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Effects of Plant Biomass, Plant Diversity, and Water Content on Bacterial Communities in Soil Lysimeters: Implications for the Determinants of Bacterial Diversity▽†

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Soils may comprise tens of thousands to millions of bacterial species. It is still unclear whether this high level of diversity is governed by functional redundancy or by a multitude of ecological niches. In order to address this question, we analyzed the reproducibility of bacterial community composition after different experimental manipulations. Soil lysimeters were planted with four different types of plant communities, and the water content was adjusted. Group-specific phylogenetic fingerprinting by PCR-denaturing gradient gel electrophoresis revealed clear differences in the composition of *Alphaproteobacteria*, *Betaproteobacteria*, *Bacteroidetes*, *Chloroflexi*, *Planctomycetes*, and *Verrucomicrobia* populations in soils without plants compared to that of populations in planted soils, whereas no influence of plant species composition on bacterial diversity could be discerned. These results indicate that the presence of higher plant species affects the species composition of bacterial groups in a reproducible manner and even outside of the rhizosphere. In contrast, the environmental factors tested did not affect the composition of *Acidobacteria*, *Actinobacteria*, *Archaea*, and *Firmicutes* populations. One-third (52 out of 160) of the sequence types were found to be specifically and reproducibly associated with the absence or presence of plants. Unexpectedly, this was also true for numerous minor constituents of the soil bacterial assemblage. Subsequently, one of the low-abundance phylotypes (beta10) was selected for studying the interdependence under particular experimental conditions and the underlying causes in more detail. This so-far-uncultured phylotype of the *Betaproteobacteria* species represented up to 0.18% of all bacterial cells in planted lysimeters compared to 0.017% in unplanted systems. A cultured representative of this phylotype exhibited high physiological flexibility and was capable of utilizing major constituents of root exudates. Our results suggest that the bacterial species composition in soil is determined to a significant extent by abiotic and biotic factors, rather than by mere chance, thereby reflecting a multitude of distinct ecological niches.

Soils harbor highly diverse bacterial communities with up to 50,000 (54) or even up to millions (18) of different 16S rRNA gene sequences. So far, it has remained unclear whether functional redundancy or a multitude of ecological niches and adaptive mechanisms governs the composition of soil bacterial communities (22, 63). In the present study, this question was assessed by analyzing the reproducibility of bacterial community composition with different experimental manipulations of soil lysimeters and by subsequently studying the bacteria associated with particular experimental conditions by use of cultivation-based methods. The variables selected for environmental manipulations were the absence or presence of higher plants species, plant diversity, and water content.

Above-ground plant diversity in particular has long been suggested to drive below-ground microbial diversity. Thus, the diversity of bacterial communities in grassland soil samples has been shown to be affected by the numbers of plant species present (23). In the rhizosphere, plants select for a specific composition of bacterial communities (32, 34, 57) depending on the type and amount of organic root exudates and of nutrients released from senescent or dead roots (25, 52). However, plant rhizosphere effects have been found to be of little significance for the composition of total community structure in grasslands whereas liming and nitrogen addition alters overall soil bacterial community structure (30). In addition, a change of tillage and crop residue management practice has been observed to lead to pronounced changes in the composition of the soil bacterial community as documented by fatty acid methyl ester analysis (51). Finally, soil bacterial diversity was found to be dependent on soil pH but to be unrelated to site temperature, latitude, organic carbon content, C:N ratio, or plant diversity in a recent continent-scale study (17).

Several of the above-cited studies were found to have been limited by the resolution of the culture-independent methods used (clone libraries of limited coverage, ribosomal DNA fingerprinting using eubacterial primers) (23). Supporting this conclusion, a significant correlation between particular plants and soil bacterial populations could be detected when the diazotrophic community was analyzed separately and at high

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resolution by amplifying the specific functional gene *nifH* (13); *Rhizobium* spp. were found to be less diverse in pastures planted with soybeans (11). As an additional point of concern, different factors often do not act independently in the natural setting; e.g., the presence of different plant species also causes differences in soil chemical properties (6). Besides requiring high-resolution monitoring techniques, studies of bacterial diversity therefore need to assess different environmental factors independently and under highly controlled experimental conditions.

In the present study, we conducted a systematic and high-resolution survey of soil bacterial diversity and its interdependence with environmental factors. Specifically, the effects of soil water content, season, absence or presence of higher plant species, and diversity of higher plant species on 10 bacterial groups were studied under highly controlled conditions in soil lysimeters. Of the numerous low-abundance bacterial phylotypes which exhibited a reproducible pattern in the lysimeters, we selected and isolated a representative bacterium and investigated the reasons underlying its interdependence with higher plant species.

MATERIALS AND METHODS

Lysimeter design and sample collection. Soil samples were obtained from lysimeters which had been in continuous operation since 2001 in the Ecological-Botanical Garden of the University of Bayreuth. The lysimeters were built with 1-m-deep concrete walls enclosing a surface area of 1.3 by 1.3 m. The walls had been smoothed with metal brushes and sealed twice using Inertol 49W coating material. The lysimeters were filled with endostagnic cambisol soil obtained from nearby grassland soils after homogenization of the soil by a rotor tiller and steaming at 100°C over 12 h for sterilization (31). Lysimeters were seeded in the year 2001 at a density of 2,000 shoots per m^2 with perennial grasses and forbs in order to create four different plant communities of increasing diversity: (i) lysimeters devoid of higher plant species which consisted of soil partially covered with mosses; (ii) lysimeters planted with the two grass species *Holcus lanatus* (Yorkshire fog) and *Arrhenatherum elatius* (tall oat grass); (iii) lysimeters planted with the two grass species *H. lanatus* and *A. elatius* and the two forb species *Plantago lanceolata* (ribwort) and *Geranium pratense* (meadow crane's-bill); and (iv) lysimeters planted with the four grass species *H. lanatus*, *A. elatius*, *Alopecurus pratense* (foxtail grass), and *Anthoxanthum odoratum* (sweet vernal grass) and the four forb species *P. lanceolata*, *G. pratense*, *Ranunculus acris* (tall buttercup), and *Taraxacum officinale* (dandelion blow balls). For each type of plant community, six lysimeters were set up in parallel. Weeding was performed over the growing season in one-month intervals. Following the first sampling campaign in June 2004, the water content of the soils was controlled in half of the plots of each lysimeter type in order to study the effect of different water content percentages on bacterial diversity. Irrigation was performed regularly, thereby maintaining the water content of the lysimeters at 15% (53). In the absence of irrigation, the water content of the soils dropped to 5% (7). Accordingly, four different types of treatment were available in June 2004, whereas eight different types of treatment were analyzed afterwards (four different plant covers times two types of water supply). For each treatment, three parallel lysimeters were set up. Lysimeters were mown twice a year to remove above-ground plant biomass. To compensate for the nutrients removed with plant biomass, "Blaukorn" fertilizer was added at 50 $kg \cdot ha^{-1}$.

Sampling of the upper 10 cm of each soil was conducted on 9 June, 2 August, and 1 September 2004. This yielded a total of 20 different types of samples (4 from June and 8 each from August and September). In order to account for spatial nonhomogeneity within individual lysimeters, three subsamples were collected from each lysimeter by coring different locations between the plants. Each core yielded between 107 and 172 g wet weight of soil. In order to account for variations between similar lysimeters, three parallel lysimeters of the same treatment group were sampled. Subsequently, all nine soil samples of the same lysimeter type were pooled and sieved through a 0.5-mm-mesh sieve. Each pooled sample was then split in half. One half was wrapped in sterile aluminum foil and immediately frozen in liquid N_2 for molecular analysis. The other half of the sample was transferred to a sterile plastic bag and kept at 4°C for subsequent enumeration and isolation of bacteria.

Total bacterial cell counts. Soil slurries were prepared as 1:100 (wt/vol) dilutions in a buffer consisting of 10 mM HEPES, 10 mM pyrophosphate, and 0.08% Tween (final concentrations). Aliquots of the slurries were subsequently fixed with 2% glutaraldehyde, and 30 μl of each sample was diluted in 5 ml of buffer and stained with SYBR green II (Molecular Probes, Eugene, OR) for 10 min in the dark. Subsamples were then filtered onto polycarbonate filters (Nuclepore Track-Etch membrane; Whatman, Springfield Mill, United Kingdom) (0.1 μm pore size, 25 mm diameter), and the filters were dried, embedded in DABCO antifading solution (25 mg of 1,4-diazabicyclo [2.2.2]octane in 1 ml of phosphate-buffered saline buffer plus 9 ml of glycerol), and subsequently examined by epifluorescence microscopy (Zeiss Axiolab microscope) (lamp, HBO 50; filter set, Zeiss Ex 450–490, FT 510, and LP 515) at a magnification of ×1,000. At least 20 fields were counted for each sample.

