Bacterial Diversity in Agricultural Soils during Litter Decomposition

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Denaturing gradient gel electrophoresis (DGGE) of amplified fragments of genes coding for 16S rRNA was used to study the development of bacterial communities during decomposition of crop residues in agricultural soils. Ten strains were tested, and eight of these strains produced a single band. Furthermore, a mixture of strains yielded distinguishable bands. Thus, DGGE DNA band patterns were used to estimate bacterial diversity. A field experiment performed with litter in nylon bags was used to evaluate the bacterial diversity during the decomposition of readily degradable rye and more refractory wheat material in comparable luvisols and cambisols in northern, central, and southern Germany. The amount of bacterial DNA in the fresh litter was small. The DNA content increased rapidly after the litter was added to the soil, particularly in the rapidly decomposing rye material. Concurrently, diversity indices, such as the Shannon-Weaver index, evenness, and equitability, which were calculated from the number and relative abundance (intensity) of the bacterial DNA bands amplified from genes coding for 16S rRNA, increased during the course of decomposition. This general trend was not significant for evenness and equitability at any time. The indices were higher for the more degradation-resistant wheat straw than for the more easily decomposed rye grass. Thus, the DNA band patterns indicated that there was increasing bacterial diversity as decomposition proceeded and substrate quality decreased. The bacterial diversity differed for the sites in northern, central, and southern Germany, where the same litter material was buried in the soil. This shows that in addition to litter type climate, vegetation, and indigenous microbes in the surrounding soil affected the development of the bacterial communities in the litter.

Plant residues are a crucial source of nutrients in both natural and agricultural ecosystems, where synchronous plant growth and residue decomposition are essential for soil fertility. Fresh plant material (e.g., litter) represents a readily available substrate for both soil fauna and soil microorganisms. The main mineralization activity is performed by soil microbial communities, and the specific quality of the organic residues controls the decomposition rate and the related release of nutrients (21).

Litter quality generally decreases during the course of decomposition due to the loss of readily available C and the accumulation of refractory compounds (13). Simultaneously, the soil microbial biomass decreases, and the C use efficiency increases (6). The change in the quality of the organic matter induces a succession of microbial communities, which has been studied by using cultivation techniques in litter bag studies (7, 27) and in vertical soil horizons (34) in forest ecosystems. Based on their functions and ecological strategies, different dominant genera and species of microorganisms are present in biotopes (34). During the decomposition process, the r strategists dominate during the early stages and are replaced later by k strategists due to growth-limiting substrate concentrations (27, 29). Theoretically, diversity should increase during succession (1). The combination of adequate biotic diversity and heterogeneity is considered to be necessary for long-term ecological functioning and resilience of ecosystems (3). Higher biodiversity within a community is thought to reduce the spatial and temporal variations in functional activities of the communities, to mitigate the risk of loss of functions after extreme environmental conditions, and thus to preserve the average rates of related ecological processes (9).

It has been reported that only a small fraction of microorganisms in nature are cultivable. Therefore, to study the total microbial community involved in organic matter decomposition, methods which include both culturable and nonculturable microorganisms are needed.

Molecular biological techniques offer new opportunities for analysis of the structure and species composition of a microbial community (20). In particular, sequence variation in rRNA genes has been exploited for inferring phylogenetic relationships among microorganisms (33) and may be used to estimate the genetic diversity of complex microbial communities in natural ecosystems (15, 17, 19, 22). Denaturing gradient gel electrophoresis (DGGE) allows one to directly determine the presence and the relative levels of different 16S rRNA amplicons and, thus, to profile the corresponding microbial populations in both a qualitative way and a semiquantitative way (4, 11, 12, 28, 32). The diversity can be estimated from the number of 16S rRNA gene sequence similarity groups (i.e., the number of DNA bands on the DGGE gel) (15). Each band is assumed to represent an operational taxonomic unit, which is called a species for simplicity (14). We tested the relationship between the number and intensity of DNA bands and the relationship between the number and relative levels of different bacterial isolates (species) in a preliminary experiment by adding known amounts of different bacteria to sterilized soil. This should have shown that DGGE can be used for semiquantitative comparison (20).
The main aim of this investigation was to determine the diversity of bacterial communities during litter decomposition in soil based on analysis of directly extracted DNA. Two types of litter were buried in comparable soil types exposed to different climates and types of vegetation in northern, central, and southern Germany. Using Odum’s system-theoretical hypotheses (23, 24), we tested whether microbial diversity increases during the course of litter decomposition (hypothesis 1), whether refractory straw supports microbial communities with diversity lower than the diversity of microbial communities supported by readily available rye grass (hypothesis 2), and whether microbial diversity is different in the same litter at different locations (hypothesis 3). Hypothesis 2 was formulated because accelerated decomposition of readily available litter is correlated with a higher diversity of soil decomposer communities (10).

