Response of Predominant Soil Bacteria to Grassland Succession as Monitored by Ribosomal RNA Analyses

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Stellingen

1. Assessing activity and abundance of uncultured microbes in the environment by nucleic acids analysis provides a better insight into the microbial community than conventional cultivation-based methods.

2. Different environments require different, adapted protocols for optimal nucleic acids extraction, often causing a considerable lack of comparability of studies based on environmental DNA.

3. Molecular fingerprinting of bacterial communities can be further improved by analyzing genes other than 16S rRNA genes.

4. Current taxonomy and phylogenetic analysis of prokaryotes are mainly linked to the evolution of rRNA genes which is a simplifying expedient and may be a misleading limitation.

5. Although rRNA could be detected in endospores, it is rather unlikely that the observed predominance of *Bacillus*-ribosomes in soil originates from *Bacillus*-endospores.
   This thesis, chapter 10.
   Fischer, K., D. Hahn, W. Hönerlage, F. Schönholzer, and J. Zeyer. 1995. *In situ* detection of spores and vegetative cells of *Bacillus megaterium* in soil by whole cell hybridization. System. Appl. Microbiol. 18:265-273.

6. Shifts in the composition of closely related members of the *Bacillus benzoevorans* cluster in soil and the *Prochlorococcus* cluster in water could in part be explained by evolution of microdiversity.
   This thesis, chapter 9.
   Moore, L.R., G. Rocap, and S.W. Chisholm. 1998. Physiology and molecular phylogeny of coexisting *Prochlorococcus* ecotypes. Nature 393:404-411.

7. Communication in science is improved by preparing a Ph.D. thesis from cumulative publications rather than publishing results after such a thesis has been approved.

8. Young scientists need to study for a few years in a foreign country. Human diversity is almost as interesting as microbial diversity.

Stellingen behorende bij het proefschrift "Response of predominant soil bacteria in Drentse A soil during grassland succession and the quantification of their ribosomal RNA".

Andreas Felske

Wageningen, 26 april 1999

Contents

Chapter 1

General Introduction

General Introduction

Although microorganisms are the main operators of chemical transformations in the biosphere, our understanding of the composition of and the interactions and dynamics within microbial ecosystems is rather poor. Within the biogeochemical cycles of life-relevant elements, mainly carbon, nitrogen and oxygen, we recognize on the one hand the creation of biogenic molecules mainly by photosynthesis. The reversion to anorganic matter on the other hand is mainly effected by heterotrophic degraders such as those present in soil. This heterogeneous mixture of minerals and organic residues is brimming of bacterial life, but we are still not able to study the metabolic functions of the relevant bacterial species. However, developments in molecular microbial ecology allowed to partially access these mysterious rulers of biogeochemistry by analyzing their cellular components. The molecular access to environmental bacteria appears to be supreme to conventional cultivation methods, since the apparent selectivity of culture media limits the comprehensive detection of environmental bacterial communities. This is the main reason why probably only a tiny fraction of all bacterial species are cultured and systematically described. Although countless numbers of different culture media have been developed and often applied to enrich and purify bacterial strains from environmental samples, they only faintly touched upon the wealth of microbial life on earth that is estimated to include more than 10^{30} prokaryotic cells (Whitman *et al.*, 1998). Up to date, by far most of bacterial cells in the environment remained to be not accessible for cultivation methods (Rosswall & Kvillner. 1978, Williams *et al.*, 1984; Brock, 1987; Ward *et al.*, 1990; Amann *et al.*, 1995). This can on the one hand be explained with the non-natural competition and selection conditions in culture media but on the other hand also with the inactivity of resting bacterial cells. For instance, it has been found that bacterial cells in terrestrial and aquatic samples are much smaller than their cultured counterparts (Roszak *et al.*, 1984; Bakken & Olsen, 1987). Therefore, it has been speculated that most bacterial cells are not viable and consequently not cultivable. The chemical and physical complexity and diversity of terrestrian environments also suggested hitherto unidentified, specific media-demands for the according native bacterial species. Since terrestrial sediments can be considered as one of the most complex habitats, all these problems especially apply to soil microbiology. Initial, culture-independent surveys revealed a tremendous diversity of bacteria in soil (Torsvik *et al.*, 1990). These suggested that already one single sample of a few grammes of soil might contain more different species than today's man-made culture collections of several thousand bacterial species.

The recent progress in molecular biology made it possible to detect uncultured microbes by the analysis of universal intracellular components. One of the most successful approaches has been the development of a molecular taxonomy for prokaryotes, based on comparative sequence analysis of the slowly evolving ribosomal RNA (Woese, 1987). Since the ribosomal RNA (rRNA) is applied for taxonomic analysis, there is no need anymore to firstly grow bacterial colonies in pure culture. Irrespective of their culturability, bacteria can be identified at the species-level by analysis of DNA or RNA. These nucleic acids components of the bacterial

cell can be directly extracted from environmental samples. Therefore, it has been an obvious choice to apply this molecular approach to study bacterial communities in soil.

In recent years, the grassland succession within the Dutch Drentse A agricultural research area near Anloo, The Netherlands (Fig. 1) has been subject of several ecological studies concerning the reaction of fauna and flora to the progressing nutrient exhaustion (Bakker, 1989; Olff, 1992; Brussaard *et al.*, 1993, Stienstra *et al.*, 1994). However, any information on the composition of the bacterial community was lacking. The work compiled in this thesis presents an attempt to fill this gap by revealing the predominant bacteria and their response to grassland succession by quantifying a particular marker for cellular activity, the bacterial 70S ribosome. Since thousands of different bacterial species are expected to be present in soil, we focused only on the ones which are predominantly active. This, together with the complexity of the environment, required the design and validation of novel, quantitative rRNA approaches to monitor the predominant bacteria in different stages of grassland development. Therefore, the following paragraphs will not only briefly summarize the relevant results from former ecological studies on the Drentse A grassland succession, but also give an overview of the principles of the rRNA approach and finally introduce the methodology of nucleic acids quantification via polymerase chain reaction (PCR).

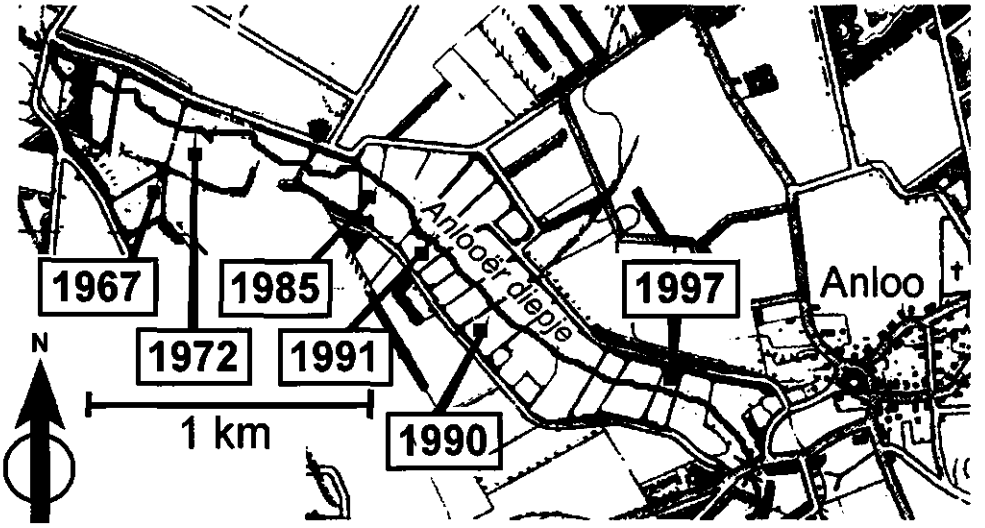

Fig. 1. The Drentse A agricultural research area near Anloo consists of several plots along the Anlooër Diepje brook. Six plots are highlighted by the year when the agricultural production stopped and restoration management started.

1. ECOLOGY OF THE DRENTSE A GRASSLANDS

1.1 History of the Drentse A grasslands

In the history of the Drentse A grasslands three different periods have been distinguished (Olff, 1992). These periods have been characterized by changing hay-making and fertilization practice. In the first phase of agricultural use, until the 1930's, these grasslands showed a species-rich vegetation and were cut once or twice a year for hay production without application of chemical fertilizers. Since the 1930's agriculture was intensified by increasing the cutting frequency and raising the hay productivity by applying artificial mineral fertilizers. This resulted in a domination of high-yield grass species and an overall decrease of species richness. However, the last decades of agricultural overproduction in Western Europe forced the European Community and the national authorities to release more and more land from agricultural production. Lots of hay meadows for cattle feeding were left for non-profit management. The further treatment of these areas, as supported by the European Community, aimed to restore the former species-rich vegetation by non-extensive hay-making of moderate frequency. Now, in the third stage of grassland management, hay is taken off only once a year without any fertilizer application. This restoration process was initiated in the late 1960's and has been extended to more and more meadows in the Drentse A area (Fig. 1). The plots selected for this study were still fertilized in 1997 or taken out of production in the years 1991, 1990, 1985, 1972 and 1967. From long-term observations of vegetation and soil properties on permanent plots it is known that these plots indeed represent the temporal successional sequence (Bakker, 1989).

1.2 Vegetation of the Drentse A grasslands

The lack of fertilization caused a change of vegetation and species diversity increased. Today different sequences of this succession can be observed, since over the decades several plots were added to this restoration management. After the fertilization stopped, the meadows showed a declining hay productivity, mainly caused by nitrogen and potassium limitations and at the later stages also phosphate depletion. This shift from little nutrient limitation in fertilized meadows to a multiple nutrient limitation in not-fertilized grassland, changed the selection pressure on the vegetation. The fast-growing plant species of high nutrient demand, like the Perennial rye-grass (*Lolium perenne*), once supreme in the battle for light, were now lacking nutrients and became out-competed by species which were better adapted to nutrient limitation. Since grass species showed quite different responses to nutrients, the nutrient decrease resulted in an increased plant species diversity (Olff, 1992). This process was accelerated by the yearly cut, leveling all tribes to the same starting position for the next vegetative period. The reduction

of the cutting frequency was also a selective force in the first years of restoration, since it is known that less intensive cutting reduces possible interference with flowering periods and the demand for fast growth (Olff, 1992). On the fertilized Drentse A meadows the Perennial rye-grass (*Lolium perenne*), Yorkshire fog (*Holcus lanatus*), Rough meadow-grass (*Poa trivialis*) and Creeping bent (*Agrostis stolonifera*) were predominant. While *Lolium perenne* faded within a few years after fertilization stopped, the others could last more than one decade on a similar level before being completely replaced within less than five years (Olff & Bakker, 1991) by raising populations of Keck (*Anthriscus sylvestris*), Common sorrel (*Rumex acetosa*) and especially Creeping buttercup (*Ranunculus repens*). In later succession stages, species like Sweet vernal-grass (*Anthoxanthum odoratum*), Red fescue (*Festuca rubra*) and Field wood-rush (*Luzula campestris*) appeared.

1.3 Culturable bacteria in Drentse A grassland soils

In a preliminary, culture-dependent study on Bacilli, Pseudomonads and Actinomycetes in the Drentse A grasslands, difference in bacterial counts could be observed between plots taken out of production 1967 and 1990 (Wopereis, 1995). Around 2×10^7 (1990), respectively 5×10^6 (1967) bacterial colony forming units (cfu) g^{-1} soil could be detected on casein agar medium. More than half of these colonies represented Gram-negative organisms, and fluorescent Pseudomonads appeared to be a major fraction of them with approximately $1 - 3 \times 10^6$ cfu g^{-1} soil. A major difference between the soils appeared to be the higher amount of Gram-negatives in an early stage of grassland succession (1990). Actinomycetes were abundant in the 1990-plot of with approximately $1 - 3 \times 10^6$ cfu g^{-1} soil, but they could hardly be detected in the progressed grassland succession (1967). Vegetative Bacilli were present in the 1967-plot with approximately 4×10^5 cfu g^{-1} soil, but they could not be detected in the 1990-plot. However, *Bacillus* endospores were found in similar amounts in each plot (4×10^6 cfu g^{-1} soil). Finally, *Arthrobacter* strains could frequently be isolated, and a culture collection of 120 *Arthrobacter* strains from Drentse A grassland soils was set up at the Laboratory for Microbiology in Wageningen (de Vrijer, unpublished results). Apparently, the genus *Arthrobacter*, belonging to the coryneform bacteria, is a predominant representant of the culturable soil bacteria.

Another approach based on most probable number enumerations was applied to quantify ammonium-oxidizing bacteria, in order to follow nitrogen utilization during grassland succession (Stienstra *et al.*, 1994). In the fields that had been without fertilization for three or seven years, the numbers of ammonium-oxidizing bacteria were not significantly different (approximately 10^6 cfu per g soil). Beyond this period the ammonium-oxidizing bacteria decreased significantly. Here approximately 10^4 or 10^3 cfu per g soil were estimated in the fields that had been without fertilization for 20 or 46 years, respectively. Although these studies could not be expected to reveal the predominant species or the real ratios between different

groups, they are indicating particular changes of the bacterial community during grassland succession.

2. RIBOSOMAL RNA AND MOLECULAR MICROBIAL ECOLOGY

During the last years, microbiologists switched gradually to molecular methods in order to characterize prokaryotes, because bacterial diversity cannot only be described by morphological and physiological properties. The latter can be common for bacteria from phylogenetically distant taxa. However, closely related genera may show a rich morphological and physiological diversity. The modern molecular taxonomy of bacteria is following the polyphasic strategy of classification by applying different approaches simultaneously (Colwell, 1970; Vandamme *et al.*, 1996). Chemotaxonomical information is collected by detecting the composition of particular cell constituents like peptidoglycans, fatty acids, polar lipids and others. Another common approach is based on the DNA base composition. Firstly, DNA-DNA reassociation kinetics are determined to indicate similarities between closely related species (Stackebrandt & Kandler, 1979). These are used as definition limit in the species concept of prokaryotes (Schleifer & Stackebrandt, 1983). Secondly, the phylogeny of bacteria is correlated to the phylogeny of their rRNA, according to Woese & Fox (1977). Determination of the nucleotide sequence of the rRNAs offers the possibility to identify bacteria by only this one cellular component. Consequently, the identification of bacteria by molecular techniques based on nucleic acids has spread dramatically. The principle strategy of the rRNA approach, as presented in Fig. 2, is to extract nucleic acids directly from environmental samples, to isolate the rDNA or rRNA and to reveal their sequence. With this sequence information, the organisms can be tracked back in the environment by whole cell hybridization with specific oligonucleotide probes (Amann *et al.*, 1995). During the processing of environmental nucleic acids, 16S rRNA fingerprinting techniques are helpful tools to assess and select the sequences of interest (Muyzer & Smalla, 1998).

In their fascinating reviews, Olsen *et al.* (1986) and Pace *et al.* (1986) outlined for the first time the application of the rRNA approach to study the composition of environmental bacterial communities. The following pioneer work on the Octopus Spring microbial mats (Ward *et al.*, 1990, 1998) and Sargasso Sea bacterioplancton (Giovannoni *et al.*, 1990) demonstrated the urgent need of this approach: the majority of present microbes could not be grown in culture but only be revealed by extracting nucleic acids directly from the environmental matrix. With cultivation approaches and their inherent high selectivity, we can detect only a small minority of present bacterial cells in the environment (Amann *et al.*, 1995). This so-called 'great plate count anomaly' was also indicated by microscopic cell counts on samples from aquatic and terrestrial habitats (Staley & Konopka, 1985). Meanwhile, numerous molecular studies on environmental microbial communities revealed a vast spectrum of bacteria in nature, far beyond the several thousand bacterial strains stored in our culture collections. A dramatic example of our lack of knowledge about bacteria in the environment is

represented by the microbial communities in soil that show a diversity which was far beyond previous expectations (Torsvik *et al.*, 1990). Therefore, the rRNA approach was recognised as the most promising tool to study environmental microbial communities (Ward *et al.*, 1992).

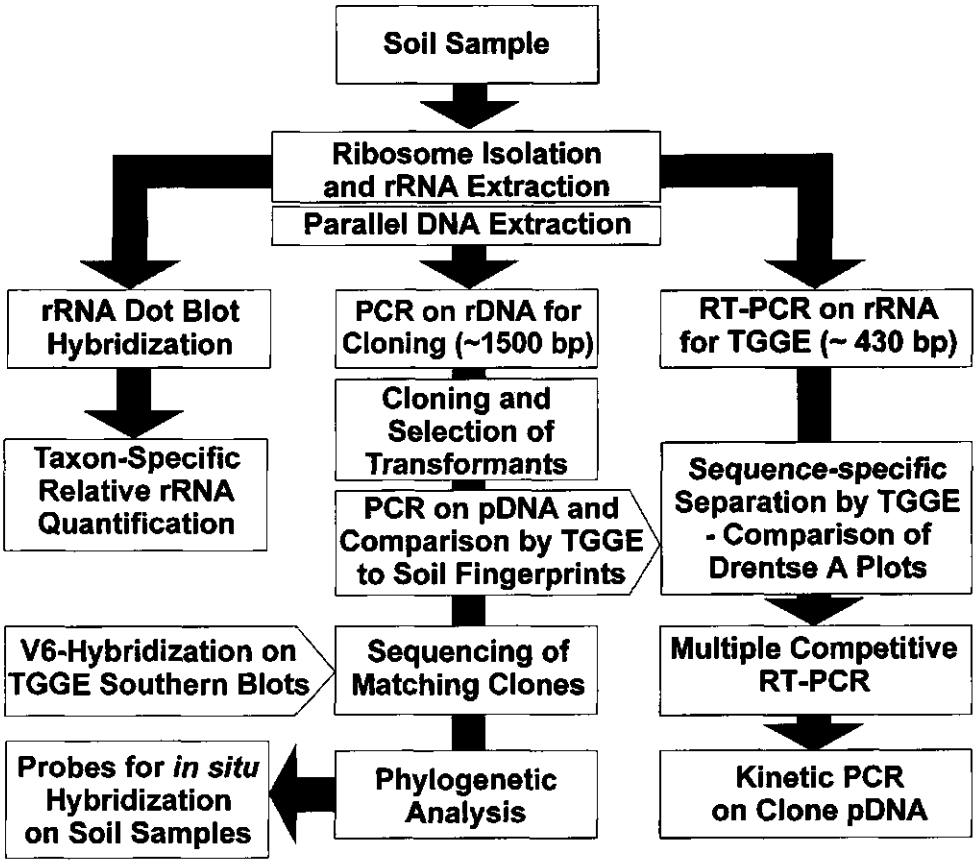

Fig. 2. The rRNA approach applied in the Drentse A survey. The starting point at the top of the scheme is the extraction of ribosomes and genomic DNA from soil samples. On the left-hand side are the different oligonucleotide hybridization techniques, in the middle the cloning and sequence analysis approach, and on the right-hand side the TGGE-based methods.

2.1 Studying microbial communities in soil by 16S rDNA

During the past six years, several culture-independent surveys have been performed to reveal the present bacteria in soil. The first of these studies was concerning the present 16S rDNA sequences in a soil sample from Mount Coot-tha, Australia (Liesack & Stackebrandt, 1992). This study was following the basic strategy to firstly amplify the 16S rDNA from directly extracted soil DNA by PCR with universal primers. These amplicons were subsequently used to generate a clone library for sequencing analysis. In the following years, habitats all over the

world were also investigated, for instance, Japanese soybean fields (Ueda *et al.*, 1995), agricultural soil from USA (Borneman *et al.*, 1996; Kuske *et al.*, 1997), Amazonian forested soil (Borneman & Triplett, 1997), rice microcosms (Großkopf *et al.*, 1998) and Hawaiian soil (Nüsslein & Tiedje, 1998). All these studies were based on similar approaches, but unfortunately each study applied different cell lysis protocols, PCR conditions, primer sets and cloning strategies. Although the comparability is limited, all these sequences provide a first snapshot on microbial species richness in soil. However, there are particular problems to describe the composition of soil bacteria community in an ecologically correct manner. The basic step is to estimate the 'species richness' by estimating all present species (Begon *et al.*, 1990). However, all these surveys are only preliminary, incomplete counts of present species and therefore, we are far away from detecting them all. Bacterial communities in soil appear to be extremely complex. Several thousands of different bacterial genomes in 1 g soil are indicated by reassociation kinetics of soil DNA (Torsvik *et al.*, 1990). Hence, we can not expect to reveal species richness by only sequence analysis of a few hundred 16S rDNA clones. Therefore, it is reasonable to focus studies of such complex bacterial communities to more specific goals like revealing novel, phylogenetic taxa (Rheims *et al.*, 1996; Ludwig *et al.*, 1997) or special physiological groups like the most active bacteria (the present Drentse A study - see below). In the context of microbial ecology quite often appears the term 'microbial diversity of soils'. However, from the description of microbial diversity in soil we are even more far away. The ecologist describes 'diversity' not only by counting all species but additionally by their commonness or rarity (Begon *et al.*, 1990). None of the mentioned studies is providing data about specific cell numbers. The molecular technique of choice for counting cell numbers is based on whole-cell hybridization with 16S rRNA-targeted probes (Giovannoni *et al.*, 1988; Amann *et al.*, 1990; Zarda *et al.*, 1997).

All 16S rDNA clone libraries from soil DNA demonstrated the presence of bacteria only remotely related to known strains present in the rDNA data bases. Because only a minority of these sequences could be closely related to cultured organisms, the bacterial communities in soil are likely to be composed of yet uncultured species. Quite often these sequences represented novel, major taxa within the phylogeny of bacteria. Several independent lines of descent within the 16S rRNA phylogeny of bacteria could be proposed, based on the analysis of the according ribotypes from soil. Recently, we learned about the *Holophaga/Acidobacterium*-cluster (Ludwig *et al.*, 1997), which appears very abundantly in clone libraries all over the world. Another frequently detected major taxon is the *Verrucomicrobiales*-cluster (Ward-Rainey *et al.*, 1995), only remotely affiliated to the Planctomycetes-line of descent. Rheims *et al.* (1996) demonstrated, that there are still major lines of descent to discover within the *Firmicutes* of high G+C content. Also for the archaeal domain, novel 16S rRNA clusters have been demonstrated in soil (Bintrim *et al.*, 1997; Munson *et al.*, 1997).

Facing the vast bacterial diversity in soil, novel molecular approaches can be of great benefit. Among these techniques, the Denaturing Gradient Gel Electrophoresis (DGGE; Fischer & Lerman, 1979) or its variant Temperature Gradient Gel Electrophoresis (TGGE; Rosenbaum

& Riesner, 1987), provided the possibility to separate complex mixtures of different 16S rRNA sequences. Following the introduction of DGGE in molecular microbial ecology (Muyzer *et al.*, 1993), Muyzer & Ramsing (1995) consequently put it into the center of their rRNA approach. Although this variant of the rRNA approach is quite popular, it has been applied to soil studies only sporadically (Felske *et al.*, 1996; Großkopf *et al.*, 1998; Nüsslein & Tiedje, 1998; Øvreås *et al.*, 1998). An interesting alternative to DGGE might be the rDNA intergenic spacer analysis (RISA) as briefly demonstrated on soil samples from Amazonia by Borneman & Triplett (1997). Here PCR amplicons are generated from the highly variable intergenic spacer between the 16S and 23S rDNA sequence. In contrast to the 16S rRNA, the intergenic spacer is highly variable in length, and amplicons can be separated into fingerprints by conventional electrophoresis. The option of identifying bacterial groups with taxon-specific probes, normally done by dot blot hybridization, can to some extend be replaced by Southern blot hybridization on DGGE gels. This novel approach has only recently been performed successfully to identify ammonia-oxidizing bacteria in DGGE fingerprints from successional grasslands (Stephen *et al.*, 1998). Another interesting approach to study microbial communities in soil with improved resolution is the separation of the extracted soil DNA by G+C content and separated analysis of the different fractions by cloning and sequencing (Nüsslein & Tiedje, 1998). The application of oligonucleotide probe hybridization techniques are often problematic, because they require the isolation of or the *in situ* access to bacterial rRNA in soil. Tracking back specific sequences in the environment is very important to confirm the PCR-based results of the rRNA approach. If not, it can not be excluded that the data are severely distorted by bias of PCR or DNA extraction (Wintzingerode *et al.*, 1997). Although the soil matrix is rather recalcitrant to whole-cell hybridization approaches or rRNA extraction for quantitative dot blot hybridization, this verification of 16S rRNA sequence data is essential. Recently, it has been demonstrated that these and other hybridization techniques are also applicable to soil (Felske *et al.*, 1996; Ludwig *et al.*, 1997; Zarda *et al.*, 1997).

2.2 Microbial community analysis by ribosomes

The ribosomes are one of the most essential components of the living cell, regarding to quantity as well to quality. They are the operators of mRNA translation and protein synthesis, constituting the central location of translating the genomic code into polypeptide molecules. All metabolic activity in cells, as catalyzed by enzymes, depends on the protein synthesis of the ribosomes. Therefore, ribosomes are present in all actively metabolizing organisms on our planet, showing highly conserved structural and functional properties in their proteins and nucleic acids. This allows us to use rRNA as universal phylogenetic marker even for evolutionary distant relations. The rRNA sequences can be obtained either from rRNA or from the according genes located in the genomic DNA (also referred to as rDNA). Although representing the same sequence, there are relevant qualitative and quantitative differences between rRNA and rDNA, as described below.

2.2.1 Influence of posttranscriptional processing of rRNA

Dealing with ribosomes, it must be considered that rRNA contains posttranscriptionally modified ribonucleotides (Lane *et al.*, 1995; Ofengand *et al.*, 1995; Noon *et al.*, 1998). Those altered nucleotides might interfere with molecular techniques like reverse transcription. Therefore, a lower efficiency of reverse transcription-PCR (RT-PCR) in comparison to PCR, or even inability of a proper reverse transcription on rRNA must be considered. This is the reason why in the presented strategy (Fig. 2) the clone library has been generated from genomic 16S rDNA templates, while all other data have been based on ribosome isolation and the isolated 16S rRNA. There are two practical reasons to generate 16S rDNA clone libraries rather from rDNA than from rRNA. Firstly, the 16S rDNA sequence includes a stretch of more or less 1500 nucleotides, and this can be a limiting size for efficient RT-PCR, that furthermore requires optimal template quality. However, rRNA from environmental samples must be expected to be of suboptimal purity, especially if it is retrieved from soil. The more important, second problem is the possibility of unwanted termination of the reverse transcriptase activity. Premature termination of reverse transcription is known since the early days of 16S rRNA cloning. For instance, the termination trap at *E. coli* position 966/967 has troubled several researchers (Weller *et al.*, 1991). It is also not accidentally that the 5'-primer that generates amplicons to be separated on TGGE starts at position 968 and therefore just escapes this critical nucleotide modification. With the primer pair GC968/1401 (Nübel *et al.*, 1996) we never observed any suspicious failures of the RT-PCR on a broad range of different pure culture 16S rRNAs. The post-transcriptional processing of rRNA might also cause fragmentation, the ultimate obstacle for reverse transcription, as was reported for the 23S rRNA of particular α-Proteobacteria (Selenska-Pobell and Evguenieva-Hackenberg 1995).

The post-transcriptional processing of the rRNA is a considerable source of bias and the main disadvantage of using rRNA instead of rDNA. Therefore, the reverse transcription step should be carefully evaluated for every applied primer. In the presented approach (Fig. 2) the primer 1401 was used for reverse transcription and the subsequent PCR was continued together with primer GC968. The equal efficiency of reverse transcription for different rRNAs can be checked with competitive RT-PCR, as demonstrated for the primer pair GC968/1401 (Chapter 4).

2.2.2 Quantitative aspects: rDNA versus rRNA

The initial assumption underlying rDNA and rRNA quantifications was that the number of rDNA sequences in a bacterial community would reflect the bacteria present, while the rRNA fraction would reflect the activity of the bacterial community (Ward *et al.*, 1992). However, only to some extent the pool of *rrn* operons reflects the composition of the bacterial community by the present species. The activity of the bacteria can not be measured, because DNA can also be retrieved from starving or dead cells (Josephson *et al.*, 1993) and even from inorganic components of the environmental matrix which once adsorbed DNA from lysed cells (Lorenz

& Wackernagel, 1987). Moreover, the wide variation of 1 - 14 operons per genomic unit among bacteria limits the quantification of bacterial cells by quantifying 16S rDNA sequences (Farrelly *et al.*, 1995; Lee *et al.*, 1996).

Instead of using rDNA, the presented rRNA approach intends to reveal the metabolically most active members of the bacterial community in soil. An essential step is the isolation of rRNA and this was realized by the direct isolation of ribosomes from environmental soil samples and the subsequent purification of their rRNA (Felske *et al.*, 1996). Since the ribosome yield can hardly be related to cell numbers, a particular definition of bacterial activity is necessary, as is detailed below.

2.2.3 The hypothesis: Ribosomes reflect metabolic activity

In what way yields the measurement of ribosome amounts (or their rRNA) useful quantitative information? Ward *et al.* (1992) supposed that the abundance of ribosomes in the environment should be a species-dependent function of the number of individual cells and their growth rates. Concerning the whole bacterial community, this should provide data of the relative contribution of each species to the entire protein synthesis capacity of the community. This leads to the fundamental methodological hypothesis of this thesis:

The relative ribosome amount in a given environmental matrix of one particular ribotype reflects the contribution of the according bacteria to the total activity of the microbial community.

Two terms in this hypothesis demand a further definition. What are the *according bacteria* that relate to the ribotype? It is not accurate to say that a specific rRNA sequence is characteristic of a single species. The modern molecular taxonomy of bacteria is following the polyphasic strategy of classification (Vandamme *et al.*, 1996), where 16S rDNA sequencing is only one of the approaches applied simultaneously. Therefore, the actual species concept of bacteria allows the existence of multiple species with identical rRNA sequence. On the other hand, it is possible that one bacterial strain contains more than one ribotype. This has been demonstrated for *Haloarcula marismortui* (Mylvaganam & Dennis, 1992), *Clostridium paradoxum* (Rainey *et al.*, 1996) and *Paenibacillus polymyxa* with more than 2% difference among the nucleotide sequences in one strain (Nübel *et al.*, 1996). Therefore, two closely related 16S rRNA sequences might indeed originate from the same bacterial strain. However, according to the latest data, this case appears to be relatively rare among bacteria, at least among the coryneformes (Felske *et al.*, 1999).

Since the hypothesis lacks any link to bacterial cell numbers, also the term *total activity* must be explained in detail. Following the suggestions of Ward *et al.* (1992), the amount of ribosomes is measured by total ribosome amounts from unknown cell numbers. Theoretically, the same amount of ribosomes can be retrieved either from a population of low cell number

with high activity or another population of high cell number with low activity. According to this definition of total activity, both completely different populations are regarded as equally active. This is not contradictory because a large, low-activity population can produce the same amount of proteins as the small, high-activity population. As a consequence, both populations will indeed contribute equally to the total activity of the bacterial community.

Challenging the assumption that ribosome content is proportional to growth activity, recent studies demonstrated a considerable temporary delay of ribosome degradation during starvation of a marine *Vibrio* species (Flaerdh *et al.*, 1992) or nocturnal inactivity of cyanobacterial mats (Nold & Ward, 1997). Extending the previous total activity definition to the time scale could circumvent this problem. The appearance of excess ribosomes is most likely an adaptation to short-time rhythms of growth and starvation, respectively activity and inactivity like day and night rhythms of photosynthesis. Here, the ribosomes in excess can boost the start of a new growth/activity cycle. Considering the total activity in time, the organism with excess ribosomes shows indeed a higher total protein production than an organism which always has to dismantle and recover its ribosome stock.

2.2.4 The Ribosome

Although the general composition and structure of ribosomes is the same for all organisms, there is a general size difference between eukaryotic and the smaller prokaryotic ribosomes. This is illustrated by the sedimentation coefficient during density gradient centrifugation, which is 70S for prokaryotic ribosomes and 80S for eukaryotic ribosomes. An actively growing *E. coli* cell contains approximately 20.000 ribosomes per genomic units. A bacterial 70S ribosome consists for 66% of rRNA and for 34% of ribosomal proteins, summing up to 80% of total RNA and 10% of total protein per cell (Voet & Voet, 1992). The ribosomes can be seen within bacterial cells via transmission electron microscopy as free globular particles of approximately 25 nm diameter or as polysomes associated to mRNA. Their particular molecular weight of $2{,}5 \times 10^6$ Dalton and the sedimentation coefficient of 70S allow the separation from other cellular proteins and nucleic acids. The 70S ribosome consists of two subunits. The small subunit contains a 16S rRNA molecule and 21 different proteins, while the large subunit contains the 5S and the 23S rRNA and 32 different proteins (Voet & Voet, 1992).

The isolation of ribosomes is usually based on ultracentrifugation (Tissiéres *et al.*, 1959; Sykes, 1971; Spedding, 1990). Since ribosomes constitute one of the largest intracellular particles, they can be purified from other cellular components by their weight with differential centrifugation. This principle has not only been applied for pure cell cultures but also for ribosome isolation from environmental samples (Weller *et al.*, 1991; Felske *et al.*, 1996 and 1998). The protocol for ribosome isolation from soil combines adapted ribosome isolation with the use of blocking reagents to protect the released ribosomes from being trapped by adhesive mineral particles. The blocking reagents are also applied to remove humic acid-like

contaminants. Such substances are known to be powerful inhibitors for enzymatic reactions like PCR, but they can be precipitated by polyvinylpyrrolidone (Young *et al.*, 1993). This polymer is added to the original ribosome buffer and remains present during subsequent differential centrifugation to cover mineral surfaces and to bind to polyphenolic substances like humic acids. Within the first steps of differential centrifugation the main part of precipitates are sedimented together with insoluble soil components. The ultracentrifugation separates the sedimenting ribosomes from soluble soil components, and subsequent phenol extractions and ethanol precipitations remove the remaining contaminants. This protocol and the parallel DNA isolation is the method applied in this study to yield bacterial 16S rRNA from soil (Fig. 2). It has been considered to be superior to direct 16S rRNA extraction in reflecting activity of bacteria, because ribosome isolation only considers intact 70S ribosomal particles (Hahn *et al.*, 1990; Tsai *et al.*, 1991; Moran *et al.*, 1993; Purdy *et al.*, 1996). Furthermore, the high purity of the isolated rRNA appeared to be essential for the analysis of the Drentse A soil samples (Felske *et al.*, 1998).

3. QUANTIFICATION OF 16S rRNA SEQUENCES

Analyses based on the PCR are widely used to detect nucleic acid sequences with high specificity and sensitivity. Its sophisticated design allows the enzyme-catalyzed copy of nucleic acid fragments, where the newly generated product DNA is denatured to serve again as template. By repeating the duplication again and again, the amounts of DNA template are increased after each reaction, consequently causing an increasing product output in each following reaction. This amplification theoretically continues under constant conditions with a constant multiplication factor in each step, constituting an exponential amplification of DNA fragments. Therefore, this technique is the ultimate choice to specifically track DNA sequences, which are present in very low concentration. Where any other method meets its limit of sensitivity, the PCR can amplify the DNA fragment and make it accessible for further analyses. For these cases where PCR is the only key to detect nucleic acids, quantitative PCR methods have been developed (Gilliland *et al.*, 1990). However, PCR is not straightforward to determine the original template DNA amount. The principal drawback exactly is this exponential amplification mechanism, where a small difference in amplification efficiency of two different targets will cause a deviation, that multiplies exponentially as well and might cause a dramatic bias. Another kind of bias is caused by substrate exhaustion within the PCR. In the early cycles of the PCR the amount of product is amplified exponentially with a constant multiplication factor. However, due to the depletion of substrates, the reaction will level off during the last cycles. This effect is hard to anticipate, because its presence depends on the initial amount of template DNA. This effect not only hampers the comparison between single PCR tubes, it apparently also introduces bias to the ratio of different sequences within one PCR

by causing a preferential amplification of less abundant sequences (Suzuki & Giovannoni, 1996; also discussed in chapter 4).

Beyond the obvious technical sources of errors, like variations in reaction mix composition or thermocycler performance which will likely cause differences from experiment to experiment, or also from tube to tube within one experiment, also sequence-specific bias must be considered. The melting behaviour of the template DNA during denaturation or its reannealing, considerably depends on the G + C content and indeed has been reported to influence amplification efficiency (Reysenbach *et al.*, 1992; Dutton *et al.*, 1993). Another possible factor concerns the formation of secondary structures within the DNA template. Although not thoroughly investigated yet, these secondary structures might hamper the performance of DNA polymerase or influence the primer annealing process. In addition, two amplicons of different size may amplify with different efficiencies (Stolovitzky & Cecchi, 1996). A shorter DNA fragment can be amplified with higher efficiency, because the release of the DNA polymerase and consequently the premature termination of the DNA polymerization are less likely .