DNA extraction and purification. Soil DNA was extracted using an UltraClean Mega Prep soil DNA kit (Mo Bio Laboratories, Inc., Solana Beach, CA) according to the instructions of the manufacturer but including a sonification step (3 min in continuous mode; Branson Sonifier B15 cell disruptor). The eluate was precipitated with ethanol and further purified using a 100/G genomic tip (QIAGEN GmbH, Hilden, Germany). Finally, the DNA was dialyzed using a Centricon-50 dialysis filtration unit (Millipore, Amicon, Bedford, MA) and washed twice using 2 ml Tris-EDTA buffer. DNA concentrations were determined by dye binding with PicoGreen (Molecular Probes, Eugene, OR).

For extraction of chromosomal DNA from cultured bacteria, cells in microtiter plates were harvested by centrifugation (20 min at 15.000 × g, 4°C). Cell pellets were lysed by six cycles of freezing and thawing with each cycle consisting of an incubation for 3 min at −80°C followed by an incubation for 3 min at 100°C. Aliquots of 1 to 3 μl of the resulting crude extracts were used directly in PCR amplifications.

PCR amplification, enterobacterial repetitive intergenic consensus PCR (ERIC-PCR), and denaturing gradient gel electrophoresis (DGGE) fingerprinting. The 16S rRNA gene fragments of members of nine phyla or subphyla of the *Bacteria* and of members of the *Archaea* were amplified by PCR employing the primers and cycling conditions listed in Table 1. For the amplification of 16S rRNA genes of some of the phylogenetic groups, primer mixtures had to be employed (see wobble positions in the primers listed in Table 1). By using DNA of pure laboratory cultures, we tested whether amplification with these primer mixes resulted in multiple fingerprints of one phylotype; however, multiple bands were never observed (compare also reference 49).

Amplification reactions were performed with a Gene Amp PCR 9700 system (Applied Biosystems, Foster City, CA). The reaction mixture contained the following ingredients per 50 μl: 100 ng of soil DNA, 5 μl of 10× PCR buffer containing 15 μM $MgCl_2$ (QIAGEN GmbH, Hilden, Germany), 5 μl of Q solution (QIAGEN), 40 μg of bovine serum albumin (Sigma-Aldrich, Steinheim, Germany), 500 nM $MgCl_2$, 200 nM deoxynucleoside triphosphates, a 1 μM concentration of each primer, and 1.25 units of *Taq* polymerase (QIAGEN). For each primer set targeting 16S rRNA gene sequences, one of the primers contained a 40-bp-long GC clamp at its 5′ end (Table 1) in order to obtain stable melting behavior of the generated DNA fragments during subsequent DGGE. The genetic diversity of isolated cultures was studied by ERIC-PCR (62). PCR products were analyzed by standard agarose electrophoresis.

Denaturing gradient gel electrophoresis was carried out in an Ingeny phorU system (Ingeny International BV, Goes, The Netherlands) employing 6% (wt/vol) polyacrylamide gels in 1× Tris-acetate-EDTA (pH 8.0). Denaturing gradients ranged from 30 to 70% (for *Archaea*, *Alphaproteobacteria*, *Betaproteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Planctomycete* species), from 30 to 80% (for *Acidobacteria* species), from 40 to 80% (for *Chloroflexi* and *Verrucomicrobia* species), or from 55 to 80% (for *Actinobacteria* species), where 100% denaturant is defined as 7 M urea and 40% (vol/vol) formamide (42). Gels were stained for 45 min with SYBR gold (MoBiTec, Göttingen, Germany) (1:10,000 dilution), visualized on a UV transilluminator (LTF Labortechnik, Wasserburg, Germany), and photographed (Visitron Systems GmBH, Puchheim, Germany). DNA bands were excised from the gel with a sterile scalpel and transferred to a 1.5 ml Eppendorf tube containing 25 μl of 10 mM Tris-HCl buffer (pH 8.0), and the DNA was eluted for 2 h at 65°C. Reamplifications were conducted using the corresponding primers (but without a GC clamp). PCR products were separated from free PCR primers by use of a QIAquick Spin kit (QIAGEN).

Quantitative analysis of bacterial diversity. The generated DGGE profiles were analyzed using ONE-Dscan electrophoresis analysis software (Scanalytics, Billerica, MA). After automated background subtraction, the lanes were normalized against each other. For each DNA band, the relative intensity value and position were recorded and incorporated in a matrix. Similarities were calculated

TABLE 1. Summary of primer sets used and cycling conditions

Target and primer set[a]	Primer sequence (5′ → 3′)	Cycling conditions[b]	Reference
Alphaproteobacteria (some *Gammaproteobacteria*)			
GC517f	GTGCCAGCAGCCGCGG	30 s at 94°C, 45 s at 68°C, 1 min at 72°C (10 cycles); 30 s at	35
Alf968r	GGTAAGGTTCTGCGCGTT	94°C, 45 s at 62°C, 1 min at 72°C (20 cycles)	45
Betaproteobacteria			
GC1055r	AGCTGACGACAGCCAT	30 s at 94°C, 1 min at 56°C, 1 min at 72°C (10 cycles); 30 s	3
Beta680f	CRCGTGTAGCAGTGA	at 94°C, 1 min at 51°C, 1 min at 72°C (20 cycles)	49
Acidobacteria			
GC341r	CTGCTGCCTCCCGTAGG	30 s at 94°C, 1 min at 62°C, 1 min at 72°C (10 cycles); 30 s	42
Acido31f	GATCCTGGCTCAGAATC	at 94°C, 1 min at 57°C, 1 min at 72°C (20 cycles)	4
Actinobacteria			
GCAB1165r	ACCTTCCTCCGAGTTRAC	30 s at 94°C, 45 s at 62°C, 1 min at 72°C (10 cycles); 30 s at	37
926f	AAACTCAAAGGAATTGACGG	94°C, 45 s at 57°C, 1 min at 72°C (20 cycles)	35
Archaea			
GC344f	ACGGGGNGCAGCCGCGA	20 s at 96°C, 45 s at 54°C, 12 s at 72°C (35 cycles)	60
ARCH785r	GGACTACVSGGGTATCTAAT		35
Bacteroidetes			
GCCFB319f	GTACTGAGACACGGACCA	30 s at 94°C, 45 s at 65°C, 1 min at 72°C (10 cycles); 30 s at	28
907r	CCGTCAATTCCTTTGAGTTT	94°C, 45 s at 60°C, 1 min at 72°C (25 cycles)	42
Chloroflexi			
GC1340r	CGCGGTTACTAGCAAC	30 s at 94°C, 45 s at 58°C, 1 min at 72°C (10 cycles); 30 s at	19
GNSB941f	AGCGGAGCGTGTGGTTT	94°C, 45 s at 53°C, 1 min at 72°C (25 cycles)	
Firmicutes			
GCLGC354f	GCAGTAGGGAATCTTCSR	30 s at 94°C, 45 s at 58°C, 1 min at 72°C (10 cycles); 30 s at	40
907r	CCGTCAATTCCTTTGAGTTT	94°C, 45 s at 53°C, 1 min at 72°C (20 cycles)	43
Planctomycetes			
GCEUB338IIf	ACACCTACGGGTGGCTGC	30 s at 94°C, 45 s at 69°C, 1 min at 72°C (10 cycles); 30 s at	12
Pla886r	GCCTTGCGACCATACTCCC	94°C, 45 s at 64°C, 1 min at 72°C (20 cycles)	46
Verrucomicrobia			
GCEUBIII338r	GCTGCCACCCGTAGGTGT	30 s at 94°C, 45 s at 63°C, 1 min at 72°C (10 cycles); 30 s at	55
Ver40f	CGGCGTGGWTAAGACATGCA	94°C, 45 s at 58°C, 1 min at 72°C (20 cycles)	This study
Enterobacterial repetitive consensus sequences			
ERIC1R	ATGTAAGCTCCTGGGGATTCAC	30 s at 94°C, 1 min at 52°C, 8 min at 70°C (30 cycles)	62
ERIC2	AAGTAAGTGACTGGGGTGAGCG		
Betaproteobacterium strain byr23-80			
Mas1139F	TTGAGCACTCTAATGAGAC	qPCR: 30s at 95°C, 45s at 56°C, 1 min at 72°C (40 cycles),	This study
Mas1281R	CTACGATACACTTTCTGGG	melt curve from 55 to 95°C	

[a] GC primers contain a 40-bp-GC clamp (CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCC) at the 5′ end.
[b] Each protocol included a single 4-min cycle at 96°C at the beginning and terminated with a 10-min cycle at 72°C.

with the SIMGEND program of NTSYS-pc software (Exeter Software, New York, NY) and expressed as Nei coefficients (47). Based on these values, dendrograms were constructed using the SAHN program of the package and applying the unweighted-pair group method using average linkages. The relative intensity values of DGGE bands were also utilized to calculate the diversity of each bacterial target group established in the lysimeter soil employing the Shannon-Weaver index of diversity H' according to the following formula (56):

$$H' = -\Sigma p_i \ln p_i$$

where the relative intensity value assigned to each individual band i was used as an indicator of the relative abundance p_i of the corresponding melting type.

