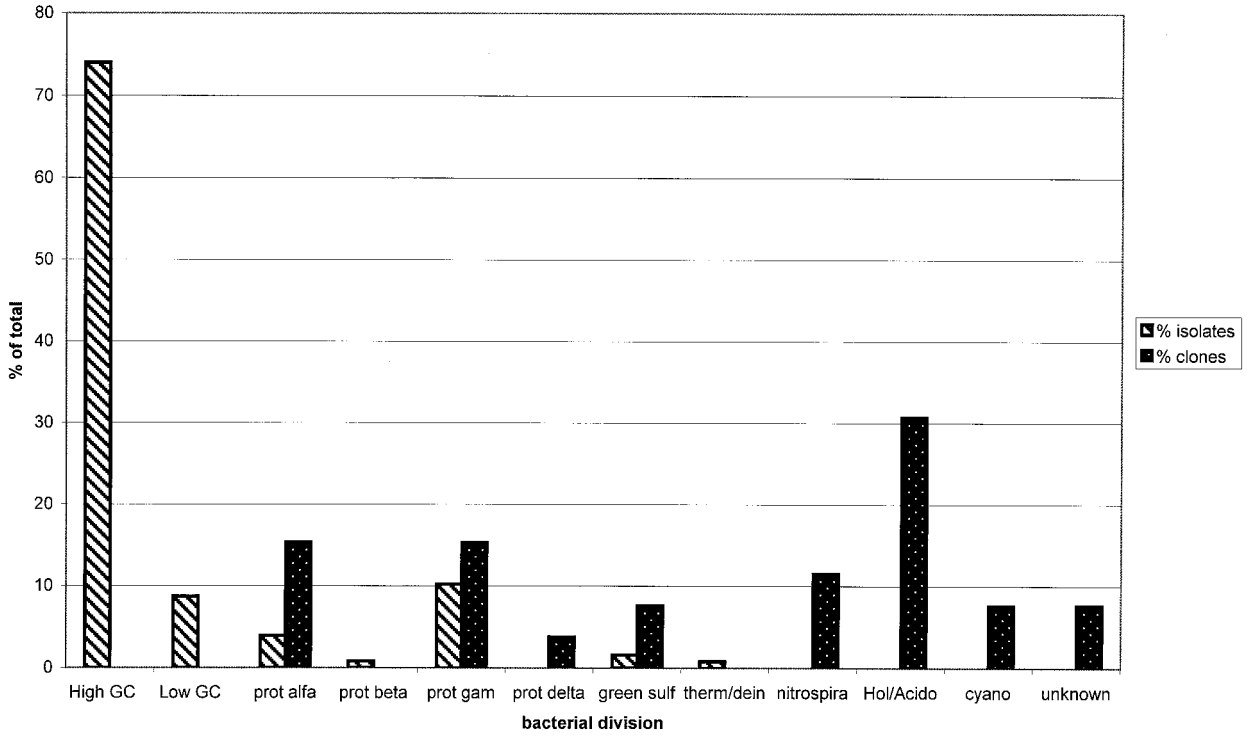

FIG. 3. Distribution of the 16S rDNA sequences (Fig. 2) and the isolates (Table 1) obtained from Lovinkhoeve soil samples over various bacterial divisions. prot alfa, α-*Proteobacteria*; green sulf, green sulfur bacteria; therm/dein, *Thermococcus*/*Deinococcus*; Hol/Acido, *Holophaga*/*Acidobacterium*; cyano, cyanobacteria.

decline (40). The culturable bacterial population in July seems to be different from those on the other months, since only gram-positive bacteria were detected (Table 1; Fig. 1). The disappearance of the pseudomonads from the isolates coincides with the appearance of several bacilli in July. Figure 1 was made to obtain a more comprehensive view of the distribution of the isolates over the major bacterial divisions in the different months. When the distribution of the isolates over the bacterial divisions is considered, May and September have high values, seven and five divisions, respectively, as opposed to January and July, which have members in, respectively, three and two divisions (Fig. 1). These high values might be attributed to the fertilization, ploughing, and harvest. Bloem and coworkers (4) showed enhanced microbial activity and levels of organic C in Lovinkhoeve soil in spring and autumn. In both periods, nutrients become available for microorganisms from fertilization (spring) and from decaying plant material left in the soil after harvest (autumn). The relatively high diversity values in September and May suggest that various different microorganisms profit from these nutrients.