Another factor is the impact of sequence mismatches on primer annealing efficiency. In the first cycles of PCR the primers must anneal to the original template. The efficiency of this process might be reduced by one or more nucleotide mismatches in the primer-annealing site. Since the primer becomes a part of the amplicon, this effect disappears with advancing cycle number. Therefore, this problem might only be present within the initial cycles, where the DNA amounts are still too low to be detected directly.

Due to all these potential problems, special precautions and PCR approaches are needed to quantify the original DNA template amount. Competitive PCR circumvents many of these problems since it only considers reactions where standard and target sequences are present in comparable amounts and amplified equally. Competitive PCR was initially developed for mRNA from eukaryotic cells (Gilliland *et al.*, 1990). Recently, particular genes from bacterial genomic DNA as retrieved from soil and sediments have been quantified using competitive PCR (Hallier-Soulier *et al.*, 1996; Wikström *et al.*, 1996; Möller & Jansson, 1997). In the first attempt to apply competitive PCR to quantify environmental bacteria by their 16S rDNA, the quantitativeness was disputable, because the amount of 16S rDNA sequences per cell could not be estimated (Lee *et al.*, 1996). As described in 2.2.2, the variable numbers of *rrn* operons and genome sizes for different species are unpredictable parameters. Consequently, the 16S rDNA amplification of different bacterial strains does neither reflect cell numbers nor ratios of nucleic acid amounts (Farrelly *et al.*, 1995). As an alternative to genomic 16S rDNA we quantified the bacterial ribosomes by their 16S rRNA to monitor spatial shifts of bacterial activity in soil (Chapter 4 and 9).

4. AIM OF THE PROJECT

The aim of this thesis was to reveal the predominant bacteria in the grassland soil and to monitor the influence of grassland succession on the bacterial community. Suitable strategies and methods had to be developed for this approach. As described in the previous sections, many microbial ecologists found that an overwhelming majority of environmental bacteria apparently are not accessible to the cultivation methods of today. The rRNA approach offers a culture-independent alternative. Considering also unculturable cells, it may allow an improved view on bacterial communities. Although this approach is used frequently, this thesis describes for the first time a combination of different molecular approaches applied on the ecological subject of grassland succession. The cloning and sequence analysis of 16S rDNA, different hybridization techniques and TGGE are combined to a polyphasic network of rRNA analysis (Fig. 2). In this approach, the ribosome is used as a marker for metabolic activity of bacteria. Microbial activity is defined as total activity of the species and not as activity per cell (see 2.2.3). Following this introduction, the methodological aspects of the polyphasic rRNA approach are described in Chapters 2 to 4. These include the problematic aspects of the cultivation approach (Chapter 2) that contrasts with the high reproducibility of the rRNA approach (Chapter 3). Chapter 4 describes a novel method based on multiple competitive RT-PCR, to quantify environmental 16S rRNAs. Following these methodological Chapters, the description of the results on the Drentse A starts with a general overview of the predominant 16S rRNA sequences (Chapter 5). The following Chapters 6 to 8 are focused on particular bacterial groups, featuring the application of novel detection techniques like V6-hybridization (Chapters 6 and 7) and multiple competitive RT-PCR (Chapter 8). Chapter 9 summarizes the findings in the light of grassland succession. The success of the rRNA approach is testified by the *in situ* detection of the predominant bacterial cells after their sequence has been revealed (Chapter 10). The summary and concluding remarks are presented in Chapter 11.

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Chapter 2

Searching for Predominant Soil Bacteria: 16S rDNA Cloning versus Strain Cultivation

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Searching for Predominant Soil Bacteria: 16S rDNA Cloning versus Strain Cultivation

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The predominant bacteria in Dutch grassland soils were characterized by RT-PCR amplification of partial 16S rRNA sequences and subsequent separation by temperature gradient gel electrophoresis (TGGE). This characterization included on the one hand the cloning of bacterial 16S rDNA sequences amplified from DNA directly isolated from soil samples. Amplicons of the cloned inserts were then separated by TGGE and compared to those obtained by RT-PCR from 16S rRNA isolated from ribosomes obtained from soil. The identity of amplicon sequences derived from the clone library and those in the matching soil fingerprint bands was confirmed by Southern blot hybridization with V6-probes. The clone library reflected quite well the predominant ribotypes in the TGGE fingerprint from soil 16S rRNA. On the other hand, we investigated the presence of the bacteria belonging to the observed ribotypes by a cultivation approach. Four ribotypes were selected that were found to be predominant both in the TGGE fingerprints and clone libraries: two closely related *Bacillus*-like sequences, a representative from the *Verrucomicrobiales* cluster and a member of the Actinobacteria. Using a variety of cultivation approaches a total of 659 pure cultures were isolated. Based on an initial PCR/TGGE screen approximately 8% of all isolates matched one of these ribotypes. However, sequence analysis indicated that none of the 54 candidates contained 16S rDNA that was identical to the cloned sequences representing the fingerprint bands.

1. Introduction

During the last years it has become generally accepted that culture-dependent surveys suffer from the 'great plate count anomaly' [28]. Most environmental bacterial cells were shown to be refractory to cultivation [1,25]. It has been estimated that less than 1% of all bacterial cells in soil can be cultured in the current types of nutrient media [1]. The culture-independent approach based on direct recovery of bacterial 16S rDNA from soil indicated the predominance of many different uncultured species [3,4,15,17,18,23,31]. This has been explained by the presence of a large number of very small and probably inactive cells not able to recover anymore [2,26]. Another explanation is the high selectivity or stress imposed by the culture media. It is feasible that the yet uncultured types of bacteria might be grown under laboratory conditions if just the right nutrients are applied. However, the possibility exists that the culturable bacteria represent the viable cells, while cloned 16S rDNA sequences are retrieved from a huge background of inactive cells. If so, this would imply that analysis of cloned 16S rDNA provides information of mainly taxonomic value, with no hint to the main bacterial operators of the biogeochemical processes in soil.

A recent molecular study provided the metabolically most active members of a bacterial community in soil [10]. The approach used was based on direct ribosome isolation from soil and purification of 16S rRNA [8]. Subsequently, the most active bacteria were detected by temperature gradient gel electrophoresis (TGGE) of the rRNA amplicons obtained by RT-PCR that were compared to those obtained from a cloning approach. It has been demonstrated that 16S rDNA clone libraries fairly well reflected the predominant environmental ribosomes, although appearance of less abundant sequences has been observed [9]. The main goal of the present study was to demonstrate how the predominant bacteria were recovered by the cultivation approach. Subsequently, the cultivation of the predominant bacteria would allow assessing their actual function in the environment. We focused on four environmental ribotypes to investigate to what extent the results of the 16S rRNA approach could be reproduced by classical cultivation techniques. According to TGGE analysis, they represented prominent ribosome amounts in Drentse A grassland soils [10]. The predominant bacteria in this habitat were represented by the *Bacillus*-like ribotype DA001, which could also be detected in soil by whole-cell *in situ* hybridization as apparently active, vegetative rods [13]. Furthermore, we selected another *Bacillus*-like ribotype DA011, ribotype DA079 that was closely related to some uncultured Actinobacteria from German peat [9], and sequence DA101 that fits into the *Verrucomicrobiales*-cluster [11].

2. Materials and methods

Soil sampling. The investigated site was located in the Drentse A agricultural research area in the Netherlands (06°41′E, 53°03′N), representing a 1.5 km stretch of grassland along the Anlooër Diepje Brook. The different cultivation history of the Drentse A plots was considered

by sampling six plots representing different years of last fertilization for agricultural hay production. Details of the soil properties have been published [29]. In total 360 surface samples (<10 cm depth) were taken in March and October 1996. Soil cores of approximately 50 g were taken with a drill (0-10 cm depth) and transferred into sterile sample bags. The single samples of each plot were pooled to representative samples by sieving and mixing 10 single samples (5 g input each).

Enrichment and cultivation of soil bacteria. Soil samples (1 g) were suspended in sterile 0,85% NaCl solution and diluted in tenfold steps. Agar media were inoculated with 100 µl of these suspensions, corresponding to 10^{-6} - 10^{-9} g soil per plate. Media inoculated with soil suspensions were accompanied by a parallel inoculated with pasteurized soil suspensions (heated for 15 min at 80°C) to select for *Bacillus*-endospores. Different types of media were applied. Nutrient broths like DSM medium 1, DSM medium 6, DSM medium 16 and DSM medium 78 [7] were used in the original composition and in tenfold dilution. Since the predominant environmental ribotype DA001 was closely related to *Bacillus benzoevorans* (97,3 % sequence similarity), we also applied selective media for *B. benzoevorans* [20, 21]. These consisted of a mineral medium containing 0,2 % of a specific carbon source. Selective enrichment of *B. benzoevorans* was reported using mineral media containing as sole carbon sources benzoate, its derivatives, or other phenylated compounds. Media were applied containing different carbon sources including acetate, benzoate, casein, chitin, corn steep liquor, gelatine, glucose, glycerol, humic acids, malt extract, meat extract, methyl benzoate, m-hydroxybenzoate, nothing, polyvinylpyrolidone, starch, tannine and tryptose. Another type of medium was prepared in soil extract instead of water [33]. Here we used on carbon sources (2 g l^{-1}) acetate, casein, nothing and tryptose either in pure soil extract or in mineral medium (see above) prepared with soil extract. All media contained 15 g l^{-1} agar and 50 mg l^{-1} cycloheximide to prevent fungal growth and had a pH of 7. A parallel version of all media was prepared with a pH of 4.0, which is corresponding to the soil pH. All cultures were incubated at 20°C and sampled after four days, two weeks, four weeks and three month.

Screening of isolates for matching sequences. Total DNA was extracted from Drentse A isolates by taking up single clone colonies with sterile toothpicks and transferring these into 1.5 ml micro-centrifuge tubes containing 50 µl TE buffer. The tubes were heated for 15 min at 95°C to lyse the cells and then chilled on ice. Amplification of 16S rDNA sequences was performed with a GeneAmp PCR System 2400 thermocycler (Perkin-Elmer-Cetus), using 35 cycles of 94°C for 10 s, 54°C for 20 s and 68°C for 40 s. The PCR reactions (10 µl) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, 0.05% detergent W-1 (LifeTechnologies), 50 µM each of dATP, dCTP, dGTP, and dTTP, 30 pmol of primer GC968f and 1401r, 0.5 units of Taq DNA polymerase (LifeTechnologies), and 1 µl cell lysate. Amplification products were confirmed by 1.4% agarose gel electrophoresis.

The Diagen TGGE system (Diagen, Düsseldorf, Germany) was used for sequence-specific separation of PCR products [24]. Electrophoresis took place in a 0.8 mm-polyacrylamide gel (6% w/v acrylamide, 0.1% w/v bis-acrylamide, 8 M urea, 20% v/v formamide, 2% v/v glycerol) with 1 x TA buffer (40 mM Tris-Acetate, pH = 8.0) at a fixed current of 9 mA (approximately 120 V) for 16 h. The manufacturer's gel casting setup produces 27 slots of approximately 7.5 µl sample capacity. For the preliminary TGGE screen a slotformer (64-slot blunt end comb) from the Li-Cor 4000L sequencer (Li-Cor, Lincoln, USA) was applied, providing 70 slots of approximately 1 µl sample capacity. A temperature gradient was built up in electrophoresis direction from 37°C to 46°C. After the run gels were silver-stained [27]. Subsequently, the gels could be inspected for matches between the clone signals and the bands of the isolates from soil. Visually matches were confirmed by a second TGGE analysis (conventional 27 slots), where PCR products from the matching isolate and the according clones were loaded in the same slot. The isolates that yielded amplicons covering those from a clone, resulting in a single band, were selected for partial sequencing.

Sequencing of PCR products from isolates. Amplification of 16S rDNA sequences was performed with a thermocycler (as above) using 30 cycles of 94°C for 10 s, 46°C for 20 s and 68°C for 100 s. The PCR reactions (2 × 100 µl) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, 150 µM each of dATP, dCTP, dGTP, and dTTP, 100 pmol of primer 7 and 1512 [9], 2.5 units of Taq DNA polymerase (LifeTechnologies), and 1µl cell lysate (see above). PCR products were purified and concentrated (from 200 to 50 µl) with glass fiber spin columns following the manufacturer's instructions (High Pure PCR Product Purification Kit, Boehringer-Mannheim, Germany). Purified DNA was eluted from the columns with 50 µl deionized water. The sequencing was done by using a Sequenase(T7)-sequencing kit (Amersham, Slough - England). Each 4-µl reaction (A, C, G and T) contained 2.5 µl template, 0.5 µl labeled primer (Infra-Red Dye 41, MWG-Biotech, Ebersberg, Germany) and 1 µl reaction mix (A, C, G or T, Amersham). Inserts were read with primer seq968 (primer U968-GC without GC-clamp), sequencing the last approximately 500 bases of the sequence. The reaction was performed in a thermocycler (as above) with 35 cycles at 94°C for 5 s, 56°C for 10 s and 68°C for 10 s. After addition of 3 µl loading dye (Amersham) the reactions were run on a Li-Cor Sequencer 4000L.

Sequence analyses. Phylogenetic analysis of the sequences was performed by alignment of the partial isolate sequences to the according clone sequence and also to the EMBL database of 16S rRNA sequences. The used software programs were BestFit and FASTA from the GCG software package [6]. The computer-aided simulation of melting behavior and migration of the amplicons during TGGE was performed with the software Poland V1.0 [16,22].

3. Results and discussion

3.1. Analysis of the cloned 16S rDNA by TGGE. In order to compare the 16S rDNA cloning with the cultivation, we applied the same screening method to both approaches. The cloned 16S rDNA inserts of the directly extracted soil DNA were amplified with the same primer pair used to analyze the isolates or the directly extracted rRNA fraction from soil. In this way, the TGGE fingerprints generated from 16S rRNA of directly extracted ribosomes from soil could be compared with the clone library or the cultivated strains. Previous results have demonstrated that 16S rDNA cloning did quite well to reveal the predominant ribotypes [9, 10]. Comparing the cloned insert bands with the TGGE fingerprints, matches were found with the most intense bands in the soil fingerprint [10]. The clone library contained 128 different 16S rDNA sequences and additionally 37 redundant sequences. Of all 165 insert-containing clones, 42 clones matched the 15 most intense TGGE fingerprint bands (Fig. 1). Most of the redundant clones indeed represented intense bands in the TGGE fingerprints. This correlation indicated that both approaches detected the same predominant soil sequences. The amplicons of approximately 40 clones appeared to match with some faint bands in the TGGE pattern, and several of these have been sequenced [10]. However, all remaining clones could not be affiliated to any bands. It appeared that the less abundant sequences which could not give visible bands in a TGGE fingerprint could be retrieved by the cloning approach if they accounted for a considerable part of the total ribosome fraction from soil. This was supported by the previous finding that all the ribotypes matching to clearly visible fingerprint bands only represented approximately half of all ribosomes [12]. The other half of ribosomes might have originated from a large number of rare bacteria, possibly hundreds or more of different bacteria [30]. The four selected ribotypes DA001, DA011, DA079, DA101 accounted for 17 of 165 cloned 16S rRNA sequences. They were also representing some of the strongest bands in the TGGE fingerprints from soil ribosomes [10]. Therefore, they appeared to be major contributors to the ribosome fraction in soil, and therefore should be considerably active.

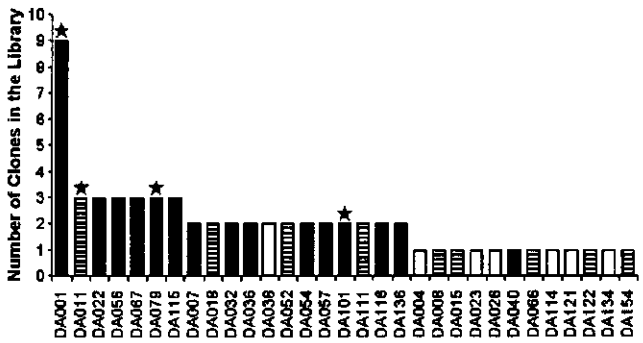

Fig. 1. Sequence redundancy in the 16S rDNA clone library. The most abundant clones correlated with the most intense bands in TGGE fingerprints (black bars). Others matched with some weaker bands (striped bars) or did not match at all (white bars). The stars indicate the four ribotypes of this study.

3.2. Isolation of microorganisms from Drentse A soil. Since the optimal growth conditions for the four bacteria containing the four ribotypes were unknown, various types of media with different carbon sources were selected. Since the predominant ribotype DA001 was closely related to *Bacillus benzoevorans* (97.3% sequence similarity), we applied three types of media containing the carbon sources benzoate or its derivatives m-hydroxybenzoate or methyl benzoate that have been used to grow *B. benzoevorans*. However, no fast-growing colonies or *B. benzoevorans*-like cell types as described by Pichinoty (20) were found. All the other media yielded the growth of colonies consisting of cell types of typical *Bacillus* appearance and endospore formation. Many agar plates were dominated by very rapidly growing *B. cereus*-like colonies showing a mycoides-phenotype. In this way 659 isolates were obtained that were subsequently screened by analyzing their rDNA amplicons via TGGE.

.3. TGGE screen of the bacterial strains isolated from soil. Amplicons obtained by PCR of the 16S rDNA of the 659 isolated strains were screened by TGGE for possible matches with either of the investigated ribotypes. Next to the isolates, we loaded markers on the TGGE gel consisting of equal amounts of PCR products generated from the cloned 16S rDNA of clones DA001, DA011, DA079 and DA101 (Fig. 2, lane M).

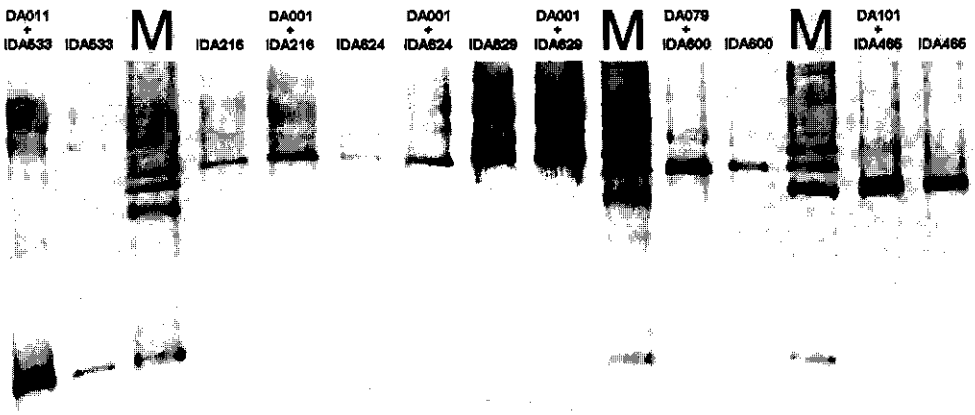

Fig. 2. Verification screen of isolates on TGGE. The 'M' indicates the marker lanes. Of 54 checked isolates 6 are presented on this gel. The marker consists of a mixture of four PCR products from DA001, DA079, DA101 and DA011, following the order from top to bottom. -, PCR product of the isolate. +, PCR product of the isolate and the according cloned environmental sequences IDA533, IDA216, IDA624, IDA629, IDA600 and IDA465.

Within the first TGGE screen we found that 54 of the 659 isolate signals might match to one of the clone signals. If the PCR amplicons are all running next to each other, the visual resolution of differences is not much better than 1 mm. Therefore, we added a second TGGE screen for verification of the approximately matching isolates. Here, the PCR products from the clone and

from one possibly matching isolate were loaded in the same slot (Fig. 2). In this way also very small migration differences could be detected since this would result in two bands. Fourteen of the 54 tested strains showed no identified migration difference and ten were selected for subsequent partial sequence analysis. The other four, matching to DA001 and DA079, were identified as Bacilli by endospore formation and consequently excluded from further analysis. The promising strains preferentially appeared at neutral pH on tenfold diluted DSM1 medium or mineral medium with acetate as carbon source (Tab. 1).

Isolated Strain	sequence similarity to clone	sequence similarity to next related species
IDA113 no	93.7% to DA001	99.0% to *Bacillus cereus*
IDA216 ac	98.2% to DA001	96.4% to *Bacillus megaterium*
IDA234 csl	76.4% to DA079	96.7% to *Bacillus megaterium**
IDA465 ac +p	87.5% to DA101	99.0% to *Bacillus cereus*
IDA533 dsm 1	85.2% to DA011	96.3% to *Arthrobacter nicotinovorans*
IDA600 ac	76.4% to DA079	96.7% to *Bacillus megaterium**
IDA624 ac	95.7% to DA001	94.8% to *Bacillus cohnii*
IDA627 ac	94.8% to DA001	94.8% to *Bacillus benzoevorans*
IDA629 ac	97.6% to DA001	94.6% to *Bacillus benzoevorans*
IDA647 dsm 1/10	93.8% to DA001	95.4% to *Bacillus pseudomegaterium*

* identical sequences

Tab. 1. Sequence analysis results of ten isolates that showed exactly the same migration distance like the according clones. ac, grown on mineral medium with acetic acid; csl, grown on mineral medium with corn steep liquor; dsm 1, grown on medium DSM 1; dsm 1/10, grown on tenfold diluted medium DSM 1; no, grown on mineral medium without carbon source; st, grown on mineral medium with starch; +p, pasteurized soil suspension.

3.4. Sequence analysis and phylogenetic assignment of isolates. Although matching on TGGE, the partial 16S rRNA sequences of the isolated bacterial strains were not identical to the according sequences of the cloned amplicons. All isolates showing equal migration distance on TGGE with the *B. benzoevorans*-relatives DA001and DA011 could be identified as *Bacillus* strains by sequence analysis and microscopic detection of endospores. Here the best fit was found with isolate IDA216 showing 98.2% sequence similarity to clone DA001 and equal migration distance on TGGE (Tab. 1). The sequence differences between clone DA001 and isolate IDA216 are unlikely to be due to PCR errors or reading errors during sequence analysis, because the base differences are all located in the highly variable regions of the 16S rRNA, which is the most likely location of base exchanges. Furthermore, the average sequence reading error for the applied protocol has been estimated to be less than 0.5% of all nucleotides [10]. In contrast, the isolates with rDNA amplicons showing equal migration following TGGE as that

of DA011, DA079 and DA101, showed only less than 90% sequence similarity to their matches. While DA079 and DA101 are located in the Actinobacteria and the *Verrucomicrobiales* cluster, the matching isolates were found to belong to *Bacillus* and *Arthrobacter* spp. These genera were already known to appear frequently during former cultivation approaches with Drentse A soil, and a culture collection of 120 different *Arthrobacter* strains from Drentse A grassland soils was previously set up (de Vrijer, unpublished data). The conventional cultivation approaches apparently had a biased preference for *Bacillus* and *Arthrobacter* strains from the Drentse A soil samples. Due to the strong appearance of *Bacillus*-strains one might expect that a strain collection of similar size like for *Arthrobacter* is also achievable for the genus *Bacillus*.

3.5. Simulation of the 16S rDNA melting process during TGGE. The apparently equal migration distance of completely different sequences on TGGE demanded for further investigation, since this might be a major source of bias when the TGGE approach is used to screen 16S rDNA sequences retrieved by cloning or isolating bacterial strains. Some of the isolates giving identical TGGE signals like the four cloned sequences were quite similar to the corresponding clone while others were completely different (Tab. 1). The theoretical migration of the amplicons was investigated by computer simulations. Following the computation algorithm of Poland [22], the melting behavior could be predicted by the nucleotide sequence of the GC968/1401 amplicon, which is used for TGGE. Here, the three completely different sequences from the *Bacillus* DA001, the Actinobacteria-relative DA079 and the *Verrucomicrobiales*-relative DA101 showed a quite similar melting behavior (Fig. 3a) and were consequently running quite close to each other (Fig. 2). The amplicon of DA001 was melting first and stopped approximately 1.5 mm earlier than the amplicon of DA079, which stopped about 2 mm earlier than DA101 (Fig. 2). These sequences were clearly separated on TGGE. More difficult are sequences closely related to each other. Figure 3b shows the melting behavior of DA001 and the three closest isolate sequences IDA216 (98.2% sequence similarity to DA001), IDA624 (95.7%) and IDA629 (97.6%). These sequences showed almost identical melting curves and could not be separated on TGGE (Fig. 2). However, another closely related sequence, DA011 (96.8% sequence similarity to DA001), showed a completely different melting behavior and migrated much further into the gel (Fig. 2). Indeed, closely related sequences might run to the same position [5, 32]. However, the distance between two bands on a gel is not proportional to the sequence difference as demonstrated in figure 3a and by amplicon DA011 (Fig. 3b).

The isolates with rDNA amplicons that matched those of clones DA079 and DA101 upon TGGE, differed considerably with respect to their 16S rRNA sequences. The melting curve of the *Bacillus* sequence IDA234/IDA600 was almost perfectly identical to DA079, although the sequence similarity was only 76.4% (Tab. 1). Remarkable are the sequences DA101 and IDA465, that generated amplicons that both showed the same migration speed but a quite different melting behavior (Fig.3c). In the beginning, the IDA465 amplicon appeared to

slow down earlier than DA101, but due to a subsequent more dramatic pausing of DA101, IDA465 might close up again or even overtake. With increasing temperature, the amplicon DA101 showed a little bit faster migration, and finally they apparently ended at the same position (Fig. 2). This peculiar difference in melting behavior could be proved by premature termination of the TGGE, at the time both sequences were indeed still separated (data not shown). In summary, slightly different amplicons might be separated with a resolution down to one base pair difference [19] or not at all (Fig. 3b). Completely different amplicons are likely to show different migration following TGGE, but by accident they might end at exactly the same position (Fig. 3c).

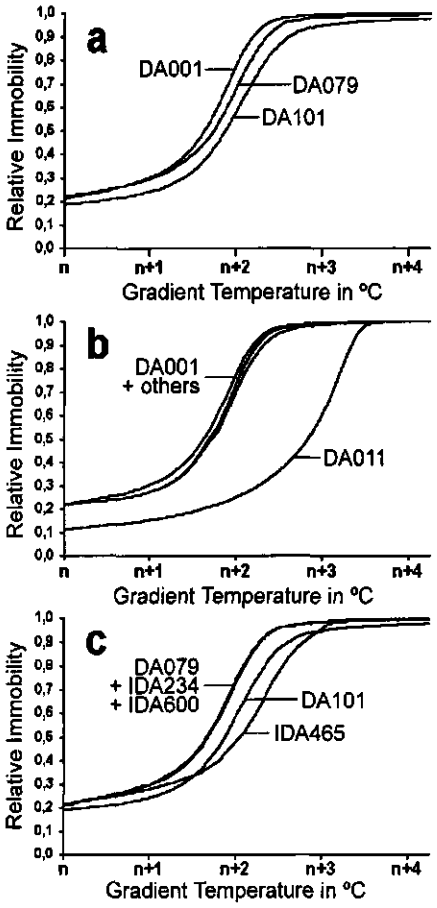

Fig. 3. Computer simulation of amplicon migration speeds in TGGE. A relative immobility of 0 is free mobility, while a relative immobility of 1 is no mobility at all. a, The three completely different sequences DA001, DA079 and DA101 show a quite similar melting behaviour. b, The highly similar sequences DA001, IDA216, IDA624 and IDA 629 show an almost identical melting behaviour while the also closely related sequence DA011 is melting at much higher temperatures. c, The sequence DA079 and its TGGE matches show an identical melting behaviour. DA101 and IDA 465 show different, crossing melting curves, but on TGGE they end on the same position.

Conclusions. TGGE-supported screening of isolated strains was found to be a convenient and efficient way to process large numbers of colonies. This study clearly demonstrated that completely different sequences from the same source samples might show the same migration speed during TGGE. Therefore, the assignment of isolates to matching bands of according environmental fingerprints without an additional check is not acceptable. This additional confirmation can be given by sequence comparison of the rDNA amplicons or by V6 probe hybridization, which does not require previous sequencing of the 16S rDNA fragments [9,14]. Cloning of environmental 16S rDNA yielded phylogenetic information that highly correlated to the TGGE analysis of environmental 16S rRNA. The most abundant clones were identical in sequence to the most intense bands in environmental TGGE fingerprints. The predominant bacteria in Drentse A grassland soils remained uncultured. This might be due to hitherto unknown nutrition and growth requirements, while other possibilities are low growth rates of the predominant soil bacteria or the inhibition by other microorganisms during growth on agar.

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Chapter 3

Spatial Homogeneity of Abundant Bacterial 16S rRNA Molecules in Grassland Soils

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Spatial Homogeneity of Abundant Bacterial 16S rRNA Molecules in Grassland Soils

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ABSTRACT

The variability of prominent bacterial 16S rRNA molecules from environmental soil samples was investigated. Ribosomes and genomic DNA were extracted from 160 soil samples derived from three different test fields in the Drentse A grasslands (The Netherlands). After amplification of bacterial 16S rRNA molecules by reverse transcription and PCR, the products were separated by temperature-gradient gel electrophoresis. Characteristic and complex band patterns were obtained, indicating high bacterial diversity. The fingerprints from soil samples from plots, taken in regular patterns, were almost identical. Reproducible differences between the three test fields of different history were obtained. A parallel approach with PCR-amplified genomic 16S rDNA led to similar results. The presence and activity of prominent bacteria in test fields of several hundred m^2 were constant. Only one gram of soil was needed to represent the prominent bacteria in large homogeneous grassland areas. The spatial distribution of bacterial ribosomes in soil at this site was homogeneous, suggesting the presence and activity of the dominant soil bacteria was the same.

Introduction

In recent years, analysis of bacterial 16S rRNA molecules has rapidly become a tool to describe diversity in environmental bacterial communities [4, 11, 18, 25, 26, 34, 41]. Direct isolation of rRNA or rDNA molecules from microbial communities circumvents selective and potentially ineffective cell cultivation. Nevertheless, particular problems of reproducibility exist in using target molecule isolation from environmental samples, such as soil. Related surveys have already proven the suitability of rRNA approaches to detect the vertical distribution of bacteria in aquatic environments [13, 16, 28, 37]. So far, no detailed molecular studies have been reported on this approach in soil. A lot of work has been done to detect microbial activity in soil by measuring metabolites or cell components, but the taxonomic determination of the organisms involved is rather limited [14]. Other, culture-dependent methods to describe bacterial communities [7] apparently suffered from the 'great plate-count anomaly' [36]. Most environmental bacterial cells were not accessible using cultivation methods [1, 31]. In our

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current research, we investigated the influence of grassland succession on the bacterial community in soils [35]. The studies using bacterial 16S rRNA sequences to reveal the most active bacterial species (A. Felske, unpublished results) and to describe their spatial distribution. Could bacterial 16S rRNA from a one-gram soil sample represent the bacterial community within a homogeneous area of several 100 m^2? Such small amounts of soil are normally used for nucleic acid isolation [15, 20, 27, 33]. In this study, the ribosome content of cells is used to indicate metabolic activity. Genomic 16S rDNA appeared to be less useful, because detection of DNA only reflects the presence of bacteria. In bacterial cultures, the amount of rRNA per cell is roughly proportional to metabolic activity [39]. Hence, the ribosome approach should select active bacteria, and neglect inactive cells (which have minimized their ribosome content). Direct isolation of ribosomes from soil, and subsequent purification of their rRNA, excludes free nucleic acids outside the living cells and focuses on 16S rRNA from intact ribosomes [8].

Recent studies, mainly based on 16S rDNA, have demonstrated that PCR and subsequent temperature-gradient gel electrophoresis (TGGE [30]), or comparable denaturing gradient gel electrophoresis (DGGE), are useful tools to investigate environmental nucleic acids [6, 10, 21, 22, 23, 27, 29, 40]. The amplicons of different target molecules can be separated by electrophoresis, and will produce a band pattern of the different amplified sequences. This pattern constitutes a fingerprint for the various sequences, and, in case of bacteria-specific PCR-primers, a fingerprint of the existing bacterial community. Many different samples can be compared easily by loading the amplicons next to each other on a TGGE gel. Hence, TGGE is a convenient technique to monitor spatial variation of bacterial communities in high sample numbers. However, few data are currently available regarding the spatial variation of bacterial molecules in the environment. Some work ([12] and B. Engelen, unpublished results) concerning bacterial genomic 16S rDNA from soil has already indicated a high reproducibility of such fingerprints at the DNA level. In the present study, we investigated the spatial distribution of bacterial 16S rRNAs in soil by using the ribosome approach [8], where ribosomes were extracted from 1-g soil samples. The purified 16S rRNA was amplified by RT-PCR, and analyzed by TGGE. This was compared with the DNA approach, where genomic 16S rDNA from the same samples was amplified by PCR for TGGE-analysis.

Materials and Methods

Collection of Soil Samples

Three different, peaty, acid, agricultural grassland test fields (A, F, and K) of the Drentse A agricultural research fields next to the Anlooër diepje river, The Netherlands (06°41'E, 53°03'N), were the sites of sample collection. Details of the soil properties have been published [35].

Test field F is a fertilized, agricultural grassland; fields A and K have not been fertilized since 1991 and 1967, respectively. Distances between the test fields were several hundred meters, with a maximum of about 1.5 km between test fields F and K. About 120 undisturbed surface samples (0–10 cm depth) were taken during March, 1996. Each test field was sampled at eight points, at intervals of 5 m. Each of these points consisted of five sites, a 1 m distance. Soil cores of about 50 g were taken with a drill (0–10 cm depth), and transferred into sterile bags. Two types of samples were prepared from this soil: First, undisturbed soil particles were taken for ribosome and DNA isolation. Samples from test field A were used solely to check variability of the 16S rRNA and rDNA community fingerprints at one-meter distances. Another 40 samples of 30–40 cm depth were taken on test field A, and processed in the same way, to assess the influence of sampling-depth.

The second type of samples were homogenized and pooled to compare the different test fields. The 40 samples from each test field were pooled to 4 samples by sieving and mixing 10 single samples (5 g each).

Amplification of 16S rRNA from Soil

Ribosomes and rRNA were isolated from Drentse A soil samples (1 g), following a previously described protocol [8]. RT-PCR was performed with the rTth DNA polymerase and buffer kit from Perkin-Elmer Cetus. RT reactions (10 µl) contained 10 mM Tris-HCl (pH 8.3); 90 mM KCl; 1 mM $MnCl_2$; 200 µM each of dATP, dCTP, dGTP, and dTTP; 15 pmol of primer L1401; and 2.5 units of rTth DNA polymerase. After addition of 1 µl of sample (about 10 ng rRNA), the mixtures were incubated 15 min at 68°C. Following the RT reaction, 40 µL of the PCR additive, containing 10 mM Tris-HCl (pH 8.3), 100 mM KCl, 3 mM $MgCl_2$, 0.75 mM ethylenebis (oxyethylenenitrilo)tetraacetic acid (EGTA), 5% (v/v) glycerol, and 15 pmol of primer U968-GC, were added. The samples (50 µl) were amplified with a GeneAmp PCR System 2400 thermocycler (Perkin-Elmer-Cetus), using 35 cycles at 94°C for 10 s, 56°C for 20 s and 68°C for 40 s. The oligonucleotide primers used were specific for bacterial 16S rRNA. The numbers in the primer names indicate the position in the 16S rRNA of E. coli [5]. Primer U968/GC: 5'-(GC-clamp)-AACGCGAAGAACCTTAC-3'; primer L1401: 5'-CGGTGTGTACAAGACCC-3' [24]. GC-clamp: 5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCA-CGGGGGG-3'—this 40mer is useful for accurate separation of PCR products in the gradient gel electrophoresis [22].