Sequencing and phylogenetic analysis. Double-pass sequencing of the 16S rRNA gene fragments was performed, employing an ABI Prism BigDye Terminator cycle sequencing ready-reaction kit (Applied Biosystems GmbH, Weiterstadt, Germany) and an ABI Prism 310 genetic analyzer (Applied Biosystems). In cases in which DNA bands from DGGE gels yielded multiple sequences, the latter were separated by cloning using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA). After being plated on selective LB agar plates, recombinants were picked randomly and the plasmids were extracted from an overnight culture in liquid LB media with a QIAprep Spin Miniprep kit (QIAGEN). Plasmids were then differentiated by enzymatic digestion with EcoRI (Fermentas GmbH, St. Leon-Rot, Germany), and different clones were subsequently sequenced. All 16S rRNA gene sequences obtained in the present study were checked for possible chimeras by use of the CHIMERA-CHECK online analysis program of the RDP-II database (38).

The 16S rRNA sequences were analyzed using ARB software (http://www.mikro.biologie.tu-muenchen.de). Sequences of the closest relative were retrieved from the GenBank database by use of BLAST 2.0.4 software (1) and

TABLE 2. Soil parameters, total bacterial cell counts, and culturability values for the different lysimeters

No. of plant species and lysimeter condition[a]	$H_2O/CaCl_2$ pH values for 2004 sampling date[b]			Root biomass[c] [g C · m^{-2}] (% of total organic C) value for sampling date 3 June 2004	Total soil organic C[c] [g C · m^{-2}] value for sampling date 3 June 2004	Total cell counts [× 10^9 cells · (g $soil^{-1}$)] value for 2004 sampling date:			% Culturability value for 2 August 2004 sampling date	
	9 June	2 August	1 September			9 June	2 August	1 September	Microdrop	Soil slurry dilution
0										
Preirrigation	5.6/4.8			0 (0)	3,239 ± 36	6.5 ± 2.5			0.021 ± 0.015	0.010 ± 0.010
$-H_2O$		5.9/4.8	6.2/5.1				1.7 ± 0.4	1.5 ± 0.2		
$+H_2O$		5.7/4.6	5.9/4.7				1.2 ± 0.2	1.2 ± 0.2		
2										
Preirrigation	5.8/4.7			296 ± 15 (7.7)	3,860 ± 69	9.1 ± 2.7			0.328 ± 0.061	0.085 ± 0.030
$-H_2O$		6.2/4.7	6.2/5.2				1.3 ± 0.3	1.5 ± 0.3		
$+H_2O$		6.3/4.8	6.2/5.1				0.9 ± 0.2	2.7 ± 0.3		
4										
Preirrigation	5.8/4.7			349 ± 22 (8.8)	3,973 ± 104	4.1 ± 1.0			0.010 ± 0.010	0.267 ± 0.055
$-H_2O$		6.2/4.7	6.3/5.2				1.5 ± 0.3	2.4 ± 0.3		
$+H_2O$		6.3/5.2	6.4/5.3				1.6 ± 0.5	4.1 ± 0.9		
8										
Preirrigation	5.8/4.8			318 ± 13 (7.9)	4,042 ± 138	2.3 ± 0.6			1.606 ± 0.160	0.042 ± 0.021
$-H_2O$		6.1/4.9	6.1/5.2				1.8 ± 0.4	2.6 ± 0.6		
$+H_2O$		6.2/4.9	6.4/5.4				1.6 ± 0.6	4.8 ± 0.9		

[a] $-H_2O$, nonirrigated lysimeters; $+H_2O$, irrigated lysimeters.
[b] pH for $CaCl_2$ determined after suspension of soil in 10 mM $CaCl_2$.
[c] Reported in reference 53.

imported into the ARB database. Through the use of the integrated Fast Aligner Version 1.03 tool, the sequences were automatically aligned and the alignment was corrected manually according to secondary-structure information. First, only sequences longer than 1,300 bp were used to construct a tree by employing the maximum likelihood algorithm (Fast DNA_ML). Afterwards, the shorter environmental sequences as obtained from the DGGE bands were inserted without changing the overall tree topology, employing the parsimony interactive tool implemented in the ARB software package. Bootstrap values were calculated from 100 bootstrap resamplings.

qPCR. Quantitative PCR (qPCR) was conducted to quantify the abundance of *Betaproteobacterium* sp. strain byr23-80 in the different lysimeters. Oligonucleotide primers specific for this phylotype were generated using ARB software; specificity was confirmed by using RDP-II Probe Match software (http://rdp.cme.msu.edu/probematch/search.jsp), by using PCR assays with different other *Betaproteobacteria* species as negative controls, and finally by sequencing qPCR products generated from soil DNA. qPCR was conducted in an iQ multicolor real-time PCR detection system (Bio-Rad, Hercules, CA), using 250 nM concentrations each of primers Mas1139F and Mas1281R (Table 1), 5 to 10 ng of DNA template, 20 μg of bovine serum albumin (Sigma-Aldrich), and 12.5 μl of iQ SYBR green Supermix (Bio-Rad) in a volume of 25 μl. The optimized cycling protocol (Table 1) resulted in a highly specific 142-bp amplicon. Genomic DNA of strain byr23-80 was used to generate a standard curve ranging from 1.6 to 1.6 · 10^{-5} ng per well. All determinations were run in triplicate, and cell numbers of strain byr23-80 were estimated based on a mean content of 4.7 · 10^{-15} g DNA · $cell^{-1}$.

Cultivation and isolation of soil bacteria. The culturability of soil bacteria was assessed in samples from August 2004. Since we were mainly interested in the effects of plant diversity on bacterial culturability, irrigated and nonirrigated lysimeter soil samples of each lysimeter type were mixed in equal portions for this purpose. Soil-solution-equivalent medium (3) (pH 7.0) buffered with 10 mM HEPES was employed. One liter of this basal medium was supplemented with artificial root exudates (33), yeast extract (0.01% wt/vol), and inducers (a 10 μM concentration each of cyclic AMP, AMP, *N*-oxohexanoyl-DL-homoserine lactone (OHHL), and L-homoserine lactone; see reference 8). Basal medium without substrates served as a control.

High-throughput cultivation was performed using sterile 96-well polystyrene microtiter plates (Corning Inc., Corning, NY). Each well received 180 μl of growth medium and 50 bacterial cells. In the first approach, the plates were inoculated by employing MicroDrop AutoDrop microdispenser system version 5.50 (MicroDrop GmbH, Norderstedt, Germany) as described previously (8, 21). In order to prevent clogging of the microdispenser pipette, soil suspensions (see above) were prefiltered through 12-μm-pore-size polycarbonate filters prior to inoculation. The second approach was used to cultivate bacteria firmly associated with particles of more than 12 μm. In this case, cultures were set up by manually inoculating liquid cultures with aliquots of the soil suspensions by use of a conventional multipipette. On each microtiter plate, 12 wells were left without inoculation and served as controls for contamination. Microtiter plates were incubated at 15°C for 6 to 8 weeks, with monitoring by visual inspection of turbidity.

Bacterial strains were isolated by streaking selected liquid cultures onto the medium described above after solidification by use of 8 g · $liter^{-1}$ gellan gum (Sigma-Aldrich) (27). Strains were characterized by biochemical and physiological tests, including growth tests with 51 individual sugars, 36 organic acids, 4 keto acids, 22 amino acids, 8 alcohols, and 4 complex substrates, as described previously (20).

Nucleotide sequence accession numbers. The 16S rRNA gene sequences have been deposited in the GenBank database under accession numbers AM749495 through AM749665 and EF546777.

RESULTS

Total cell numbers and culturability of soil bacteria. Within the study period, cell numbers in the lysimeters ranged from (0.9 ± 0.2) × 10^9 to (9.1 ± 2.7) × 10^9 cells · g^{-1} (Table 2). In June 2004, the highest values were determined for samples obtained from the lysimeters which were devoid of higher plant species or from lysimeters planted with two plant species (Table 2). Two months later, cell counts had decreased and were similar in all lysimeter samples. In September 2004, bacterial numbers had increased in lysimeters planted with four or eight plant species. In this month, an increased water supply resulted in significantly increased bacterial cell numbers in all lysimeters stocked with higher plant species compared to those seen in the nonirrigated parallels (*t* test; $P < 0.05$). With respect to the soil pH, only minor differences were observed between the different lysimeters. No significant differences in bacterial cell volumes were detected (data not shown).