Seasonal changes in microbial community diversity were also visualized by the DGGE banding profiles. Cluster analysis of the profiles revealed distinct differences between soil samples of the different months, while duplicate samples clustered closely together (Fig. 4). The July profiles were very different from those from the other samples, while both the May and September patterns cluster together. Results from the statistical analysis of the banding profiles confirm that the observed differences exceed the variations in banding patterns present in duplicate samples. This observation was in line with the seasonal changes observed in the isolates, although probably a completely different part of the community was sampled by both methods. Results on seasonal fluctuations obtained by cultivation-based methods might be more sensitive to detect changes, since the culturable part of the community might react more rapidly to changes in temperature or humidity. Changes in cell physiology resulting from differences in environmental conditions could select for a certain fraction of the microbial community which might outgrow the rest of the community. Ferroni and Kaminski (15) found a relationship between seasonal temperature changes and the number of psychrophylic and mesophylic isolates from the sediment-wa-

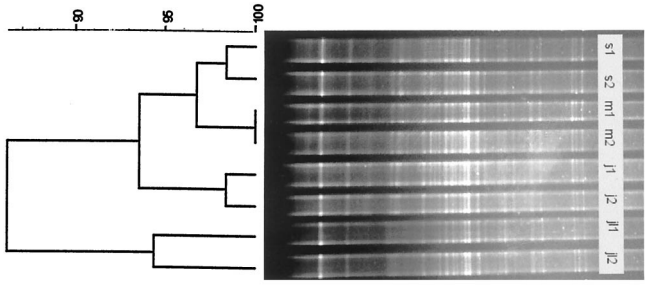

FIG. 4. DGGE banding patterns representing the most abundant bacteria in duplicate Lovinkhoeve soil samples taken in September (s1, s2), January (j1, j2), May (m1, m2), and July (jl1, jl2). At the left a dendrogram is given, representing the similarity between the patterns according to cluster analysis based on Dice's algorithm.

ter interface. However, in soil other parameters such as humidity and nutrient supply could also play a crucial role.

Studying bacterial diversity by molecular methods revealed a different population from that found by cultivation-based methods. In order to compare our results with those obtained by cultivation, diversity of the data was reduced by grouping the clones into the major bacterial divisions (Fig. 3). Both methods are able to detect bacteria belonging to the α- and γ-*Proteobacteria*. The high percentage of high-GC gram-positive bacteria which is detected by cultivation is remarkable as opposed to a more even distribution of the rDNA sequences over several bacterial divisions. Apparently, these bacteria are only a minor fraction of the total community, since no sequences of high-GC gram-positive bacteria were found. Other work has shown that the DNA extraction method combined with the primers used in this study can detect gram-positive bacteria (14, 26, 29). These results suggest that the culturable fraction of the community is only a small part of the total community.

Phylogenetic analysis of the 16S rDNA sequences showed that most clones have a high homology to isolates or clones previously obtained from various soil types (Fig. 2).

There are only a few studies that present data on phylogenetic analysis of 16S rDNA sequences from soil allowing a comparison to the data presented in this work (6, 11, 20, 25). A large proportion of the sequences from Lovinkhoeve soil, approximately 30%, belonged to the *Acidobacterium* division, of which sequences are detected in soil worldwide (2, 11, 17, 20). Approximately 35% of our clones belong to the *Proteobacteria*, and no gram-positive bacteria were found. Dunbar and coworkers (11) found that approximately 50% of their clones isolated from natural soil belonged to the *Acidobacterium* division, 10% to the gram-positive organisms, and 10% to the *Proteobacteria*. McCaig et al. (25) analyzed clones from intensively fertilized grassland and found that 13% of their clones belonged to the gram-positive bacteria, 50% belonged to the *Proteobacteria*, and 7% to the *Acidobacterium* division. Similarly, Borneman et al. (6) detected only 16% *Proteobacteria* in relatively oligotrophic grass pasture soil; however, the *Acidobacterium* division was at that time not yet recognized as a separate division. In our search to discover more general ecological relationships, the ratio between the *Proteobacteria* and the *Acidobacterium* division was calculated in order to compare our data with results found in literature. This ratio might be indicative for the nutrient status of the soil ecosystem, since the ratio was 0.16 in oligotrophic soil (11, 20); 0.34 in low-input agricultural soil (6); 0.46 in this work, which also represents a low-nutrient system; and 0.87 in a high-input agricultural system (25). However, in the study by McCaig and coworkers (25), no major differences between community diversities of improved and unimproved grassland soil were found. Although the assumption that the ratio between the *Proteobacteria* and the *Acidobacterium* division is dependent on the trophic level of the soil is based on only a few studies, results from another investigation seem to confirm this conclusion. Marilley and Aragno (24) found that the rhizosphere, which is a relatively nutrient-rich niche for bacteria, has a positive selection for the *Proteobacteria* and reduced the percentage of the *Acidobacterium* division. However, it must be noted that small differences in the percentages could occur, since the methods used for DNA extraction and primers for PCR differ among the various studies.

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