Applying molecular techniques to monitor microbial communities in composting processes

S. Koschinsky, S. Peters, F. Schwieger, C.C. Tebbe

Institut für Agrarökologie, Bundesforschungsanstalt für Landwirtschaft (FAL), Bundesallee 50, 38116 Braunschweig, Germany

ABSTRACT

Microorganisms participating in the self-heating phase of composting material, which consisted of marker gene tagged corn (*Zea mays*), horse dung and wood chips, were characterized by DNA fingerprinting (ARDRA) of cells cultured at 50°C and by analyzing DNA that was directly extracted from compost samples. Cultivation dependent methods detected five major groups at the end of the self-heating phase (after 18 d). Three groups showed high homology to bacteria from the genus *Bacillus* at the 16S rRNA gene level. Straightforward PCR amplification of bacterial target genes from compost extracted DNA was inhibited because of the high background of plant DNA and presence of humic acid-like compounds. By use of humic acid resistant, heat-stable DNA polymerases in combination with single strand, DNA binding, T4 gene 32 protein, PCR inhibition could be completely overcome. After 6 d of composting, the marker gene of the corn plants was degraded below the threshold of detection ($< 10^{2.6}$ copies per g of compost). The diversity of bacterial and fungal communities in the compost was analyzed by single-strand-conformation polymorphism (SSCP) of approx. 400 bp PCR products, which were amplified with universal primers for 16S rRNA (bacteria) and 18S rRNA genes (fungi), with compost DNA as a template. The generated patterns showed a succession of different members of the microbial community during the self-heating phase.

Introduction

Molecular, nucleic acid-based characterization techniques are a promising approach to increase our knowledge about succession and function of microbial communities during composting processes. DNA-DNA hybridization with gene probes has been used to monitor the fate of specific bacterial strains, such as *E. coli* or *Salmonella* in composts [4]. The use of PCR amplification of 16S rRNA genes of cultivated isolates followed by restriction enzyme analysis of the amplified products (ARDRA) is an efficient way to differentiate bacterial isolates from compost and obtain information about their phylogenetic position. Specific microorganisms from compost can also be identified by PCR detection of characteristic genes [15].

Directly extracted DNA from composting material can be used as a template for PCR amplification and detection of specific microorganisms without the need of cultivation. The *lacZ* gene of *E. coli* [10] has been targeted in PCR reactions with compost DNA. Other target sequences can include 16S rRNA genes using universal primers for bacteria or 18S rRNA genes for fungi. From the mixture of PCR products of almost identical size, which will be generated from such a community DNA, patterns can be generated by

Microbial Biosystems: New Frontiers
Proceedings of the 8th International Symposium on Microbial Ecology
Bell CR, Brylinsky M, Johnson-Green P (eds)
Atlantic Canada Society for Microbial Ecology, Halifax, Canada, 1999.

electrophoretic techniques. Such genetic "fingerprints" should reflect the structure of the microbial community or a specific fraction of it. DNA-based direct community analysis is important since cultivation dependent methods intrinsically favor the growth of specific community members. A variety of techniques has recently been developed for community analysis: denaturing gradient gel electrophoresis (DGGE) [9], terminal restriction fragment length polymorphism (tRFLP) [8] and DNA single-strand-conformation polymorphism (SSCP) [7,12].

In the present study we investigated the self-heating phase of compost material with DNA based detection methods. The stability of DNA from plant material was analyzed by following the persistence of a recombinant plant marker gene. These results have relevance for recommendations for a waste recycling process for genetically engineered plants. The diversity of cultivated bacteria that were present at the end of the self-heating phase after 18 d of composting was determined by ARDRA. The succession of bacterial and fungal communities was monitored during the composting process by SSCP of PCR amplified regions of the 16S and 18S rRNA genes from total compost DNA.

Materials and Methods

Composting Process and Sampling

Two compost piles were set up: One compost pile contained corn plants (*Zea mays*) which were genetically engineered (GMO) by the chromosomal insertion of the phosphinothricin acetyl transferase (*pat*) gene (Anjou 285 Prestige LL, AgrEvo, Frankfurt, Germany). This gene confers resistance to the herbicide BASTA® (AgrEvo). The other pile contained corn plants without the *pat* gene. Each pile consisted of 750 kg of shredded plant material, 0.5 m^3 wood chips and 10 % straw-bedded horse manure. The components were mixed, wetted and filled into wooden boxes. The hot composting stage was stopped after 18 d. Samples were taken from the 60 cm depth. For DNA extraction samples were frozen at -20°C.