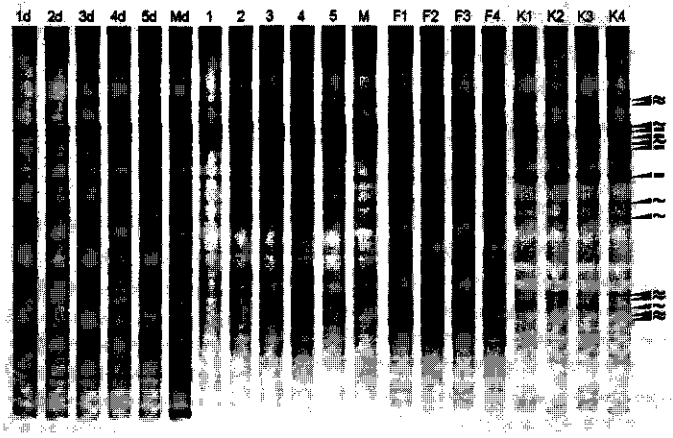

Fig. 1. rDNA amplicon fingerprints on a silver-stained TGGE gel, representing five single sampling points of one-meter distance, from test field A; and pooled samples from test fields A, F, and K, composed of 10 different single samples each. 1d–5d, PCR products from test field A samples of 30–40 cm depth; Md, PCR products from a pooled test field A sample of 30–40 cm depth; 1–5, PCR products from test field A samples (<10 cm); M, PCR product from a pooled test field A sample (<10 cm); F1–F4, PCR products from pooled samples of test field F; K1–K4, PCR products from pooled samples of test field K. Prominent band-positions are marked with an arrow. Some showed a similar intensity on all test fields (–), others differed in intensity (–).

Amplification of 16S rDNA from Soil

Genomic DNA was isolated from the same soil samples that were used for ribosome isolation [8]. Soil 16S rDNA was also amplified with primers U968/GC and L1401, to obtain a soil band pattern for TGGE. One µl of tenfold diluted DNA solution (about 10 pg) was amplified with a GeneAmp PCR System 2400 thermocycler (Perkin-Elmer-Cetus), using 35 cycles at 94°C for 10 s, 56°C for 20 s, and 68°C for 40 s. The PCR reactions (50 µl) contained 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 3 mM $MgCl_2$; 50 µM each of dATP, dCTP, dGTP, and dTTP; 0.05% detergent W-1 (Life Technologies); 100 pmol of primer U968-GC and L1401 (as above); and 1.25 units of *Taq* DNA polymerase (Life Technologies).

Temperature gradient gel electrophoresis (TGGE)

The Diagen TGGE system (Diagen GmbH, Düsseldorf, Germany) was used for sequence-specific separation of PCR products. The band separation range of the TGGE was optimized by adjusting the temperature gradient to 9°C difference. Electrophoresis took place in a 0.8 mm-polyacrylamide gel (6% acrylamide, 0.1% bisacrylamide, 8 M urea, 20% formamide, and 2% glycerol), with 1× TA buffer (40 mM Tris-Acetate, pH = 8.0) at a fixed current of 9 mA (about 120 V), for 16 h. A temperature gradient of 37°C to 46°C built up in electrophoresis direction. Twelve µl of each amplification product was separated by TGGE. After electrophoresis, the gels were silver-stained [9].

Results and Discussion

Direct ribosome isolation yielded 1–3 µg purified rRNA g^{-1} soil; these samples could be used for RT-PCR, with bacteria-specific primers. Parallel extraction of soil DNA yielded purified genomic DNA, also suitable for PCR, with the same primers. These partial 16S rRNA and rDNA amplicons were separated by temperature-gradient gel electrophoresis. Complex band patterns gave specific fingerprints of the 16S rRNA sequences (Figs. 1–4). Prominent bands within the fingerprints should consist of the most abundant molecules, although other important members of the microbial community could have been underrepresented. Their signals might be weaker or even absent due to possible PCR biases (primer specificity) and unknown cell lysis efficiencies.

Theoretically, the 16S rRNA fingerprints reflect the sequences of the most active species combined; the 16S rDNA fingerprints represent the individual species. The 16S rDNA fingerprints show several very strong bands, some bands of lower intensity, and an additional number of weak bands (sometimes resulting in a smear). Consequently, it is not possible to estimate the total number of different 16S rDNA molecules present, despite the high resolution power of TGGE. This is not surprising, because thousands of different bacterial genomes can be expected in one gram soil [38]. Hence, this approach reflects the diversity of amplifiable prominent sequences. Environmental microbial communities usually contain a few prominent species with many individuals, and a lot of species of low abundance [2]. This also seems to be true for the bacterial community in Drentse A grassland soils.

Comparison of 16S rDNA and rRNA fingerprints revealed the presence of common prominent bands. Fingerprints originating from 16S rRNA appear less dense than the 16S rDNA fingerprints (Fig. 4), indicating a lower number of 16S rRNA sequences. This is logical, because rDNA molecules do not require microorganisms to be active. Previous

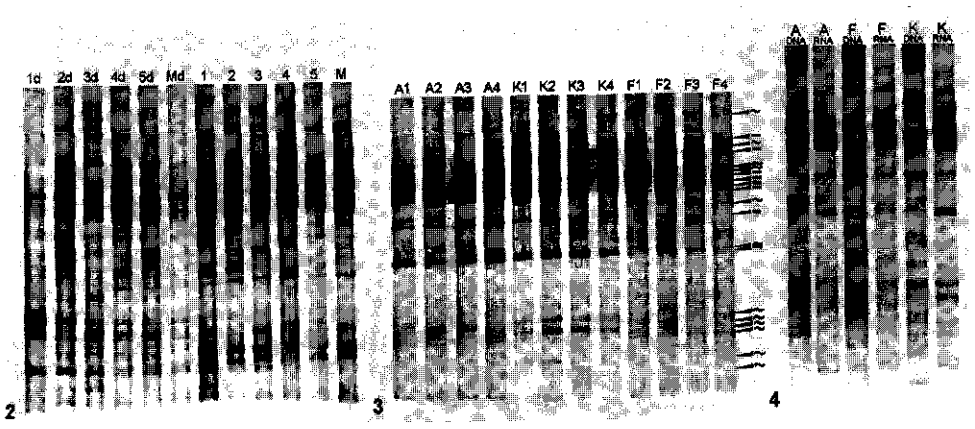

Fig. 2. Test field A: rRNA amplicon fingerprints on a silver-stained TGGE gel, representing five sampling points of one-meter distance. 1d–5d, RT-PCR products from soil samples of 30–40 cm depth; Md, RT-PCR products from a pooled sample of 30–40 cm depth; 1–5, RT-PCR products from surface soil samples (<10 cm); M, RT-PCR products from a pooled surface sample (<10 cm).

Fig. 3. Test field A, K, and F: rRNA amplicon fingerprints on a silver-stained TGGE gel, representing pooled samples composed of 10 different single samples each. A1–A4, RT-PCR products from pooled samples of test field A; K1–K4, RT-PCR products from pooled samples of test field K; F1–F4, RT-PCR products from pooled samples of test field F. Prominent band-positions are marked with an arrow. Some showed a similar intensity on all test fields (–), others differed in intensity (–).

Fig. 4. rRNA and DNA amplicon fingerprints on a silver-stained TGGE gel, representing test fields F, A, and K: RNA, RT-PCR products from pooled samples; DNA, PCR products from pooled samples.

investigations had already indicated that a large fraction of environmental microbial communities are resting or in a stage of low activity [3, 32]. DNA obtained from environmental samples could originate from such dormant cells, from dead cells [17], or even from free DNA. After lysis of the source organism and adsorption of DNA at mineral surfaces, especially in soils, nucleic acids could remain more-or-less intact for a long time [19]. In contrast, extracted ribosomes and their 16S rRNA should represent the most active bacteria in the environment [41].

Reproducibility of Test Field A Fingerprints from 1 m Distance

The soil samples taken 1 m from each other in test field A, represented by undisturbed soil particles of 1 g total input, yielded highly reproducible 16S rDNA fingerprints (Fig. 1, lanes 1d–M). The 16S rRNA yielded similar TGGE fingerprints (Fig. 2). The prominent bands can be found in all lanes with the same intensity. The variability of the community composition from 1 g undisturbed soil was apparently very low at 1 m distance. The presence and relative abundance of the prominent bacteria was similar in all samples. The 16S rRNA fraction, representing the most active bacteria, showed a higher variation (Fig. 2). This is likely to be caused by the higher variability of activity compared to presence. When environmental conditions change, soil bacteria respond by altering their metabolic activity instead of their spatial position. The differences observed between the rRNA samples probably reflect some microheterogeneity of the environmental conditions in the different soil samples. This microheterogeneity might become obvious by focusing on the composition of bacterial community via sample size reduction or through the use of group-specific primers. By increasing the sample size, an average fingerprint can be obtained. This has been achieved by pooling samples from each test field.

Variability of Fingerprints from Different Depths in Test Field A

Comparison of samples from the same position at 0–10 and 30–40 cm depth in test field A revealed differences (Figs. 1 and 2). A reproducible shift within the microbial community was observed by increasing sampling depth. A minority of bands appeared to be depth-specific; others showed variations in intensity. Most of the prominent bands could be found in all lanes. The bacterial communities in test field A, at 0–10 and at 30–40 cm depth, were similar.

Variability of Pooled Fingerprints from Different Test Fields

Pooling the soil samples yielded fingerprints of high reproducibility, almost identical to each other (Figs. 1 and 3). This was expected, because the individual samples were very similar. Many of the prominent bands are present in all lanes. The distribution of the dominant bacteria, as represented by the strongest fingerprint bands (Fig. 3), appeared to be relatively homogeneous. Only a minority of the strong bands were specific to an individual test field. Most variable bands showed reproducible variations in intensity, but seemed to be present everywhere. The fraction of weak bands in the TGGE fingerprints of genomic DNA showed more variety (Fig. 1), suggesting that major differences between the microbial communities of the test fields might be found in less abundant species. Without an extensive fraction of weak bands, the fingerprints from ribosomes looked much more alike (Fig. 3 and 4). Within a distance of a few hundred meters, despite different vegetation and agricultural history, the composition of the dominant active bacteria in test fields A, F, and K were similar. Single, test field–specific bands indicated that differences between the test fields are due to reactions of particular species instead of general shifts within the whole bacterial community.

Conclusions

Temperature-gradient gel electrophoresis is a suitable tool to test the reproducibility of extracted, native nucleic acids. The TGGE band patterns yielded a comprehensive overview of the main 16S rRNA molecules. Although the band patterns were complex, the reproducibility was high. Analyzing 16S rRNA can be used to examine a homogeneous environment with sample sizes orders of magnitude smaller than the investigated area. However, the degree of diversity generally depends on sample size. Microheterogeneity is likely present in our soil, and would probably become visible by drastic sample size reduction. Our aim was the opposite, i.e., to define average TGGE fingerprints of bacterial 16S rDNA and rRNA for each test field. This could be achieved by pooling samples or otherwise increasing sample size. This principle can be applied to a variety of other environments, but an increasing loss of information will probably occur in heterogeneous environments. Our results demonstrated that the diversity of prominent bacterial 16S rRNA molecules in a homogeneous test field of several hundred m^2 was comprehensively represented in a one-gram soil sample. The similarity of the 16S rRNA fingerprints of the three test fields indicate that long-distance (even kilometers long) spatial shifts of bacterial communities may not be dramatic, despite a heterogeneous history of cultivation and fertilization.

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Chapter 4

Quantification of 16S rRNAs in Complex Bacterial Communities by Multiple Competitive Reverse Transcription-PCR in Temperature Gradient Gel Electrophoresis Fingerprints

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Quantification of 16S rRNAs in Complex Bacterial Communities by Multiple Competitive Reverse Transcription-PCR in Temperature Gradient Gel Electrophoresis Fingerprints

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A novel approach was developed to quantify rRNA sequences in complex bacterial communities. The main bacterial 16S rRNAs in Drentse A grassland soils (The Netherlands) were amplified by reverse transcription (RT)-PCR with bacterium-specific primers and were separated by temperature gradient gel electrophoresis (TGGE). The primer pair used (primers U968-GC and L1401) was found to amplify with the same efficiency 16S rRNAs from bacterial cultures containing different taxa and cloned 16S ribosomal DNA amplicons from uncultured soil bacteria. The sequence-specific efficiency of amplification was determined by monitoring the amplification kinetics by kinetic PCR. The primer-specific amplification efficiency was assessed by competitive PCR and RT-PCR, and identical input amounts of different 16S rRNAs resulted in identical amplicon yields. The sequence-specific detection system used for competitive amplifications was TGGE, which also has been found to be suitable for simultaneous quantification of more than one sequence. We demonstrate that this approach can be applied to TGGE fingerprints of soil bacteria to estimate the ratios of the bacterial 16S rRNAs.

Since its initial application to environmental 16S ribosomal DNA (rDNA) by Muyzer et al. (20), denaturing gradient gel electrophoresis (DGGE) has been an attractive technique in molecular microbial ecology. Various workers have described microbial diversity as assessed by DGGE for a variety of different ecosystems. In spite of the growing interest in this technique, little attention has been given to the quantitative aspects of the fingerprints of bacterial communities. In most studies the workers investigated uncultured bacteria which were detected in environmental nucleic acid extracts by 16S rDNA fingerprints generated either by temperature gradient gel electrophoresis (TGGE) (25) or DGGE (13). Since such fingerprints were a result of PCR amplification of nucleic acid sequences, quantification of the signals had to be based on the principles of the quantitative PCR approach. In spite of the wide application of PCR, the quantitative use of PCR is not straightforward. Since the DNA molecules are amplified during PCR, the amount of initial target molecules can be estimated only by presuming that amplification efficiency is reproducible. The exponential nature of the amplification process is highly sensitive to any disturbance of amplification efficiency, which can easily result in major PCR bias. The main reason to use PCR for quantification is its sensitivity and specificity in comparison to the sensitivity and specificity of other techniques.

The three main methods used for quantitative analysis by PCR (or reverse transcription [RT]-PCR) are the limiting dilution PCR (23, 29), the kinetic PCR (1, 4, 7, 31), and the competitive PCR (3, 14, 33). The limiting dilution PCR approach is based on simple dilution of the template. For the other two methods a standard template of known concentration is required. This standard must be similar to the target to ensure equal amplification of both templates. The kinetic PCR determines the increase in the number of amplicons with time by measuring the absolute amount of DNA per cycle. On the one hand, this technique monitors the amplification efficiency (i.e., the exponential increase in the amount of PCR product). On the other hand, the time shift in the exponential growth curve between the target and the standard allows calculation of the unknown template DNA concentration in the target sample. An easier and more convenient method is the competitive PCR. In this method the standard and the target have different sequences to distinguish them and are amplified in the same reaction tube. This eliminates bias caused by the thermocycler or the reaction mixture. Defined serial dilutions of the standard template in a couple of parallel PCR mixtures are prepared to compete with the target sequence. The reaction in which the amounts of the PCR products of the standard and target are the same indicates the concentration of the original target template. The crucial point is to design a standard sequence that can be easily distinguished from the target after amplification. Since TGGE and DGGE are tools that are used to separate amplicons on the basis of their sequences, they are also suitable detection tools for quantitative PCR.

Competitive PCR initially was developed and used for mRNA obtained from target cells growing in pure culture (3, 14, 33), not for nucleic acids obtained from uncultured environmental bacteria. Recently, the amounts of particular genes in bacterial genomic DNA retrieved from soil and sediments have been determined (15, 19, 35). The application of kinetic PCR to 16S rDNA sequences (4) and the first attempt to perform a competitive PCR with environmental 16S rDNA (17) have been described only recently. In the latter study, the application of quantitative PCR to 16S rDNA of uncultured bacteria could be disputed, because the amount of 16S rDNA sequences per cell could not be estimated. It has been observed previously that the variable numbers of *rrn* operons and the genome sizes of different species are crucial parameters, and

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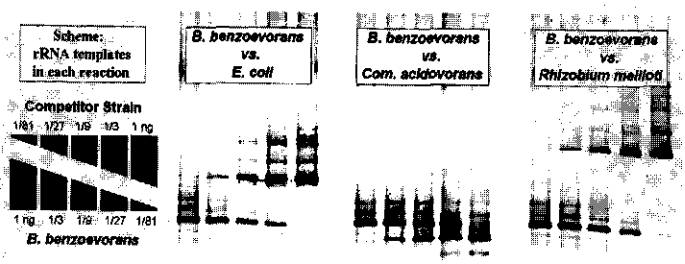

FIG. 1. Competitive RT-PCR performed with rRNA standards from different bacterial taxa. The scheme on the left shows the order of rRNA input. In the third of the five reactions equal amounts of the two competitor rRNAs are present. This ratio is also reflected by band intensities after separation of the amplicons by TGGE and detection by silver staining (2 μl of RT-PCR product per lane). The faint bands accompanying the main bands were RT-PCR side products and were not included in the quantitative analysis. We used more RT-PCR product than necessary to visualize traces of the out-competed sequence. However, the highly sensitive silver staining method also detected some RT-PCR side products, most likely side products representing a DNA polymerization bias.

consequently, 16S rDNA amplification of different bacterial strains reflected neither cell numbers nor ratios of nucleic acid amounts (8). As an alternative approach, we quantified bacterial ribosomes by using their 16S rRNA in order to monitor spatial changes in bacterial activity in soil (9, 10, 12). Ribosomes can be used as a marker for bacterial activity (34), because the amounts of ribosomes (and their rRNA) per cell were found to be roughly proportional to the growth activity of bacteria in pure culture (32).

In a previous study, the predominant 16S rRNAs of a bacterial community in soil were revealed by TGGE, hybridization, cloning, and sequencing (12). This study focused on rRNA to identify the most active bacteria. After direct ribosome isolation from soil, part of the bacterial 16S rRNA was amplified by RT-PCR. Sequence-specific separation of partial 16S rRNA amplicons by TGGE yielded reproducible, soil-specific fingerprints. The predominant bands of these fingerprints were identified by using a clone library of 16S rDNA amplicons, which resulted in characterization by sequence analysis. Here we describe a novel approach to quantify the 16S rRNA of uncultured bacteria by quantitative RT-PCR and evaluation of the amplification step. Careful evaluation of the amplification efficiencies of the sequences concerned was necessary, as demonstrated by different model experiments.

MATERIALS AND METHODS

Soil sampling. We selected a plot with an area of several 100 m^2 in the Drentse A agricultural research area in The Netherlands (06°41'E, 53°03'N) for sample collection. This grassland plot had not been fertilized since 1990 and was described as type A in a previous study (12). Details of the soil properties have been published previously (26). A total of 40 surface samples (depth, <10 cm) were taken in March 1996. Soil cores weighing approximately 50 g were obtained with a drill (depth, 0 to 10 cm) and then were transferred into sterile sample bags and stored at 4°C for a maximum of 48 h before nucleic acid extraction.

Bacterial strains. Several rRNA standards were prepared by extracting rRNA from laboratory cultures of the following strains: *Alcaligenes faecalis* DSM 30030, *Arthrobacter atrocyaneus* DSM 20127, *Azospirillum brasiliense* DSM 1690, *Bacillus benzoevorans* DSM 6385, *Bacillus subtilis* DSM 10, *Comamonas acidovorans* DSM 50251, *Escherichia coli* NM 522, *Pseudomonas fluorescens* DSM 50090, *Rhizobium meliloti* DSM 1981, and *Streptomyces griseus* DSM 773. All of the strains were grown as recommended by the distributors (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; Promega, Madison, Wis.).

Preparation of rRNA standards from pure cultures. Twenty-milliliter bacterial batch cultures at the end of the logarithmic growth phase were harvested by centrifugation for 10 min at 5,000 × g (Sorvall model RC24 superspeed centrifuge equipped with a type SM24 rotor). Each supernatant was discarded, and the bacterial pellet was resuspended in 8 ml of TN150 buffer (10 mM Tris-HCl [pH 8.0], 150 mM sodium chloride). Subsequently, 1 ml of TE-buffered phenol and 1 ml of chloroform-isoamyl alcohol (24:1) were added to a sterilized 12-ml cell homogenizer tube containing 3 g of glass beads (diameter, 110 μm). This tube was closed tightly and treated for 1 min in an MSK cell homogenizer (Braun-Melsungen, Melsungen, Germany) at 4,000 rpm. Then the glass beads, phenol, and precipitated cell debris were separated by centrifugation at 5,000 × *g* for 5 min. The aqueous phase was transferred into a 50-ml centrifuge tube, and after 2 volumes of ice-cold ethanol was added, the nucleic acids were precipitated by incubation for 30 min at −20°C and were collected by centrifugation for 20 min at 10,000 × *g*. The pellet was washed with 5 ml of 70% ethanol, air dried, and then resuspended in 500 μl of TMC buffer (10 mM Tris-HCl [pH 7.5], 5 mM magnesium chloride, 0.1 mM cesium chloride). After transfer into a 1.5-ml microcentrifuge tube, the DNA was digested for 15 min at 37°C with 5 μl of RNase-free DNase (RQ1; Promega). The reaction was terminated by adding 400 μl of water-saturated phenol-chloroform-isoamyl alcohol (25:24:1). The tube was vortexed for 1 min and centrifuged in a microcentrifuge for 1 min at full speed. The extraction procedure was repeated with 400 μl of chloroform-isoamyl alcohol (24:1). Ethanol precipitation was done as described above, and the purified rRNA was resuspended in 500 μl of Tris buffer (10 mM Tris-HCl, pH 8.0). The yields were up to 1 mg per culture, as estimated by UV spectrophotometry. Solutions containing 1 μg of rRNA per ml of Tris buffer-glycerol (1:1, vol/vol) were prepared as standards for subsequent competitive RT-PCR experiments. The glycerol allowed unfrozen storage at −20°C, which is optimal for multiple use (11).

Ribosome isolation from soil and bacterial rRNA yield estimation. Soil rRNA was obtained by isolating ribosomes from Drentse A soil samples by a previously described protocol (9). Briefly, ribosomes were released from the soil (1 g) by treatment with a bead beater in the presence of ribosome buffer. Subsequent centrifugations removed cell debris and soil particles from the suspension. Then the ribosomes were precipitated by centrifugation for 2 h at 100,000 × *g*. The rRNA was isolated and purified by phenol extraction, ethanol precipitation, and DNase digestion. rRNA solutions were prepared in Tris buffer-glycerol (1:1, vol/vol) for subsequent competitive RT-PCR experiments. The *Bacteria*-specific probe EUB338 (1) was used to estimate the amount of bacterial rRNA per gram of soil by dot blot hybridization. Soil rRNA was blotted and fixed onto a nylon membrane (Hybond-N+; Amersham, Rainham, United Kingdom) as described previously (2). The EUB338 oligonucleotide was 5′ labeled by using phage T4 polynucleotide kinase (Promega) and 30 μCi of [γ-^{32}P]ATP. Prehybridization, hybridization, and stringent washing were performed as described by Manz et al. (18). The signals of the radioactively labeled probe were analyzed with a PhosphorImager SF (Molecular Dynamics, Oakland, Mass.). Soil rRNA signals were related to signals obtained with *E. coli* rRNA standards of known concentrations to calculate the soil rRNA content.

Competitive RT-PCR performed with rRNA and primers U968-GC and L1401. The competitive RT-PCR was performed with an rTth DNA polymerase kit (Perkin-Elmer Cetus, Norwalk, Conn.). The RT reaction mixtures (10 μl) contained 10 mM Tris-HCl (pH 8.3), 90 mM KCl, 1 mM $MnCl_2$, 200 μM dATP, 200 μM dCTP, 200 μM dGTP, 200 μM dTTP, 750 nM primer L1401 (22), 2.5 U of rTth DNA polymerase, and 2 μl of rRNA from each competitor. After incubation for 15 min at 68°C, 40 μl of a PCR mixture containing 10 mM Tris-HCl (pH 8.3), 100 mM KCl, 0.75 mM EGTA, 0.05% Tween 20, 3.75 mM $MgCl_2$, 50 μM dATP, 50 μM dCTP, 50 μM dGTP, 50 μM dTTP, and 190 nM primer U968-GC (22) was added. Amplification was performed with a model 2400 GeneAmp PCR System thermocycler by using 35 cycles consisting of 94°C for 10 s, 56°C for 20 s, and 68°C for 40 s. Adjusted rRNA solutions obtained from the 10 bacterial standard strains (see above) were compared with each other in an experiment consisting of 45 competitive RT-PCR assays. Each competitive RT-PCR experiment consisted of five reaction mixtures containing decreasing gradients of competitor rRNA (Fig. 1). For multiple-competitor RT-PCR, the first compet-

itor was always the *E. coli* rRNA standard, while the second competitor was a defined mixture containing the other bacterial 16S rRNA standards or soil rRNA.

A Diagen TGGE system (Diagen, Düsseldorf, Germany) was used for sequence-specific separation of competitor amplicons after RT-PCR. Electrophoresis was performed in a 0.8-mm polyacrylamide gel (6% [wt/vol] acrylamide, 0.1% [wt/vol] bisacrylamide, 8 *M* urea, 20% [vol/vol] formamide, 2% [vol/vol] glycerol) with 1× TA buffer (40 mM Tris-acetate, pH 8.0) at a fixed current of 9 mA (about 120 V) for 16 h. A temperature gradient from 37 to 46°C was built up in the direction of electrophoresis. After electrophoresis the gels were silver stained (6). The gels were analyzed with MolecularAnalyst/PC fingerprinting software (Bio-Rad, Hercules, Calif.).

Preparation of DNA standards for kinetic PCR. The 10 bacterial strains which were used as rRNA standards were checked for equal amplification efficiency by kinetic PCR, and the 20 environmental cloned ribotypes representing the predominant band signals in the TGGE fingerprints from Drentse A soil were also checked (12). Uniform DNA templates were generated by PCR to overcome the problem of different numbers of 16S rDNA target sequences per amount of DNA. This could vary between different bacterial genomes (8), and the plasmid DNA of the transformants provided a much higher 16S rDNA target sequence concentration than genomic DNA provided. After the bacterial standard strains and the transformants containing the environmental sequences were grown on solid medium, single colonies were transferred into 1.5-ml microcentrifuge tubes containing 50 μl of TE buffer. The tubes were heated for 15 min at 95°C to lyse the cells and then chilled on ice. The 16S rDNA sequences were amplified by using 35 cycles consisting of 94°C for 10 s, 48°C for 20 s, and 68°C for 2 min. Each PCR mixture (50 μl) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, 150 μM dATP, 150 μM dCTP, 150 μM dGTP, 150 μM dTTP, 30 pmol of each primer, 2.5 U of *Taq* DNA polymerase (Life Technologies, Paisley, United Kingdom), and 1 μl of cell lysate. Bacterium-specific primers 8f and 1512r (10) were used for the cultured bacteria, and vector-specific primers T7 and SP6 (16) were used for the cloned sequences. Rough estimates of the DNA amplification yields were obtained by 1.4% agarose gel electrophoresis, and the preparations were diluted to concentrations of approximately 1 ng of DNA μl^{-1}.

Kinetic PCR. The 16S rDNA PCR products obtained from the 10 standard bacteria and the 20 environmental ribotypes (see above) were used as uniform templates for kinetic PCR. Fivefold dilutions (approximately 200 and 40 pg μl^{-1}) were prepared from the template solutions (approximately 1 ng of DNA μl^{-1}) in order to determine the influence of template concentration on amplification efficiency. The preparations containing the three different DNA concentrations were amplified with an Amplitron II thermocycler (Barnstead/Thermolyne, Dubuque, Iowa) by using 10 to 26 cycles consisting of 94°C for 10 s, 56°C for 20 s, and 68°C for 40 s. Each PCR mixture (eight mixtures, 20 μl per mixture) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, 50 μM dATP, 50 μM dCTP, 50 μM dGTP, 50 μM dTTP, 100 pmol of labeled primers U968-GC/Biot. and L1401/TBR (4), and 0.5 U of *Taq* DNA polymerase (Life Technologies). After a 160-μl reaction mixture containing 8 μl of template DNA was prepared, the mixture was distributed into eight tubes (20 μl per tube). The eight replicates per sample were removed from the thermocycler one after another when the 10th, 12th, 14th, etc., cycles were completed (see Fig. 3). Ten microliters of PCR product from each reaction mixture was mixed with 40 μl of 1.25× QPCR buffer (12.5 mM Tris-HCl [pH 8.3], 62.5 mM KCl) in a separate QPCR sample tube for measurement of the electrochemiluminescence signal with a QPCR System 5000 instrument (Perkin-Elmer Cetus) as described previously (4). After addition of 15 μl of a 2-mg ml^{-1} preparation of streptavidin-coated paramagnetic beads (Perkin-Elmer Cetus), the biotin-labeled PCR products were captured during 30 min of shaking incubation at 1,400 rpm. After capture, 340 μl of QPCR assay buffer (Perkin-Elmer Cetus) was added, and the mixture was analyzed with the QPCR System 5000 instrument. The slopes of the amplification kinetics lines were calculated by performing a linear regression analysis with the computer software QPCR ANALYSIS V 0.63 (Perkin-Elmer Cetus). In this process the correlation coefficient, r^2, was increased to >0.99 by removing one or two first datum points (if they were below the lower detection limit) and/or one or two last datum points (if they were in the stationary phase of the PCR). Data sets were normalized by considering the last value of each kinetic used as 100% and calculating the previous values as a part of this value. From this slope the multiplication factor (m) per cycle was estimated by using the following formula: $c_n = mc_{n-1}$, where c is the DNA yield and n is the cycle number.

TGGE analysis of soil DNA by limiting dilution PCR. PCR assays performed with diluted template DNA were used to search for sequences that exhibited reduced amplification efficiency. At the detection limit of a template dilution series, the most abundant sequence, not the sequence which exhibits the best amplification efficiency, was predominant. DNA was used instead of rRNA in order to detect lower target concentrations. The *Taq* DNA polymerase required much lower amounts of target DNA sequences than the rTth DNA polymerase used for rRNA targets required (21). Soil DNA was isolated as described previously (11). The concentration was adjusted to approximately 100 pg μl^{-1} after a rough estimate was obtained on an ethidium bromide-stained agarose gel. Then serial twofold dilutions were prepared in 12 steps. The resulting samples were the templates used for PCR and to check the TGGE results as described above.

RESULTS

Competitive RT-PCR with primers U968-GC and L1401. Equal amplification of different 16S rRNAs was verified with 10 cultured bacterial strains belonging to diverse taxa. Corresponding rRNA standards containing 1 ng μl^{-1} and subsequent threefold dilutions were prepared for each strain and compared with each other in a competitive RT-PCR experiment. After performing reactions in which both rRNA competitors were present at the same concentration we observed approximately identical band intensities on TGGE gels (Fig. 1). Similar results were obtained when 16S rDNA amplicons were used as competitors in competitive PCR. 16S rDNA amplicon preparations were adjusted to equal concentrations and were used as templates for competitive PCR in order to compare the cloned environmental sequences to each other and to cultured strains (data not shown). Some bacterial sequences exhibited a few minor mismatches with the primer sequence (G-T or A-C mismatches), but we did not observe any amplification bias related to this, even when the annealing temperature was increased from 56 to 60 or 64°C.

Sequence-specific amplification efficiency for cultured and uncultured bacteria. The sequence-specific amplification efficiency was measured by monitoring the amplification kinetics by kinetic PCR. We used only kinetic PCR performed with 16S rDNA amplicons as the targets to directly compare the cloned 16S rDNA sequences of the uncultured soil bacteria and cultured strains. Our comparison of amplicons from cultured strains with amplicons from cloned inserts of environmental 16S rDNA did not reveal any significantly different amplification kinetics (Fig. 2). All of the bacterial strains tested and the cloned 16S rDNAs from soil exhibited the same amplification kinetics. The slope of the exponential DNA increase during PCR allowed us to calculate the average amplification efficiency. For all of the bacterial sequences the measured multiplication factor per PCR cycle was approximately 1.34 (for a primer annealing temperature of 56°C). This indicates that the DNA polymerization process was properly initialized and completed with 34% of all template molecules in each cycle. The multiplication factor varied for different annealing temperatures between approximately 1.5 (48°C) and 1.2 (64°C).

Multiple quantification of rRNAs in TGGE fingerprints. The method described above (competitive RT-PCR and subsequent detection by TGGE) could also be used to quantify each of several different sequences in one sample. In defined artificial rRNA mixtures containing rRNA from four species, the signals of the individual competitors could be quantified by identifying the reaction in which one particular target signal and the standard band had the same intensity. After quantitative image analysis, the values could be used to relate the target concentration to the known template rRNA concentration of the standard. The values obtained with the rRNA standard indeed reflected the theoretical template input (Fig. 3). These results indicated that this approach might also be used with environmental fingerprints. However, we could not check to determine whether the amplification efficiencies of all the sequences present were identical. Important but unknown 16S rRNA sequences could produce faint bands or even be absent from the TGGE band pattern if their amplification efficiencies were much lower than the amplification efficiencies of the other sequences. Abundant sequences which cannot compete with other sequences might be detected if amplification template concentrations were reduced. At the highest dilutions competition is reduced and amplification is limited to only the most abundant sequences. Indeed, for the Drentse A fingerprints no signals other than the strongest bands in the original

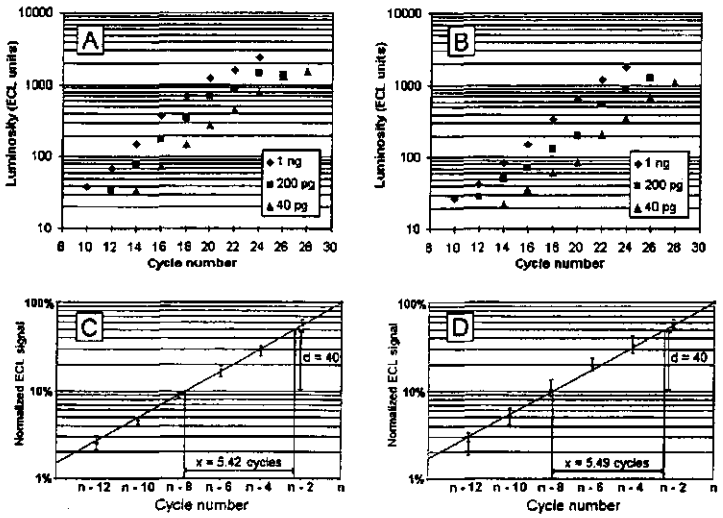

FIG. 2. Amplification kinetics of 30 different 16S rDNA sequences: detection signal value versus PCR cycle number. The templates used were different 16S rDNA amplicon samples, and three different amounts (1 ng, 200 pg, and 40 pg) were tested. (A) The target was a 16S rDNA amplicon from *E. coli*. (B) The target was a 16S rDNA amplicon of clone DA001. (C) Normalized results of all experiments and parallel experiments performed with 10 pure-culture organisms (30 kinetics experiments). (D) Normalized results of all experiments and parallel experiments performed with 20 environmental sequences (60 kinetics experiments). The slopes were used to calculate the amplification factor per cycle (1.341 in panel C and 1.346 in panel D). The error bars indicate the minimal and maximal deviations in the data sets.

band pattern remained at the highest dilutions (Fig. 4). This possibility was checked by performing PCR with soil DNA, because the RT-PCR product began to disappear at rRNA levels below approximately 10 pg. Since this level corresponded to approximately 10^6 target sequences, the band pattern shifts could not be observed or anticipated. On the basis of all of this evidence for equal amplification of the different sequences, we used an rRNA standard for the soil rRNA to perform multiple-competitor RT-PCR. In order to find a suitable rRNA standard for the fingerprints, we had to select a bacterial strain that produced a TGGE signal somewhere in a bandless gap in the environmental fingerprints. For the Drentse A fingerprints *E. coli* rRNA was a suitable choice (Fig. 5). First, the 20 most prominent sequences were quantified absolutely by using the principles of conventional competitive PCR, and values of about 20 to 200 ng per ribotype were obtained (Fig. 6A). Then the specific rRNA yields were related to the corresponding total rRNA yield from the soil sample as estimated by quantitative dot blot hybridization with *Bacteria*-specific probe EUB338 (Fig. 6B). The average yield from test plot A was 2.5 ± 0.6 μg of rRNA g of soil^{-1}; the minimum and maximum yields were 1.8 and 3.2 μg g^{-1}, respectively. The sum of all of the values estimated for the 20 predominant sequences accounted for 48% ± 16% of the total rRNA yield.