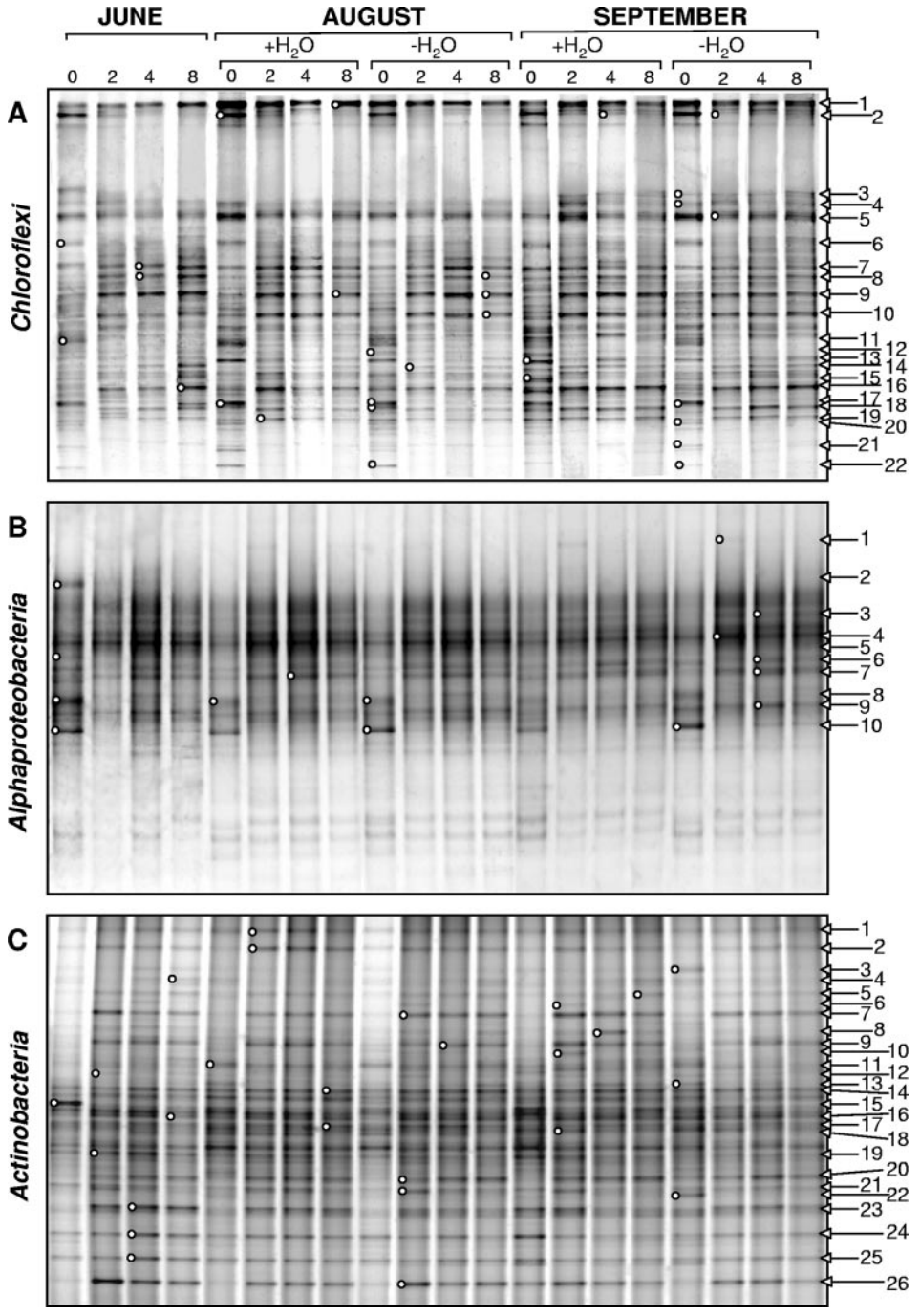

FIG. 1. Three examples of the DGGE analyses of PCR-amplified 16S rRNA gene fragments. Lanes 0, 2, 4, and 8 represent samples from lysimeters with the respective numbers of higher plant species; the $-H_2O$ and $+H_2O$ lanes correspond to the nonirrigated and irrigated lysimeters; lanes June, August, and September correspond to the three soil sampling dates. (A) *Chloroflexi* species. (B) *Alphaproteobacteria* species. (C) *Actinobacteria* species. For each bacterial group, circles denote the bands which were excised and sequenced, and arrows with consecutive numbering indicate the different melting types analyzed. Negative images of SYBR gold-stained DGGE gels are shown.

The cultivation efficiency determined for the August 2004 samples ranged between 0.01 and 1.6% of total cell counts (Table 2). No consistent trend was observed with respect to plant cover or the two cultivation techniques used.

Comparative analysis of bacterial diversity in the different lysimeters. The compositions of the prokaryotic communities in the 20 different soil samples were compared by phylogenetic fingerprinting employing PCR-DGGE. In order to increase the resolution of this analysis (21, 49), 16S rRNA gene fragments were selectively amplified for each of the 10 target groups of bacteria. For the specific amplification of *Verrucomicrobia* species, we initially applied previously published primers. How-

TABLE 3. Summary of the analysis of environmental and cultured DGGE melting types and phylotypes

Target group	Environmental samples							Cultured samples		
	Total no. of melting types detected	No. of DNA bands sequenced[b]	No. of melting types analyzed (no. with multiple sequences)[c]	No. of phylotypes recovered	No. of sequences matching sequences in the databases at a similarity of:			No. of melting types	No. of melting types matching environmental melting types	No. of phylotypes matching environmental phylotypes
					>97%	93%–97%	<93%			
Chloroflexi	71	29	22 (3)	27	7	14	6	0	0	0
Acidobacteria	23	20	20 (0)	20	8	12	0	3	0	0
Verrucomicrobia[a]	54	10	10 (4)	17	5	10	2	12	1	0
Planctomycetes[a]	36	11	11 (3)	14	3	10	1	8	3	0
Alphaproteobacteria	24	15	10 (0)	10	7	3	0	6	4	0
Betaproteobacteria	29	14	10 (0)	10	8	2	0	6	2	1
Bacteroidetes	26	22	12 (0)	12	9	3	0	8	0	0
Actinobacteria	38	26	26 (0)	26	14	8	4	17	1	1
Firmicutes	19	13	12 (0)	12	6	6	0	6	2	0
Archaea	16	14	12 (0)	12	6	6	0	0	0	0
Total	336	174	145 (10)	160	73	74	13	66	13	2

[a] Several DGGE bands generated for the *Verrucomicrobia* and *Planctomycetes* species were found to contain up to four different 16S rRNA gene sequences which had to be separated by cloning prior to sequencing. Therefore, the phylogenetic analysis of these two groups was limited to the 10 major bands, which yielded a total of 17 phylotypes.

[b] Corresponding to bands denoted by circles in Fig. 1.

[c] Indicated by numbered arrows in Fig. 1.

ever, all primer combinations tested were found to be too unspecific, since they also yielded sequences of *Planctomycetes*, *Bacteroidetes*, and *Proteobacteria* species (data not shown). Consequently, a novel primer suitable for the PCR-DGGE approach was designed (primer Ver40f; Table 1) which, in combination with primer EUBIII338R, permitted a selective amplification of *Verrucomicrobia* sequences.

Adding up the group-specific fingerprints detected in the 20 samples, our PCR-DGGE analysis yielded total numbers of different melting types between 16 and 71 for the 10 target groups (three examples are depicted in Fig. 1; all results are summarized in Table 3). The number of fingerprints was highest for members of the phyla *Chloroflexi* (Fig. 1A) and *Verrucomicrobia*, with 71 and 54 distinguishable melting types, respectively. In contrast, between 23 and 38 melting types were detected for the *Alphaproteobacteria*, *Betaproteobacteria*, *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, and *Planctomycetes* species. Of all groups analyzed, the *Firmicutes* and the *Archaea* species yielded the lowest numbers of distinct melting types (19 and 16, respectively) (Table 3). The signal intensity of DNA fingerprints was evenly distributed for some groups, like the *Actinobacteria* species (Fig. 1C), whereas fingerprint patterns were dominated by some bands in other cases (Fig. 1B). In order to account not only for the total number of bands but also for the evenness of this intensity distribution, the DGGE fingerprint patterns of the individual groups were analyzed by densitometry. After determination of the relative signal intensities of the fingerprints in each lane, the Shannon-Weaver index of diversity was calculated from the number of fingerprints and their relative signal intensities. The results confirmed that *Chloroflexi* species reached the highest diversity, while the *Bacteroidetes* and *Archaea* species were the least diverse among all groups investigated (Table 4).

For each of the 10 target groups, the fingerprint patterns generated from the set of 20 different lysimeter samples resembled each other (see Fig. 1A to C), indicating that the manipulation of plant communities in the lysimeters did not lead to completely altered bacterial communities. However, our analysis also revealed a differential response of some bacteria to the presence or absence of higher plant species. For example, DGGE profiling of the *Chloroflexi* species (Fig. 1A) yielded several DNA fragments which were detected exclusively (bands 11, 17, and 22) or at higher abundance (bands 2 and 6) in barren lysimeters whereas others (bands 9 and 10) were detected at higher signal intensities in the planted lysimeters. Similarly, bands 8 and 10 of the *Alphaproteobacteria* species (Fig. 1B) were detected exclusively or at a higher signal strength in the barren lysimeters whereas bands 7, 9, and 20 of the *Actinobacteria* species (Fig. 1C) were found in planted lysimeters. Only few fingerprints, e.g., band 2 of the *Alphaproteobacteria* species, were unique to single soil samples.