**Materials and Methods**

**Addition of bacteria to soil.** To determine if each bacterial genotype (species) produced one band and if the intensity of the band was proportional to the number of cells, strains of the following 10 bacteria isolated from soil (18) were used: Alcaligenes sp., Arthrobacter sp., Bacillus sp., Corynebacterium sp., Enterobacter cloacae, Flavobacterium sp., Nocardia sp., Pseudomonas cepacia, Psychrobacter sp., and Rhodococcus sp. The strains were added to a sandy soil that was autoclaved twice for 30 min at 120 °C, with 24 h between the two sterilization procedures. Suspensions of the separate bacterial strains at an optical density at 600 nm of 1.5 were added to sterilized soil; the amount added was 0.5 ml per 9.5 g of sterilized soil. Before addition of the bacteria the dry matter content of the soil was 92.1% (wt/wt).

To determine the relationship between band intensity and relative abundance, we performed an additional experiment with three strains which were added to sterilized soil in three different ratios. The ratios were based on optical densities. Cell numbers were measured by image analysis afterwards. An optical density at 600 nm of 1 corresponded to densities of 33.1 ml of 1.5 were added to sterilized soil; the amount added was 0.5 ml per 9.5 g of sterilized soil. Before addition of the bacteria the dry matter content of the soil was 92.1% (wt/wt).

To determine the relationship between band intensity and relative abundance, we performed an additional experiment with three strains which were added to sterilized soil in three different ratios. The ratios were based on optical densities. Cell numbers were measured by image analysis afterwards. An optical density at 600 nm of 1 corresponded to densities of 33.1 ml of sterilized MilliQ water, 5 μl of Mg-containing buffer, 2 μl of skim milk, 0.05 μl of T4 gen, 10 μl of a deoxyribonucleic triphosphate mixture, 1 μl of primer 1, 1 μl of primer 2, 1 μl of the DNA solution, and 0.75 μl of Expand High Fidelity DNA polymerase (Laroch). The polymerase was added after a hot-start procedure (5 min at 94°C, followed by 5 min at 80°C). PCR was performed with a Perkin-Elmer 9600 thermocycler by using the following protocol: 1 min at 94°C (denaturation), 1 min at 65°C (annealing), and 3 min at 72°C (elongation) with a 1°C rise every second cycle during annealing for 20 cycles, followed by 20 cycles with an annealing temperature of 55°C and a final cycle consisting of 10 min at 72°C.

**Litter extraction.** Genomic DNA was extracted directly from litter with a FastDNA Spin kit for soil (Bio 101, Carlsbad, Calif.) (www.bio101.com). Litter (0.05 to 0.25 g [fresh weight], corresponding to approximately 0.05 g [dry weight]) was extracted by bead beating with a FastPrep instrument (Bio 101) for 30 s at level 5.5. The supernatant was transferred, and the DNA was precipitated with a protein precipitation solution (250 μl). The DNA was purified by the GeneClean procedure (Bio 101).