DISCUSSION

General problems of quantitative PCR. Since PCR is a process that involves exponential amplification, correct calculation of the original number of target sequences on the basis of the amount of the final PCR product can be massively distorted by experimental bias. The first obvious problem with PCR-based

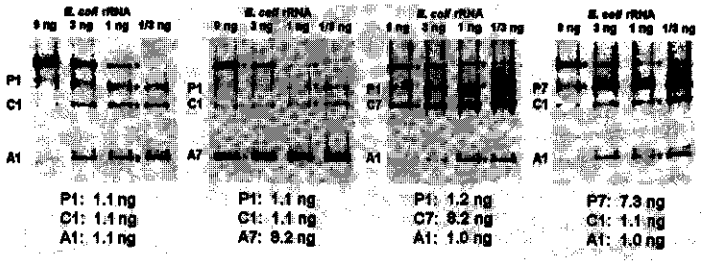

FIG. 3. Four multiple-competitor RT-PCR of rRNA with four competing rRNAs resolved by TGGE and detected by silver staining (2 μl of RT-PCR product per lane). *E. coli* rRNA was applied at different dilutions, as indicated. Signals A1 and A7 represented 1 and 7 ng of rRNA from *Arthrobacter atrocyaneus*. Signals C1 and C7 represented *Comamonas acidovorans*, and signals P1 and P7 represented *Pseudomonas fluorescens*. Each rRNA was quantified in the lane in which the intensity of the corresponding *E. coli* signal (indicated by an asterisk) was most similar in relation to the amount of rRNA represented by the *E. coli* signal. Results are given below the fingerprints.

FIG. 4. Silver-stained TGGE gel with PCR products from soil DNA from sample A1 (12 μl of RT-PCR product per lane). Lane 1 contained the PCR product generated from 100 pg of template DNA. Lanes 2 through 8 contained twofold serial dilutions of template DNA. Lane 8 contained approximately 0.8 pg of template DNA, which might represent a few hundred genomic units of soil bacteria.

quantitative assays is inherent to amplification itself. In the early cycles of the PCR the amount of product increases exponentially, but due to the depletion of substrates the amount might level off during the last cycles. It has been demonstrated that this change in amplification efficiency results in preferential amplification of less abundant sequences (28). This can be explained by an increased annealing competition effect (24). During the annealing phase the primer target sites could be

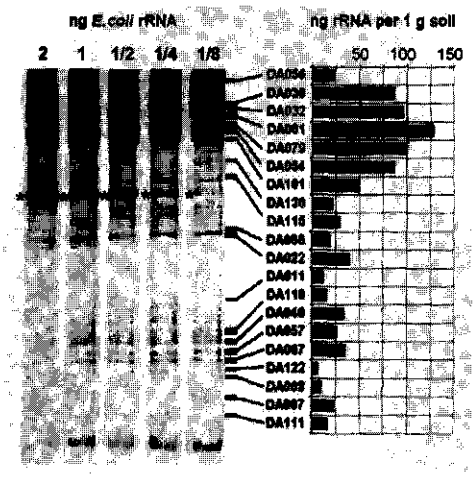

FIG. 5. Multiple-competitor RT-PCR of rRNA from soil sample A1 resolved by TGGE and detected by silver staining (12 μl of RT-PCR product per lane). The 20 signals selected for quantification are indicated; the designations have been described previously (12). The 20 sequences were quantified with image analysis software. Each sequence was quantified in the lane in which the intensity of the corresponding *E. coli* signal (indicated with an asterisk) was most similar in relation to the amount of rRNA represented by the *E. coli* signal and the amount of soil (10 mg) represented by the soil rRNA template.

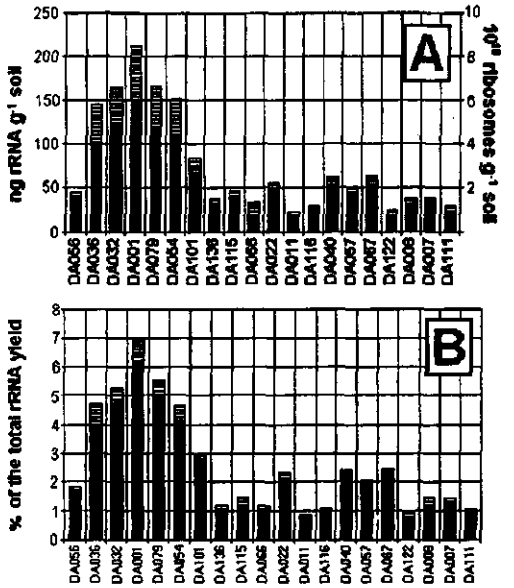

FIG. 6. Average rRNA yields for the 20 sequences in Fig. 5, based on 40 soil samples. The striped columns indicate the standard deviations. (A) Total amounts of rRNA. (B) Relative amounts as part of the total rRNA yield as estimated by quantitative dot blot hybridization with *Bacteria*-specific probe EUB338.

found by the primers or could rehybridize with their complements on the complementary DNA strand. In the early cycles of PCR this annealing competition is dominated by the huge excess of primer molecules, and proper DNA polymerization can be initialized. Since the primers become part of the PCR product, the number of free primers is significantly reduced in the late cycles of PCR. In contrast, the competitor, the complementary strand of the PCR product, is amplified exponentially. Therefore, the template DNA rehybridization process might become a serious competitor for primer annealing and prevent initialization of DNA polymerization. This inhibition should be most efficient for the abundant sequences, because their amplicon/primer ratio is less favorable than the ratio for the less abundant sequences. Therefore, specialized PCR procedures are needed to determine the amount of the original DNA template. In competitive PCR this bias is eliminated by analyzing only reaction mixtures in which the standard and target are present in similar amounts and are amplified almost equally. In kinetic PCR the breakdown of exponential amplification can be identified and the resulting data points can be neglected. The latter approach is also useful for directly detecting amplification efficiency. In this case sequence-specific factors, such as G+C content, secondary structures, and, especially, the size of the amplicon, might cause some bias which increases exponentially during the PCR. Another significant factor is primer annealing efficiency. In the first cycles of PCR the primers must anneal to the original template. The efficiency of this process might be reduced by some sequence mismatches in the primer target site. Since the primer becomes part of the amplicon and introduces its own sequence, this

effect disappears as the cycle number increases. Therefore, the bias in the initial cycles might be not detectable by kinetic PCR. Only preliminary quantitative PCR experiments performed with known template concentrations could reveal this deviation.

Competitive RT-PCR on TGGE. Primers U968-GC and L1401 have been used successfully for equal amplification of 16S rRNAs from bacterial cultures of different taxa and also cloned 16S rDNA amplicons from uncultured Drentse A bacteria. Sequence-specific amplification efficiency was assessed by monitoring the amplification kinetics by kinetic PCR. Primer-specific amplification efficiency was checked by competitive PCR and RT-PCR in which different templates and annealing temperatures were used. TGGE with subsequent silver staining and image analysis proved to be the optimal detection system for competitive amplification. The ability of this system to clearly separate sequences that differed by as little as one nucleotide (22) meant that it was possible to use standards having the same molecule length and almost identical sequences as targets. Such standards were the best competitors for equal coamplification with the target sequence. In contrast, the common approach of using standards of different lengths (14) introduces the danger of bias due to unequal amplification efficiencies (27). In the case of rRNA there is also no need to artificially construct a standard; the natural rRNA of another bacterial strain could meet all demands. This was experimentally confirmed by the equal sequence-specific amplification efficiencies of all of the different target sequences and the *E. coli* rRNA standard used.

Multiple-competitor RT-PCR for environmental 16S rRNAs. We found that the TGGE detection approach was also suitable for simultaneous quantification of several different sequences. We could quantify with one competitive RT-PCR assay numerous predominant bacterial rRNA sequences from complex bacterial communities. In the resulting complex TGGE fingerprints (Fig. 5) the clear signals were the most reliable signals and the many faint signals were less reliable. It should also be verified that one band indeed represents only one sequence and not several different sequences with the same migration speed (10).

Absolute quantification of rRNA sequences (Fig. 6A) is of questionable value, because it cannot be expected that all target molecules can be released from complex environments like soil. As estimated for inoculated sterilized soils, the ribosome isolation method which we used might result in a loss of about 50% of all ribosomes to the soil matrix (9). Much greater losses were estimated for other methods of nucleic acid extraction (17). Therefore, we preferred to use the ratio PCR proposed by Raeymaekers (24). This strategy was first used to analyze the expression of the $GABA_A$ receptor gene family on the basis of its mRNA (5). In this approach the variable expression of a gene can be related to constant mRNA levels of housekeeping genes. In this way uncertain absolute quantification can be replaced by a relative estimate of the change in gene expression. This reasoning may be applied to 16S rRNAs from bacterial communities. Bacteria which react to environmental changes in space or time by changing their ribosome levels can be related to the average or total amount of ribosomes for all bacteria. For the individual predominant ribotypes in soil we calculated values of 20 to 200 ng of rRNA g of $soil^{-1}$, which correspond to approximately 10^{10} to 10^{11} ribosomes g^{-1} (Fig. 6A) since one ribosome contains approximately 2.5×10^{-12} μg of rRNA (approximately 4,500 nucleotides). Of course, we did not expect that the 20 predominant sequences quantified represent all of the rRNA types present in complex soil environments (30). Therefore, we related the values obtained to the total rRNA yield estimated by another method (Fig. 6B). We found that the 20 predominant sequences represented approximately one-half of all of the rRNA extracted from the soil. On the one hand, this demonstrated that a considerable amount of bacterial ribosomes did not give strong signals in the TGGE fingerprints. This should have been due to a huge number of less active species which contributed a high total amount of ribosomes, but the individual different 16S rRNA sequences were too rare to compete successfully during RT-PCR. On the other hand, the major part of the total rRNA represented by the 20 sequences selected indicated that these sequences indeed originated from (at least most of) the predominant members of the bacterial community.

Relative quantification of multiple-competitor RT-PCR mixtures separated on high-resolution TGGE gels meets the demands of molecular microbial ecology for studying numerous species. Moreover, detection by TGGE allows workers to use quantification standards with optimal properties. The possibility of PCR amplification bias was investigated and eliminated for primers U968-GC and L1401 by performing kinetic PCR with the sequences concerned, limiting dilution PCR with soil DNA, and finally simulations of (multiple) competitive RT-PCR assays with defined rRNA standards and artificial rRNA mixtures. In addition, particular uncertainties must always be considered when complex, mainly unknown environmental microbial communities are the subject of investigation. In natural samples we might encounter extended lysis resistance of cells, adsorption and loss of nucleic acids to the environmental matrix, and previously unknown types of 16S rRNA sequences. Therefore, caution is required when conclusions are drawn from competitive PCR performed with environmental samples. At the moment we recommend limiting this approach to rRNA, mRNA, or plasmid DNA target molecules and following the cautious approach of ratio PCR.

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Chapter 5

Phylogeny of the Main Bacterial 16S rRNA Sequences in Drentse A Grassland Soils (The Netherlands)

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Phylogeny of the Main Bacterial 16S rRNA Sequences in Drentse A Grassland Soils (The Netherlands)

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The main bacteria in peaty, acid grassland soils in the Netherlands were investigated by ribosome isolation, temperature gradient gel electrophoresis, hybridization, cloning, and sequencing. Instead of using only 16S rDNA to determine the sequences present, we focused on rRNA to classify and quantify the most active bacteria. After direct ribosome isolation from soil, a partial amplicon of bacterial 16S rRNA was generated by reverse transcription-PCR. The sequence-specific separation by temperature gradient gel electrophoresis yielded soil-specific fingerprints, which were compared to signals from a clone library of genes coding for 16S rRNA. Cloned 16S rDNA sequences matching with intense bands in the fingerprint were sequenced. The relationships of the sequences to those of cultured organisms of known phylogeny were determined. Most of the amplicons originated from organisms closely related to *Bacillus* species. Such sequences were also detected by direct dot blot hybridization on soil rRNA: a probe specific for *Firmicutes* with low G+C content counted for about 50% of all bacterial rRNA. The bacterial activity in Drentse A grassland soil could be estimated by direct dot blot hybridization and sequencing of clones; it was found that about 65% of all the bacterial ribosomes originated from *Firmicutes*. The most active bacteria apparently were *Bacillus* species, from which about half of the sequences derived. Other sequences similar to those of gram-positive bacteria were only remotely related to known *Firmicutes* with a high G+C content. Other sequences were related to *Proteobacteria*, mainly the alpha subclass.

During the last few years, microbial ecologists have switched more and more to molecular strategies to study the distribution and activity of microorganisms in the environment. The earlier culture-dependent surveys used to describe bacterial communities were suspected of suffering from the "great plate count anomaly" (48). Most natural bacterial cells apparently were not accessible for the cultivation methods used today (2, 42). Explanations for these observations have fluctuated between the presence of cells which were not viable (nonculturability) and the hitherto unknown specific medium requirements of most bacteria (not yet cultured). Supporting both of these explanations, recent molecular studies of terrestrial and aquatic environments indicated on the one hand the presence of extremely small, possibly nonviable cells (3, 43) but on the other hand the presence of rRNA sequences of unknown species which have never been described as a cultured strain.

Thousands of different bacterial genomes per gram of soil were estimated to occur in terrestrial environments (52). Even comprehensive culture collections could hardly compete with such an extensive bacterial diversity in soil. Around the world, several culture-independent surveys of the microbial diversity in soil had been performed (5, 6, 23–25, 29, 39, 40, 44, 46, 53). They all were based principally on the PCR amplification of the small-subunit (SSU) rDNA from directly extracted soil DNA with universal primers. These amplicons were used for the subsequent generation of more or less comprehensive SSU rDNA clone libraries, allowing subsequent sequencing analysis. Unfortunately, all the studies used different cell lysis methods and primer sets. Although the comparability is thus limited, all these sequences provide the first indication of microbial diversity based on "real environmental" 16S rDNA data. Analysis of such 16S rDNA clone libraries demonstrated the presence of hitherto unidentified bacteria that were only remotely related to known strains (5, 6, 24, 25, 29, 40, 57). In fact, only a minority of sequences retrieved from directly isolated soil DNA could be closely related to cultured organisms. The major conclusion was that bacterial communities in the environment were composed mainly of uncultured species. Hence, the structure and function of bacterial communities in terrestrial and aquatic environments must have been mainly unknown. To date, this fact has prevented deeper insights into most basic nutrient fluxes in the ecosystems, where bacteria are suspected to contribute major functions.

Beyond the present collection of 16S rDNA sequences, our investigations are intended to reveal the metabolically most active members of the bacterial community in soil. A promising strategy for this had to be based on the direct isolation of suitable marker molecules. Genomic DNA could not be considered suitable, because detection of the DNA neither indicated activity nor proved the viability or even the presence of the corresponding cells (20, 28). The ribosome appeared to be a more useful marker, since the amount of ribosomes (and their rRNA) per cell was found to be roughly proportional to the growth activity of bacteria in pure culture (55). The 16S rRNA sequences were used as a marker for bacterial activity (59), a providing universal presence (in all cellular organisms) and species-specific sequence information (36, 37, 56). Starting point of our strategy consequently was the direct isolation of ribosomes and the subsequent purification of their rRNA from environmental soil samples (8). Then the major taxa represented by this ribosome fraction were identified by the application of different group-specific probes to the membrane-bound rRNA samples. Subsequent quantification of the probe

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signals and their comparison to a universal *Bacteria* probe yielded relative quantities of taxa. Another, more specific approach was used to compare the rRNA fraction with a 16S rDNA clone library generated from directly extracted soil DNA. Universal *Bacteria* primers were used to amplify the 16S rRNA target by reverse transcription-PCR (RT-PCR) and cloned 16S rDNA amplicons by PCR. The resulting amplicons were separated into a banding pattern of single sequences by temperature gradient gel electrophoresis (TGGE) (41), a technique that detects single changes in sequences. This technique, like the comparable denaturing gradient gel electrophoresis (DGGE) (15), was useful to reveal sequence diversity by generating fingerprints specific for the bacterial community (14, 33, 51). Comparison of single clones to the ribosomal soil band pattern indicated possible matches to particular bands within the soil rRNA fingerprints. Subsequent sequencing allowed initial interpretation of the organisms from which the single bands in the soil band pattern were derived. Then a detailed phylogenetic analysis could be added, because the clones were obtained by amplification of the almost complete 16S rDNA sequence (58).

This paper comprises the results of a comprehensive survey of most of the active bacteria in soil. The site we used is located in the Drentse A research area in The Netherlands and consisted of fields of peaty, acid grassland. Based on the approach focusing on metabolic activity, we identify most prominent bacteria in the upper soil layer and their distribution among major taxa, as indicated by their 16S rRNA sequences.

MATERIALS AND METHODS

Soil sampling. Peaty, acid grasslands of the Drentse A agricultural research area in The Netherlands (06°41'E, 53°03'N), were the sites of sample collection. They covered a geologically homogeneous stretch of approximately 1.5 km along the Anloerr Diepje River. The different cultivation history of the Drentse A plots was taken into account by sampling six plots representing different final years of fertilization for agricultural hay production. One plot was within the still fertilized area (type F), while another plot was part of an area that had not been fertilized since 1967 (type K). On the other plots, the fertilization stopped between 1985 and 1991 (type A). Details of the soil properties have been published (49). In total, 360 surface samples (<10 cm deep) were obtained in March and October 1996. Soil cores of approximately 50 g were obtained with a drill (0 to 10 cm deep) and transferred into sterile sample bags. Two types of samples were prepared: single soil samples were used for ribosome isolation to check the variability of the 16S rRNA community fingerprints per plot (11), and homogenized, pooled samples were used to compare the different areas. The pooled samples were obtained by pooling the single samples from each plot by sieving and mixing 10 single samples (5 g each) to end up with four samples.

Isolation of ribosomes and rRNA purification. Ribosomes were isolated from Drentse A soil samples by a previously described method (8). Briefly, the ribosomes were released from 1 g of soil by bead beater treatment in the presence of ribosome buffer. Subsequent centrifugations cleared the suspension of cell debris and soil particles. Then the ribosomes were precipitated by an ultra-high-speed centrifugation (2 h at 100,000 × *g*). The rRNA was extracted and purified by phenol extractions, ethanol precipitations, and DNase digestion to obtain suitable templates for RT-PCR. From 1 g of soil, we eventually obtained 100 μl of solution containing approximately 15 ng of rRNA per μl.

Specific quantitation of rRNA by dot blot hybridization. The *Bacteria*-specific probe EUB338 (1) was used to estimate the amount of bacterial rRNA per gram of soil in 24 rRNA samples (4 per plot). The average value was taken as 100% for subsequent comparison. Probe EUK1379 was used to detect eukaryotic SSU rRNA (18), and probe ARC915 was used to detect SSU rRNA of *Archaea* (47). The probes ALF1b, BET42a, and GAM42a were applied to quantify rRNA of the alpha, beta, and gamma classes of the *Proteobacteria*, respectively (31). Probe HGC was specific for high-G+C Gram-positive organisms (54). The LGC probe set has been applied to quantify Gram-positive organisms with a low content of G and C nucleotides (32). Another probe set called PLA has been applied to quantify *Planctomycetes* (34). The procedures have been published previously (31, 32, 34).

Partial amplification of 16S rRNA. RT-PCR was performed with the rTth DNA polymerase kit from Perkin-Elmer Cetus. RT reaction mixtures (10 μl) contained 10 mM Tris-HCl (pH 8.3), 90 mM KCl, 1 mM $MnCl_2$, 200 μM each dATP, dCTP, dGTP, and dTTP, 750 nM primer L1401 (35), 2.5 U of rTth DNA polymerase, and 1 μl of 10-fold-diluted template RNA (approximately 1.5 ng). After incubation for 15 min at 68°C, 40 μl of the PCR additive containing 10 mM Tris-HCl (pH 8.3), 100 mM KCl, 0.75 mM EGTA, 0.05% (vol/vol) Tween 20, 3.75 mM $MgCl_2$, 50 μM each dATP, dCTP, dGTP, and dTTP, 190 nM primer U968-GC (35) was added. Amplification was performed in a GeneAmp PCR System 2400 thermocycler (Perkin-Elmer Cetus), with 35 cycles of 94°C for 10 s, 56°C for 20 s, and 68°C for 40 s.

Screening of a 16S rDNA clone library for matching sequences. Total DNA was isolated from Drentse A soil samples as previously described (8). 16S rDNA sequences were amplified with a GeneAmp PCR System 2400 thermocycler, using 35 cycles of 94°C for 10 s, 54°C for 20 s, and 68°C for 2 min. The PCR mixtures (50 μl) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, 0.05% detergent W-1 (Life Technologies), 150 μM each dATP, dCTP, dGTP, and dTTP, 30 pmol of primers 8f and 1512r (10), 2.5 U of *Taq* DNA polymerase (Life Technologies), and 1 μl of template DNA (approximately 10 pg). The amplification products were confirmed by agarose gel electrophoresis (1.4% agarose) and then separated from primers and deoxynucleoside triphosphates on a low-melting-point agarose gel. Then they were cloned in pGEM-T linear plasmid vector and *Escherichia coli* JM109 competent cells as specified by the manufacturer (Promega, Madison, Wis.). After the transformants were grown overnight, single-clone colonies were taken up with sterile toothpicks and transferred into 1.5-ml microcentrifuge tubes containing 50 μl of TE buffer. The tubes were heated for 15 min at 95°C to lyse the cells and then chilled on ice. Insert sequences were amplified with a thermocycler (as above), using 25 cycles of 94°C for 10 s, 46°C for 20 s, and 68°C for 100 s. The PCR mixtures (10 μl) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, 150 μM each dATP, dCTP, dGTP, and dTTP, 3 pmol of primers T7 and SP6 (21), 0.25 U of *Taq* DNA polymerase (Life Technologies), and 1 μl of cell lysate. The vector-specific primers T7 and SP6 amplified the region between the multiple cloning sites where the amplicons should be inserted. Clones providing an amplicon of the correct size (approximately 1.6 kb) were identified by agarose gel electrophoresis. Cell lysates of positively identified clones were again amplified with a thermocycler (as above), using 25 cycles of 94°C for 10 s, 56°C for 20 s, and 68°C for 40 s. The PCR mixtures (10 μl) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, 50 μM each dATP, dCTP, dGTP, and dTTP, 1 pmol of primer U968-GC and L1401 (35), 0.25 U of *Taq* DNA polymerase (Life Technologies) and 1 μl of template DNA (approximately 10 pg). A 1-μl sample of each amplification product was separated by TGGE next to RT-PCR amplicons from soil rRNA (see above).

The Diagen TGGE system (Diagen, Düsseldorf, Germany) was used for sequence-specific separation of PCR products. Electrophoresis took place in a 0.8-mm polyacrylamide gel (6% [wt/vol] acrylamide, 0.1% [wt/vol] bisacrylamide, 8 M urea, 20% [vol/vol] formamide, 2% [vol/vol] glycerol) with 1x TA buffer (40 mM Tris acetate [pH 8.0]) at a fixed current of 9 mA (approximately 120 V) for 16 h. A temperature gradient from 37 to 46°C was built up in the direction of electrophoresis. After the run, the gels were silver stained (7). Then the gels could be screened for matches between clone signals and the bands of the RT-PCR fingerprints from soil. Apparent visual matches were confirmed with clone-specific V6 probe Southern blot hybridizations (16, 17). RT-PCR fingerprints from soil and clone signals were transferred to a nylon membrane. A clone-specific probe was used to detect the cloned sequence within the RT-PCR fingerprints from soil. The detailed procedure has been published previously (10).

Sequencing of PCR products from cloned inserts. Insert sequences were amplified with a thermocycler (as above), using 30 cycles of 94°C for 10 s, 46°C for 20 s, and 68°C for 100 s. The PCR mixtures (two 100-μl samples) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, 150 μM each dATP, dCTP, dGTP, and dTTP, 100 pmol of primer T7 and SP6, 2.5 U of *Taq* DNA polymerase (Life Technologies), and 1 μl of cell lysate (see above). The PCR products were purified and concentrated (from 200 to 50 μl) on fiberglass spin columns as specified by the manufacturer (High Pure PCR Product purification kit; Boehringer, Mannheim, Germany). Purified DNA was eluted from the columns with 50 μl of deionized water. The sequencing was done with a Sequenase (T7) sequencing kit (Amersham, Slough, England). Each 4-μl reaction mixture (A, C, G, and T) contained 2.5 μl of template, 0.5 μl of labelled primer (Infra-Red Dye 41; MWG-Biotech, Ebersberg, Germany), and 1 μl of reaction mix (A, C, G, or T; Amersham). The inserts were read in two directions: primer seqT7 and seqSP6 (sequence-like primers T7 and SP6) read from the plasmid into the insert, and primers seq515 (5'-ATCGTATTACCGCGGCTGCTGGCA-3'), seq338 (inverted sequence of probe EUB338), and seq968 (primer U968-GC without the GC clamp) read from inside the insert to its borders. The reaction was performed in a thermocycler (as above) with 35 cycles at 94°C for 5 s, 56°C for 10 s, and 68°C for 10 s. After the addition of 3 μl of loading dye (Amersham), the reactions were run on a no. 4000L sequencer (Li-Cor, Lincoln, Neb.).

Phylogenetic analyses. The environmental sequences were analyzed with ARB software (50). The ARB package is a combination of alignment and dendrogram tools, allowing alignments to a comprehensive SSU rDNA database (of 8,000 sequences) and detailed phylogenetic analysis. Distance matrices were calculated by the neighbor-joining method (45), and phylogenetic trees were constructed by using maximum parsimony criteria with nearest-neighbor optimization. Sequences with less than 90% similarity to any other known sequence were checked for chimera formation with the CHECK_CHIMERA software of the Ribosomal Database Project (30).

Nucleotide sequence accession numbers. The sequences of the soil rDNA clones were deposited in the EMBL database. Clones of other clone libraries usually were cited as a short combination of site-specific letters and clone numbers. Clones from forested soil from the Mount Coot-tha region in Australia were assigned MC sequence numbers (24, 25); those from a peat bog sample from Germany were assigned TM sequence numbers (40); those from a soybean field in Japan were assigned FIE or PAD sequence numbers (53); those from the Amazonia rainforest soil were assigned P or M sequence numbers (6). The new DA sequences and their accession numbers are as follows: DA001 (X99967), DA004 (Y07647), DA007 (Y07583), DA008 (Y12597), DA011 (Y07580), DA014 (Y07585), DA015 (Y07605), DA016 (Y07606), DA018 (Y07581), DA022 (Y07579), DA023 (Y07586), DA032 (Y07574), DA036 (AJ000981), DA038 (AJ000986), DA040 (AJ000985), DA052 (Y07646), DA054 (Y07575), DA056 (X99966), DA057 (AJ000988), DA066 (AJ000982), DA067 (Y07582), DA079 (Y11555), DA101 (Y07576), DA111 (Y12596), DA114 (AJ000980), DA115 (Y07578), DA116 (AJ000984), DA134 (AJ000983), DA136 (Y07577), and DA154 (AJ001222).

RESULTS

Ribosome isolation. The rRNA yield from Drentse A grassland soil samples was estimated by direct dot blot hybridization with the *Bacteria*-specific EUB338 probe to be approximately 1.5 ± 0.6 μg of bacterial rRNA per g (dry weight) of soil. The original ribosome isolation protocol (8) was modified so that the amount of soil material used was reduced from 1.5 to 1.0 g to reduce the size of the ultracentrifugation pellets and their resistance to resuspension. It has been found that soil input reductions can overcome such overloading problems with precipitates (9). The final rRNA solutions (100 μl per g of soil) were of suitable purity for dot blot hybridization (10 μl of input per dot). A 10-fold-diluted solution for RT-PCR (1 μl of input) was used to generate amplicons of reproducibly high yield and quality (data not shown). The rRNA solutions for RT-PCR were successfully checked for the absence of genomic DNA as previously described (8).

Group-specific quantification of soil rRNA. The hybridization experiments gave the first indications of the most active bacterial groups in Drentse A grassland soils. The *Bacteria*-specific EUB338 probe gave, for all plots, an average value of 1.55 μg of bacterial rRNA per g (dry weight) of soil, which was taken to be 100% for subsequent comparisons. The *Archaea* probe ARC915 gave only 0.5% ± 0.2%, and the *Eucarya* probe EUK1379 gave between 0 and 2%. As a theoretical part of the EUB338 signal, the ALF1b probe for the alpha *Proteobacteria* detected 22% ± 5% of all bacterial rRNA. The probe BET42a for the beta *Proteobacteria* found 1.5%, and the GAM42a probe for the gamma *Proteobacteria* gave no rRNA. The probe HGC for *Firmicutes* with a high G+C content counted approximately 19% ± 6%. The *Planctomycetes* probe set PLA gave between 0 and 4%. For the PLA probes and also for the *Eucarya* probe, the separation between background and signal was not clearly significant. The strongest signals appeared with the probe set LGC for *Firmicutes* with a low G+C content. With 49% ± 11%, about half of all bacterial ribosomes in the Drentse A grassland soils appeared to be from gram-positive bacteria with a low G+C content. The results from the different plots showed slight but not significant differences within the ratio of the ALF1b, HGC, and LGC signals (data not shown).

Identification of cloned 16S rRNA sequences in RT-PCR fingerprints from soil. TGGE analysis of RT-PCR products gave specific fingerprints for the rRNA population in soil. In a previous study (11), it was demonstrated that selected plots of the Drentse A area gave highly reproducible fingerprints. During our studies, three types of fingerprints could be distinguished for the surface soil layer (<10 cm deep) of Drentse A grasslands (Fig. 1). Fingerprints of type F originated from the still cultivated section of the Drentse A area. Type K was found in a plot taken out of production in 1967. The most abundant type, type A, represented the areas where fertilization stopped between 1985 and 1991.

Many of the predominat bands can be found in types K, A, and F. The distribution of the main bacteria appeared to be relatively homogeneous. Only a minority of the strong bands were area specific; most variable bands showed reproducible variations in intensity but were present everywhere.

Clone signals matching soil fingerprint bands indicated the identity of the sequences within the clone library and the soil fingerprints. Also, clone redundancy was indicated by TGGE analysis, where several clones showed the same migration distance. Redundant clones were most commonly found for clones matching the most intense fingerprint bands. Redundancy of the presented sequences is indicated in the phylogenetic trees (Fig. 2 to 5). For example, clone DA001 matched the most intense band of the RT-PCR fingerprint from soil (Fig. 1) and also represented another eight identical sequences of the 16S rDNA clone library (Fig. 3B). Of 165 clones, 37 could be identified as redundant by TGGE and subsequent partial sequencing (approximately 500 bp with primer seq968). The complete sequencing analysis could be limited to only different clones, which were found in the RT-PCR fingerprint from soil.

Figure 1 shows all the matches of clones with intense and also some faint fingerprint bands. Although TGGE has high resolution and the identities of clones with the same migration distance on TGGE are known, it could not be excluded that quite different sequences accidentally migrated to the same position. Hence sequence identity had to be verified by V6 probe hybridization. This approach could be used for most of the intense fingerprint bands (Fig. 1). Perfect probe specificity was demonstrated for clones DA079 and DA101 (10, 12) but could not always be achieved for the others. Due to cross-reactions, some results remained ambiguous. Weak fingerprint bands often could not clearly be identified as the matching clone sequence because the hybridization signals were too faint (data not shown).

Sequence analysis and phylogenetic assignment of clones. The partial 16S rRNA sequences covered a stretch of approximately 1500 nucleotides. About half of the sequences found in the clone library showed only slight relationships to other known sequences, while the other half were highly similar (approximately 95% sequence identity) to other database entries (mainly *Bacillus* species). The average sequencing error could be estimated by screening the latter. The cloned sequences were checked for "impossible nucleotides" by alignment to the next relatives. Less than 0.5% of all nucleotides were found to be unique within conserved regions of the cloned sequence and could almost always be related to reading errors in ambiguous regions of the sequencing gel. Sequences with less than 90% similarity to any other complete sequence of cultured organisms were checked for chimera formation. In all sequences, the beginning and the end of the sequences showed highly similar alignment results; therefore, chimera formation was not indicated. Only sequence DA052 remained questionable, because it (or any part of it) was not closely related to any other known sequence.

The sequences found in the clone library were not randomly distributed over the main 16S rRNA phylogeny clusters of bacteria. Most of the sequences fell into the cluster of low G+C gram-positive bacteria (mainly *Bacillus* relatives). Other groups were the alpha and beta *Proteobacteria*, the *Verrucomicrobiales*, the *Holophaga/Acidobacterium* cluster, and the high-G+C gram-positive bacteria (Fig. 2 to 5).

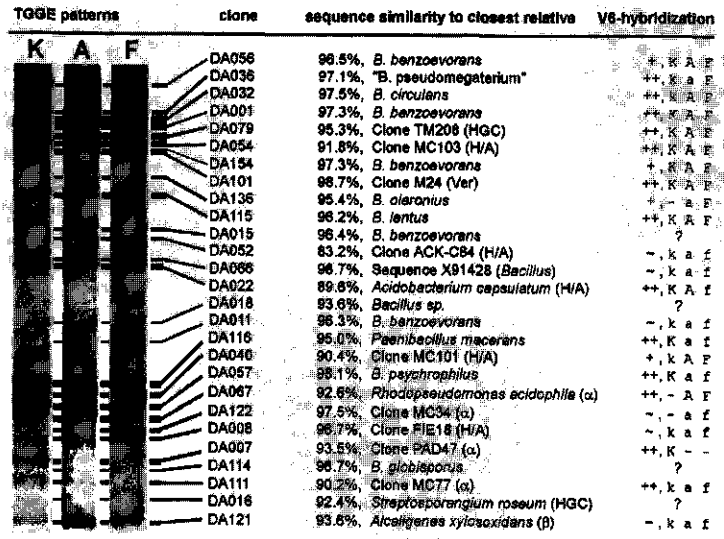

FIG. 1. Matching of clones with TGGE fingerprints of types A, F, and K generated from soil rRNA. The sequenced clones and their closest relatives are indicated. The 16S rRNA clusters are indicated in parentheses: HGC, high-G+C gram-positive bacteria; H/A, *Holophaga/Acidobacterium* cluster; Ver, *Verrucomicrobium* cluster; α and β, alpha and beta *Proteobacteria*. The column V6-hybridization summarizes the results of the V6 probe hybridization approach. Symbols: ++, positive identification by highly specific hybridization signal; +, positive identification by specific hybridization signal with minor cross-reactions; ~, tentative identification by specific hybridization signal with major cross-reactions; ?, hybridization signals within the TGGE pattern too faint; K, A, or F, prominent sequence in type K, A or F; k, a or f, less abundant sequence in type K, A or F; -, not detected by the V6 probe.

DISCUSSION

Experimental strategy. Bacterial communities in soil were found to be extremely complex (52). Hence, one could not expect to gain a serious understanding of the general bacterial diversity on the basis of only sequence analysis of a few hundred 16S rDNA clones (5, 46). This could not indicate all the bacteria present, since this would demand comprehensive clone libraries, or allow any quantitative conclusions. Surveys on such complex bacterial communities should be limited to more specific goals such as a revealing uncultured bacteria (24, 25) or investigating the diversity of particular phylogenetic taxons (29, 40) or physiological groups like the most active species (this study).

When Muyzer et al. (33) introduced the DGGE approach (which is comparable to TGGE) to molecular microbial ecology, they proposed this as an easier and much faster alternative to the sometimes tedious and expensive cloning procedure. Amplified environmental sequence populations could be specifically separated by DGGE, at once indicating the relative abundance of each sequence. Single bands could be excised, reamplified, and sequenced. However, two drawbacks had to be considered. First, the excised band would represent only a few hundred nucleotides of the target sequence. A detailed phylogenetic analysis might be hampered by this limitation. Second, it could never be excluded that one particular band might contain more than one sequence and consequently confuse the sequencing analysis. This had to be considered, especially when complex environmental bacterial communities were analyzed. Cloning of the excised and reamplified material must then be used to demonstrate its singularity. This cloning of single bands of interest and subsequent screening of the

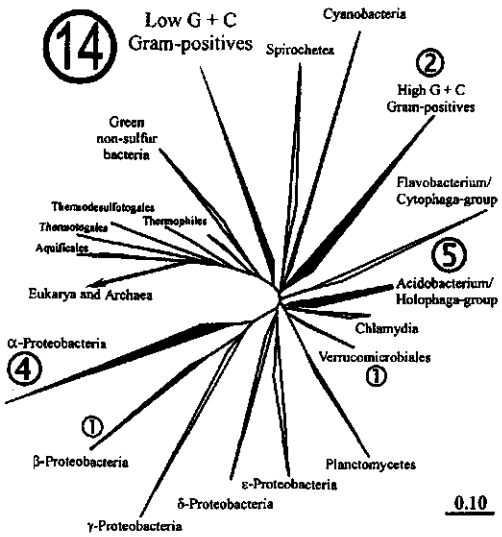

FIG. 2. Phylogenetic tree of almost 8,000 SSU rRNA sequences within the ARB database. The clusters containing the DA sequences are highlighted. The numbers indicate the distribution of the DA sequences as compiled in Fig. 1. The alpha *Proteobacteria* and *Cyanobacteria* clusters also represent mitochondrial and chloroplast sequences, respectively. The *Archaea* and *Eucarya* branches are hidden. The bar in the lower right corner indicates the branch length and represents 0.1 base substitution per nucleotide.