Factors influencing bacterial diversity in lysimeters. In order to identify the factors affecting bacterial community composition, pairwise similarity values were calculated from the fingerprints patterns by employing the Nei coefficient. A clus-

TABLE 4. Mean values and ranges of the Shannon-Weaver index of diversity (H′) calculated for the bacteria of 10 bacterial groups present in lysimeter soil

Bacterial group	H′	
	Mean	Range
Chloroflexi	3.47	2.75–6.37
Acidobacteria	2.37	1.85–2.70
Verrucomicrobia	2.67	2.39–2.86
Planctomycetes	2.78	2.56–3.01
Alphaproteobacteria	2.09	1.38–2.65
Betaproteobacteria	2.07	1.77–2.36
Bacteroidetes	1.87	1.42–2.24
Actinobacteria	2.72	1.53–3.04
Firmicutes	2.57	2.31–2.78
Archaea	1.95	0.52–2.44

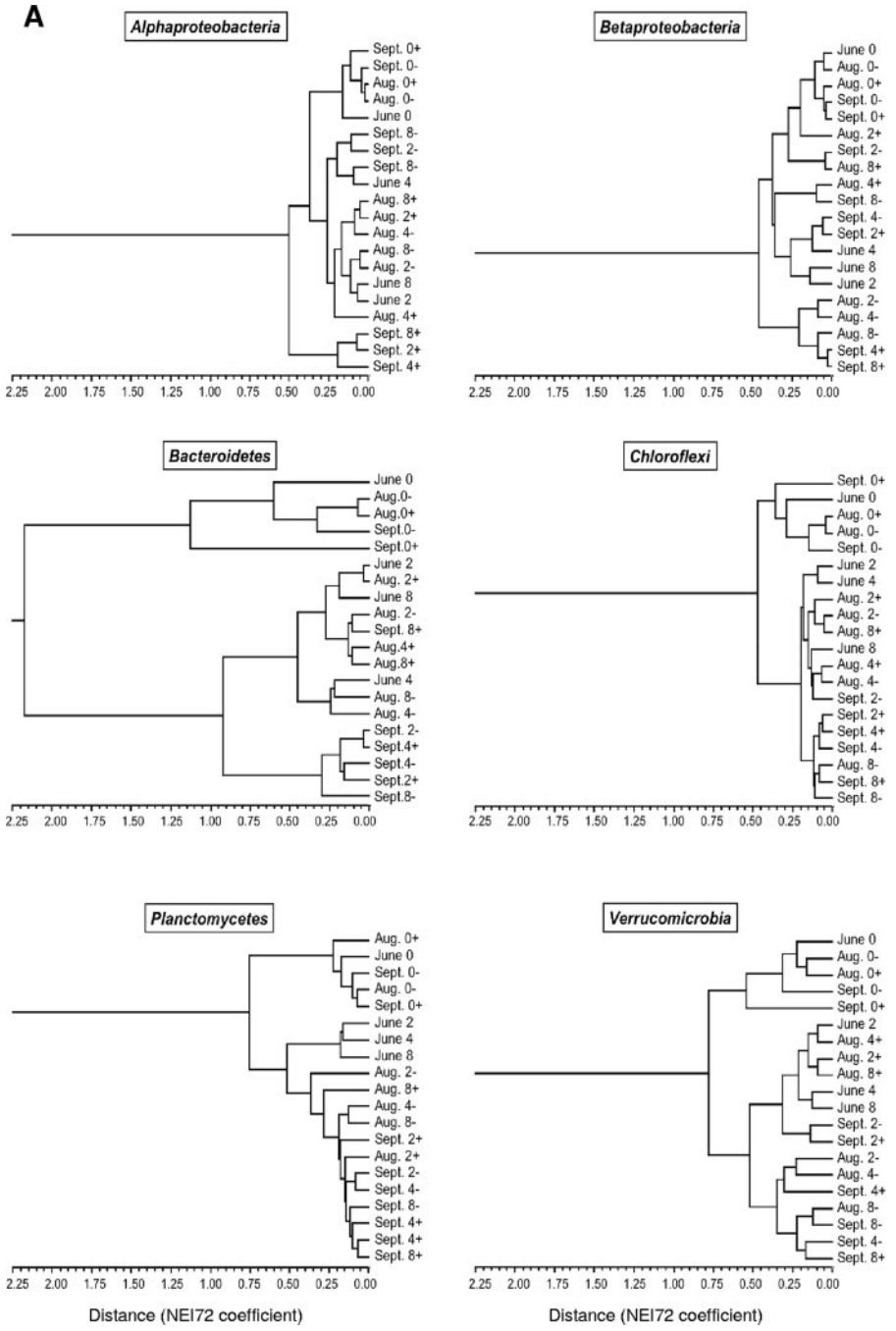

FIG. 2. Similarity of the bacterial populations of individual (sub)phyla established under different experimental conditions in the lysimeters. The analyses are based on a comparison of the PCR-DGGE fingerprint patterns. (A) Populations of *Alphaproteobacteria*, *Betaproteobacteria*, *Bacteroidetes*, *Chloroflexi*, *Planctomycetes*, and *Verrucomicrobia* species compared for each (sub)phylum separately. (B) Populations of *Acidobacteria*, *Actinobacteria*, *Firmicutes*, and *Archaea* species.

ter analysis was then performed for each bacterial target group. For 6 of the 10 groups, namely, the *Chloroflexi*, *Alphaproteobacteria*, *Betaproteobacteria*, *Bacteroidetes*, *Planctomycetes*, and *Verrucomicrobia* species (Fig. 2A), the populations established in the absence of higher plant species (labeled "0" in Fig. 2A) were more similar to each other than to the populations in other lysimeters. In the case of the *Chloroflexi*, *Bacteroidetes*, *Planctomycetes*, and *Verrucomicrobia* species, populations in the lysimeters lacking higher plant species formed the most distant subcluster of all. In contrast to the six groups named above, no distinct clustering, and hence no obvious dependence on any of the factors tested, could be observed for the *Actinobacteria*, *Acidobacteria*, *Firmicutes*, or *Archaea* species (Fig. 2B).

A comparison of the values for the Nei coefficients across all 10 bacterial groups revealed that the most pronounced changes

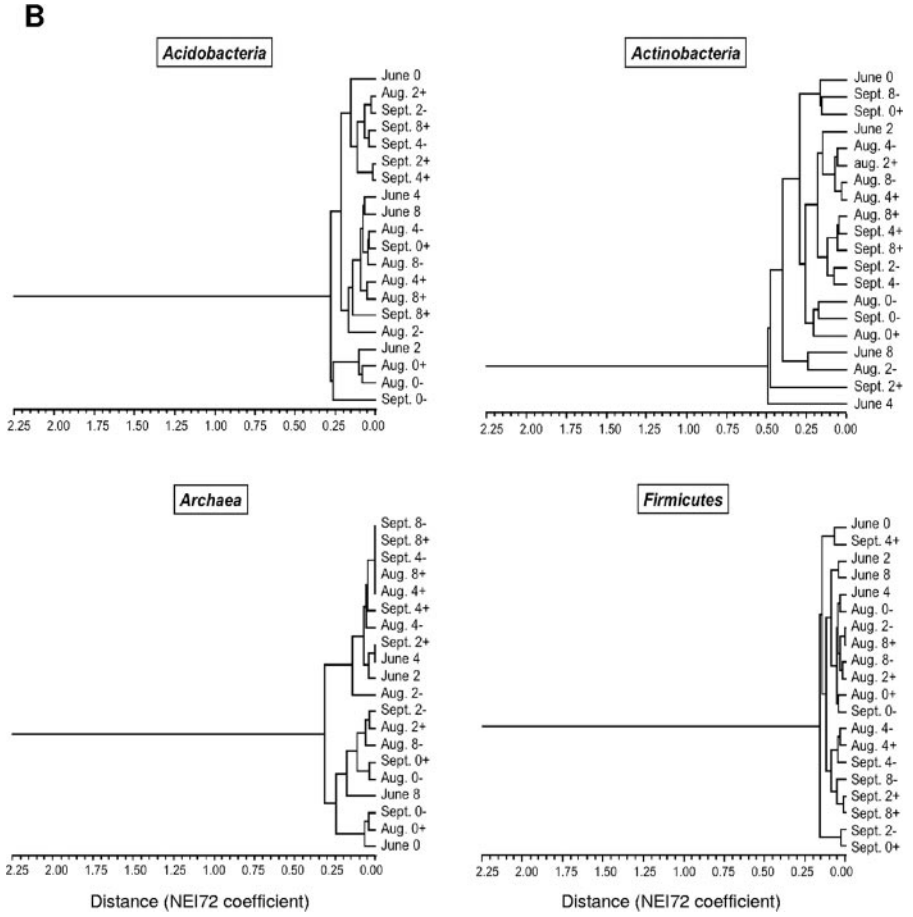

FIG. 2—*Continued.*

in composition occurred in the phylum *Bacteroidetes* (maximum Nei coefficient of 2.17; Fig. 2A). DNA fingerprinting of the *Firmicutes* species yielded the smallest changes in the community composition for all lysimeter treatments (maximum value of the Nei coefficient, 0.15; Fig. 2B), suggesting a highly stable composition of this group and only marginal effects of the experimental manipulations.

