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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].

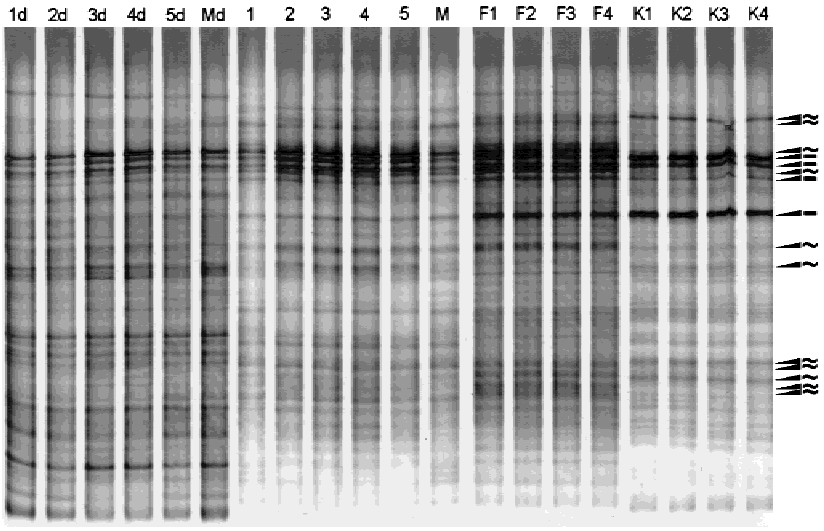

Fig. 1. rDNA amplicon fingerprints on a silver-stained TGGE gel, representing five single sampling points of one-meter distance, from test field A; and pooled samples from test fields A, F, and K, composed of 10 different single samples each. 1d–5d, PCR products from test field A samples of 30–40 cm 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

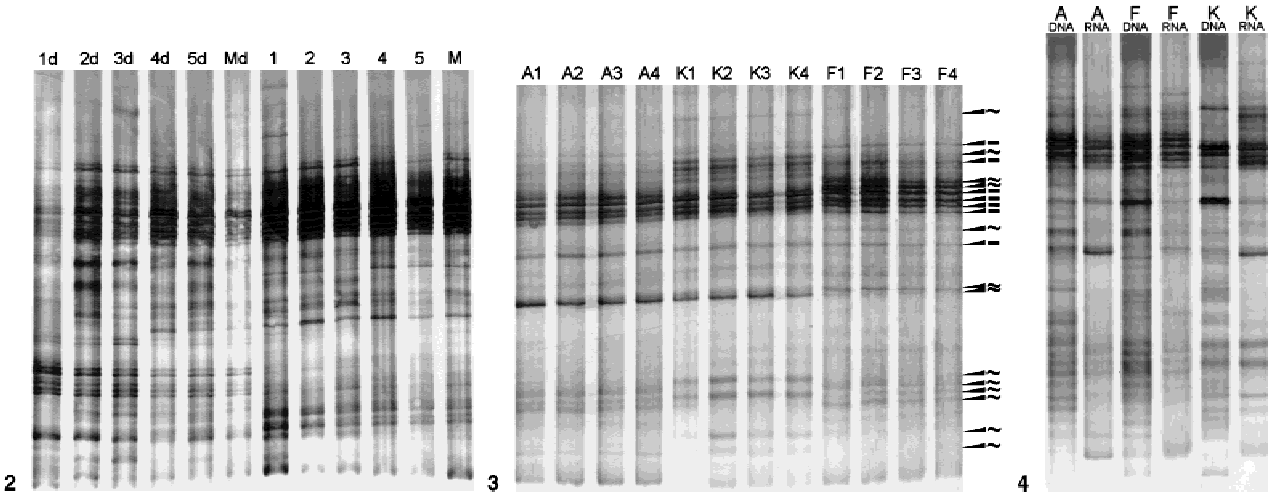

Fig. 2. Test field A: rRNA amplicon fingerprints on a silver-stained TGGE gel, representing five sampling points of one-meter distance. 1d–5d, RT-PCR products from soil samples of 30–40 cm depth; Md, RT-PCR products from a pooled sample of 30–40 cm depth; 1–5, RT-PCR products from surface soil samples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