**PCR.** The variable V3 region of 16S rRNA gene sequences from nucleotide 341 to nucleotide 534 (Escherichia coli numbering) was amplified by PCR by using primers 2 and 3 and the hot-start touchdown protocol described by Muyzer et al. (20). DNA extracted from the litter was amplified with a PCR mixture (50 μl) containing 29.2 μl of sterilized MilliQ water, 5 μl of Mg-containing buffer, 2 μl of skim milk, 0.05 μl of T4 gen, 10 μl of a deoxyribonucleic triphosphate mixture, 1 μl of primer 1, 1 μl of primer 2, 1 μl of the DNA solution, and 0.75 μl of Expand High Fidelity DNA polymerase (Laroch). The polymerase was added after a hot-start procedure (5 min at 94°C, followed by 5 min at 80°C). PCR was performed with a Perkin-Elmer 9600 thermocycler by using the following protocol: 1 min at 94°C (denaturation), 1 min at 65°C (annealing), and 3 min at 72°C (elongation) with a 1°C rise every second cycle during annealing for 20 cycles, followed by 20 cycles with an annealing temperature of 55°C and a final cycle consisting of 10 min at 72°C.

**DGGE.** Profiles of the amplified 16S rRNA gene sequences were produced by DGGE as described by Muyzer et al. (20) by using the Ingeny U-Phor system (Ingeny, Goes, The Netherlands). The PCR products were loaded onto a polyacrylamide gel (8% [wt/vol] acrylamide in 0.5× TAE buffer [4.84 g of Tris base per liter, 11.42 ml of acetic acid per liter, 20 ml of 0.5 M EDTA per liter; pH 8.0]) with a 45 to 75% denaturant gradient (100% denaturant was 7 M urea and 40% [vol/vol] denaturated formamide). The gels were loaded with equal amounts of DNA, and electrophoresis was carried out in 0.5× TAE buffer at 75 V for 16 h at 60°C. The DNA fragments were stained for 20 min in 0.5% TAE buffer with ethidium bromide (final concentration, 0.5 μg/liter). The gel was destained in distilled water for 5 min. Images of the gels were obtained by using a UV 300

**Table 1. Site and soil characteristics of the northern, central, and southern German experimental sites**

<table>
<thead>
<tr>
<th>Site</th>
<th>Location</th>
<th>Temp (°C)</th>
<th>Precipitation (mm)</th>
<th>Organic C concn (mg of C g of soil–1)</th>
<th>pH (CaCl2)</th>
<th>Texture</th>
<th>Crop in 2002</th>
<th>Soil units</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hohenschulen</td>
<td>54°19’N, 10°00’E</td>
<td>8.5</td>
<td>750</td>
<td>17</td>
<td>7.0</td>
<td>sL-tL</td>
<td>Triticum</td>
<td>Luvisols</td>
<td>b</td>
</tr>
<tr>
<td>Frankenhausen</td>
<td>51°24’N, 9°26’E</td>
<td>9.7</td>
<td>622</td>
<td>12</td>
<td>7.3</td>
<td>tU</td>
<td>Secale</td>
<td>Luvisols, antirohos, pelsools</td>
<td>a</td>
</tr>
<tr>
<td>Scheyern</td>
<td>48°29’N, 11°26’E</td>
<td>7.4</td>
<td>803</td>
<td>15</td>
<td>6.2</td>
<td>sul</td>
<td>Medicago and Lolium</td>
<td>Luvisols, cambisols</td>
<td>c</td>
</tr>
</tbody>
</table>

Notes: a Mean annual temperature. b Mean annual precipitation. c Classification according to ISSS-ISRIC-FAO (1998).
RESULTS

Number of DNA bands and number of bacterial species. Eight of ten bacterial strains isolated from soil yielded a single band. Two strains yielded more than one band. Flavobacterium sp. yielded a strong band and a weak band, and E. cloacae clearly produced five bands whose intensities were similar.

The eight strains that produced a single band were added to autoclaved soil both separately and in a mixture. The autoclaved soil did not produce any DNA band. No Nocardia and Rhodococcus DNA bands were detected (Fig. 1). For Rhodococcus the reason is not clear. The densities of Nocardia (10^5 cells g\(^{-1}\)) were probably below the detection limit of our DGGE protocol. This protocol is normally used for bacterial communities in field soils with total densities of about 10^9 bacteria g\(^{-1}\). The other six isolates each yielded a clear DNA band at a density of about 10^5 cells g\(^{-1}\). The individual isolates were clearly reflected in the DNA band pattern of the mixture. When the mixture was diluted 10-fold, the pattern was weaker but still visible. This indicates that the detection limit of our DGGE protocol is about 10^6 cells g of soil\(^{-1}\). The Arthrobacter and Corynebacterium bands were so close that they appeared to be one band in the mixture.