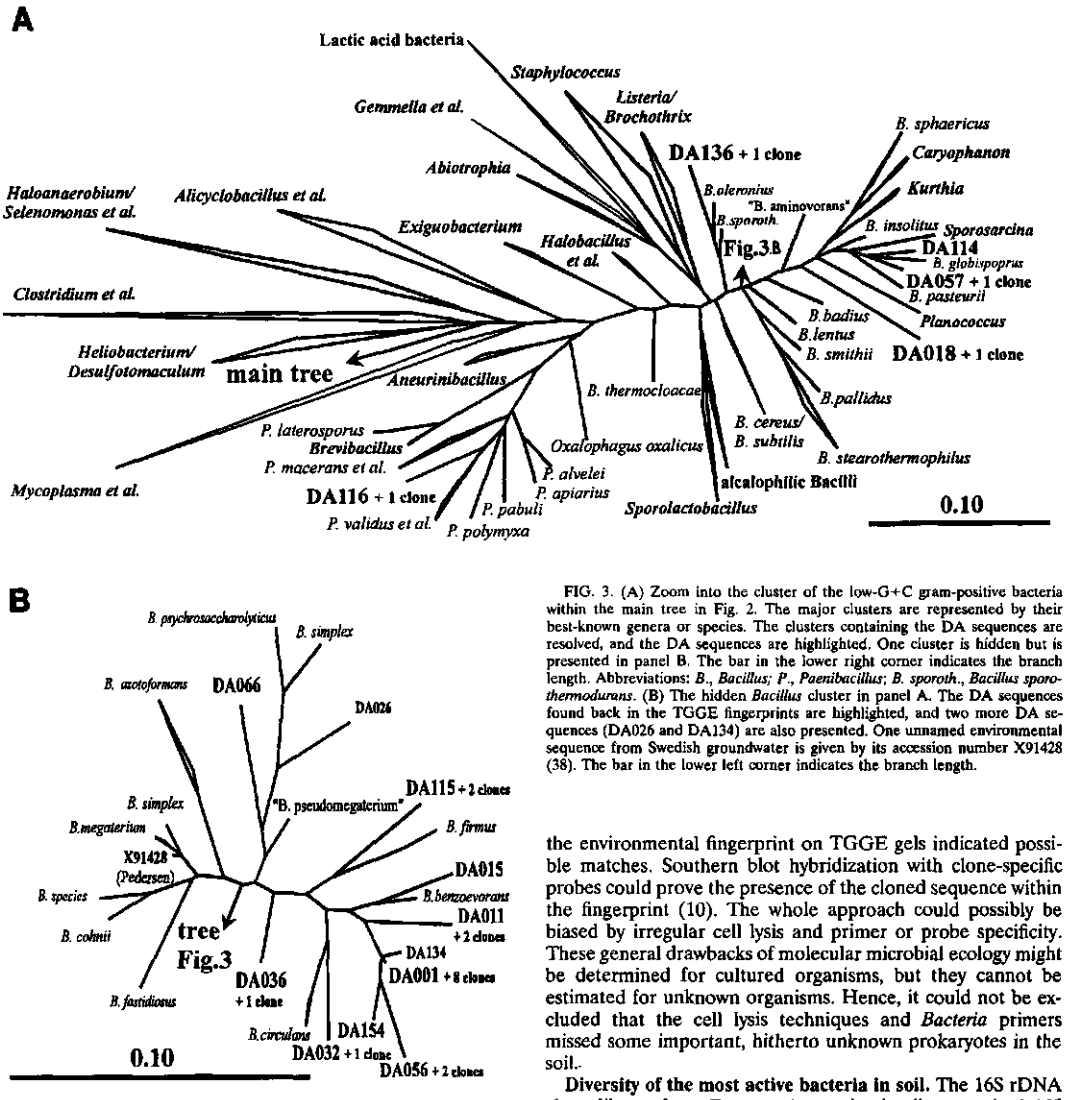

FIG. 3. (A) Zoom into the cluster of the low-G+C gram-positive bacteria within the main tree in Fig. 2. The major clusters are represented by their best-known genera or species. The clusters containing the DA sequences are resolved, and the DA sequences are highlighted. One cluster is hidden but is presented in panel B. The bar in the lower right corner indicates the branch length. Abbreviations: *B.*, *Bacillus*; *P.*, *Paenibacillus*; *B. sporoth.*, *Bacillus sporothermodurans*. (B) The hidden *Bacillus* cluster in panel A. The DA sequences found back in the TGGE fingerprints are highlighted, and two more DA sequences (DA026 and DA134) are also presented. One unnamed environmental sequence from Swedish groundwater is given by its accession number X91428 (38). The bar in the lower left corner indicates the branch length.

clones might become tedious and expensive. The only remaining advantage of TGGE or DGGE was the greatly enhanced semiquantitative assessment of sequence abundance by comparing band intensities.

We found that both approaches, cloning and TGGE, could complement each other and give a rather powerful combination if they were applied in parallel from the beginning. The possible drawbacks of TGGE and DGGE were erased because the cloned sequences were unique and represented the almost complete 16S rDNA sequences. TGGE fingerprint bands did not have to be excised, which might have been difficult when bands were very close to each other (see, e.g., the fingerprints in Fig. 1). Comparing amplicons of the cloned inserts next to the environmental fingerprint on TGGE gels indicated possible matches. Southern blot hybridization with clone-specific probes could prove the presence of the cloned sequence within the fingerprint (10). The whole approach could possibly be biased by irregular cell lysis and primer or probe specificity. These general drawbacks of molecular microbial ecology might be determined for cultured organisms, but they cannot be estimated for unknown organisms. Hence, it could not be excluded that the cell lysis techniques and *Bacteria* primers missed some important, hitherto unknown prokaryotes in the soil.

Diversity of the most active bacteria in soil. The 16S rDNA clone library from Drentse A grassland soils comprised 165 clones, representing 128 different types and 37 redundant sequences. Other studies of environmental clone libraries found a smaller number of or even no redundant clones. This was interpreted as an indication of the high bacterial diversity (5, 59). Compared to these studies, the Drentse A clone library contained a relatively high sequence redundancy. This might be the result of the combination of high-resolution TGGE clone-screening and accurate sequencing. However, it could also indicate a limited bacterial diversity caused by selective influences of the environment. More arguments for the latter possibility were the defined small number of intense bands in the TGGE fingerprint and the unequal presence of the major bacterial taxa. The clear dominance of *Bacillus* species and the limited number of other taxa (Fig. 2) were remarkable. Borne-

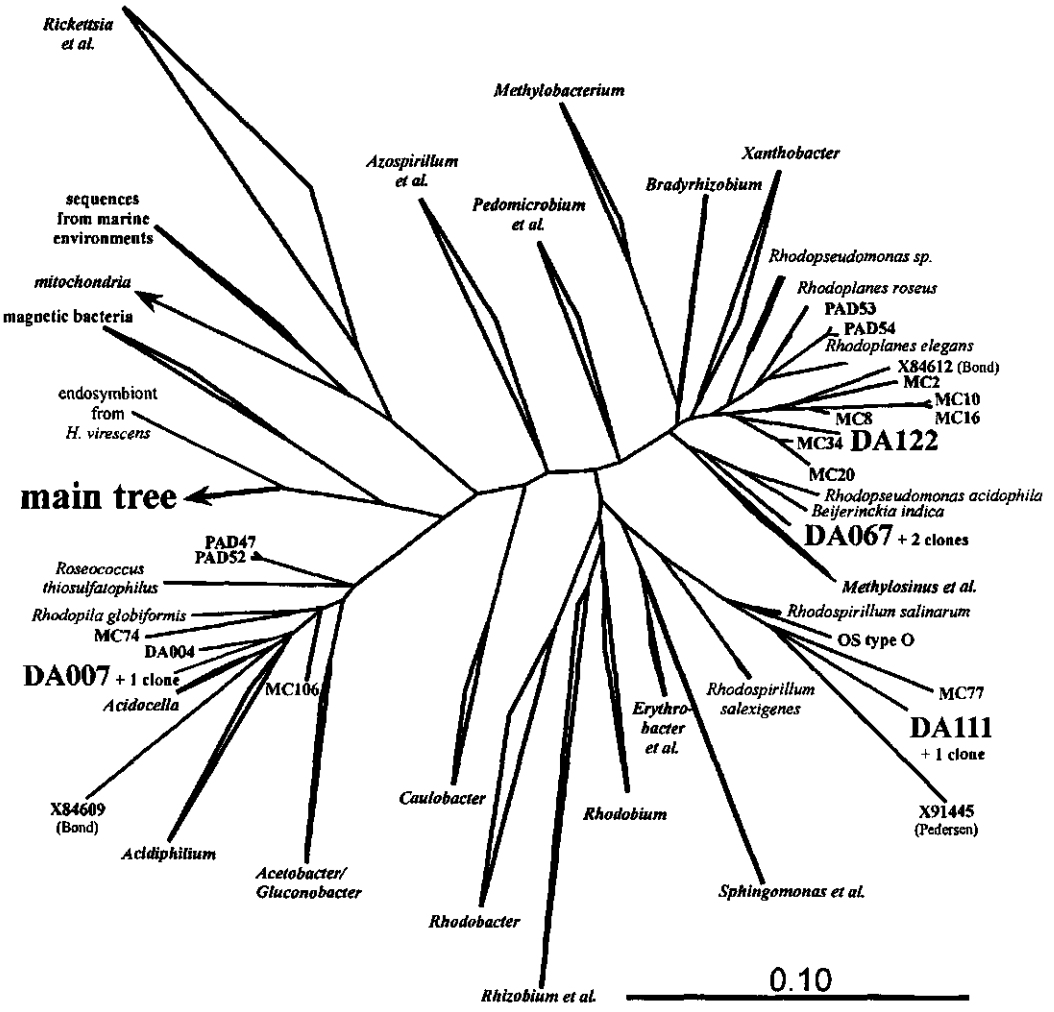

FIG. 4. Zoom into the cluster of alpha *Proteobacteria* within the main tree in Fig. 2. The major clusters are represented by their best-known genera. The clusters containing the DA sequences are resolved, and the DA sequences found back in the TGGE fingerprints are highlighted. One more DA sequence (DA004) is also presented. The mitochondrial branch is hidden. Three unnamed environmental sequences are given by their accession numbers: those from Swedish groundwater (X91445) and Australian sludge (X84609 and X84612). Sequence "OS type O" originated from an Octopus Spring cyanobacterial mat (56). The bar in the lower right corner indicates the branch length.

man et al. (5), for example, found much more diversity of cloned sequences from an agricultural soil from Wisconsin. Maybe the peaty, acid (pH ~ 4) Drentse A grasslands are a highly selective environment for bacteria. A comparable redundancy of 16S rDNA clones has been found in German peat bog samples (40). The question remained whether these acid environments caused a comparable selective pressure. The phylogeny of their 16S rDNA sequences could hardly be related to each other: only one cloned sequence from the Drentse A soil, DA079, could be related to the ones from German peat bog (10).

Dominance of *Bacillus* sequences. Of the 72 sequenced clones, 37 could be related to cultured species of the genus *Bacillus*. Most of the *Bacillus* sequences fell into one particular *Bacillus* branch of the low-G+C gram-positive organism tree (Fig. 3). Most of them were members of novel, hitherto uncultured phylogenetic lines within the *B. benzoevorans* line of descent (Fig. 3B). These *B. benzoevorans* relatives apparently were the most important group of soil bacteria. They were represented by approximately 20% of all sequenced clones, including clone DA001. This clone was the most abundant one in the 16S rDNA library (9 of 165 clones) and corresponded to the strongest band in the TGGE fingerprints (Fig. 1). The multiple appearance of closely related *B. benzoevorans*-like sequences in the TGGE fingerprint raised the question whether this could

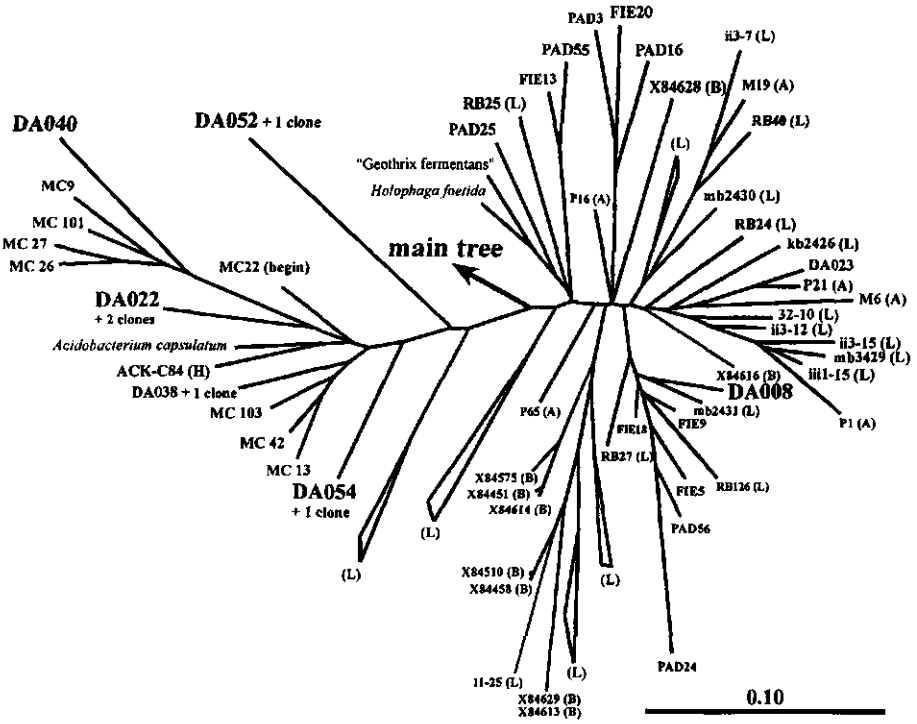

FIG. 5. Zoom into the cluster of *Holophaga* and *Acidobacterium* within the main tree in Fig. 2. The DA sequences found back in the TGGE fingerprints are highlightened, and two more DA sequences (DA023, DA038) are also presented. Beyond the MC sequences from Australian forested soil (24) and the Japanese FIE and PAD sequences (53), other environmental sequences are marked with (A) from Amazonian forested soil (6), (H) from American mountain lakes (19), (B) from Australian sludge (4), and (L) from German agricultural soil (29). The 31 (L) sequences were assigned to (L) clusters where possible. The bar in the lower right corner indicates the branch-length.

be due to 16S rDNA sequence heterogeneity, in which one bacterial strain produces more than one signal on TGGE. Such a finding was first described for *Paenibacillus polymyxa* (35). This cannot be excluded for our case, but it appears to be relatively rare among bacteria (13).

An interesting link to the *B. benzoevorans* relatives could be found in an SSU rDNA clone library of isolates from agricultural soil in Wisconsin (5). Clone DA001 had up to 99% sequence identity (270-nucleotide overlap) to four clones from Wisconsin (clones 102, 112, 122, and 132). This indicated a worldwide presence and maybe also importance of this *Bacillus* group.

Unidentified groups of environmental bacteria. Several distinct groups of unidentified environmental bacteria could be found in Drentse A grassland soils. Most of the alpha *Proteobacteria*-like sequences showed similarities to sequences from other environmental clone libraries. Clone DA007 fell into the neighborhood of *Acidiphilium*, together with two clones from Australian forested soil and another two from Japanese soybean field soil (Fig. 4). Clones from the same sources and Swedish groundwater (4) were grouped with clone DA122 within the *Rhodoplanes* line of descent. The sequence DA111 also had relatives in Australian forest soil and, surprisingly, also in the Octopus Spring microbial mat (59), all of which fell into the *Rhodospirillum* branch.

Other clones, which were not closely related to cultured organisms, fell into a group of German peat bog clones (DA079) (10), the *Verrucomicrobiales* cluster (DA101) (12), and, especially, the *Holophaga/Acidobacterium* cluster (Fig. 5), which probably represents a major taxon on its own (29). Other clones falling into this line of descent again were derived from Australian forested soil (24), Japanese soybean field soil (53), American mountain lakes (19), and, especially, the Roggenstein agricultural test fields in Germany (29). Apart from these several dozen environmental sequences, only three cultured strains were described in this cluster: *Acidobacterium capsulatum* (22), *Holophaga foetida* (26), and "*Geothrix fermentans*" (27). All these species and sequences were isolated from terrestrial environments and might represent one of the most important groups of soil bacteria. This study demonstrated their prominent presence not only in 16S rDNA clone libraries but also within the ribosome fraction from soil. Hence, they also might constitute a major part of the microbial activity in soil.

Conclusions. TGGE-supported clone screening was a convenient and efficient way to detect and retrieve the most abundant 16S rRNA sequences. This study yielded environmental sequence data of high quality, allowing detailed phylogenetic analysis of the main soil bacteria. A lot of work is done in the field of molecular microbial ecology to retrieve environmental 16S rDNA sequences as the first data about the real bacterial world. The most exciting aspect of gathering 16S rDNA sequences from environments around the world springs from the

possibility of revealing phylogenetic relationships to each other and to cultured bacteria. The use of cultured relatives of an unidentified sequence could point to selective approaches to cultivating the organism, and relationships between cloned sequences from different habitats could give the first indications of their potential importance and spatial distribution in the environment. This study once more detected unidentified bacterial lines of descent as already found on other sites of the world. Beyond that, they were also indicated as being metabolically active by their ribosomes. Now it seems likely that these uncultured bacteria are some of the most important metabolizers in soil. Their stage of activity also promises the possibility to grow them in culture. Revealing these rulers of our environment, isolating them, and finally studying them in vitro and in situ will certainly give important insights into the major nutrient fluxes of our planet.

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Chapter 6

Ribosome Analysis Reveals Prominent Activity of an Uncultured Member of the Class Actinobacteria in Grassland Soils

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Ribosome analysis reveals prominent activity of an uncultured member of the class Actinobacteria in grassland soils

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A 16S rRNA-based molecular ecological study was performed to search for dominant bacterial sequences in Drentse A grassland soils (The Netherlands). In the first step, a library of 165 clones was generated from PCR-amplified 16S rDNA. By sequence comparison, clone DA079 and two other identical clones could be affiliated to a group of recently described uncultured Actinobacteria. This group contained 16S rDNA clone sequences obtained from different environments across the world. To determine whether such uncultured organisms were part of the physiologically active population in the soil, ribosomes were isolated from the environment and 16S rRNA was partially amplified via RT-PCR using conserved primers for members of the domain Bacteria. Subsequent sequence-specific separation by temperature-gradient gel electrophoresis (TGGE) generated fingerprints of the amplicons. Such community fingerprints were compared with the TGGE pattern of PCR-amplified rDNA of clone DA079 which was generated with the same set of primers. One of the dominant fingerprint bands matched with the band obtained from the actinobacterial clone. Southern blot hybridization with a probe made from clone DA079 confirmed sequence identity of clone and fingerprint band. This is the first report that a member of the novel actinobacterial group may play a physiologically active role in a native microbial community.

Keywords: 16S rDNA library, Actinobacteria, temperature-gradient gel electrophoresis (TGGE), ribosome extraction from soil, V6 probe

INTRODUCTION

Over the last few years, molecular ecological studies on mainly terrestrial environments (Liesack & Stackebrandt, 1992a, b; Stackebrandt *et al.*, 1993; Rheims *et al.*, 1996a, b) have indicated the existence of two monophyletic groups of uncultured bacteria from the class *Actinobacteria* (Stackebrandt *et al.*, 1997). As judged from the analysis of PCR-amplified 16S rDNA these sequences showed only rather remote similarities to sequences of cultured actinobacteria. The relevant clones were obtained from an Australian forested soil taken from the Mount Coot-tha region in Brisbane, Queensland (Liesack & Stackebrandt, 1992a, b; Stackebrandt *et al.*, 1993), a peat bog sample from Germany (Rheims *et al.*, 1996a, b), geothermally heated soil from New Zealand (Rainey *et al.*, 1993) and a soil sample from Finland (Saano *et al.*, 1995). Also, short 16S rDNA clone sequences from a paddy field (Maidak *et al.*, 1994), a soybean field (Ueda *et al.*, 1995) and a marine environment (Fuhrman *et al.*, 1993) were demonstrated to cluster with these actinobacterial sequences. Thus it has been concluded that this group of uncultured organisms might contribute to ecologically important processes (Rheims *et al.*, 1996b).

The presence of rDNA sequences in a clone library proves neither activity nor abundance of the microorganism from which the DNA is represented in the library. Previous investigations already indicated that a large fraction of environmental microbial communities

Abbreviation: TGGE, temperature-gradient gel electrophoresis.

The EMBL accession number for the sequence of DA079 reported in this paper is Y11555.

is in a stage of low activity or resting (Bakken & Olsen, 1987; Roszak *et al.*, 1984). DNA obtained from environmental samples could thus originate from such dormant cells, from dead cells (Josephson *et al.*, 1993), or even from free DNA. Adsorption of DNA at mineral surfaces, especially in soils, could harbour more or less intact nucleic acids a long time after lysis of the source organism (Lorenz & Wackernagel, 1987). Our strategy to obtain information about the presence of metabolically active bacteria in the environment focuses on the analysis of 16S rRNA from isolated ribosomes. As the ribosome per cell ratio is roughly proportional to growth rate of bacteria (Wagner, 1994), rRNA is regarded as an indicator of total bacterial activity.

Here we report on the finding of a novel sequence of one of the actinobacterial lineages from the Drentse A rDNA clone library (A. Felske, A. Wolterink, R. van Lis & A. D. L. Akkermans, unpublished results). The cloned sequence DA079 was investigated for its significance in the environmental 16S rDNA population and 16S rRNA fractions of ribosomes that were isolated from the same site (Drentse A agricultural test area, The Netherlands). After direct ribosome isolation from soil samples (Felske *et al.*, 1996), rRNA was purified and used for RT-PCR with bacteria-specific primers. This partial 16S rRNA amplicon, representing the complex sequence population of the soil, was sequence-specifically separated by temperature-gradient gel electrophoresis (TGGE; Rosenbaum & Riesner, 1987). The resulting fingerprint was screened for the cloned sequence DA079 by amplifying the plasmid DNA with the same primers as those used for soil rRNA. Running this product next to the rRNA fingerprint indicated possible matches. Subsequent electrophoretic Southern blotting and hybridization with a clone-specific probe was applied to confirm the match.

METHODS

Collection of soil samples. Peaty, acid grasslands of the Drentse A agricultural research fields next to the Anlooër Diepje River, the Netherlands (06° 41′ E, 53° 03′ N), were the sites of sample collection. A total of 120 surface samples (< 10 cm depth) were taken on three different testfields during March 1996. Another 240 surface samples were taken on the same and additional three testfields in October 1996. Six different testfields (A, B, C, F, K and O) along the Anlooër Diepje River were investigated. Distances between the relevant testfields were several hundred metres, with a maximum of 1·5 km between testfields F and K. On each testfield, 40 soil cores of about 50 g were taken with a drill (0–10 cm depth) and transferred into sterile sample bags. The 40 samples of each testfield were pooled to four samples by sieving and mixing 10 single samples (5 g input each). Details of the soil properties were published by Stienstra *et al.* (1994).

Isolation, amplification, cloning and sequencing of 16S rDNA sequences. Total DNA was isolated from Drentse A soil samples by a parallel pathway during ribosome isolation (Felske *et al.*, 1996). Amplification of 16S rDNA sequences was performed with a GeneAmp PCR System 2400 thermocycler (Perkin Elmer Cetus), using 35 cycles of 94 °C for 10 s, 54 °C for 20 s and 68 °C for 2 min. The PCR reactions (100 µl) contained 10 mM Tris/HCl (pH 8·3), 50 mM KCl, 3 mM $MgCl_2$, 0·05% detergent W-1 (LifeTechnologies), 150 µM each of dATP, dCTP, dGTP and dTTP, 100 pmol primers 8f and 1512r (as below), 2·5 U *Taq* DNA polymerase (LifeTechnologies), and 1 µl template DNA. Amplification products were confirmed by 1·4% agarose gel electrophoresis and then separated from primers and dNTPs on a low-melting-point agarose gel. Subsequently they were cloned in pGEM-T linear plasmid vector and *Escherichia coli* JM109 competent cells according to the manufacturer's instructions (Promega). Isolated and purified plasmids (Wizard 373 DNA purification system; Promega) were sequenced using a Sequenase (T7)-terminator dsDNA sequencing kit (Pharmacia) on a Li-Cor Sequencer 4000L.

Isolation of ribosomes and amplification of 16S rRNA. Ribosomes were isolated from Drentse A soil samples (1 g input) as previously described (Felske *et al.*, 1996). RT-PCR was performed with the r*Tth* DNA Polymerase kit from Perkin Elmer Cetus. Reverse transcription reactions (10 µl) contained 10 mM Tris/HCl (pH 8·3), 90 mM KCl, 1 mM $MnCl_2$, 200 µM each of dATP, dCTP, dGTP and dTTP, 750 nM primer L1401 (as below), 2·5 U r*Tth* DNA polymerase and 1 µl template RNA. After incubation for 15 min at 68 °C (reverse transcription), 40 µl PCR additive containing 10 mM Tris/HCl (pH 8·3), 100 mM KCl, 0·75 mM EGTA, 0·05% (v/v) Tween 20, 3·75 mM $MgCl_2$, 50 µM each of dATP, dCTP, dGTP, and dTTP, 190 nM primer U968/GC (as below) were added. Amplification was performed in a GeneAmp PCR System 2400 thermocycler, using 35 cycles of 94 °C for 10 s, 56 °C for 20 s and 68 °C for 40 s. The correct size of amplification products was checked by electrophoresis on a 1·4% agarose gel.

Partial 16S rDNA amplification of clone DA079 for TGGE. A single DA079 clone colony (identified by sequence analysis) was taken up with a sterile toothpick and transferred to a 1·5 ml microcentrifuge tube containing 50 µl TE buffer. The tube was heated for 15 min at 95 °C and then chilled on ice. A TGGE-suitable 16S rDNA amplicon was generated with a GeneAmp PCR System 2400 thermocycler, using 25 cycles of 94 °C for 10 s, 56 °C for 20 s and 68 °C for 40 s. The PCR reactions (20 µl) contained 10 mM Tris/HCl (pH 8·3), 50 mM KCl, 3 mM $MgCl_2$, 50 µM each of dATP, dCTP, dGTP, and dTTP, 100 pmol primers U968/GC and L1401 (as below), 0·5 U *Taq* DNA polymerase and 1 µl DA079 cell lysate. Dilution series of the PCR product (2, 1, 0·5 and 0·25 µl per lane) were used for subsequent TGGE and electrophoretic Southern blotting.

Preparation of the clone-specific probe V6-DA079. A probe for the clone DA079 was generated by amplification of the highly variable V6 region of the 16S rDNA (Heuer & Smalla, 1997) with a GeneAmp PCR System 2400 thermocycler, using 30 cycles of 94 °C for 10 s, 46 °C for 20 s and 68 °C for 10 s. The PCR reaction (100 µl) contained 10 mM Tris/HCl (pH 8·3), 50 mM KCl, 3 mM $MgCl_2$, 25 µM each of dATP, dCTP, dGTP, and dTTP, 200 pmol primers V971 and R1057 (as below), 2·5 U *Taq* DNA polymerase and 1 µl DA079 cell lysate. Resulting PCR products were purified and concentrated by ethanol precipitation. The precipitated DNA was resolved in 50 µl nanopure water and then 5′-labelled using phage T4 polynucleotide kinase (Promega) and 3·7 Bq of [γ-^{32}P]ATP (370 MBq ml^{-1}; Amersham Buchler) in a 30 min reaction at 37 °C.

TGGE, electrophoretic Southern blot and hybridization. The Diagen TGGE system was used for sequence-specific separation of PCR products. The temperature gradient was optimized to 9 °C difference for improved resolution. Electro-

phoresis was performed with a 0·8 mm polyacrylamide gel (6%, w/v, acrylamide; 0·1%, w/v, bis-acrylamide; 8 M urea; 20%, v/v, formamide; 2%, v/v, glycerol) with 1 × TA buffer (40 mM Tris-acetate, pH 8·0) at a fixed current of 9 mA (about 120 V) for 16 h. A temperature gradient from 37 to 46 °C was established in the direction of electrophoresis. Samples for RT-PCR of soil rRNA and PCR of plasmid DNA were applied twice in symmetrical order.

After electrophoresis one-half of the gel was used for silver staining (Engelen *et al.*, 1995), the other half for Southern blotting. This half was shaken for 15 min in 0·5 × TBE buffer (Sambrook *et al.*, 1989). Two pieces of gel-sized Whatman filter paper and one sheet of nylon membrane (Hybond-N+; Amersham) were treated in the same way. One filter paper, the membrane, the gel and finally the other filter paper were placed above each other into a TransBlot SD Electrophoretic Transfer Cell (Bio-Rad). After closing the transfer cell a current of 400 mA was applied for 1 h. After this electrophoretic blot the membrane was briefly washed in 0·5 × TBE and placed on top of another Whatman filter paper (presoaked with 0·4 M NaOH) for 10 min. After shaking the membrane for 10 min in 2 × SSC (Sambrook *et al.*, 1989), the DNA was immobilized by baking at 120 °C for 30 min. Prehybridization (1 h at 56 °C) and hybridization (16 h at 56 °C) were performed in 5 × SSC with 2% (w/v) blocking reagent (Boehringer), 0·1% *N*-lauroylsarkosine, 0·02% SDS and 20% (v/v) formamide. For hybridization, 10 µl labelled probe V6-DA079 were added. Subsequent stringent washing steps were twice for 5 min in 2 × SSC with 0·1% SDS and twice for 15 min in 0·1 × SSC with 0·1% SDS on a shaker at room temperature. A detection screen (Molecular Dynamics) was incubated with the hybridized membrane and the probe signals were detected with a Phosphor Imager SF (Molecular Dynamics). Quantification was performed with image analysis software ImageMaster 1D Elite version 2.0 (Pharmacia).

Oligonucleotides. All oligonucleotides used in this study were specific for bacterial 16S rRNA. The numbers in the primer names indicate the position of the 5′ nucleotide in the 16S rRNA of *E. coli* (Brosius *et al.*, 1978). The sequence for primer U968/GC is 5′-(GC clamp)-AAC GCG AAG AAC CTT AC-3′, and for primer L1401 it is 5′-CGG TGT GTA CAA GAC CC-3′. These 17mers are specific for highly conserved 16S rRNA regions from bacteria (Nübel *et al.*, 1996). The sequence of the GC clamp, linked to the 5′ terminus of the PCR-amplified product is 5′-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G-3′. This 40mer is useful for accurate separation of PCR products in the gradient gel electrophoresis (Muyzer *et al.*, 1993).

The primers used for generating the clone library are were 8f, 5′-CAC GGA TCC AGA CTT TGA T(C/T)(A/C) TGG CTC AG-3′, and 1512r, 5′-GTG AAG CTT ACG G(C/T)T AGC TTG TTA CGA CTT-3′. Both are specific for highly conserved 16S rRNA regions of bacteria (taken and modified from Weisburg *et al.*, 1991).

Probe V6-DA079 was amplified with primers V971, 5′-GCG AAG AAC CTT ACC-3′, and R1057, 5′-CAT GCA GCA CCT GT-3′; both are specific for highly conserved 16S rRNA regions from bacteria (Hartung, 1996).

Phylogenetic analysis for clone DA079. The 16S rDNA sequence of clone DA079 was transferred to the alignment editor ae2 (Maidak *et al.*, 1994) and compared to the DSMZ 16S rDNA database of Actinobacteria. The sequence of DA079 was then compared to the most closely related members of uncultured peat organisms of groups II and III and their closest cultivated relatives. Sequences of clones from other environments could not be shown within the same tree (except clone MC58), as they do not have enough sequence information or overlap with the other clone sequences. For the construction of a phylogenetic tree, sequences from other more remotely related organisms were also included in the comparison.

The similarity values for these sequences were transformed into phylogenetic distance values that compensate for multiple substitutions at any given site in the sequence (Jukes & Cantor, 1969). The phylogenetic dendrogram was constructed with the neighbour-joining method included in the PHYLIP package (Felsenstein, 1993).

RESULTS AND DISCUSSION

Detection of clone DA079 rDNA and related environmental sequences in soils

Clone DA079 originates from a Drentse A 16S rDNA clone library, comprising 165 positive clones (A. Felske, A.Wolterink, R. van Lis & A. D. L. Akkermans, unpublished results). Two other clones were identical in sequence, so finally three of 165 clones represented this DA079 sequence. Some closely related German peat clones (designated TM clones) were previously found to have relatives in geographically widely separated soil environments, i.e. Australia, Finland, Japan, New Zealand, and in the Atlantic and Pacific oceans (summarized by Rheims *et al.*, 1996b). With the detection of the uncultured organism DA079 in Drentse A grassland soils another example for the wide distribution of this novel group of uncultured actinobacteria in different soil types is given.

In detail, clone DA079 is a member of peat clone group II (as defined by Rheims *et al.*, 1996b), showing a similarity of 95·3% to clone TM208 (Fig. 1). No close relationship exists between the peat clone group II (including clone DA079) and the nearest cultured relative, *Acidimicrobium ferrooxidans* (Clark & Norris, 1996). According to the current taxonomic structure of the class Actinobacteria (Stackebrandt *et al.*, 1997), even a relationship at family level seems unlikely.

Quantification of sequences in clone libraries

To draw a conclusion on the importance of these organisms, as detected by analysis of amplification products, the following factors should be considered.

Deduction of the number of organisms characterized by a unique 16S rDNA sequence in the community from the number of sequences in a 16S rDNA library is difficult. As most 16S rDNA sequences appear only once during sequence analysis of clones (Weller *et al.*, 1991; Borneman *et al.*, 1996), the question is raised whether this reflects the real abundance or whether these unique sequences were randomly recovered from a low-abundance background?

One may imagine a model in which 50% of all the 16S rDNA within a native bacterial community originates from a few dozen dominant species. The other 50% consists of thousands of rare sequences occurring with

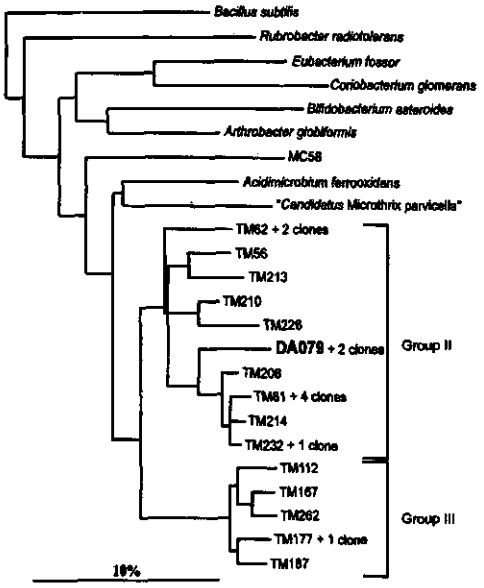

Fig. 1. Dendrogram showing phylogenetic positions of 16S rDNA related groups II and III and the clone DA079 within the Actinobacteria. The sequence of *Bacillus subtilis* served as an outgroup sequence. The analysis is based on about 1100 5′ nt of the 16S rDNA sequence (limited by the length of the sequence of clone MC58). The bar represents 10 nt substitutions per 100 nt.