Extraction of Total DNA from Compost Samples and Detection of the Recombinant pat-gene

Compost samples were ground in liquid N and subsequently DNA was extracted from 200 mg (wet weight) compost samples using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The DNA concentration was determined fluorometrically (Pico Green; Molecular Probes, Leiden, The Netherlands). The nucleotide sequence of the *pat*-gene was kindly provided by AgrEvo. PCR reactions were conducted in a total volume of 50 µl. Each reaction contained 1.5 mM $MgCl_2$, 200 µM of ATP, CTP, GTP and TTP, respectively, 0.5 µM of each primer (Table 1) and 1 unit of thermostable DNA polymerase (Expand®; Boehringer Mannheim, Mannheim, Germany). A total of 2.5 µg T4 gene 32 protein (Boehringer) was added to each PCR amplification [11]. The amplification was carried out with the following conditions: 2 min at 94°C initial denaturation followed by 35 cycles, each consisting of 40 s at 94 °C, 1 min at 72°C and a final extension for 5 min at 72°C.

Characterization of Cultivated Bacteria and of Microbial Community Structures by SSCP

Compost samples were extracted with 0.1 % sodium polyphosphate solution. Dilutions were inoculated onto plate count agar (PCA; Oxoid Unipath Ltd., Basingstoke, Hampshire, U.K.) with or without 10 mM BASTA®. Cycloheximide (final concentration 50 mg l^{-1})

was added to the media to inhibit fungal growth. Inoculated plates were incubated at 50°C for 16 h. In order to distinguish bacterial isolates, amplified ribosomal DNA restriction analysis (ARDRA) was applied [5] with primers shown in (Table 1). Each ARDRA group was defined by patterns obtained from two enzymes (*Cfo* I and *Rsa* I). PCR products of the 16S rRNA genes obtained from representative isolates were further identified by DNA sequencing (IIT Bioservice, Bielefeld, Germany). The sequences were analyzed using BLASTN 2.0.4 [1].

Table 1. Primer sequences and applications in this study

Target gene and size (base pairs) of amplified fragment	Purpose of analysis	Sequence (5'-3')**	Reference
pat gene, 377 bp	marker gene for plant DNA	Pat-1 (f): CCA CAA ACA CCA CAA GAG T Pat-2 (r): TGC CAA AAA CCA ACA TCA T	This study
16S rRNA gene, approx. 1,060 bp	ARDRA of cultivated bacterial isolates	Ec41(f): GCT CAG ATT GAA CGC TGG CG Ec1066 (r): ACA TTT CAC AAC ACG AGC TG	[8]
16S rRNA gene, 408 bp	bacterial community structure by SSCP	Com1* (f): CAG CAG CCG CGG TAA TAC Com2 (r): CCG TCA ATT CCT TTG AGT TT	[12]
18S rRNA gene, 377 bp	fungal community structure by SSCP	NS7* (f): GAG GCA ATA ACA GGT CTG TGA TGC NS8 (r): TCC GCA GGT TCA CCT ACG GA	[14]

*primer not phosphorylated at the 5' end
**forward (f) and reverse (r)

Using primers which hybridized to highly conserved regions of the bacterial 16S rRNA genes and fungal 18S rRNA genes, products of approx. 400 bp length were amplified by PCR with total compost DNA as a template (Table 1). Thermocycling and removal of the phosphorylated strands of the PCR products were done as described elsewhere [12]. The remaining DNA was separated by PAGE (0.6 x MDE, FMC Bioproducts, Rockland, Me.). Gels were silver stained [2].

Results and Discussion

Within 3 d of composting, the temperature in 20 cm depth increased from 20°C to 60°C, the maximum of 78°C was recorded after 10 d. Further on the temperature declined to 60°C until day 18. The pH decreased within the first two days from 5.4 to 4.3. Later on, during the self heating phase, the pH increased to 7.3 (18 d). Total N increased as a result of mineralization from 17 mg N kg^{-1} to 19 mg N kg^{-1}. Ammonia increased from 4 mg N kg^{-1} to 16 mg kg^{-1}. Nitrate decreased from 4 mg N kg^{-1} to levels below the detection limit (< 1 mg N kg^{-1}).

Persistence of Plant DNA during Composting

Applying the compost DNA extraction protocol developed for this study we were able to obtain 30 µg DNA from 200 mg of compost material taken at the beginning of the composting. Later on the yields decreased, indicating a degradation of total DNA in the compost. The threshold of detection for the *pat* gene was $10^{2.6}$ copies per g fresh weight. The PCR mediated detection of the *pat* gene from composting material was inhibited in non-diluted DNA extracts, probably by the presence of humic acids generated during composting. However, this inhibitory effect could be eliminated by optimizing for humic

acid resistant, heat stable DNA-polymerases and the DNA single strand binding T4 gene 32 protein [13]. A similar approach allowed sensitive detection of genes in DNA directly extracted from soil [11].