Through a combination of the intensity profiles of fingerprints of all 10 groups, an analysis of the overall similarity of the different prokaryotic communities was conducted (Fig. 3). Two main clusters were observed which separated at a cutoff value of the Nei coefficient of 0.57. Cluster 1 comprised the populations which had developed only in the planted lysimeters and cluster 2 those present in the lysimeters without higher plant species. Cluster 1 was composed of two subclusters, with subcluster 1a comprising the populations in the planted lysimeters sampled in September and subcluster 1b those in the planted lysimeters sampled in June and August. Our combined analysis therefore supports the conclusion that the presence or absence of higher plant species exerts the most pronounced effect on the overall composition of the prokaryotic community. Season was identified as a secondary control factor. In contrast, no effect of the other two factors tested, namely, the plant diversity or the water content, was observed.

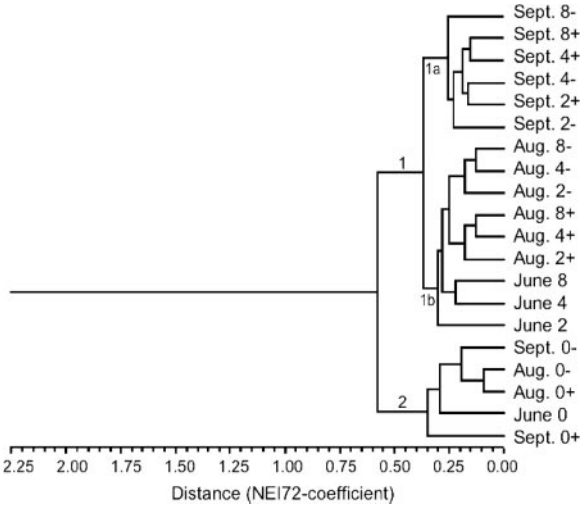

FIG. 3. Similarity of the bacterial communities established under different experimental conditions in the lysimeters. The cluster analysis is based on a comparison of the combined PCR-DGGE fingerprint patterns of all 10 (sub)phyla which were investigated in the present study.

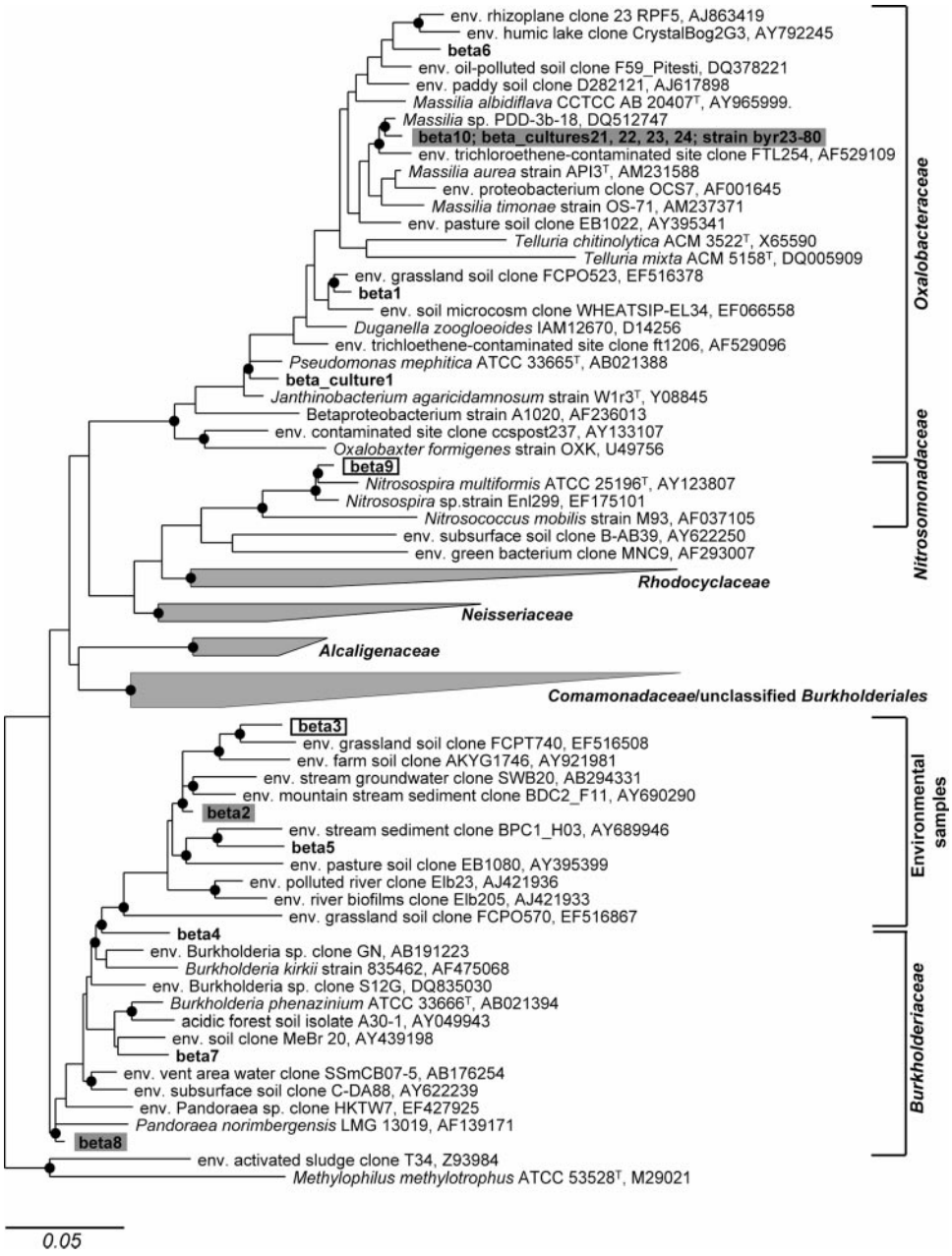

FIG. 4. Maximum likelihood phylogenetic analysis of the 16S rRNA gene sequences of *Betaproteobacteria* species obtained in the present study (shown in boldface). Sequences obtained from barren lysimeters are shown in boxes; sequences recovered only from planted lysimeters are shaded in gray. Sequences detected in both types of lysimeters are depicted in boldface only. The bar represents 0.05 fixed-point mutations per nucleotide. Nodes with a bootstrap support of ≥50% (1,000 bootstrap resamplings) are denoted by black dots.

Phylogenetic analysis of the 16S rRNA gene fingerprints. The DNA bands generated by PCR-DGGE were excised and sequenced in order to resolve the phylogenetic affiliation of the bacteria occurring in the lysimeters and to investigate whether they represented known or novel phylotypes. In order to focus on the main fingerprints and on those associated with particular experimental conditions, we selected 145 out of the total of 336 melting types (Table 3). For some melting types, several DNA bands were analyzed in order to study the reproducibility of the fingerprinting approach. Overall, 174 DNA bands were thus analyzed by sequencing.

At the outset, we investigated whether DNA bands with the same melting behavior usually contained the same 16S rRNA gene sequences. In all 29 cases tested (e.g., case 2 of the *Chloroflexi* species and case 10 of the *Alphaproteobacteria* species; Fig. 1), bands with identical melting behaviors yielded identical 16S rRNA gene sequences. We also tested whether a single melting type comprised more than one 16S rRNA gene sequence. For 7 of the 10 target groups, each DNA band analyzed yielded only a single 16S rRNA gene sequence (i.e., the number of melting types analyzed equaled the number of phylotypes recovered; Table 3). In contrast, some (14%) of the

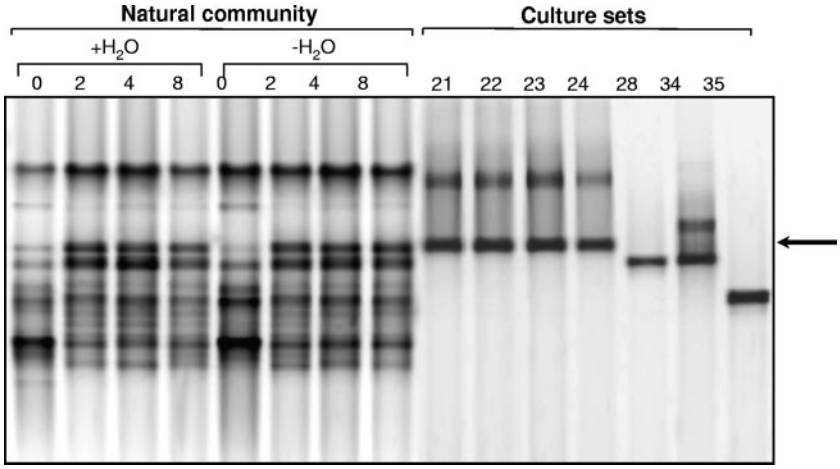

FIG. 5. Comparison of DGGE fingerprints of *Betaproteobacteria* species of seven different culture sets (five cultures combined per set) isolated from August 2004 samples with fingerprints detected in the bacterial communities in lysimeters in the same month. A negative image of a SYBR gold-stained DGGE gel is shown. The arrow indicates melting position of phylotype byr23-80.