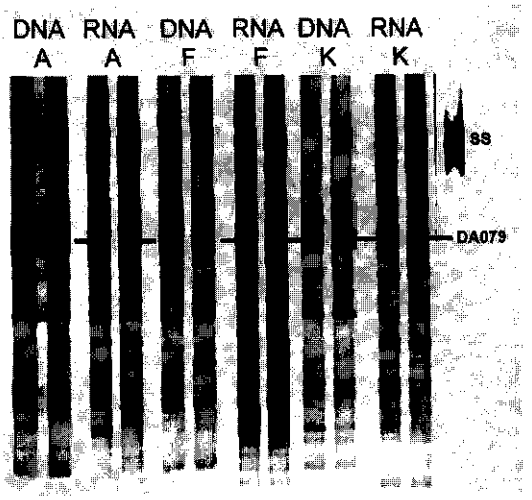

Fig. 2. Amplicon fingerprints on silver-stained TGGE gels. RT-PCR products from ribosomes and a partial 16S rDNA PCR product from genomic DNA from the same probing site, obtained with the corresponding set of primers, each show bands of equal position, matching with the DA079 signal as indicated. Fingerprints represent two of four pooled samples each from Drentse A sites A, F and K. 'SS' indicates the position and size of the single-stranded cDNA/rDNA fraction.

a very low mean abundance each. This model is not unreasonable as the presence of thousands of different bacterial species can be expected to occur in a single soil sample (Torsvik *et al.*, 1990). Extraction of DNA and subsequent PCR amplification with universal bacterial primers yields a 16S rDNA amplicon of a very complex sequence composition, optimally reflecting the native composition. In the subsequent steps of ligation and transformation, the selection of sequences is random. Hence rare sequences have a realistic chance of appearing in a successfully cloned insert. In our model, one-half of all clones will represent the few dominant species, while the other half is composed of randomly selected members of a low-abundance background. The higher the microbial diversity, the more likely is the appearance of such randomly selected rare sequences. Thus the normally limited size of environmental clone libraries (a few hundred clones) has the danger of overestimating unique clones. Multiple detection of identical clone sequences within one clone library, on the other hand, might indeed indicate abundance in the original population. In this study three identical 16S rDNA sequences of the type DA079 were identified within the Drentse A library of 165 clones.

In contrast to the uncertain meaning of sequence quantities in clone libraries, the semi-quantitative assessment of PCR amplicons in TGGE fingerprints is only based on staining intensity. A silver-stained TGGE gel would show a background of thousands of different sequences as a faint background smear, somewhere between invisibility and extremely faint bands. Hence estimation of sequence abundance in genetic material isolated from the environment is more likely to be representative by application of the TGGE approach.

Detection of sequence DA079 by TGGE in total rRNA and rDNA from soil

Direct ribosome isolation yielded purified rRNA [1·5 ± 0·6 μg (g soil)$^{-1}$] which could be used for RT-PCR with bacteria-specific primers. Ribosome isolation and subsequent RT-PCR were reproducible as demonstrated by TGGE. Partial sequences of the 16S rRNA were reversibly transcribed into cDNA, amplified by PCR and the products separated by high-resolution TGGE. The TGGE fingerprint (Fig. 2) reflects the diversity of dominant rRNA sequences of pooled soils from different sampling sites. Fingerprints from the same testfield were highly reproducible. Pooled samples from the same testfield, as presented in this study, appeared to be identical (Fig. 2). This indicates a high spatial constancy of the dominant bacteria in these grassland soils.

To determine whether one of the major bands could be affiliated to 16S rDNA of clone DA079, its plasmid insert was amplified with TGGE primers U968/GC and L1401. The migration distance of the fragment obtained from

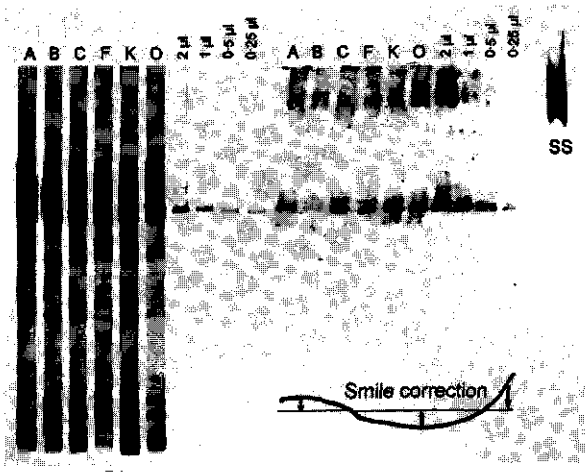

Fig. 3. Comparison between a silver-stained TGGE gel (left-hand side) and the second half of the same gel after an electrophoretic Southern blot and hybridization with the V6-DA079 probe (right-hand side). The DNA samples were loaded onto corresponding positions. RT-PCR products from ribosomes of the native bacterial community represent pooled samples from Drentse A sites A, B, C, F, K and O. Next to this, dilution series of the DA079 PCR product (2, 1, 0·5 and 0·25 µl) were applied. On top of the gel lanes, single-stranded DNA appears (position and size marked 'SS'). At this position the V6-DA079 probe gives hybridization signals as well. The 'smile correction' bar indicates an image correction carried out to compensate for distortions of the blot signals.

the DA079 clone was identical to one of the strongest bands obtained from amplified cDNA. Here the definition of total activity allows two scenarios for abundance and state of the bacterial cells: this species counts for high total activity within the community by only low activity per cell, but extraordinarily high cell number; or this species could show an extraordinarily high activity per cell, but only low cell number within the community. The TGGE fingerprints obtained from amplified genomic rDNA from soil (Fig. 2) point more to the first scenario. They indicate that sequence DA079 is not only one of the most abundant 16S rRNA but also 16S rDNA sequences. The relative abundance of DA079 in the rDNA fingerprints appears comparable to the 16S rRNA in the ribosomal RT-PCR fingerprints. Thus it seems likely that the prominent ribosome number from this species is caused more by the high number of active cells rather than by extraordinarily high activity per cell. The high reproducibility of the TGGE fingerprints further indicates a homogeneous distribution of this activity.

Confirmation of sequence DA079 by V6 probing

As even the high resolution of TGGE does not absolutely exclude the possibility that two different 16S cDNA sequences might migrate to exactly the same position in the gel, the authenticity of the two corresponding bands needed to be verified. A TGGE gel was symmetrically loaded, each half containing cDNA samples prepared from ribosomes of different sampling sites and dilutions of the PCR products obtained from 16S rDNA of clone DA079. One-half of the gel was stained with silver, while the material of the second half was blotted onto a membrane and hybridized with a DA079-specific, radioactively labelled probe (Fig. 3). Comparison of the signals of both approaches allows the conclusion that $97 \pm 5\%$ of the silver-stained fingerprint band was indeed composed of the DA079 16S rDNA fragment (Table 1).

The 95 nt probe used in Southern hybridization was derived from the 16S rDNA insert of clone DA079. Comparison of the probe sequence with the homologous region of clone sequences from highly related actinobacteria detected in a peat bog (Rheims *et al.*, 1996b) gave highest similarity values above 98% (data not shown). The question is raised whether the hybridization results indicate the presence of sequence DA079 in the ribosome fraction and rDNA clone library to the exclusion of other highly related actinobacterial sequences, as found in the peat bog clone library. Most likely, such sequences would migrate to different positions in the TGGE gel. It must be assumed that sequence DA079 is the only prominent member of this group in the ribosome fraction. Other highly related actinobacterial sequences might also be present in the minority population, but they are not detectable in the TGGE fingerprints.

Conclusions

This study suggests that the cloned sequence DA079 originated from one of the most active bacterial species in Drentse A grassland soils. The strength of the DA079 band in the TGGE fingerprints and the hybridization signal demonstrated a prominent abundance of this sequence within the ribosomal 16S rRNA and genomic 16S rDNA fractions from soil. Hence, it can be concluded that the as-yet-uncultured actinobacteria are a potentially important part of the native bacterial community in Drentse A grassland soil. This leads to the speculation that their role is similar in the other environments where their presence had been described previously. The 16S rDNA clone libraries generated in the past provided first indications of the enormous degree of prokaryotic diversity by revealing hitherto unknown sequences of unidentified organisms. After analysis of the cloned sequences we can now turn back to the environment to reveal the ecologically relevant

Table 1. Pixel volume quantification ratios for silver staining vs hybridization signals, respectively, soil fingerprint vs DA079

Lane	Image pixel volume for:		Ratio A/B (= C)	DA079 signal/soil fingerprint ratio (1·96/C)
	Silver staining (A)	Hybridization (B)		
A	114·12	57·74	1·98	0·99
B	55·54	22·59	2·46	0·80
C	116·92	59·65	1·96	1·00
F	86·92	40·65	2·14	0·92
K	93·15	51·25	1·70	1·15
O	114·32	54·67	2·09	0·94
Mean of all soil samples (SD)			2·06 (11)	0·97 (5)
2 µl	135·44	77·45	1·75	–
1 µl	99·05	50·83	1·97	–
0·5 µl	44·24	24·04	1·84	–
0·25 µl	20·35	9·00	2·26	–
Mean of all DA079 samples (SD)			1·96 (10)	–

organisms. Application of ribosome isolation, subsequent RT-PCR and separation of amplicons by TGGE, in combination with taxon-specific probing, leads to the identification of the metabolically dominant portion of the community. This allows us to more specifically discuss the composition of environmental microbial communities.

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Chapter 7

Prominent Occurrence of Ribosomes from an Uncultured Bacterium of the *Verrucomicrobiales*-Cluster in Grassland Soils

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Prominent occurrence of ribosomes from an uncultured bacterium of the Verrucomicrobiales cluster in grassland soils

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A. FELSKE AND A.D.L. AKKERMANS. 1998. An uncultured bacterium of the Verrucomicrobiales cluster was identified by its 16S rDNA sequence as a major bacterium in Dutch Drentse A grassland soils. Potential metabolic activity of the according organism was estimated by applying direct ribosome isolation from soil and partial amplification of the 16S rRNA via RT-PCR using bacteria-specific primers. Temperature gradient gel electrophoresis separated the amplicons sequence specifically into reproducible fingerprints. One of the fingerprint bands matched with the signal of clone DA101. Southern blot hybridization with a DA101-specific V6 probe confirmed sequence identity. It is the first time that an organism of the Verrucomicrobiales cluster has been indicated as a potential major metabolizer in environmental microbial communities.

INTRODUCTION

Recent molecular ecological studies on 16S rDNA from terrestrial environments have demonstrated the existence of a group of uncultured bacteria. Their presence was first reported in Australian forest soil (Liesack and Stackebrandt 1992). They were remotely related (approximately 85% 16S rRNA sequence similarity) to *Verrucomicrobium spinosum* (Ward-Rainey *et al.* 1995), fimbriate prosthecate bacteria isolated from a lake in Germany (Albrecht *et al.* 1987; Schlesner 1987), and to *Prosthecobacter* strains from different aquatic environments (Hedlund *et al.* 1996). Following these initial observations, more occurrences of such sequences have been reported. The sequences were detected in Japanese soybean fields (Ueda *et al.* 1995), in the activated sludge of Australian sequencing batch reactors (Bond *et al.* 1995), in agricultural soil from the USA (Lee *et al.* 1996), in forested soil from Amazonia (Borneman and Triplett 1997) and in mountain lakes in the USA (Hiorns *et al.* 1997). These molecular studies were based on the PCR-amplification of the SSU rDNA using universal primers on DNA that was directly extracted from the environment. The amplicons were used for the generation of SSU rDNA clone libraries and subsequent sequence analysis. Apparently, they represented a group of hitherto uncultured bacteria that is widely distributed and present in significant numbers in different environments.

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The 16S rDNA sequence DA101 was cloned from soil of the Drentse A agricultural test area (Netherlands). Its significance in the soil ribosome fractions was then investigated. The aim of these investigations was to detect the according organism within the metabolically active part of the soil bacteria. Genomic 16S rDNA could not be used for this approach because detection of the DNA would only reflect the presence of bacteria. The ribosome appeared to be more useful because it has been found for bacterial cultures that the amount of rRNA per cell was roughly proportional to growth activity (Wagner 1994). Hence, the approach using ribosomes should reflect the presence of active bacteria. Ribosomes were isolated from 360 soil samples and the 16S rRNA was partially amplified via RT-PCR using bacteria specific primers. Then, temperature gradient gel electrophoresis (TGGE; Rosenbaum and Riesner 1987) was performed to separate the amplicons sequence specifically. The amplicons were investigated for the presence of sequence DA101 by comparison on TGGE and a novel DNA probe hybridization approach applying highly specific V6 probes (Heuer and Smalla 1997).

METHODS

Soil sampling

Samples were taken from the peaty, acid grasslands of the Drentse A agricultural research area in the Netherlands (06°41′E, 53°03′N). The survey covered six test fields within

a stretch of about 1·5 km along the Anlooër Diepje River. Details of the soil properties have been published (Stienstra *et al.* 1994). In total, 360 undisturbed surface samples were taken in March and October of 1996. Soil cores of approximately 50 g were taken with a drill (0–10 cm depth) and transferred into sterile sample bags. The 40 samples from each plot were pooled to four samples by sieving and mixing 10 single samples (5 g input each).

Isolation of ribosomes, rRNA purification and amplification

Ribosomes were isolated from Drentse A soil samples following a previously described protocol (Felske *et al.* 1996). In short, ribosomes were released from 1 g of suspended soil by bead-beater treatment. Subsequent centrifugation cleared the suspension from cell debris and soil particles. The ribosomes were then precipitated by an ultra speed spin (2 h at 100 000 *g*); their rRNA was extracted and purified by phenol extraction, ethanol precipitation and DNase treatment. RT-PCR was performed using rTth DNA polymerase as described previously (Felske *et al.* 1997). The bacteria specific primers U968-GC and L1401, which were used in the reactions, covered about 430 bp of the bacterial 16S rDNA. In order to obtain accurate separation of the amplicons during gradient gel electrophoresis, a 40mer-GC-clamp was included in primer U968-GC (Muyzer *et al.* 1993).

Isolation, amplification, cloning and sequencing of 16S rDNA sequences

The procedure is the same as described previously (Felske *et al.* 1997). In short, total DNA was isolated from Drentse A soil samples by a parallel procedure during ribosome isolation (Felske *et al.* 1996). The amplification of 16S rDNA sequences was performed with primers 8f and 1512r, covering approximately 1·5 kbp of the bacterial 16S rDNA. Amplification products were purified, cloned into the plasmid pGEM-T, and sequenced on a Li-Cor Sequencer 4000l (Lincoln, USA). Clone DA101 was compared to the TGGE fingerprints from soil by amplifying the insert of DA101 pDNA with the same primers U968-GC and L1401. Serial dilutions of the PCR product (1 μl and twofold dilution steps) were used for subsequent TGGE analysis.

TGGE, Southern blot and hybridization with the clone-specific probe V6-DA101

V6-probes could be generated without previous sequencing of the target. The probe V6 DA101 was generated from DA101 pDNA via PCR with the bacteria specific primers V971 and R1057 as described previously (Felske *et al.* 1997). The primers encompassed the highly variable V6 region of the 16S rDNA that constituted the probe binding site. This PCR amplicon of approximately 90 bp was then purified and 5′-labelled with [γ-^{32}P]ATP.

The Diagen TGGE system was used for sequence specific separation of PCR products. The detailed protocol has been published previously (Felske *et al.* 1997). Samples—RT-PCR of soil rRNA and PCR of pDNA—were applied twice in symmetrical order. After electrophoresis, one half of the gel was used for silver staining and the other half, for Southern blotting. The blot membrane was hybridized with probe V6 DA101. Signals were detected with a Phosphor Imager SF (Molecular Dynamics, Hercules, USA) and quantification was performed with image analysis software ImageMaster 1D Elite V.2.0 (Pharmacia, Uppsala, Sweden).

Phylogenetic analysis of clone DA101

Alignment and phylogenetic analysis of the sequence were performed with the ARB software (Strunk and Ludwig 1995), providing an SSU rDNA database of 8000 sequences. The phylogenetic trees were constructed following maximum parsimony criteria with nearest-neighbour-optimization.

RESULTS AND DISCUSSION

Phylogeny of sequence DA101

Clone DA101 is one of two identical sequences from the Drentse A 16S rDNA clone library, comprising 165 positive clones (Felske *et al.* 1997). The partial 16S rDNA sequence of clone DA101 numbers 1528 nucleotides and is deposited in the EMBL database under accession number Y07576. The sequence DA101 falls into a distinct branch of bacterial 16S rRNA phylogeny, the Verrucomicrobiales cluster (Fig. 1). Here, four (L) sequences from Australian forested soil (Liesack and Stackebrandt 1992), five (U sequences from Japanese soybean fields (Ueda *et al.* 1995), two (B) sequences from activated sludge of Australian sequencing batch reactors (Bond *et al.* 1995), the sequence EA25 from agricultural soil in the USA (Lee *et al.* 1996), 11 (A) sequences from Amazonian forested soil (Borneman and Triplett 1997) and six (H) sequences from lake water in the USA (Hiorns *et al.* 1997) can also be found. The high similarity of sequence DA101 to clone M24 (up to 98·7% sequence homology) and to other clones indicates its authenticity. Thus, the presence of chimera structures within sequence DA101 is unlikely.

Identification of sequence DA101 in native ribosome fractions

The presence of sequence DA101 has been investigated in the ribosome fraction of the soil to demonstrate possible metabolic activity of the according organism. The approach

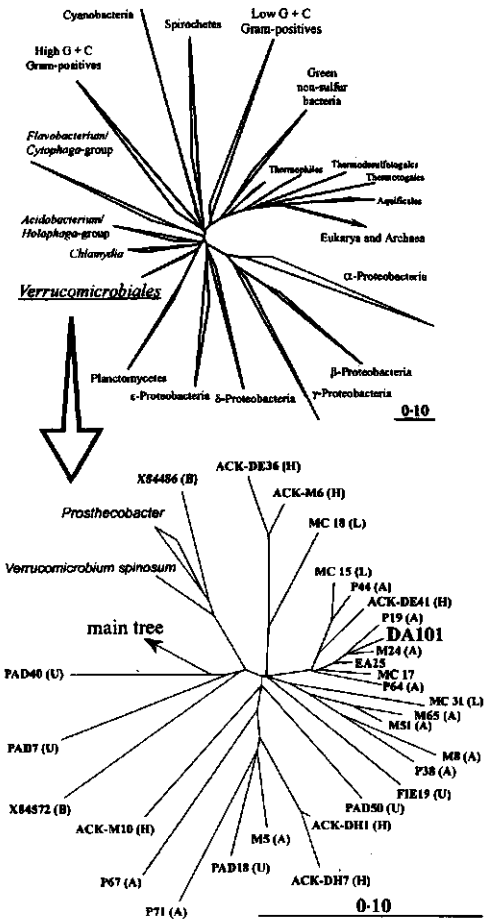


Fig. 1 Phylogenetic tree of almost 8000 SSU rRNA sequences within the ARB database and a zoom into the Verrucomicrobiales cluster. The bars in the lower right corners indicate the branch length representing 0·1 base substitutions per nucleotide

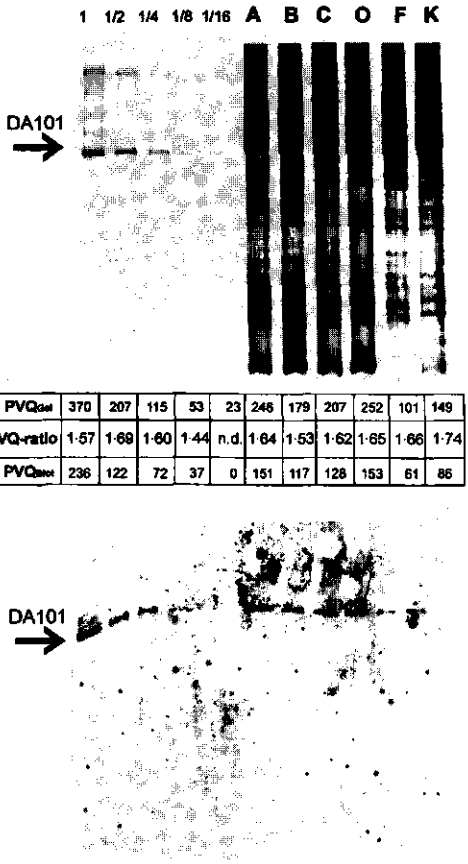
followed a strategy that has already been applied for a sequence related to the Actinobacteria (Felske *et al.* 1997). It was based on the assumption that the ribosome fraction from soil would better reflect metabolically active organisms than genomic DNA. A fingerprint of the most abundant 16S rRNA sequences was generated by RT-PCR and TGGE. Direct ribosome isolation (average yield: 1·5 ± 0·6 μg from 1 g soil), subsequent RT-PCR and TGGE were reproducible as demonstrated by a previous survey on spatial homogeneity of TGGE fingerprints from soil (Felske and Akkermans 1998). Almost identical fingerprints of samples from the same test field indicated a spatially constant distribution of the prevalent bacteria.

A match of sequence DA101 with one fingerprint band was found by amplifying the plasmid DNA with the same primers as those used for the ribosome fraction from soil, and running this product on TGGE next to the fingerprint. Subsequent Southern blotting and hybridization with a clone specific V6 probe were used to confirm the match. Both detection systems, silver staining and Southern blot hybridization could be applied in parallel by loading RT-PCR products from soil rRNA and dilution series of the DA101 product twice in symmetrical order on a TGGE gel. After TGGE, one half of the gel was used for silver staining, the other half for Southern blotting and hybridization (Fig. 2). After hybridization, the non-specific silver staining and the highly specific signals of the V6-probe were compared to ascertain that both approaches detected the same sequence. Quantification of signals was performed with image analysis software. The sequence DA101 and the matching fingerprint bands showed a constant ratio of signal strength (silver staining *vs* V6 probe, approximately: 1·6, Fig. 2). This particular band within the RT-PCR fingerprints from soil rRNA was identified as the partial sequence of clone DA101. It was the last band of a prominent band cluster that could always be found in TGGE fingerprints from all investigated test fields (Fig. 3). The intensity of the DA101 band in the TGGE fingerprints indicated a relatively high abundance of this sequence within the 16S rRNA from soil. Such semi-quantitative interpretations of amplification products could be misleading; abundant target sequences caused by mismatches in the primer binding sites or insufficient cell lysis could be missed. Although the universal bacteria primers for TGGE might have failed to amplify some important bacterial sequences, the prominence of one particular amplicon out of perhaps thousands of different target sequences (Torsvik *et al.* 1990) indicates the extraordinary abundance of this one specific rRNA molecule.

Conclusions

Muyzer *et al.* (1993) introduced the DGGE approach (which is comparable to TGGE) to molecular microbial ecology as a powerful alternative to the cloning of environmental sequence populations. We have found that this approach is enhanced when used in combination with parallel cloning and subsequent V6 hybridization. Southern blot hybridization with clone-specific V6 probes is suitable for proving the identity of a cloned sequence with particular bands in even very complex fingerprints of environmental sequences.

Our findings suggest that the cloned sequence DA101 originated from one of the most active bacterial species in Drentse A grassland soils. It is the first time that not only



PVQdet	370	207	115	53	23	246	179	207	252	101	149
PVQ-ratio	1·57	1·69	1·60	1·44	n.d.	1·64	1·53	1·62	1·65	1·66	1·74
PVQblot	236	122	72	37	0	151	117	128	153	61	86

Fig. 2 Comparison between a silver stained TGGE gel (upper part) and the second, identical half of the same gel after Southern blotting and hybridization with the V6 probe (lower part). RT-PCR products from ribosomes of the native bacterial community represented pooled soil samples from six Drentse A sites (A, B, C, O, F and K). Next to this, the DA101 PCR product (1 μl and subsequent twofold dilution) was applied. DA101 bands were quantified with image analysis software (PVQ = Pixel Volume Quantification values). The ratios between the values from the silver stained gel and the blot were calculated (PVQ ratio)

Fig. 3 Amplicon fingerprints on silver stained TGGE gels. The fingerprints represent the most different RT-PCR fingerprints obtained from the Drentse A area. All of them contained a prominent band cluster including sequence DA101

the presence of this unidentified group of bacteria has been observed in soil, but also, their metabolic significance also ascertained. These bacteria might be an important part of native bacterial communities in Drentse A grassland soil and possibly also in the other environments where they have been detected.

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Chapter 8

Quantification of 16S rRNA Levels from Uncultured Soil Bacteria of the *Holophaga/Acidobacterium*-Cluster

Andreas Felske, Willem M. de Vos, and Antoon D. L. Akkermans

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ABSTRACT

The activity of uncultured bacteria, represented by five cloned ribotypes of the *Holophaga/Acidobacterium*-cluster, was monitored in Dutch grassland soils by quantifying the numbers of their ribosomes g^{-1} soil. The importance of these bacteria was indicated by the relative abundance of 16S rRNA RT-PCR products in temperature gradient gel electrophoresis fingerprints. The levels of rRNA in soil samples were quantified with multiple competitive RT-PCR along a 1.5 km transect through the grassland. In total, five members of the *Holophaga/Acidobacterium*-cluster were estimated to contribute 4×10^{10} - 1×10^{11} ribosomes g^{-1} soil, representing 7 - 14% of all bacterial ribosomes. These results indicate that ribosomes from *Holophaga/Acidobacterium*-cluster are homogeneously distributed in soil and might contribute significantly to microbial activity in soil.

1. Introduction

Over the last years molecular ecological surveys on terrestrial environments have revealed the existence of monophyletic groups of uncultured bacteria. Based on analysis of PCR amplified 16S rDNA sequences, major clusters of bacteria have been identified that show only remote relation to cultured bacteria [3,4,16,24]. Recently, a more precise analysis on the phylogenetic affiliation of one of the most important groups of uncultured soil bacteria demonstrated that the *Holophaga/Acidobacterium-cluster* is an independent, major bacterial taxon on its own [19]. It appeared, that similar approaches of 16S rDNA cloning from environmental samples all over the world already had resulted in numerous related sequences in Australian forest soil [16], Japanese soybean field soil [29], mountain lakes in the United States [12], soils from Arizona [14], Amazonia [4] and the Netherlands [8]. However, the number of isolated bacterial strains affiliated to this cluster is very limited and only includes *Acidobacterium capsulatum* [13], *Holophaga foetida* [17] and "*Geothrix fermentans*" [18]. Moreover, these species were remote relatives of separate position within the phylogeny of this cluster [19].

A well-studied environmental bacterial community is located in the Dutch Drentse A agricultural research area. These grassland soils were the subject of various ecological studies [2,22,27], including molecular microbial approaches [6,7,8,9]. Since many different ribotypes of hitherto uncultured bacterial lines of descent were identified here, it is not surprising that also members of the *Holophaga/Acidobacterium-cluster* were found [8]. Moreover, multiple appearance of identical sequences in a 16S rDNA clone library already indicated an extraordinary abundance of these organisms. The predominant but uncultured bacteria in this area were not only revealed by their 16S rDNA sequences but also by the prominent appearance of their sequences in RT-PCR products from ribosomal RNA. The original template rRNA amounts in soil were quantified to assess the spatial distribution of the ribosomes of different bacteria from the *Holophaga/Acidobacterium-cluster* and their contribution to the total bacterial ribosome pool.

2. Materials and methods

Soil sampling. The Drentse A agricultural research area in the Netherlands (06°41'E, 53°03'N) is a grassland stretch of approximately 1.5 km along the Anlooër Diepje brook [27]. Six sampling sites were selected in distances of approximately 300 m. From each sampling site two parallels were taken, each prepared by pooling four mixed samples of 5 - 15 m distance. The mixed samples again consisted of five 50 g soil cores, which were taken with a drill (0-10 cm depth) and transferred into sterile sample bags. Pooling of samples was done by sieving and mixing single samples (5 g input each).

Preparation of ribosomal RNA. A 20 ml batch culture of *Escherichia coli* NM 522 (Promega, Madison, USA) was grown for 16 h at 37°C and used for rRNA extraction as previously

described [10]. The amount of extracted rRNA was estimated spectrophotometrically. A solution of 1 µg rRNA per ml and subsequent twofold serial dilutions were prepared in Glycerol-Tris buffer (50% Glycerol, 10 mM Tris-HCl, pH 8.0) and used as standards for subsequent multiple competitive RT-PCR experiments.

The soil rRNA was achieved via ribosome isolation from Drentse A soil samples following a previously described protocol [5]. In short, cell lysis was performed with 1 g of soil by bead-beater treatment in the presence of ribosome buffer. Differential centrifugations were applied to clear the ribosome suspension from soil residues and finally to precipitate the ribosomes. After rRNA purification the yield of bacterial rRNA per g soil was estimated with the *Bacteria*-specific EUB338 probe [1]. The procedure has been published by Manz et al. [20]. Solutions of rRNA were prepared in Glycerol-Tris buffer and used as templates for subsequent competitive RT-PCR experiments. The rRNA was prepared in a final volume of 100 µl that represented 10 mg soil μl^{-1}.

Multiple competitive RT-PCR. The RT-PCR was performed with rTth DNA Polymerase (Perkin Elmer-Cetus, Norwalk, Conn.). Five reactions per multiple competitive RT-PCR assay were prepared as described before [10], including primer pair L1401 and U968-GC [21] 3 µl template RNA (2 µl *E. coli* rRNA and 1 µl soil rRNA). Each of the five reactions contained different amounts of *E. coli* rRNA (2 ng, 1 ng, 0.5 ng, 250 pg, 125 pg). Amplification was performed in a GeneAmp PCR System 2400 thermocycler (Perkin Elmer Cetus), using 35 cycles of 94°C for 10 s, 56°C for 20 s and 68°C for 40 s.

The Diagen TGGE system (Diagen, Düsseldorf - Germany) was used for sequence-specific separation of competitor amplicons after RT-PCR. Temperature gradient gel electrophoresis [25] took place in a 0,8 mm-polyacrylamide gel (6% w/v acrylamide, 0,1% w/v bis-acrylamide, 8 M urea, 20% v/v formamide, 2% v/v glycerol) with 1 × TA buffer (40 mM Tris-Acetate, pH = 8,0) at a fixed current of 9 mA (around 120 V) for 16 h. A temperature gradient was built up in electrophoresis direction from 37°C to 46°C. After the run gels were silver-stained [26]. Gels were analysed with image analysis software MolcularAnalyst/PC fingerprinting software (Bio-Rad, Hercules, USA). In each of the multiple competitive RT-PCR assays the lane was selected were the concerning environmental ribotype appeared to be most similar to the *E. coli* standard. The *E. coli* standard has previously been demonstrated to equally co-amplify with environmental sequences like the ones from the *Holophaga/Acidobacterium*-cluster [10]. Pixel volumes of the band-images were quantified (PV) and the original rRNA amount (M) of the environmental ribotype was calculated with these values: $M_R = PV_R \times PV_{E.coli}^{-1} \times M_{E.coli}$. Since 1 µl soil rRNA input represented 10 mg original soil sample, the rRNA content g^{-1} soil could be calculated. Also the ribosome content in soil could be calculated because one ribosome contains approximately $2,5 \times 10^{-12}$ µg rRNA (more or less 4,500 nucleotides).

3. Results and Discussion

Molecular surveys on bacterial diversity in soil frequently yielded a considerable amount of sequences from the *Holophaga/Acidobacterium*-cluster in 16S rDNA clone libraries (Tab. 1). In the Drentse A grassland study of 165 clones 12 sequences could be related to this cluster (including redundant clones) [8]. These results already indicated the importance and relative abundance of the *Holophaga/Acidobacterium*-bacteria, but their appearance in a clone library did not allow any conclusions about the abundance, activity or spatial distribution of their cells.

field site	ref.	H/A-clones	all clones
Mount Coot-tha forested soil, Australia	[16]	**8**	113
Agricultural soybean field soil, Japan	[29]	**5**	17
Arlington agricultural soil, USA	[3]	**20**	124
Eastern Amazonia forested soil, Brazil	[4]	**6**	100
Roggenstein agricultural soil, Germany	[19]	**36**	144
Sunset Crater/Cosnino woodland soils, USA	[14]	**31**	60
Drentse A grassland soil, Netherlands	[8]	**12**	165

Tab. 1. Appearance of *Holophaga/Acidobacterium* (H/A)-clones in recent studies.

The TGGE fingerprinting of 16S rRNA amplicons is an excellent tool to search for spatial distributions of bacterial communities [7]. This method also provides quantitative information to measure spatial shifts of bacterial activity in soil by using the multiple competitive RT-PCR approach [10]. The Drentse A grassland study showed five ribotypes of the *Holophaga/Acidobacterium*-cluster that could be matched to TGGE fingerprint bands (Fig. 1). These TGGE fingerprints represented amplicons of 16S rRNA via RT-PCR with *Bacteria*-specific primers on directly extracted soil rRNA. Here we found that ribotype DA054 gave one of the strongest bands, apparently representing one of the most important bacteria in Drentse A grassland soils. The ribotypes DA022 and DA040 were matching to bands of medium intensity, while the ribotypes DA008 and DA052 could be related to faint bands. A multiple competitive RT-PCR assay was used to quantify the original rRNA amounts in the soil samples. The principle of this method is to apply an *E. coli* standard of known rRNA concentration to the soil rRNA, then to perform the (multiple competitive) RT-PCR and finally to separate the amplicon sequence-specifically on TGGE. Careful analysis of the multiple competitive RT-PCR revealed that signals from the decreasing *E. coli* standard concentrations were fading relatively soon (Fig. 1). Hence, the detection range for the multiple competitive RT-PCR, or for TGGE-fingerprints in general, remained relatively narrow. The difference in original template 16S rRNA molecule amounts from either the most intense or the faintest TGGE signals was found to be 10 - 100-fold. The few dozen bands detectable in the TGGE fingerprints clearly do

not reflect all present ribotypes. Indications that there might be several thousand different bacterial genomes in soil have been found even in single samples of a few gram [28]. Even if this was only true in part for the Drentse A soils, there must have been a much higher diversity of ribosomes in Drentse A soils. Most likely, countless minor ribotype populations disappeared in the TGGE fingerprint background, somewhere between faintest band and invisibility. Therefore, the TGGE fingerprints of bacterial 16S rRNA from Drentse A grassland soils could only be considered as diversity indicator for the fraction of predominant bacterial ribotypes. On the other hand, the accuracy of the rRNA quantification via multiple competitive RT-PCR greatly benefited from this narrow detection range.

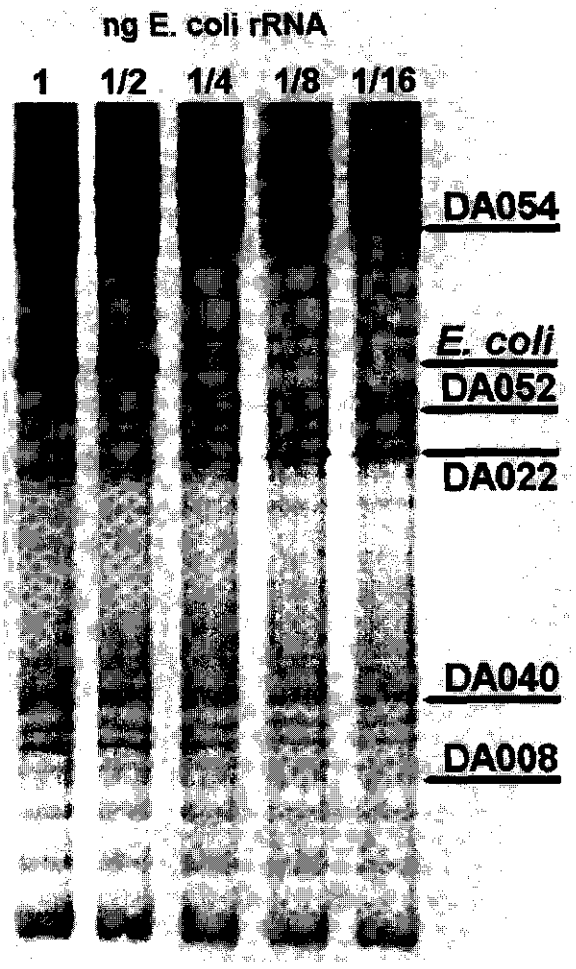

Fig. 1. Multiple competitive RT-PCR of rRNA from a soil sample of site 6 resolved by TGGE and detected by silver staining. The five ribotypes selected for quantification are marked. Quantification was performed with image analysis software. Each sequence was quantified in that lane where the according *E. coli* signal was of most similar intensity, in relation to the rRNA amount represented by this *E. coli* signal and the amount of soil represented by the soil rRNA template.