DNA band intensity and relative abundance of bacteria. The ratio of the optical densities was reflected by the ratio of the intensities of the three DNA bands in each lane (Fig. 2). The relative abundance calculated from the intensity of the DNA bands correlated with the relative abundance calculated from the optical densities (r = 0.73; P < 0.0001; n = 27) and with the relative abundance calculated from the cell numbers (r = 0.67; P < 0.0001; n = 27).

Bacterial DNA content in plant litter. The original litter material contained a small amount of DNA, and the amount increased rapidly after the litter bags were inserted into the soil (Fig. 3). The DNA content of the crude extract was generally higher for the rye grass than for the wheat straw. The DNA content of the rye litter was higher at the central German site than at the northern and southern sites on day 18. The target gene for the bacterial communities could successfully be amplified in all extracts with two exceptions: one rye litter replicate on day 180 and one fresh wheat straw replicate on day zero. These samples were not used for the statistical analyses. Equal quantities of amplified DNA were loaded into the slots of the different lanes on the gel for semiquantitative comparison of the diversity of litter-decomposing bacteria by DGGE.
DGGE. The melting profiles revealed different DNA fragments that were the same length but had different base sequences (operational taxonomic units or species) over the 180-day decomposition period (Fig. 4 and 5). Between 28 and 52 bands were distinguished in the rye litter and the wheat litter (Table 2). The lowest number was in the fresh rye litter, and the highest number was obtained after 118 days of decomposition in the wheat straw. Dominating bands were present particularly during the first part of the decomposition period on days 0, 18, and 58, in contrast to the later stages of decomposition on days 118 and 180, when the bands were more similar.

In lane 13 of Fig. 6, the main DNA bands are hardly visible even though the DNA content in this sample was similar to the DNA content in the replicate lane, lane 12 (Fig. 3). However, the difference between lanes 12 and 13 indicates that amplification of the target sequences was inhibited for unknown reasons. Therefore, lane 13 was not used for further analysis.

Diversity. The Shannon-Weaver diversity index, evenness, and equitability showed that generally microbial diversity increased as decomposition proceeded (Table 2). When evenness and equitability were examined, this trend was not significant after 18 days, particularly for wheat litter. The average number of bacterial DNA bands increased significantly from 32 on day 0 to 45 on day 180 \( (P = 0.003) \), as determined by analysis of variance. The average diversity in the wheat straw (44 DNA bands) was significantly higher than that in the rye litter (36 DNA bands) \( (P = 0.0004) \). The highest diversity was found in the wheat straw on day 118. The increasing evenness and equitability of the bacterial communities during the course of litter decomposition indicated that the species were more evenly represented during the later stages of decomposition.

On day 18, the number of DNA bands and the Shannon-Weaver diversity index were significantly higher at the central and southern German sites \( (P = 0.023) \). The evenness and equitability values were similar at the three sites.

Similarity and succession. PCA showed that the (qualitative) differences in DNA profiles among the five decomposition stages were much greater than the differences between the duplicates (Fig. 5). The rye litter and straw litter showed divergent development of bacterial communities (Fig. 7) which was associated with significantly different diversity indices. Also, the differences among the bacterial DNA profiles at the sites in northern, central, and southern Germany were much
The intensities of the DNA bands reflected the relative levels of bacterial communities developed in the decomposing rye litter that had the same origin.