DGGE bands generated from *Chloroflexi* species and between 27 and 40% of the bands generated from the *Planctomycetes* and *Verrucomicrobia* species were found to contain multiple sequences which had to be separated by an additional cloning step prior to sequencing.

The presence of the same melting behavior for different 16S rRNA gene sequences masks changes in their relative abundances and hence limits the assessment of microbial diversity based on DGGE fingerprinting as described above. Based on our sequence analysis, it can be concluded that this limitation did not apply to our analysis of bacterial diversity for the seven phylogenetic groups and was only of minor importance in the case of *Chloroflexi* species (where only 14% of the bands contained multiple sequences). However, it is likely that fingerprinting of the *Verrucomicrobia* and *Planctomycetes* species did not reveal all of the differences which existed between the different lysimeters.

Out of a total of 336 detectable melting types, 145 were analyzed which (due to the presence of multiple sequences in the same band; see above) yielded 160 unambiguous 16S rRNA gene sequences (Table 3). Based on the comparison with the GenBank database, the majority of sequences of the *Chloroflexi*, *Acidobacteria*, *Verrucomicrobia*, and *Planctomycetes* species were only distantly related to those of known phylotypes. Phylogenetic analyses revealed that only 32 of the 160 phylotypes were most closely related to cultured bacteria (Fig. 4, also see Fig. S1A to S1J in the supplemental material). Members of the phylum *Actinobacteria* were exceptional in this respect, since half of the sequences from the lysimeters were affiliated with cultured phylotypes. However, the majority of the 16S rRNA gene sequences were found to be affiliated to environmental sequences, mostly originating from soil samples.

Diversity of cultured bacteria and comparison with environmental phylotypes. In an attempt to isolate some of the phylotypes associated with particular experimental conditions in the lysimeters, improved cultivation media were inoculated by two complementary techniques using the soil samples from August. This yielded a total of 217 bacterial cultures. For rapid screening, sets of five cultures were pooled, the cells were lysed, and the 16S rRNA gene fragments of each set were amplified, employing the different group-specific primers. PCR products were analyzed by DGGE, running them beside representative amplificates of the environmental samples (Fig. 5).

Overall, 66 different DGGE melting types were detected among the 217 bacterial cultures (Table 3). Whereas no representatives of *Chloroflexi* and *Archaea* species were retrieved, the numbers of melting types detected for the other eight phylogenetic groups ranged between 3 and 17. Thirteen of the DGGE fingerprints corresponded to those of the natural community and originated from *Verrucomicrobia*, *Planctomycetes*, *Alphaproteobacteria*, *Betaproteobacteria*, *Actinobacteria*, and *Firmicutes* species. Sequence analyses of these 13 melting types revealed that one melting type of the *Betaproteobacteria* species (Fig. 5) and one melting type of the *Actinobacteria* species were identical to those of phylotypes detected by the culture-independent approach (Table 3). In the case of the *Betaproteobacteria* species, environmental sequence beta10 was detected in several culture sets (sets 21 through 24; Fig. 5). This sequence was affiliated at a sequence similarity of 99.2% to *Massilia* sp. strain PDD-3b-18 and at 99.1% to environmental clone FTL254 from a trichloroethene-contaminated site (Fig. 4). The sequences of the two actinobacterial cultures 5 and 38 matched the sequence of environmental HGC23 and were most closely related to that of *Arthrobacter* strain Ellin178 at a similarity of 97.6% (see Fig. S1G in the supplemental material).

Whereas HGC23 represented a phylotype which occurred under most experimental conditions (Fig. 1C), the intensity of the fingerprints of phylotype beta10 clearly correlated with the presence of plants (Fig. 5). Consequently, beta10 was chosen for subsequent isolation and characterization of the corresponding bacterial strains.

Physiology and abundance of the beta10 phylotype. After different liquid cultures were streaked onto solid media, four different isolates of phylotype beta10 were recovered. All isolates exhibited the same cell morphology and were similar with respect to genome structure, as revealed by genomic ERIC-PCR fingerprinting (Fig. 6). Therefore, one of the isolates was chosen for subsequent characterization. Strain byr23-80 is a 0.7- to 1.0-μm-wide and 1.5- to 2.0-μm-long motile short rod.

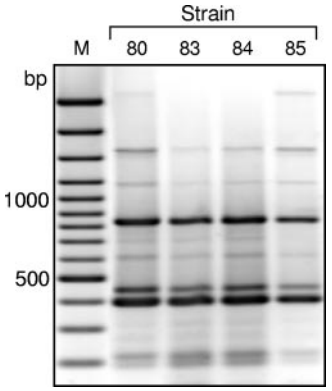

FIG. 6. Genomic fingerprints of four strains of phylotype beta10 isolated from different liquid microtiterplate cultures. Genomic fingerprints were generated by ERIC-PCR. PCR products were separated in an agarose gel and stained with ethidium bromide. M, molecular size markers (100-bp ladder).

Test results showed that it was gram negative, oxidase negative, and weakly positive for catalase. The isolate is an obligate aerobe with a range of growth conditions of 4 to 30°C, pH 6 to 10, and up to 2% NaCl (wt/vol). Optimum growth was observed at 15°C and pH 7 to 7.5. Strain byr23-80 utilized 9 out of 51 sugars or sugar derivatives (D-cellobiose, D-erythrose, L-erythrulose, D-galactose, glucose, glucose 1-phosphate, glucose 6-phosphate, maltose, and *N*-acetylglucosamine). A total of 19 of 36 organic acids tested (acetate, adipate, butyrate, crotonate, fumarate, caprylate, caproate, β-hydroxybutyrate, isovalerate, lactate, levulinate, malate, caprylate, oxaloacetate, propionate, protocatechuate, pyruvate, succinate, and valerate) and 3 keto acids (α-ketoisocaproate, α-ketoglutarate, and α-ketovalerate) as well as 13 different amino acids [L(+)alanine, L-alanylglycine, L-asparagine, L(+)asparaginate, L(+)cysteine, L(+)glutamate, L(+)isoleucine, L(+)leucine, L(+)lysine, L(+)phenylalanine, L(+)serine, L(+)threonine, and L(+)tyrosine] were utilized as single carbon and energy sources of growth. None of the eight alcohols tested served as a growth substrate. In addition, the isolate was capable of hydrolyzing Tween 20, Tween 80, starch, and casein.

Group-specific PCR-DGGE provides a rapid and sensitive yet semiquantitative means of determining the composition of complex bacterial communities. We therefore used qPCR as an independent method of greater precision to quantify the abundance of phylotype beta10 and to confirm its interdependence with the presence of higher plant species. The complete sequence of the representative isolate byr23-80 was used to establish a specific qPCR protocol for its phylotype. Based on our qPCR results, cell numbers of phylotype beta10 ranged between $1.67 \cdot 10^5$ and $4.09 \cdot 10^6$ $(\text{g soil})^{-1}$ and in most cases were significantly elevated in planted lysimeters (Fig. 7A). Phylotype beta10 thereby constituted a fraction of $1.7 \cdot 10^{-4}$ to $1.8 \cdot 10^{-3}$ (i.e., 0.017 to 0.18%) of total bacterial cells (Fig. 7B).

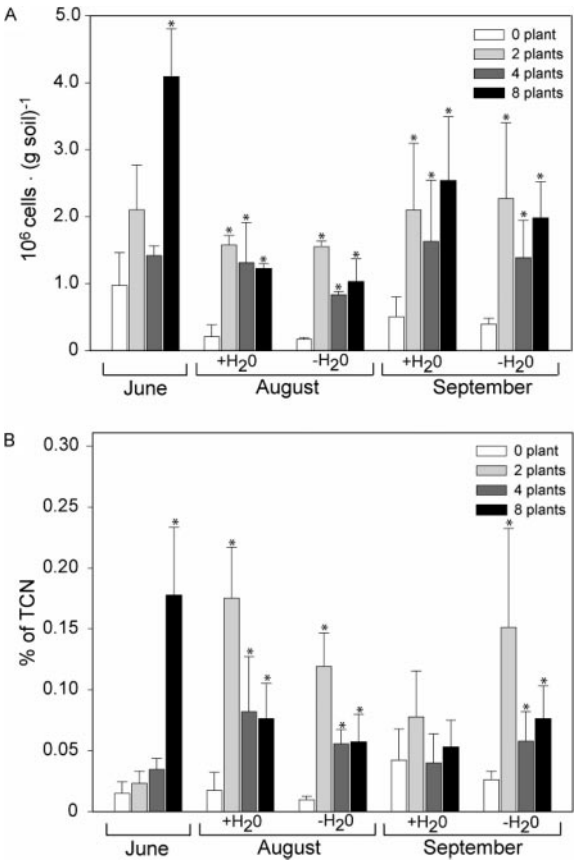

FIG. 7. Abundance (A) and relative abundance (B) (per total bacterial cell numbers [TCN]) of phylotype beta10 in the different lysimeters as quantified by specific quantitative PCR. Vertical lines indicate 1 standard deviation. *, significant differences compared to lysimeters devoid of plants at a significance level of $P < 0.05$.