The computerized analysis of scanned gel images is a routine technique to compare band intensities. Band definition and background subtraction are commonly critical steps during image analysis, and this is especially true for our complex environmental fingerprints. The quantitative comparison of TGGE bands could only be performed with signals of similar intensity. Within a multiple competitive RT-PCR assay of five reactions (Fig. 1) the *E.coli* rRNA input was reduced by factor of two from lane to lane. The resulting signal was the most intense band of the whole fingerprint at 1 ng input (Fig. 1, lane 1), indicating that all other, weaker TGGE signals of soil 16S rRNA originated from templates of less than 100 ng rRNA g^{-1} soil (sampling site 6). This was not true for sampling sites 9 and 10, where an additional reaction of 2 ng *E.coli* rRNA input had to be added. The reaction with 16-fold reduced *E.coli* rRNA input produced only a very faint band, almost at the lower detection limit of the applied image analysis system. The advantage of this quantitation method is the high specificity. In principle, the sequence-specific separation by TGGE allows to analyse single ribotypes without the use of highly specific primers and without knowledge of the sequence. In contrast, hybridization techniques with oligonucleotide probes *in situ* in soil [19] or on extracted soil rRNA [5] demand for extensive specificity tests, and even then aspecific reactions can never be excluded.

The multiple competitive RT-PCR may suffer from the common problems of quantitative PCR. The standard, in our case *E. coli* rRNA, must be prepared, adjusted and maintained with extreme care. The used *E. coli* rRNA standard has previously been demonstrated to be suitable for the environmental ribotypes from Drentse A soil and a taxonomically broad range of cultured bacteria [10]. There the *E. coli* standard sequence has been checked for equal amplification efficiency with the target sequences, with respect to primer annealing and DNA polymerization. Another, TGGE-specific, problem is that of sequence singularity. Especially in complex fingerprints, the question must be raised if one band is indeed composed of one sequence or maybe of two or even more completely different ribotypes that accidentally show identical migration speed. In case of doubt, Southern blot hybridization might be applied, for instance using the convenient V6-probes [6, 11]. V6-hybridization has been applied successfully for the ribotypes DA022, DA040 and DA054, but the faint DA008 and DA052 signals gave no clear results [8].

The estimated specific rRNA yields for the five ribotypes ranged between 1 and 135 ng rRNA g^{-1} soil, representing approximately 0.1 - 6% of all bacterial rRNA (Fig. 2). The total bacterial rRNA yields from Drentse A grassland soil samples were estimated by direct dot blot hybridization with the *Bacteria*-specific EUB338 probe. For the most important ribotype DA054 (Fig. 1) we found a quite homogeneous distribution on all sampling sites. Here we estimated a mean of 69.8 ± 15.4 ng rRNA g^{-1} soil, scoring for 4.6 ± 1.1% of all bacterial rRNA. Also ribotype DA022 gave a relatively high score with 2.1 ± 0.8% of all bacterial rRNA. Indeed, ribotypes DA054 and DA022 represented some of the most important organisms in Drentse A grassland soils. Another important ribotype was DA040, who scored 2.2 ± 0.8% of all bacterial rRNA in sampling points 3 - 12, but only 0.4% in sampling points 1 and 2. The

latter gave TGGE fingerprints that showed a reduced DA040 signal and were previously defined as type K fingerprints [8]. The standard deviations of the presented data are not mainly caused by variable TGGE patterns found in multiple competitive RT-PCR. In fact, the TGGE fingerprints from the Drentse A area showed an almost perfect reproducibility and surprisingly high similarity over the 1.5 km-stretch along the Anlooër Diepje brook [7]. The data variation was caused by the rRNA yields g^{-1} soil, which showed a considerable standard deviation (Tab. 2).

The considerable standard deviation of rRNA yields (Tab. 2) directly pointed to one of the major drawbacks of multiple competitive RT-PCR. The rRNA, respectively ribosome extraction from environmental samples could not be expected to be quantitative. The applied protocol might result in the loss of approximately half of all ribosomes during extraction [5]. The isolation efficiency of nucleic acids from environmental samples is a general problem of molecular microbial ecology, especially for such recalcitrant matter like soil. Therefore, the absolute yields in ng rRNA g^{-1} soil should not be stressed too much, the relative yields, given as part of the total rRNA, might be more reliable. The yielded rRNA amounts corresponded to up to 10^{12} ribosomes g^{-1} soil (approximately 2,5 µg rRNA). It was hard to estimate the original cell numbers by rRNA yield because the rRNA content per cell might vary a lot, depending on the stage of activity [30]. Therefore, the information retrieved from rRNA is different than from genomic DNA. The pool of *rrn* operons encoding the rRNAs qualitatively reflects the composition of the bacterial community by the present species, but this is not directly dependent on the stage of activity of the single species. Quantifying the amounts of *rrn* operons could not give very useful information since it could not be related to cell numbers [15]. This is mainly due to the wide variation of *rrn* operon numbers per genomic unit among bacteria [23]. More informative is the measurement of ribosome amounts by their rRNA. Ward *et al.* [31] supposed that the abundance of ribosomes in the environment should be a species-dependent function of the number of individual cells and their growth rates.

This survey provides the first insights into the specific proportions of bacterial activity in soil and how far members of the novel *Holophaga/Acidobacterium*-cluster might contribute to general microbial activity. This study also demonstrates major, quite homogeneously distributed amounts of ribosomes from these organisms in soil, suggesting that these uncultured bacteria are among the most important metabolizers in soil. It may be speculated that this is also the case in all the other soils where the *Holophaga/Acidobacterium*-cluster already showed prominent abundance (Tab. 1). For more detailed studies of their activity it will be of outmost importance to grow these organisms in pure culture. Although this is not achieved yet, our study demonstrated that these organisms are not recalcitrant resting stages or non-viable starving residues. They are very abundant and active in soil and therefore it should be possible to isolate them from the environmental matrix. The challenge is now to find the right culture conditions for them.

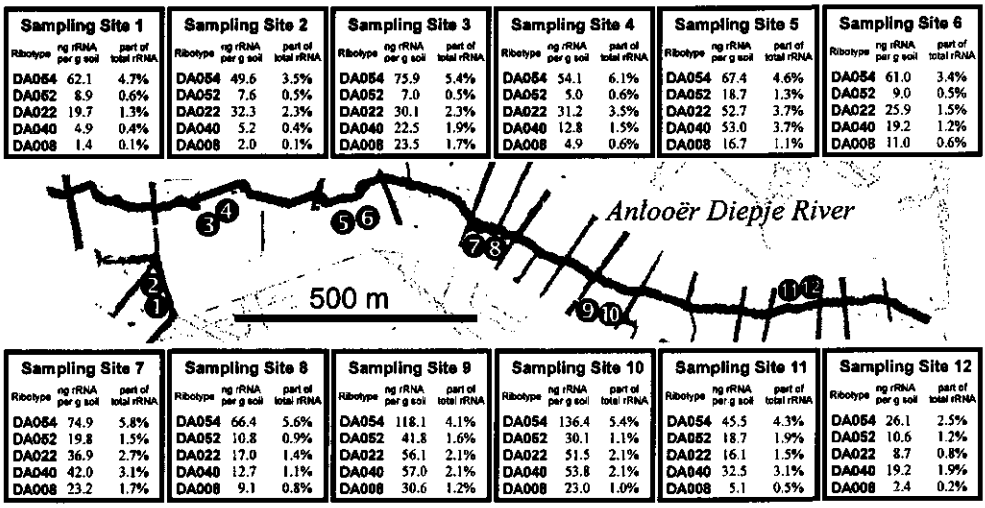

Fig. 2. Relative geographic location of the 12 sampling sites along a 1.5 km stretch of the Anlooër Diepje River. The average multiple competitive RT-PCR results for the five investigated ribotypes from each sampling site are given in the boxes. Also their part of total bacteria rRNA, as estimated by EUB338 dot blot hybridization, is presented as percentage value.

Drentse A Sampling Site	EUB338-count: total rRNA yield in ng per g soil (SD)	rRNA yield of the five H/A ribotypes in ng per g soil (SD)	10^{10} ribosomes of the five H/A ribotypes per g soil (SD)	part of all five H/A ribotypes of the total bacterial rRNA
1	1426(180)	97(1)	3.9(1)	6.8%
2	1355(176)	106(10)	4.2(4)	7.8%
3	1409(561)	159(18)	6.4(7)	11.3%
4	872(91)	108(19)	4.3(8)	12.4%
5	1719(479)	208(49)	8.3(2.0)	12.1%
6	1657(410)	126(28)	5.0(1.0)	7.6%
7	1345(146)	197(64)	7.9(2.6)	14.6%
8	1292(160)	116(50)	4.6(2.0)	9.0%
9	2488(570)	304(55)	12.2(2.2)	12.2%
10	2569(670)	295(60)	11.8(2.4)	11.5%
11	1120(272)	118(54)	4.7(2.2)	10.5%
12	1172(307)	107(47)	4.3(1.9)	9.1%

Tab. 2. Compilation of multiple competitive RT-PCR results for all five ribotypes and comparison to the total bacteria rRNA yield, as estimated by EUB338 dot blot hybridization.

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Chapter 9

Specific Response of a Soil Bacteria Community to Grassland Succession as monitored by 16S rRNA Levels of the Predominant Ribotypes

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ANDREAS FELSKE*, ARTHUR WOLTERINK, ROBERT VAN LIS, WILLEM M. DE VOS AND ANTOON D. L. AKKERMANS

ABSTRACT

The shifting composition of soil bacteria community during grassland succession was investigated in the Dutch Drentse A area. Six meadows, taken out of agricultural production at different time points, represented different stages of grassland succession. Since the stop of fertilization and agricultural production the six plots showed a constant decline of nutrients and vegetation changes. The predominant bacteria had been revealed previously by sequencing of cloned 16S rDNA from soil. Here their activity was monitored by direct ribosome isolation from soil and temperature gradient gel electrophoresis of RT-PCR products generated from bacterial 16S rRNA. The 16S rRNA amounts g^{-1} soil of 20 predominant ribotypes were monitored via multiple competitive RT-PCR in six plots of different succession stage. These ribotypes mainly represented *Bacillus*, members of the *Holophaga/Acidobacterium*-cluster and α-Proteobacteria. The 20 monitored 16S rRNA molecules represented approximately half of all bacterial soil rRNA. The grasslands showed highly reproducible shifts of bacterial ribotype composition. The relative proportion of some bacteria decreased while others increased their rRNA levels during progressing succession. The total bacterial ribosome level increased during the first years after agricultural production and fertilization stopped. This correlated with the collapse of the dominant *Lolium perenne* population and an increased rate of mineralization of organic matter. The results indicate a true correlation between the total activity of the bacterial community in soil and the amount of their ribosomes.

The agricultural overproduction of the last decades in Western Europe resulted in the release of more and more land from agricultural management and many meadows providing hay for cattle feeding were left as unfertilized grassland. The further management of these areas aimed to restore the former species-rich vegetation by non-extensive hay making of moderate frequency. A well-studied model system to follow this process is the Drentse A agricultural research area. These grasslands represent different stages of real successional changes, since plots of same quality were taken out of production at different time points (3). A constant reduction of nutrient matter in the soil was driven by the vegetation, since limiting nutrients like nitrogen, potassium or phosphate were removed with the biomass during the yearly cutting. This process induced unfavorable conditions for fast-growing species with high nutrient demand, like *Lolium perenne*. With the decline of this dominant high-yield vegetation, an increasing diversity of plant and animal species could be observed (17). All these effects were documented by studying the successional changes of the plant community composition, but the impact of this process on the bacterial community in soil remained widely unexplored. In one study a reduction of culturable ammonium-oxidizing bacteria during grassland succession was observed, which was correlated to the reduced availability of nitrogen in the soil (24). However, the effect of the grassland succession on the bacterial community in general remained unclear, since a vast majority of soil bacteria must be considered as unculturable (2). For instance, in soils all over the world bacteria of the *Holophaga/Acidobacterium* cluster are abundantly detected by molecular markers (12), but remain uncultured. Such bacteria can be detected by extracting nucleic acids directly from soil samples and identifying the nucleotide sequences of PCR-amplified 16S rRNA genes (27). The predominant bacteria in Drentse A grasslands were previously identified by the main bacterial 16S rRNA sequences in these soils (10), mainly representing *Bacillus*-related organisms, α-Proteobacteria, the *Holophaga/Acidobacterium* cluster (12), *Verrucomicrobiales* (28) and uncultured peat-Actinobacteria (19). In this study, the shifts of the bacterial community were monitored on the one hand at the level of major bacteria taxa with quantitative dot blot hybridization (22). On the other hand, the specificity of quantification was increased to a resolution of specific 16S rRNA sequences by applying multiple competitive RT-PCR (11) to monitor shifts of the predominant ribotypes.

MATERIALS AND METHODS

Field site.

The Drentse A agricultural research area in the Netherlands (06°41′E, 53°03′N) is a stretch of grassland meadows on a glacial sand plain along the Anlooër Diepje brook. The soil is a loamy fine sand of peaty appearance, normally quite wet and with high content of organic matter, usually 10-15% (24). The climate is Atlantic with a mean annual temperature of 8.5-9.0°C and 800-850 mm rain $year^{-1}$ (5). Soil humidity and pH were estimated as described by Stienstra et

al. (24). The water content was quite variable over the fields, ranging from 16-37%, with a total mean of 28% ± 7%. The pH of the soils was between 4.2 and 4.9. Six sampling sites were selected in distances of approximately 300 m. From each sampling site four parallels were taken, each prepared by pooling two mixed samples of 10 m distance. One mixed sample again consisted of five 50 g soil cores that were taken with a drill (0-10 cm depth) in 1 m-distances and transferred into sterile sample bags. Pooling of samples was done by sieving (2 mm mesh) and mixing single samples (5 g input each). In the history of the Drentse A grasslands three different periods have been distinguished (16). These periods have been characterized by changing hay making and fertilization practices. Until the 1930's, these grasslands showed a species-rich vegetation and were cut once or twice a year for hay production without application of chemical fertilizers. At that time the agricultural use was intensified by increasing the cutting frequency and raising the hay productivity by applying artificial mineral fertilizers. This process lead to a domination of high-yield grass species and an overall decrease of species richness. In the late 1960's and following decades part of the land was released from agricultural production to restore the former species-rich vegetation. The Dutch State Forestry Commission, as the new tenant of the released meadows, recorded the further fate of the single plots and it was assured that hay was taken off only once a year without any fertilizer application. Today, different sequences of this succession can be observed, since over the years and decades more and more plots were added to this restoration management. At the time of sampling, the plots selected for this study were still fertilized (1997) or taken out of production in the years 1991, 1990, 1985, 1972 and 1967, respectively. From long-term observations of vegetation and soil properties on permanent plots it is known that these plots indeed represent the temporal successional sequence (3).

Preparation of ribosomal RNA.

Several rRNA standards are prepared by rRNA extractions from laboratory cultures using the following strains: *Arthrobacter atrocyaneus* DSM 20127, *Bacillus benzoevorans* DSM 6385, *Escherichia coli* NM 522, and *Rhizobium meliloti* DSM 1981. All strains were grown in culture as described by the distributors (DSMZ, Braunschweig, Germany; Promega, Madison, Wis.) and used for rRNA extraction as previously described (11). The amount of extracted rRNA was estimated spectrophotometrically. A solution of 1 μg rRNA per ml and subsequent twofold serial dilutions were prepared in Glycerol-Tris buffer (50% Glycerol, 10 mM Tris-HCl, pH 8.0) and used as standards for quantitative dot blot hybridization or multiple competitive RT-PCR (only *E. coli*).

The soil rRNA was prepared by ribosome isolation from Drentse A soil samples as previously described (6, 9). In short, the bacteria in 1 g of soil were lysed in ribosome buffer by bead-beater treatment. Differential centrifugations cleared the ribosome suspension from soil particles, humic acids contaminations, and cell debris. After precipitation of the ribosomes by ultracentrifugation the rRNA was purified by DNase digestion, phenol extractions, and ethanol

precipitations. Solutions of rRNA were prepared in Glycerol-Tris buffer in a final volume of 100 µl, representing 10 mg soil μl^{-1}.

Quantitative Dot Blot Hybridization.

Taxon-specific quantification of rRNA was done by dot blot hybridization on soil rRNA and according rRNA standards for *Bacteria*, α-Proteobacteria, high and low G+C Gram positives from pure cultures (see above). The soil rRNA was prepared from 24 pooled soil samples (4 per plot). The total amounts of bacterial rRNA g^{-1} soil has been estimated with the *Bacteria*-specific EUB338 probe (1). The mean values were the 100%-reference to calculate the multiple competitive RT-PCR data and the group specific probes signals as a part of it. The probe ALF1b was applied to quantify rRNA of α-Proteobacteria (13). Probe HGC was specific for High G+C Gram positives (25). The LGC-b probe has been applied to quantify Gram-positives with low content of G/C nucleotides (14). Dot blot hybridization experiments were performed on nylon membranes (HybondN+, Amersham, Slough, England). Following a standard protocol (21) 10 µl rRNA per dot were applied and immobilized by baking 30 min at 120°C. Oligonucleotide probes were 5'-labelled using phage T4 polynucleotide kinase (Promega) and 30 µCi of [γ-^{32}P]ATP (3000Ci/mmol; Amersham). Prehybridization, hybridization and stringent washing steps were performed as described by Manz et al. (13) or by Mayer for the LGC-b probe (14).

Multiple competitive RT-PCR.

The multiple competitive RT-PCR was performed as previously described (11). In short, five RT reactions per multiple competitive RT-PCR assay were prepared. They were all containing the same amount of soil rRNA (representing 10 mg soil) but different, known amounts of *E. coli* rRNA standard. A joint master mix for five RT reactions was prepared, 5 µl soil rRNA was added and this mixture was divided to five reaction tubes. The *E. coli* rRNA standards were added and the RT-PCR (35 cycles) was performed with the primers L1401 and U968-GC (15). The *E. coli* standards have previously been demonstrated to equally co-amplify with the Drentse A sequences by using these primers (11).

The Diagen TGGE system (Diagen, Düsseldorf, Germany) was used for sequence-specific analysis by temperature gradient gel electrophoresis (20) after multiple competitive RT-PCR as described previously (7). The silver-stained gels were analysed with image analysis software MolcularAnalyst/PC fingerprinting software (Bio-Rad, Hercules, USA). The *E. coli* bands and the according environmental ribotypes of most similar signal strength were quantified by estimating the pixel volumes (PV) of the band images. The original rRNA amount (M) of the environmental ribotypes (R) was calculated: $M_R = PV_R \times PV_{E.coli}^{-1} \times M_{E.coli}$. Since the soil rRNA input per reaction represented 10 mg original soil sample, the individual rRNA amounts g^{-1} soil could be calculated. The absolute rRNA values were transformed to relative quantities to overcome rRNA extraction bias (11), because the applied ribosome isolation method was expected not to release all present ribosomes from soil (6).

RESULTS

Quantitative dot blot hybridization.

The *Bacteria*-specific EUB338 probe was used to quantify bacterial rRNA g^{-1} soil (dry weight) in the six plots representing different stages of grassland succession (Tab. 1). Significantly higher rRNA yields from the 1991-plot and also the slightly increased yields from the 1990- and the 1985-plot. The hybridization experiments also gave general indications for the most active bacterial groups in Drentse A grassland soils. In comparison to the *Bacteria*-specific EUB338 probe the Firmicutes with low G+C-content were detected as the dominant major taxon by the LGC probe. Approximately half of all bacterial ribosomes in Drentse A grassland soils appeared to be from this taxon (Tab. 1). Calculated as a part of the EUB338-signal, the ALF1b probe for α-Proteobacteria and the probe HGC for Firmicutes with high G+C-content each detected approximately 20% of all bacterial ribosomes. The results from the different plots showed slight but not significant differences within the ratio of the ALF1b, HGC and LGC probe signals (Tab. 1). Clear grassland succession tendencies could not be identified on this taxonomic level.

	Plot 1997	Plot 1991	Plot 1990	Plot 1985	Plot 1972	Plot 1967
Bacteria rRNA g^{-1} soil	1.1 µg (0.3)	2.5 µg (0.6)	1.6 µg (0.5)	1.7 µg (0.4)	1.1 µg (0.5)	1.3 µg (0.2)
α-Proteobacteria	18.2% (11.4)	19.2% (8.2)	29.4% (6.1)	26.1% (5.2)	20.0% (4.0)	20.8% (5.4)
LGC Firmicutes	47.1% (14.4)	56.6% (2.9)	40.0% (5.4)	44.3% (11.2)	53.0% (7.5)	50.3% (15.3)
HGC Firmicutes	13.1% (2.6)	22.4% (3.1)	14.9% (2.8)	20.6% (4.2)	25.4% (9.0)	18.6% (7.7)

Tab. 1. Quantitative dot blot hybridization results: The first data row shows the bacterial rRNA amount g^{-1} soil. The values of the taxon-specific probes below were calculated as a part of the total bacterial rRNA yield (in % of the EUB338 probe results). The standard deviation is shown in parenthesis.

Multiple competitive RT-PCR.

Since no obvious response to grassland succession was observed at the level of major bacterial taxa, the distribution of the main ribotypes was determined sequence-specifically by multiple competitive RT-PCR for TGGE fingerprints (11). The 20 most intense signals of known sequence identity (10) were selected (Fig. 1).

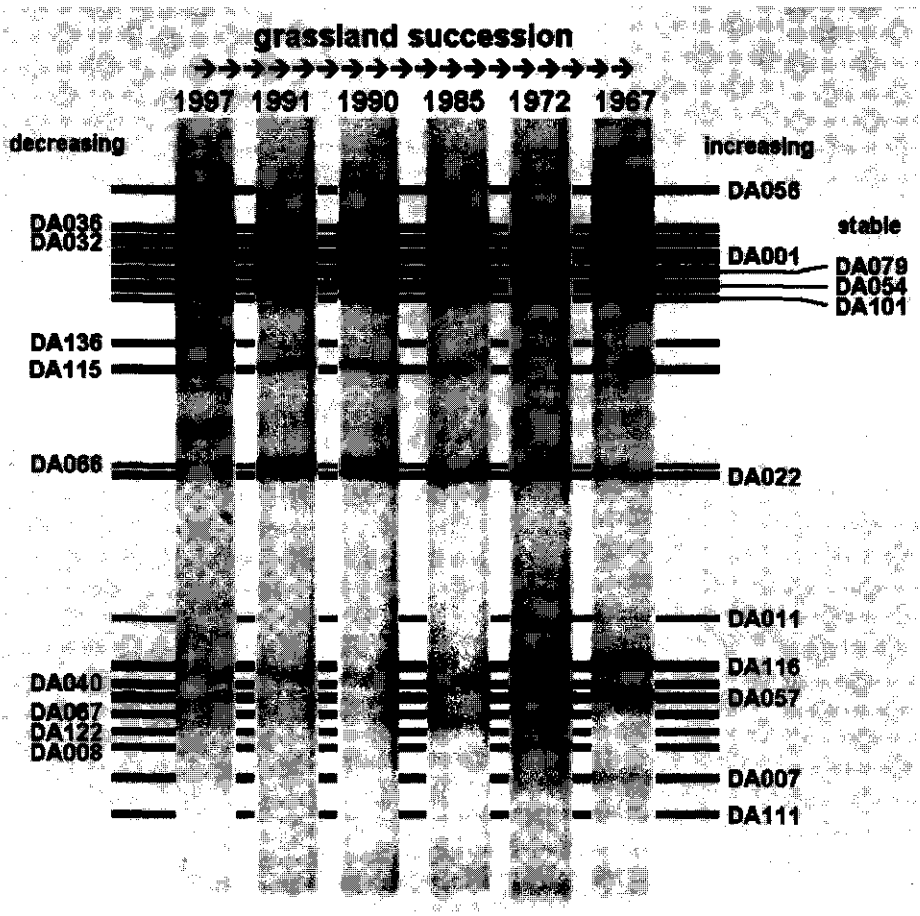

Fig. 1. Representative TGGE fingerprints for the six plots in the artificial temporal order from the initial situation on plot 1997 to the advanced succession stage of plot 1967. The ribotypes, which are decreasing during succession, are indicated on the left-hand side, the increasing ones on the right-hand side. Three ribotypes remained stable without obvious relative changes.

Since the 20 most intense bands appeared with high reproducibility in the different samples of one plot (8), the preparation of four pooled samples was sufficient for each plot. The absolute rRNA quantities g^{-1} soil of those 20 most prominent sequences were determined and found to represent approximately 50% of all bacterial rRNA, as quantified by the EUB338 probe (Fig. 2). Here the data indicated that within the first years after fertilization has stopped, the bacterial rRNA amount increased about twofold and subsequently dropped down again.

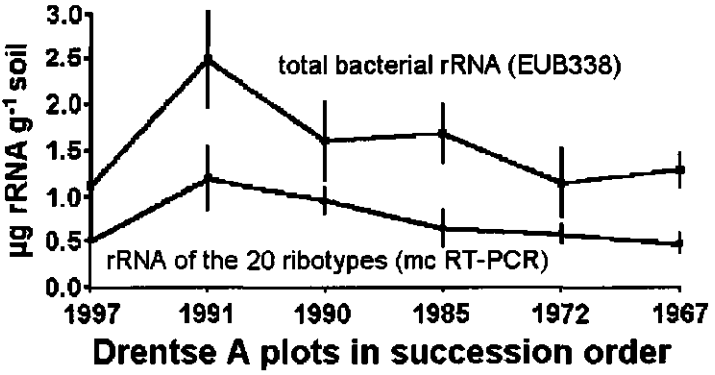

Fig. 2. Comparison of rRNA quantification by dot blot hybridization and multiple competitive RT-PCR. The upper graph shows the total bacterial rRNA yield g^{-1} soil as estimated by dot blot hybridization with the EUB338 probe. The lower graph represents the sums of the single values for the 20 ribotypes as calculated by multiple competitive RT-PCR. The vertical bars indicate the standard deviation.

The individual responses of the ribotypes gave a more specific picture (Fig. 3a-e). All individual rRNA amounts were normalized to the value of 1997 to highlight the specific tendencies. Their particular rRNA level changes allowed the definition of five categories. The first category includes the signals of increased intensity in the TGGE fingerprints of the 1967-plot. These were the ribotypes that increased in the latest stage of succession (Fig. 3a). This positive response was demonstrated for the α-Proteobacterium DA007 and four *Bacillus*-like ribotypes. The second category of ribotypes only showed an intermediate positive tendency followed by a lasting high level (Fig. 3b). Here we find one representant each of the prominent taxa *Bacillus*, α-Proteobactea and the *Holophaga/Acidobacterium* cluster. Three of the strongest TGGE-bands represented the third group of signals, remaining with the similar relative intensity in all fingerprints, not clearly deviating from the general tendency (Fig. 3c). Here we find the representative of the *Verrucomicrobiales*, peat-Actinobacteria, and one from the *Holophaga/Acidobacterium* cluster. Other ribotypes of the fourth category followed an indistinct tendency, finally ending at a low level in the 1967-plot (Fig. 3b). Here we find again a member of the *Holophaga/Acidobacterium* cluster and three *Bacillus*-relatives. Finally, the TGGE signals that appeared most intense in the 1997-plot (Fig. 1), represented the ribotypes which were clearly decreasing during grassland succession (Fig. 3e). Here we find two representants each of the taxa *Bacillus* and α-Proteobactea and another one of the *Holophaga/Acidobacterium* cluster. All their rRNA levels were drastically decreased in the 1967-plot, while the 1997-plot and the 1967-plot showed comparable total rRNA amounts (Fig. 2).

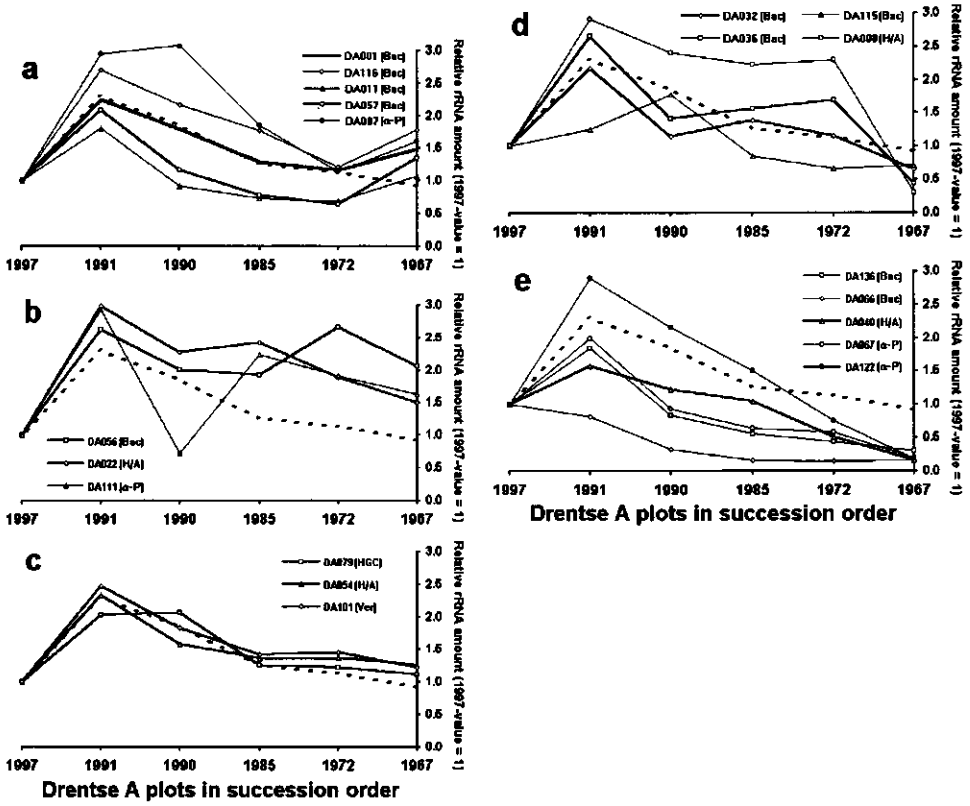

Fig. 3. Multiple competitive RT-PCR results of the 20 ribotypes in the six plots. All 1997-values were set to 1 and all other data points were calculated as a part of them. The dotted graph represents the average tendency of rRNA levels during grassland succession corresponding to the sum of all 20 ribotypes (Fig. 2).The 16S rRNA-clusters are indicated in parentheses: Bac, *Bacillus*; HGC, high G+C Gram-positive bacteria; H/A, *Holophaga/Acidobacterium*-cluster; Ver, *Verrucomicrobium*-cluster; α-P, alpha *Proteobacteria*. a, the ribotypes growing stronger at the later stages of grassland succession. b, the intermediate winners of grassland succession. c, rRNA levels of minute deviation from the average. d, inconstantly loosing ribotypes. e, dramatically declining rRNA levels during grassland succession.

DISCUSSION

The impact of grassland succession and vegetation changes on the soil bacteria.

This study was aimed to provide insight to the effects of grassland succession on the composition of the soil bacteria community in the Drentse A agricultural research area. In general, the rRNA levels were approximately doubled a few years after fertilization has stopped. This increase of activity probably was correlated to an abrupt change of the vegetation, namely the collapse of the once dominant *Lolium perenne* population. While

Lolium perenne faded within a few years after fertilization stopped, the other predominant species like Yorkshire fog (*Holcus lanatus*), Rough meadow-grass (*Poa trivialis*) and Creeping bent (*Agrostis stolonifera*) could last more than one decade on a similar level before being completely replaced within less than five years (17) by raising populations of Keck (*Anthriscus sylvestris*), Common sorrel (*Rumex acetosa*) and notably Creeping buttercup (*Ranunculus repens*). In later succession stages, species like Sweet vernal-grass (*Anthoxanthum odoratum*), Red fescue (*Festuca rubra*) and Field wood-rush (*Luzula campestris*) appeared. Correlating these vegetation shifts to the changes in the bacterial community is highly speculative, but it appears plausible to suppose a link between the two most dramatic events, the peak of bacterial ribosomes and the disappearance of *Lolium perenne*. Decaying plant residues like the lower shot-parts and the root system might have provided an increased nutrient input and supported bacterial activity. For instance, an increased turnover of organic matter was also indicated by earthworm activity. In a study of 1992 it has been demonstrated that the 1991-plot contained a mean of 308 earthworms in one m^2, the 1985-plot (at this time not fertilized since seven years) 808, the 1972-plot only 233 (4). The same survey also indicated similar peaks for carbon mineralization and microbial biomass. Another study found a boost of nitrogen mineralization from the plot not fertilized since two years to the plot not fertilized since seven years (16). The nitrogen mineralization increased from 124 to 176 kg ha^{-1} yr^{-1} and dropped again on older fields. Since all these parameters are linked to bacterial activity, their correlation to the results of the multiple competitor RT-PCR indicate a dependency between the total activity of bacterial communities in soil and the amount of ribosomes in soil.

Almost all 20 ribotypes followed the general tendency of a bacterial ribosome-level increase in the first years after fertilization-stop. After the increase in the 1991-plot the rRNA amounts were decreasing during the following stages of grassland succession. Here the 20 ribotypes showed different reactions. Some of them were decreasing much more than the average while others increased their rRNA levels during progressing succession. These were the ribotypes causing the differences in the TGGE fingerprints from different plots. The five defined categories of response could not be significantly correlated to the phylogeny of their ribotypes. The predominant taxa are not limited to particular categories. This is in accordance to the results of the dot blot hybridization, which indicated that the response to the grassland succession is not specific on the level of the major bacterial taxa.

Long-term development of bacterial communities in soil.

The high spatial reproducibility of TGGE fingerprints of the Drentse A grassland soils was impressive (8) despite of the different vegetation of the six plots. This raised the question if the predominant soil bacteria were responding to the composition of the vegetation at all. Apparently the bacteria were more influenced by homogeneous abiotic soil properties. Maybe our approach with its resolution on the 16S rRNA level missed some important community shifts. A ribotype might originate from one single strain, a couple of strains from the same species or from different, closely related species. While a single, consistent TGGE band

represented bacteria of close phylogenetic relation, they might exhibit quite different physiology. Nevertheless, the homogeneous distribution of a ribotype, although not excluding heterogeneity of physiology, must be explained. This striking homogeneity observed for the predominant bacteria should be caused by homogeneous soil properties like the geochemical composition of the soil matrix. The distribution of bacterial cells in a given matrix is driven by proliferation and subsequent spatial dissemination. Local bursts of growth are equalized by active or passive migration of the descending cells through the matrix. The higher the ratio between dissemination rate and proliferation speed is, the more homogeneous should be the composition of the total bacterial community. Considering the difficulties of cell migration through the heterogeneous soil matrix, the time scales must be extended for dissemination and even more for proliferation. Therefore, the high homogeneity of the microbial community is requiring a slow growth rate of the predominant bacteria. This is also supported by the fact that the microbial community was investigated by its rRNA, reflecting the activity of the bacteria (26,27). Monitoring the activity rather than presence was promising a higher resolution for heterogeneities, but still we observed a surprising reluctance of the soil bacteria community to change its composition in space and time. Although we are used to recognize bacteria as high-speed lifeforms which demonstrated generation times as short as 20 minutes under laboratory conditions, we must consider that particular microorganisms might count their generation time in weeks or months. Maybe here is a key to define the term 'unculturability'. It might be possible, that the predominant soil bacteria are not physiologically capable to grow on nutrient broth within considerable time. Since the 'great plate count anomaly' indicated that the vast majority of environmental bacteria are not culturable (23), this supposition might be true for almost all bacteria. Within the vast richness of bacterial strains there might be only a minute minority of fast-growing organisms, the latter captured in our culture collections.

Conclusions.

Multiple competitive RT-PCR succeeded to reveal the tendencies of activity shifts for the predominant soil bacteria during Drentse A grassland succession, while quantitative dot blot hybridization failed to detect differences on a higher taxon level. Although the nutritious matter was depleting more and more and the vegetation clearly changed, there was no correspondingly drastic reaction of the microbial community. We could quantify reproducible shifts of ribosome levels, but the composition of the bacterial community remained remarkably stable. Evidence for severe competition and major replacement of species, as apparent in the grass vegetation, could not be found.

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Chapter 10

In Situ Detection of an Uncultured Predominant *Bacillus* in Dutch Grassland Soils

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In Situ Detection of an Uncultured Predominant Bacillus in Dutch Grassland Soils

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Uncultured predominant *Bacillus* ribotype DA001 in Dutch Drentse A grassland soils, as revealed by its 16S rRNA sequence, was detected in soil by fluorescent whole-cell in situ hybridization. A prominent rod-shaped cell type was identified in bacterial suspensions prepared from soil by a multiple 16S rRNA probing approach.