DISCUSSION

The DNA band pattern obtained by DGGE is an attractive way to study complex communities in environmental samples because most of the bacterial genotypes (species) gave one band in our experiments with species added to a sterilized soil. The intensities of the DNA bands reflected the relative levels of the bacterial strains, and the bands for most individual strains were also present in the DNA band pattern of the mixture. However, both under- and overestimation of the real number of genotypes could occur. Although we occasionally observed missing and overlapping bands which may have complicated the interpretation of band patterns, bacterial DNA profiles obtained by DGGE can be used as a semiquantitative measure of bacterial diversity, and we therefore used this method to study bacterial diversity during litter decomposition in agricultural soils.

The DNA band patterns obtained from amplified 16S rRNA gene sequences and DGGE indicated that the structure and diversity of bacterial communities changed significantly during 180 days of litter decomposition in an agricultural soil. The number of bacterial DNA bands increased from 28 to 40 in rye litter and from 36 to 52 in wheat litter (Table 2). The numbers of bands in the litter were in the range found in rhizosphere and bulk soil by Duineveld et al. (8) but were lower than the numbers of bands in mineral soils in northern Germany (more than 50 bands [unpublished data]) and in The Netherlands (about 50 bands [4]). Compared to the DGGE patterns for rhizosphere soil, the DGGE patterns for bulk soil are generally complex, with many distinct bands (30).

The number of DNA bands increased as litter decomposition proceeded, whereas the microbial biomass and activity decreased (6). This can be regarded as due to bacterial adaptation to more heterogeneous environmental conditions and the complex composition of the remaining organic matter. Soil microbial communities have extreme phenotypic and genotypic diversity (28). The increase in diversity as the activity decreased appeared to reflect the conversion of litter to soil organic matter and the concomitant development of diverse microbial communities adapted to lower availability of nutrients. The disappearance of dominating bands and the subsequent development of a more uniform band pattern as decomposition proceeded can be interpreted as follows: r strategists (opportunists) that prevailed on fresh litter were replaced by a variety of K strategists (persisters) related to resistant organic matter and humic substances (1). The development of a higher level of diversity is probably related to an increased importance of biotic interactions within the community (25, 26).

Only approximately 30% of the wheat straw had been decomposed after 180 days, whereas more than 80% of the rye litter had disappeared (data not shown). The bacterial communities in the slowly decomposing wheat straw appeared to be more diverse than those in the rapidly decomposing rye litter. Apparently, more species (or genotypes) were required for decomposition or were able to grow when the litter quality was low. The low nutritional quality of the wheat straw was reflected by the content of bacterial DNA, which was much lower for wheat straw than for rye litter (Fig. 3). In the rapidly decomposing rye grass litter with higher nutritional value, the amount of bacterial DNA increased rapidly and the bacterial DNA was dominated by fewer organisms, as indicated by the lower number of bands and lower diversity.

Previous studies on decomposition of various litter types showed that there were litter-specific biomass pools (21), decomposition-stage-dependent changes in biomass, respiration, and enzyme activity (6), and also specific community compositions of culturable bacteria and fungi (7). This study showed that the total communities, including both cultivable and non-culturable bacteria, became more diverse during litter decomposition in agricultural soils, as revealed by DGGE of specifically amplified PCR products. More information concerning the identities of the dominant members of the bacterial community could be obtained by excision of DNA bands from the gels, followed by cloning and sequencing of PCR-amplified gene fragments, as has been done for ammonia-oxidizing communities by Laverman et al. (16). Identification of species was
beyond the scope of this study, in which we focused on quantification of diversity during litter decomposition.

Based on band pattern and bacterial diversity indices in the same litter investigated in soils in northern, central, and southern Germany, site-specific differences for the same litter were found, and such differences had to be expected. However, we acknowledge that common soil components may form complexes with proteins and may inhibit the PCR by interaction with Taq DNA polymerase (31).

In conclusion, microbial diversity increased during the course of litter decomposition (hypothesis 1 was confirmed), in accordance with the system-theoretical hypotheses of Odum (23, 24). Compared to the microbial communities in readily available rye grass, refractory straw enabled development of microbial communities with greater diversity (hypothesis 2 was rejected). Bacterial diversity differed in the same litter buried in similar soils at different locations (hypothesis 3 was con-
firmed). Thus, not only the origin of the litter but also the surrounding soil affected the development of bacteria in the litter.

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