DISCUSSION

Representation of bacterial diversity by group-specific PCR-DGGE fingerprinting. PCR-DGGE analyses which target *Bacteria* species as a single group typically yield about 30 individual melting types (41) and are capable of detecting phylotypes only when they constitute at least 1 to 9% of all phylotypes present in a bacterial community (42, 61). The total number of phylotypes detected by the 10 different primer sets employed in the present study was 336. By use of the group-specific PCR-DGGE protocol, phylotype beta10 could be discerned in samples where this phylotype constituted as little as 0.017% of all bacterial cells (compare the leftmost lane in Fig. 5 with the corresponding value in Fig. 7B). This detection limit corresponded to 3,170 cells or 15 pg of genomic DNA of the beta 10 phylotype per PCR. Thus, the approach used in the present study was suitable to rapid assessment of the structure of bacterial communities at an improved resolution. It should be emphasized that, since our PCR-DGGE analyses are based on the amplification of 16S rRNA genes (and not rRNA), they provide information on changes in the composition of the entire bacterial community rather than focusing on differences in the physiologically active fraction of soil bacteria.

Bacterial diversity in the lysimeters. None of the phylotypes detected in the present study matched 16S rRNA gene sequences in the databases. The sequences recovered from four of the target groups (*Chloroflexi*, *Acidobacteria*, *Verrucomicrobia*, and *Planctomycetes* species) were only distantly related to any of the sequences available in the databases. Many phylotypes occurred

in different lysimeters as well as in the same lysimeter on different sampling dates and in addition were found to be most closely related to other environmental sequences from soil. Together, these observations indicate that the bacteria identified by our phylogenetic fingerprinting are members of bacterial clades which were indigenous and typical for the soil environment. Although different grassland bacterial communities have been studied extensively over the past decade (see, e.g., references 4, 9, 10, 27, 34, 37, 39, and 57), a significant fraction of the indigenous bacterial community obviously remains to be discovered. Based on our results, this applies in particular to the *Chloroflexi* and *Verrucomicrobia* species. Our data are in line with the recent discovery of numerous previously unknown *Chloroflexi* species in other soil ecosystems (10). Although the *Chloroflexi* and *Verrucomicrobia* species typically represent rather small fractions of soil bacterial communities (7 and 3.2% of all cloned 16S rRNA genes, respectively; see reference 26), they represented the most diverse of the 10 groups investigated in the current study. Only 16 phylotypes of *Archaea* species were recovered by PCR-DGGE, indicating a low diversity of this group in soil. These results correspond to the low diversity of *Archaea* species in various soils of Norway and Indiana (44).

Factors controlling soil bacterial diversity. Microbial biomass has been shown to significantly correlate with plant diversity in experimental fields planted with 1 to 16 plant species (64). This correlation was attributed to the higher level of primary production associated with higher plant species diversity. In our study, no interdependence of bacterial cell numbers and plant diversity was observed. Also, total bacterial cell counts did not correlate with root biomass or total soil organic carbon numbers (see values for June 2004 in Table 2). Accordingly, our study focused on the interrelation of bacterial community composition with the presence or absence of plants, plant diversity, season, and water content.

Numerous previous reports did not reveal a correlation between the species composition of plants and the bacterial diversity in soils (32). No effect of plant community composition on the relative abundances of bacterial phyla on a Michigan long-term ecological research site (9) and a Dutch grassland site (16) was detected. Similarly, other studies showed that plant species composition had little direct effect on bacterial community composition (30, 48), while the diversity of bacterial community in another study showed a correlation to plant diversity in grassland soils (23). Our work confirms that a coupling between the overall diversity of soil bacteria and the diversity of above-ground plant communities does not exist. This indicates that the interdependence between the absence or presence of plants and the abundance of various bacteria which we observed in the lysimeters features rather low specificity.

Specific associations between particular plant species and soil bacterial populations have so far been documented for a few individual groups, like diazotrophic (13, 24) or dissimilatory (24) nitrate-reducing bacteria. In the current study, however, 20 out of the 160 sequence types analyzed were found at increased abundance in lysimeters devoid of higher plant species. In contrast, the abundance of 32 phylotypes coincided with the presence of higher plant species in the lysimeters. Of these 32 phylotypes, only a very few were closely related to known rhizosphere bacteria. Changes in the composition were observed for six different bacterial (sub)phyla. Furthermore, our analyses were limited to bulk soil. Root exudates and the microenvironments created by plants primarily affect the diversity of bacterial communities in the rhizosphere (32, 57). Based on the fact that about one-third of the bacterial sequence types in bulk soil were found to correlate with the absence or presence of plants, the influence of plants must extend significantly beyond the rhizosphere and must be of relevance to many different and previously unknown types of soil bacteria. These results are in contrast to observations of other grassland systems where plant rhizosphere effects were of little significance in the composition of total community structures (30). Our different results can be attributed to the higher resolution of the methods for diversity assessment employed in the current study.

Most notably, the DGGE fingerprint patterns observed for many of the minor constituents of the soil bacterial assemblage correlated with particular environmental conditions within the lysimeters. This observation also suggests that even low-abundance bacterial phylotypes reproducibly occupy particular ecological niches in soil. This indicates that the composition of soil bacterial communities is determined to a considerable extent by environmental conditions rather than being mostly the result of mere chance. Using a large-scale cultivation approach, we targeted a representative of the low-abundance phylotypes, quantified its abundance in response to environmental conditions, and obtained further insight into its ecological niche in order to test this hypothesis.

Insights from culture-based studies. Analyses of the 16S rRNA gene sequences of the 217 bacterial cultures revealed that only about 3% represented environmental phylotypes detected by the culture-independent approach. In several previous studies, none of the environmental phylotypes could be recovered by cultivation (14, 36, 58). Various cultivation approaches have been shown to selectively favor the growth of *Actinobacteria* species over those of the dominant *Proteobacteria*, *Acidobacteria*, or *Verrucomicrobia* species (14, 58). Similarly, the diversity of *Actinobacteria* species was overrepresented in our culture collection in comparison to the bacterial community composition in the lysimeters. Yet sequences of two of the phylotypes cultured did match sequences detected in the natural bacterial community. Obviously, dominant phylotypes grow less readily in artificial growth media than rare ones. Consequently, the number of cultures established per soil sample needs to be increased in order to improve the chances of isolating representative bacteria from the soil environment.

The betaproteobacterial isolate byr23-80 was studied in detail, since the corresponding beta10 sequence represented that of one of the low-abundance phylotypes which displayed a distinct response towards the presence of higher plant species. Strain byr23-80 was identified as a novel lineage within the genus *Massilia*. In another study, addition of fresh plant organic matter to a calcareous silty-clay soil resulted in a pronounced and specific stimulation of *Beta-* and *Gammaproteobacteria* species over *Actinobacteria*, *Cyanobacteria*, *Gemmatimonadetes*, and *Planctomycetes* species (5). Remarkably, the largest number of additional sequences detected in that study after the addition of the fresh organic matter be-

longed to the *Massilia* group. However, the correlation between the abundance of phylotype beta10 and the presence of living plants suggests a closer interaction between the two. Physiological characterization revealed that isolate byr23-80 is a highly versatile bacterium capable of utilizing a wide spectrum of organic compounds as single carbon and energy sources. Root exudates consist of organic acids, amino acids, and sugars. Strain byr23-80 was found to be capable of using at least eight (glucose, fumarate, succinate, malate, glutamate, alanine, leucine, and serine) of the 21 (33) major constituents of root exudates. Using culture-independent stable isotope techniques, bacteria of the genus *Massilia* have been shown to be active in soil and to rapidly respire glucose but not phenol, naphthalene, or caffeine (50).

Implications for the assessment of soil microbial diversity. DNA reassociation studies indicate that soil bacterial communities harbor up 50,000 (54) or even up to millions (18) of different 16S rRNA gene sequences. From these large numbers it has to be deduced that the diversity of complex microbial communities resides mostly in low-abundance species. Indeed, thousands of low-abundance populations were found to account for most of the unexpectedly high diversity of deep-sea bacterioplankton communities (59).

The high diversity of soil bacterial communities could be (i) due to a multitude of ecological niches and adaptive mechanisms (15, 29) and/or (ii) caused by high functional redundancy (22, 63) of the soil bacteria. One-third (52 out of 160) of the sequence types analyzed in the present study showed a distinct response to a single environmental factor tested, namely, the presence or absence of plants. As demonstrated for one of them (beta10), at least some of these phylotypes constitute only a very small fraction (0.017 to 0.18% of total cell numbers) of the soil microbial community in the lysimeter samples. Still, the occurrence and response of these phylotypes followed a reproducible pattern in independent lysimeters. Our results suggest that the bacterial species composition in soil is determined to a significant extent by abiotic and biotic factors rather than mere chance. The observation of high reproducibility of bacterial fingerprint patterns in independent lysimeters also contradicts the general assumption of high functional redundancy in soil and indicates the presence of a multitude of distinct ecological niches.

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