One of the strategies currently used to identify the predominant bacteria in the environment is to extract nucleic acids from environmental samples and detect the nucleotide sequences of PCR-amplified 16S rRNA genes (11). Since this approach might suffer from bias in DNA extraction, PCR, and cloning efficiency, how representative the data obtained really are is questionable. One of the techniques currently used to detect bacterial cells in the environment is in situ hybridization of rRNA with fluorescent oligonucleotide probes (1). The great sensitivity of this technique allows workers to detect single cells in such complex and recalcitrant environments as soil (2).

Here we report on the in situ detection of an uncultured member of the genus *Bacillus* that is predominant in grassland soils in the Drentse A agricultural research area (The Netherlands). This organism is known to contain ribotype DA001 16S rRNA, which was discovered during a molecular survey of the main 16S rRNA sequences in these soils (4). A total of 165 clones of a 16S ribosomal DNA (rDNA) library constructed from soil DNA were examined, and ribotype DA001 was found nine times; this sequence was by far the most abundant 16S rDNA sequence. Ribotype DA001 was closely related to the 16S rRNA sequences of cultured *Bacillus benzoevorans* strains (sequence similarity, 97.3%) and to several other sequences in the same Drentse A 16S rDNA clone library (Fig. 1). Moreover, the presence of four very closely related cloned sequences in an agricultural soil in Wisconsin (3) suggests that this *Bacillus* line of descent is distributed worldwide.

To detect predominant *Bacillus* ribotype DA001, bacteria were extracted from soil (10) and fixed and pretreated for in situ hybridization as described previously (5). A total of 24 homogenized and pooled soil samples obtained from different areas of the Drentse A grasslands were investigated (4). First, the soil samples were homogenized by mechanical treatment and washed with sterile deionized water to release bacterial cells attached to the soil matrix. The released bacteria were separated by differential centrifugation and resuspended in approximately 5 volumes of phosphate-buffered saline, and then they were fixed with paraformaldehyde at 4°C for 16 h (1). After the cells were applied to gelatin-coated slides (1), they were permeabilized with a combination of sodium dodecyl sulfate and dithiothreitol and then pretreated with lysozyme in order to detect bacilli and their endospores (5). Hybridizations were performed in 8 μl of hybridization buffer (630 mM NaCl, 10 mM Tris-HCl, 0.01% sodium dodecyl sulfate; pH 7.2) in the presence of 20% formamide, 5× Denhardt's reagent, 10 pmol of oligonucleotide probe REX72 (5'-TGGGAGCAAGCTCC CAAAG-3'), and 10 pmol of oligonucleotide probe LGC353b (5'-GCGGAAGATTCCCTACTGC-3') at 45°C for 4 h. After hybridization the slides were incubated with hybridization buffer at 45°C twice for 20 min, and then they were washed with deionized water and air dried. Subsequent staining with a solution containing 1 μg of 4',6-diamidino-2-phenylindole (DAPI) per ml was performed as described by Hahn et al. (6). Fluorescent signals were detected with an Axioplan microscope (Zeiss, Oberkochen, Germany) fitted with filter sets for simultaneous detection and individual detection of DAPI, Cy3, and fluorescein.

Relatively weak fluorescent signals were observed for bacteria in soil compared to the fluorescent signals for laboratory cultures, as observed by other workers (2). Using a single fluorescent probe did not yield clearly convincing results. Due to the high background signal of nonbacterial soil particles, it was difficult to demonstrate the difference between positive and negative signals. Fluorescein-labeled probe REX72 produced weak green signals against a dark greenish to brownish background due to aspecific probe attachment to and autofluorescence of soil particles. Therefore, we used a multiple staining approach in which we labeled the positive cells with three fluorescent indicators having different colors in order to intensify the total signal and to introduce a color contrast between positive cells and the background (2, 7). The first indicator was DAPI, a fluorescent dye for DNA, which was used to visualize all microorganisms with its blue signal (6). We used a 10-fold-lower DAPI concentration than we used previously (6) to equalize the strengths of the signals and to reduce the background DAPI fluorescence. In our experiments DAPI detected about 10^8 bacterium-like particles per g of soil. The second marker used was specific for the most important *Bacillus* species and some other low-G+C-content organisms and targeted a 16S rRNA region previously described as specific for gram-

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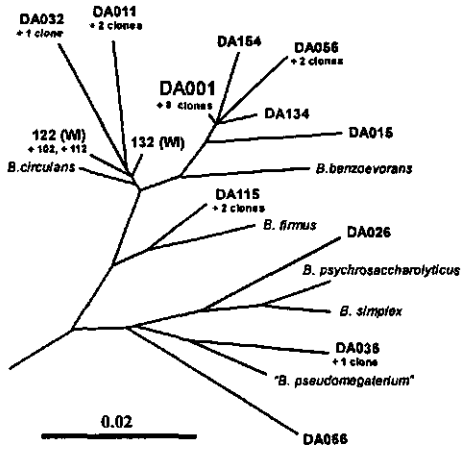

E. coli-position	72	90
DA001 target site	5'--CTTTGGGAGCTTGCTCCCA--3'	
DA011+ 134	5'--|||||||||||||||||G--3'	
DA015 + 056 + 154	5'--||GA|||||||||||||--3'	
DA115	5'--|AA|A||||||||||T|--3'	
DA032	5'--A|||A|||||||||T||--3'	
Bacillus benzoevorans	5'--TG||||||||||||||||--3'	
Bacillus circulans	5'--|||AAA|||||||nTTn--3'	
Bacillus firmus	5'--GA|-|||||||||||||--3'	
Bacillus subtilis	5'--GA|-||||||||||||T--3'	
Bacillus globisporus	5'--GA|-|A||||||||T|T--3'	
Bacillus cereus	5'--GA||AA|||||||||TT|--3'	
Bacillus megaterium	5'--GA||A|A|||||||T|T|--3'	

FIG. 1. Tree showing the phylogenetic positions of DA001 and related sequences from Drentse A soils. The positions of environmental sequences from Wisconsin agricultural soil are also indicated (WI). The bar indicates the branch length corresponding to 0.1 base substitution per nucleotide. The phylogenetic tree was constructed with the ARB software by using approximately 8,000 small-subunit rRNA sequences, maximum parsimony criteria, and nearest-neighbor optimization (9). The accession numbers of the sequences are given elsewhere (3, 4). The annealing site of probe REX72 is shown for the cloned Drentse A sequences and some *Bacillus* species. *B, Bacillus*.

positive bacteria with low G+C contents (8). This Cy3-labeled oligonucleotide probe, LGC353b, was used to detect *Bacillus* cells and endospores with its red signal (bright purple when it was added to the DAPI blue signal). Approximately 40% of all of the DAPI-detected particles were also detected by LGC353b; these particles were mainly globular configurations less than 1 μm in diameter and sometimes were arranged in clusters (Fig. 2a). The third marker used was highly specific fluorescein-labeled probe REX72 for *Bacillus* ribotype DA001. Highly specific 16S rRNA sequence regions of a particular *Bacillus* ribotype were not easy to find, since *Bacillus* species exhibited relatively high levels of 16S rRNA sequence similarity to each other. The most promising nucleotide stretch was located in highly variable region V1. In the stretch from *Escherichia coli* position 72 to position 90 (Fig. 1) all cultured *Bacillus* species exhibited various mismatches with ribotype DA001, and only closely related ribotypes from the Drentse A clone library exhibited considerable similarity. With the multiple staining approach, ribotype DA001 cells should have been stained blue by DAPI on the DNA, red by LGC353b on the *Bacillus*-specific target site in the 16S rRNA, and green by fluorescein-labeled probe REX72. Since blue light, red light, and green light together produce white light (Fig. 2b), DA001 cells yielded bright signals that were clearly distinguishable from the other bacterial and background signals. Approximately 5% of all of the DAPI-detected particles were rods approximately 2 μm long that were simultaneously detected by all three indicators (Fig. 2a). In some cases, dot-shaped signals were detected; these signals could have originated from endospores or could have been vertical views of cells.

The specificity of the oligonucleotide probes was checked by using 30 *Bacillus* isolates from Drentse A grassland soil, including some *Bacillus cereus*-like strains. All of the strains tested (and their endospores, if present) gave positive signals with probe LGC353b (Fig. 2c) and DAPI (Fig. 2d), but none of them was detected by REX72. This experiment demonstrated the reproducibility of cell fixation and pretreatment, DAPI staining, and LGC353b hybridization for different *Bacillus* strains. However, the specificity of probe REX72 could be checked only indirectly. A search of the ARB database of 8,000 small-subunit rRNA sequences (9) revealed that the only matching organism was ribotype DA001, but this ribotype is not available in pure culture. Therefore, there was no positive control for the specificity tests. Moreover, the other Drentse A ribotypes which were most likely to cross-react (Fig. 1) have not been cultured. The high specificity of probe REX72 for in situ reactions in soil containing all of the different uncultured bacteria could not be verified. Hence, we cannot exclude the possibility that some other uncultured DA001 relatives in soils (Fig. 1) might have reacted with probe REX72. The best indications that REX72 was highly specific were the shape and size of the cells detected and the clear difference from the results obtained with the more universal signal of LGC353b.

Although the REX72-hybridizing cells were prominent and abundant, we had problems detecting the positive signals. To do this, we had to adjust the concentration of bacteriumlike particles in the suspension. When the bacterial pellet from soil was diluted less than 200-fold (after differential centrifugation [see above]) we could not detect the positive signals among all of the bright fluorescent signals. When a preparation was diluted more than 1,000-fold, the number of signals was too low for representative photographic documentation. This dilution range corresponded to approximately 10^3 to 10^4 bacteria μl^{-1}.

Our results indicated that ribotype DA001 indeed originated from one of the most abundant bacteria in Drentse A grassland soils. The shape and size of the positive signals indicated that vegetative *Bacillus* ribotype DA001 cells were present, suggesting that these cells were metabolically active in the soil. However, our attempts to cultivate this *Bacillus* type failed. Such cultivation is essential for investigating the function and activity of the bacteria and the geochemically relevant biodegradation processes carried out by the active bacterial community in the soil. A better understanding of the metabolism of these bacilli is important, since these organisms are potentially important parts of native bacterial communities not only in Drentse A grassland soil but possibly also in other environments, such as an agricultural soil in Wisconsin (3), where they have been detected. Actually, there is no evidence that the Wisconsin bacilli are similar to our bacteria except for the 16S rRNA sequence. The metabolic properties of the bacteria might be completely different, even if the 16S rRNA are almost identical.

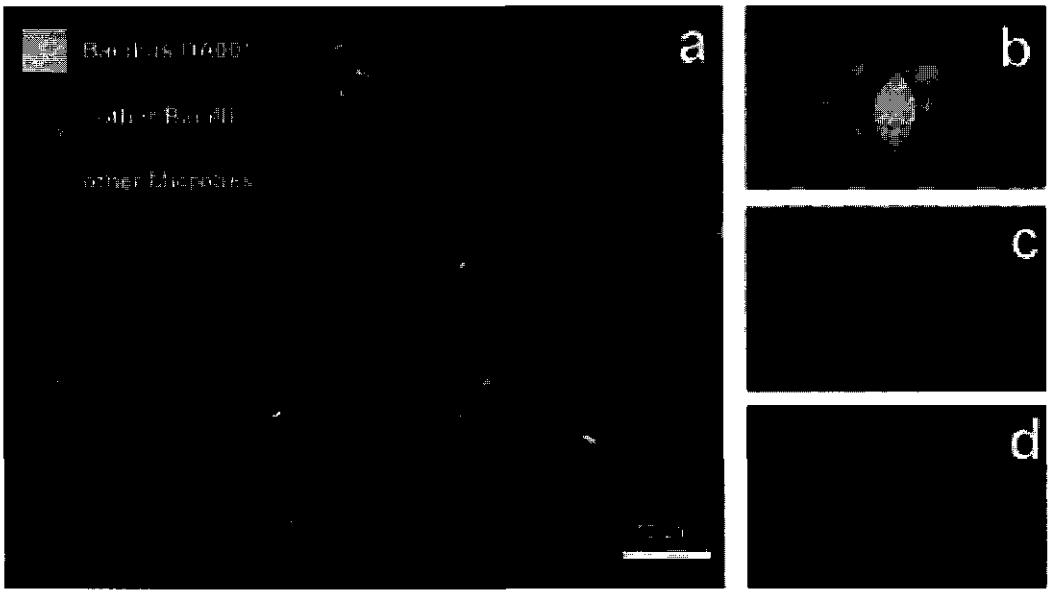

FIG. 2. In situ hybridization results. (a) Detection of *Bacillus* ribotype DA001 with triple staining in a 320-fold-diluted microparticle soil extract. Magnification, ×1,200. (b) Colors theoretically expected with triple staining. (c) Reaction of probe LGC353b with a fixed sample of a *B. cereus* isolate to prove permeabilization of endospores. Magnification, ×1,600. (d) DAPI staining of the sample in panel c.

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Please find Fig. 2 in color on the back cover

Chapter 11

Summary and Concluding Remarks

The research described in this thesis was aimed to provide insight into the effects of grassland succession on the composition of the soil bacteria community in the Drentse A agricultural research area. The Drentse A meadows represent grassland succession at different stages. Since 30 years particular plots have been taken out of intense agricultural production and were not fertilized anymore. However, the grass-vegetation was continuously removed once per year. This caused a progressive depletion of nutrients in the soil. In order to reveal the effect of grassland succession on soil microbes, the main bacteria in Drentse A grassland soils were identified by a molecular strategy based on detection and quantification of 16S rRNA. Instead of only using genomic 16S rDNA to reveal present sequences, we focused on rRNA to quantify the activity of the predominant bacteria. The ribosome is considered to be a useful marker for the overall metabolic activity of bacteria. In bacterial cultures the amount of ribosomes per cell has been found to be roughly proportional to growth activity. In our approach the activity is defined as total activity of one ribotype in relation to the bacterial community and not as activity per cell. Hence, bacteria of low activity per cell but extraordinary high cell number might be assessed as very active. Following direct ribosome isolation from soil, several different methods like RT-PCR, separation of amplicons by temperature gradient gel electrophoresis (TGGE), different hybridization methods, cloning and sequencing were applied simultaneously to reveal the predominant 16S rRNA sequences for taxonomic identification. Quantitative dot blot hybridization with taxon-specific oligonucleotide probes revealed dominance of low G+C Gram-positives while other important groups appeared to be α-Proteobacteria and high G+C Gram-positives (Chapter 5). However, this approach did not demonstrate clear tendencies of community structure shifts by quantifying the rRNA of the major taxa. Therefore, a more sensitive method has been chosen, based on RT-PCR amplicons of bacterial 16S rRNA. The sequence-specific separation of these amplicons by TGGE reproducibly yielded characteristic band patterns from hundreds of soil samples (Chapter 3). Although the TGGE signals were very complex due to the high bacterial diversity in soil, different 16S rRNA fingerprints from a single plot were highly similar, while reproducible differences between plots of different history were observed. A parallel approach with PCR-amplified genomic 16S rDNA led to similar results. The presence and activity of prominent bacteria in test fields of several hundreds m^2 were found to be quite homogeneous. Only one gram of soil was found to be representative for the predominant bacteria in large homogeneous grassland areas. After the high reproducibility of presence and activity was demonstrated for

the main soil bacteria, representative TGGE fingerprints were compared to TGGE signals from a clone library of genes coding for 16S rRNA (Chapter 5). Cloned 16S rDNA amplicons matching the intense bands in the fingerprint were sequenced. The relationship of these sequences to those of cultured organisms of known phylogeny were determined. Approximately one half of the amplicons represented sequences closely related to those of cultured *Bacillus*-species, indicating that most of the active bacteria apparently belonged to the genus *Bacillus*. Other sequences similar to Gram-positive bacteria with high G+C-content were only remotely related to those of cultured bacteria, as is illustrated by clone DA079 that could be affiliated to uncultured Actinobacteria from peat (Chapter 6). Another important group of sequences was related to Proteobacteria, mainly the α-subclass. Several sequences could not be related to cultured organisms but to the *Holophaga/Acidobacterium*- or the *Verrucomicrobiales*-cluster (Chapters 7 and 8).

The parallel application of RT-PCR/TGGE and 16S rDNA-cloning to reveal the most abundant 16S rRNA sequences was found to be a powerful combination. The clone screening on TGGE was convenient and efficient, offering access to the almost complete 16S rRNA sequence. The subsequently performed V6-hybridization was a relatively simple approach to prove the identity of bands even in complex fingerprints (Chapter 6). The most predominant *Bacillus*-like ribotype DA001 in Drentse A grassland soils could also be detected by fluorescent whole-cell *in situ* hybridization (Chapter 10). Prominent rod-shaped cells of approximately 2 μm length could be identified in bacterial suspensions from soil with a multiple 16S rRNA probing approach. The specific DA001-signals represented about 5% of all microbial particles, which were visualized by the universal DNA-dye DAPI. Indeed, the sequences detected by the PCR-based methods represented abundant bacteria in soil. The most predominant *Bacillus*-like 16S rRNA sequence DA001 apparently originated from active, vegetative cells and not from endospores.

The possibility to draw quantitative information about the microbial community from the complex TGGE fingerprints has been explored. A novel approach has been developed to quantify rRNA sequences in complex bacterial communities by multiple competitive RT-PCR and subsequent TGGE analysis (Chapter 4). The used primer pair (U968-GC and L1401) was carefully tested and found to amplify with the same efficiency 16S rRNAs from bacterial cultures of different taxa as well as the cloned 16S rDNA amplicons from soil samples. The sequence-specific efficiency of amplification was followed by monitoring the amplification kinetics via kinetic PCR. The primer-specific amplification efficiency was assessed by competitive PCR and RT-PCR, and identical input amounts of different 16S rRNAs were found to result in equal amplicon yields. We applied this method as multiple competitive RT-PCR to TGGE fingerprints from soil bacteria to estimate the ratios of their 16S rRNAs (Chapter 9). This was done for different stages of grassland succession in the Dutch Drentse A area. The 16S rRNA amounts g^{-1} soil of 20 predominant ribotypes were monitored via multiple competitive RT-PCR in six plots of different succession stage. The 20 monitored 16S rRNA levels represented approximately half of all bacterial soil rRNA. The different Drentse A

meadows, representing progressing stages of grassland succession, showed highly reproducible shifts of ribotype composition. In general, the rRNA levels were found to be doubled after the first years without fertilization. During the further progression of grassland succession the rRNA amounts were found to decline again. The 20 ribotypes showed remarkably different succession histories, causing the differences in TGGE fingerprints from different plots. While organic carbon and available nitrogen were declining during grassland succession, some bacteria were apparently suffering much more than the average. However, other bacteria showed an increased contribution to the bacterial rRNA pool, indicating that some bacteria could improve their position when less nutrients were available. The general increase in bacterial ribosomes in the first years after fertilization-stop was correlating to the increase of other parameters related to bacterial activity, i. e. carbon mineralization and microbial biomass. This suggested a true correlation between the total activity of bacterial communities in soil and the amount of ribosomes. This study provides extended information about uncultured bacteria in soil and describes the application and evaluation of several novel approaches in molecular microbial ecology. The following six conclusions are highlighting the major achievements and findings:

1. Representative rRNA and rDNA fingerprints can be generated for homogeneous landscapes of large scale. This demonstrates that the often tiny sample size in molecular studies has not to be a limitation for microbial ecology. Nucleic acid extraction from small soil samples can be applied to characterize a several magnitudes larger environmental matrix. The composition of bacterial communities might be quite homogeneous for kilometers of grassland with heterogeneous vegetation and cultivation history. This conclusion is of general importance for molecular microbial ecology and landscape ecology.

2. The rRNA cycle (see Introduction, Fig. 2) for the predominant soil *Bacillus* recognized as ribotype DA001 has been completed. Its rRNA not only has been identified as predominant in the isolated fraction of soil ribosomes, but was also detected in an abundant type of bacterial cells by whole-cell hybridization to a fluorescently labeled, 16S rRNA-targeted oligonucleotide probe.

3. The genus *Bacillus* appears to be dominant in the microbial community of Drentse A grassland soils. Uncultured members of the *B. benzoevorans*-line of descent are predominant among these bacilli. This group of *Bacillus*-ribotypes accounted for approximately 20% of all bacterial ribosomes in Drentse A soil. Such a predominant cluster of very closely related bacteria has never been observed before in soils. The reasons and circumstances of this special community composition remained unexplored.

4. Prominent clusters of hitherto uncultured environmental bacteria were also detected in Drentse A soils. The *Holophaga/Acidobacterium*-cluster, the *Verrucomicrobiales* and a peat-

related Actinobacteria-cluster had already been known from different locations all over the world. It is the first time that these hitherto uncultured bacteria were identified as prominent contributors to the ribosome fraction in soil. Since these organisms apparently contain considerable amounts of intact ribosomes they are likely to be metabolically active. Some of these novel ribotypes belong to the most intense bands in the TGGE fingerprints, which may suggest a major role in environmental nutrient fluxes. This finding also contributes to the discussion about the unculturability of environmental bacteria, since based on the presented results it appears unlikely that the reason for their unculturability is a lack of viability. It is more probable that suitable culture conditions are still not found yet. Since these bacteria are abundantly detected all over the world, future research should be aimed to attempt to culture them.

5. A novel approach has been developed to quantify the predominant rRNA molecules of environmental bacteria communities. The multiple competitive RT-PCR allowed to quantify in a highly-specific way many different rRNA molecules within one assay. The RT-PCR-related possibilities of bias were investigated and excluded for the applied primer pair. Therefore, amplification by RT-PCR could be excluded as a major source of bias in this study. Other uncertainties are the selectivity of the applied primers and probes and the cell lysis efficiency. The selection of all the oligonucleotide probes and primers is based on only a few thousand 16S rRNA sequences of different length and quality. Although the available 16S rRNA sequence data are limited, the presence of hitherto unknown bacteria with novel 16S rRNA sequences not matching the used primers is not indicated. The combined results of cloning, TGGE and dot blot hybridization, all achieved with different probes or primers, do not reveal possibly neglected groups of organisms. A serious bias caused by incomplete cell lysis may not be excluded. However, the majority of ribosomes originated from Gram-positives, indicating the lysis of bacteria with resistant cell walls. The possibility that highly resistant resting stages like endospores might have been missed is not relevant, since this study aimed to detect the most active bacteria.

6. Multiple competitive RT-PCR revealed activity shifts for the predominant soil bacteria during Drentse A grassland succession. Some species responded to the nutrient depletion during grassland succession. Though the depleting nutritious matter and the changing vegetation, the overall impact of grassland succession did not cause a correspondingly drastic impact on the microbial community composition. Reproducible shifts of ribosome levels could be demonstrated, but the composition of the bacterial community remained remarkably stable. Evidence for major competition or replacement of species could not be found.

Samenvatting

Het doel van het hier beschreven onderzoek is inzicht te geven in de effecten van graslandsuccessie op de samenstelling van de bodemmicroflora in graslanden in het Drentse A gebied (Nederland). In dit gebied bevinden zich een aantal graslanden in verschillende ontwikkelingsstadia. Gedurende de afgelopen 30 jaar werden op verschillende tijdstippen percelen grasland uit produktie genomen en hierna niet meer bemest. Eenmaal per jaar werd gemaaid en werd het hooi afgevoerd. Teneinde het effect hiervan op de bodemmicroflora te bepalen, werden de dominante bacteriën geïdentificeerd met behulp van moleculaire technieken gebaseerd op het detecteren en kwantificeren van 16S rRNA. Naast het genomische 16S rDNA werd het rRNA gebruikt voor het kwantificeren van de activiteit van de meest voorkomende bacteriën. Het ribosoom is een bruikbare marker voor de totale metabole activiteit van de meest voorkomende bacteriën. In een reinkultuur is het aantal ribosomen per cel ongeveer evenredig met de groeisnelheid van de bacteriën. In onze benadering wordt activiteit gedefinieerd als de totale activiteit van een bacteriegroep in de grond en niet als activiteit per cel. Dit betekent dat bacteriën met een lage activiteit per cel die in groot aantal aanwezig zijn als erg actief worden beschouwd. Na een rechtstreekse isolatie van ribosomen werden de meest voorkomende 16S rRNA sequenties verkregen voor identificatie. Hiertoe werden verschillende moleculaire technieken gebruikt, zoals de reverse transcriptase-polymerase chain reaction (RT-PCR), scheiding van de PCR produkten via temperatuur gradient gel electroforese (TGGE), verschillende hybridisatie methoden, kloneren en DNA-sequencen. Uit kwantitatieve analyse van dot-blot hybridisatie met groep-specifieke oligonucleotiden probes bleek dat de meeste bacteriën in de onderzochte gronden tot de Gram-positieve bacteriën met laag G+C gehalte behoren. Andere veel voorkomende groepen behoren tot de alfa Proteobacteria en de Gram-positieve bacteriën met hoog G+C gehalte (Hoofdstuk 5). Daar met deze grove benadering geen duidelijke verschuiving kon worden waargenomen, werd een meer gevoelige methode gekozen, gebaseerd op de analyse van via RT-PCR vermenigvuldigd bacterieel 16S rRNA. De sequentie-specifieke scheiding van deze PCR produkten via TGGE resulteerde in karakteristieke bandpatronen van verschillende bodemmonsters (Hoofdstuk 3). Hoewel de TGGE patronen erg complex waren vanwege de hoge bacteriële diversiteit in de bodem, bleken de patronen van monsters uit eenzelfde perceel zeer identiek en significant verschillend van monsters afkomstig uit percelen met een verschillende voorgeschiedenis. Dezelfde resultaten werden verkregen na analyse van 16S rDNA. Zowel de aanwezigheid als de activiteit van de veel voorkomende bacterie-typen in de proefvelden van enkele honderden vierkante meters bleek zeer homogeen. Slechts een gram grond was voldoende om de aanwezigheid van de meest voorkomende bacteriën te bepalen. Nadat de hoge reproduceerbaarheid van TGGE bandpatronen was aangetoond, werden deze vergeleken met TGGE signalen van de gekloneerde 16S rRNA genen (Hoofdstuk 5). De 16S rRNA produkten die een overeenkomstige bandpositie gaven in de TGGE patronen werden vervolgens gesequenced. Hierdoor kon de verwantschap van deze sequentie worden bepaald met sequenties van

gekweekte organismen. Ongeveer de helft van alle gekloneerde PCR-produkten bleek verwant te zijn met die van gekweekte *Bacillus*-soorten. Hieruit werd afgeleid dat de meeste actieve bacterietypen behoren tot het geslacht *Bacillus*. Andere sequenties die leken op die van Gram-positieve bacteriën met hoog G+C gehalte bleken slechts ver verwant met die van kweekbare groepen. Een voorbeeld hiervan is kloon DA079 die gelijkt op sequenties van PCR produkten afkomstig uit veengrond (Hoofdstuk 6). Een andere belangrijke groep van sequenties was verwant met de alfa-Proteobacteria. Andere sequenties bleken niet nauw verwant met bekende gekweekte organismen, maar vielen binnen de *Holophaga/Acidobacterium* of de *Verrucomicrobiales*-kluster (Hoofdstuk 7 en 8).

De combinatie van de analyse van RT-PCR/TGGE en gekloneerd 16S rDNA bleek een goede manier om de meest voorkomende 16S rDNA sequenties te detecteren en te identificeren. Het screenen van klonen via TGGE bleek een eenvoudige en efficiënte manier om de complete 16S rRNA sequentie te bepalen. Met behulp van de uitgevoerde V6-hybridizatie kon de identiteit van de band zelfs in complexe fingerprints aangetoond worden (Hoofdstuk 6). De meest voorkomende *Bacillus*-sequentie van kloon DA001 werd in cellen aangetoond in de grond door middel van "fluorescent whole-cell *in situ* hybridization" (Hoofdstuk 10). Ongeveer 5% van alle bodembacteriën die met de universele DNA-kleurstof reageerden, bleken *Bacillus*-achtige staafvormige cellen met een lengte van 2 um. Tevens bleek dat deze sequentie detecteerbaar was in actieve, vegetatieve cellen, en niet uitsluitend in endosporen.

In Hoofdstuk 4 werd de mogelijkheid onderzocht kwantitatieve informatie te verkrijgen uit complexe TGGE bandpatronen. Een nieuwe methode werd ontwikkeld om rRNA sequenties in complexe bacterie-gemeenschappen te kwantificeren met behulp van competitieve RT-PCR en TGGE analyse. Aangetoond werd dat de gebruikte primers (U968-GC en L1401) in de PCR met dezelfde efficiëntie 16S rRNA's amplificeerden van bacterie cultures van verschillende taxa. Dit gold ook voor de amplificatie van gekloneerd 16S rDNA fragmenten. De sequentie-specifieke efficiëntie van de amplificatie werd bepaald door de amplificatiekinetiek te volgen via kinetische PCR. De primer-specifieke amplificatie efficiëntie werd bepaald via competitieve PCR en RT-PCR en identieke uitgangshoeveelheden van verschillende 16S rRNA's resulteerden in gelijke ampliconopbrengsten. We pasten deze methode toe als multipele competitieve RT-PCR op TGGE fingerprints van bodembacteriën teneinde de relatieve hoeveelheden van het 16S rRNA van deze bacteriën te bepalen (Hoofdstuk 9). Dit werd uitgevoerd voor grondmonsters afkomstig uit verschillende ontwikkelingsstadia in de grasland-successie. Van 20 veel voorkomende bacterie-ribotypen werd het 16S rRNA gehalte per gram grond bepaald in 6 verschillende graslanden met een verschillend ontwikkelingsstadium. De 20 veel voorkomende ribotypen vormden ongeveer de helft van de totale hoeveelheid bacterieel bodem-rRNA. De verschillende graslanden met opeenvolgende ontwikkelingsstadia van de vegetatie, toonden een zeer reproduceerbare verschuiving in de samenstelling van de ribotypen. Het bleek dat het rRNA gehalte enkele jaren na beëindigen van de bemesting verdubbeld was. Gedurende de hierna volgende ontwikkelingsstadia daalden de rRNA gehaltes weer. De TGGE bandpatronen toonden aan dat de 20 meest voorkomende

ribotypen duidelijk verschillende verschuivingen vertoonden in de tijd. Terwijl de hoeveelheid organische stof en de beschikbare hoeveelheid stikstof afnamen gedurende de grasland-successie, bleken sommige ribotypen sneller af te nemen dan het gemiddelde en bleken andere bacterietypen een hogere bijdrage te leveren tot de totale bacteriële rRNA pool. Dit betekent dat deze bacterie-typen een relatief voordeel hebben bij verminderde nutrient-beschikbaarheid. De toename in de hoeveelheid bacterie-ribosomen tijdens de eerste jaren na beëindigen van bemesten was gecorreleerd met een eerder waargenomen toename in parameters die gerelateerd zijn aan bacteriële activiteit, nl. koolstofmineralizatie en microbiële biomassa. Dit suggereert een werkelijke correlatie tussen de totale activiteit van een bacteriële gemeenschap in de bodem en de hoeveelheid bacterie-ribosomen.

De resultaten van dit onderzoek kunnen als volgt worden samengevat:

1. Representatieve rRNA en rDNA fingerprints kunnen worden verkregen voor homogene graslanden. Hiermee is aangetoond dat de veelal geringe grootte van de monsters nodig voor moleculair onderzoek geen beperking hoeft te zijn in de microbiële ecologie. Extractie van nucleïnezuren uit kleine grondmonsters kan toegepast worden voor de karakterisering van een groot gebied. De samenstelling van de bacterie-gemeenschap kan zeer homogeen zijn in een groot gebied zelfs met verschillen in vegetatie en voorgeschiedenis. Deze conclusie is van algemeen belang voor zowel de microbiële ecologie als ook de landschaps ecologie.

2. De rRNA cyclus (zie Fig. 2 , Hoofdstuk 1) voor de algemeen voorkomende sequentie van kloon DA001 van de bodem *Bacillus* is rond. Dit rRNA was niet alleen dominant vertegenwoordigd in de geïsoleerde fractie van ribosomen uit de grond, maar kon ook aangetoond worden in een veelvuldig voorkomend type bacteriecellen via in situ hybridisatie van cellen met een fluorescerende probe gericht tegen het 16S rRNA.

3. *Bacillus*-typen domineren in de microbiële gemeenschap in de Drentse A grasland-grond. Van de bacilli bleken de niet-gekweekte verwanten van *B. benzoevorans* het meest voor te komen. In totaal vormde de *Bacillus*-typen ongeveer 20% van alle ribosomen in de Drentse A grond. Een dergelijk veel voorkomend cluster van zeer verwante bacteriën is tot nu toe nog niet eerder in een grond waargenomen.

4. In de Drentse A grond werden ook clusters van niet te cultiveren bacteriën veelvuldig aangetroffen. Hiervan werden het *Holophaga/Acidobacterium*-cluster, het *Verruco-microbiales*-cluster en het met veengrond geassocieerde Actinobacteria-cluster reeds eerder aangetroffen in gronden verspreid over de gehele wereld. Ons onderzoek heeft voor het eerst aangetoond dat de sequenties van deze niet-gecultiveerde bacteriën algemeen voorkomen in de ribosoom-fractie van de grond. Daar deze organismen kennelijk een significant aantal ribosomen bevatten is het aannemelijk dat zij ook metabolisch actief zijn. Sommige van

deze nieuwe ribotypen behoren tot de meest intense banden in de TGGE patronen, hetgeen veronderstelt dat zij een belangrijke rol spelen in de nutriënten-flux in de bodem. Hieruit kunnen we concluderen dat deze organismen niet oncultiveerbaar zijn omdat ze inactief zouden zijn. Aannemelijker is dat de kweekomstandigheden thans nog onbekend zijn.

5. Een nieuwe methode werd ontwikkeld om veel voorkomende rRNA moleculen in bacterie-gemeenschappen te kwantificeren. Met behulp van de "multipele competitieve RT-PCR" kunnen vele verschillende rRNA moleculen op een zeer specifieke manier in een analyse bepaald worden. Aangetoond werd dat voor de gebruikte set primers belangrijke fouten tijdens de RT-PCR uitgesloten konden worden. Onderzocht werd ook of de selectiviteit van de primers en probes en de efficiëntie van cel-lyses van invloed kunnen zijn op de resultaten. De selectie van alle primers en probes is gebaseerd op slechts enkele duizenden beschikbare 16S rRNA sequenties en er zijn geen 16S rRNA sequenties bekend die niet overeenkwamen met de gebruikte primers. De gecombineerde resultaten van kloneren, sequencen en dot-blot hybridisatie, allen uitgevoerd met verschillende probes en primers, geven geen aanleiding te veronderstellen dat er bepaalde groepen organismen vergeten zijn. Een serieuze foutenbron blijft echter onvolledige cel-lyses. De mogelijkheid dat zeer recalcitrante cellen, zoals endosporen, niet gedetecteerd worden is niet relevant, omdat ons onderzoek tot doel had de meest actieve bacteriën te detecteren.

6. Met behulp van de "multipele competitieve PCR" werden in de grasland successie verschuivingen waargenomen in de activiteit van een aantal veelvoorkomende bodembacteriën. Sommige groepen reageerden sterk op de nutriënten-uitputting tijdens de grasland-successie. Desondanks leidden nutriënten-uitputting en verandering in de vegetatie tijdens de grasland-successie niet tot drastische veranderingen in de samenstelling van de microbiele gemeenschap.

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After all, it was a good time, and I soon started to miss it when I left. It will always be one of my best memories. However, I am not willing to terminate the Drentse A story yet. There are still a lot of things to do, and one day we might strike again...

Curriculum vitae

The author of this thesis was born on the 18th of July 1969 in Gifhorn, Germany. He studied Biology at the Technical University of Braunschweig and specialized in Microbiology, Biochemistry and Genetics. In 1994 he joined the Institute of Biochemistry and Plant Virology of the BBA in Braunschweig to complete the studies. He obtained the M.Sc. in 1995 and his thesis was on "Extraction, amplification and separation of ribosomal RNA from bacterial communities in soil" under the supervision of Dr. Backhaus and Prof. Stackebrandt. In August 1995 he started a Ph.D. at the Department of Microbiology, Wageningen Agricultural University, The Netherlands, under the supervision of Prof. de Vos and Dr. Akkermans. The results of this study, which was completed in 1998, are presented in this thesis. In 1998 he started at the Institute for Natural Resources and Agrobiology in Sevilla (Spain) to work on molecular methods for studying environmental bacteria and their role in biodeterioration of prehistoric rock paintings.

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