The decay of plant DNA was monitored by PCR detection of the *pat* gene in 10-fold dilutions of compost DNA (Fig. 1). In 10^2 dilutions the *pat* gene could only be amplified from samples taken after 1 d. Tenfold dilutions contained detectable amounts of *pat* for 3 d. The decay of the *pat* gene to levels below the detection limit in non-diluted DNA was reached after 8 d. Thus, DNA was quickly degraded during the self-heating phase.

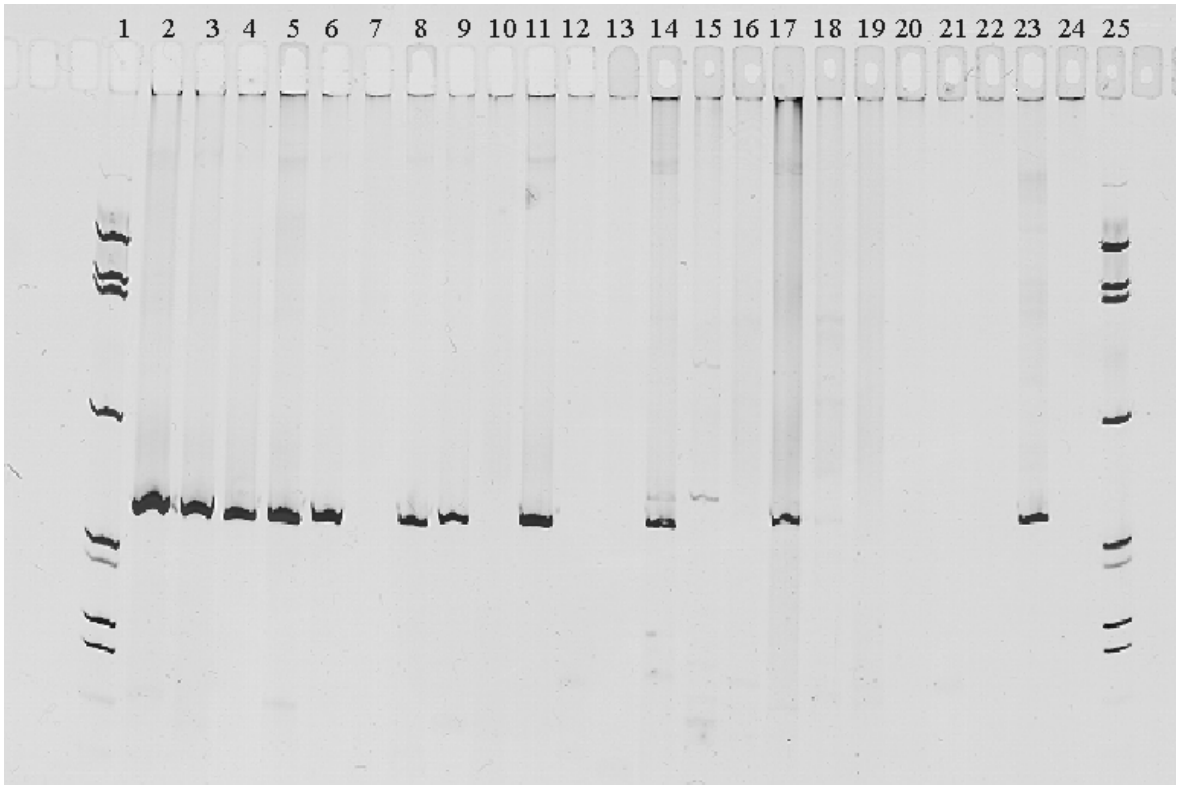

Fig. 1.

Diversity of Cultivated Bacteria at the End of the Hot Composting Stage

ARDRA analysis of the composition of bacterial strains at the end of the self-heating phase indicated that the majority of 600 isolates could be grouped into one of five ARDRA types. DNA sequencing of the 16S rRNA genes showed that three groups showed highest similarities (> 98 %) to bacteria of the genus *Bacillus*. This is in accordance to a recent study [3] where several *Bacillus* species were isolated from hot compost. The only group which was detectable on BASTA amended medium was related to *Pseudomonas stutzeri* (similarities, 96 to 97.3 %). The fifth group could not be assigned with high similarity ($\geq$ 96 %) to any sequence in the database. This indicated that even on basis of cultivation methods the diversity of bacteria in hot composts is not well characterized yet.

SSCP Analyses of Microbial Communities during the Self-heating Phase

Our new protocol [12] based on separation of single stranded DNA molecules obtained from 16S or 18S rRNA gene targeted sequences was suitable for both pure culture and

community analysis. Profiles of 16S rRNA amplified genes from compost DNA increased in their complexity during the composting process (Fig. 2). This indicated that the bacterial diversity increased. Products obtained from pure culture isolates could also be detected in the pattern obtained from the 18 d community DNA sample. However, in order to assign each dominating band in the community patterns to specific microorganisms, further subcloning and DNA sequencing would be needed. The changing profiles obtained with community DNA demonstrated that community members changed during composting.

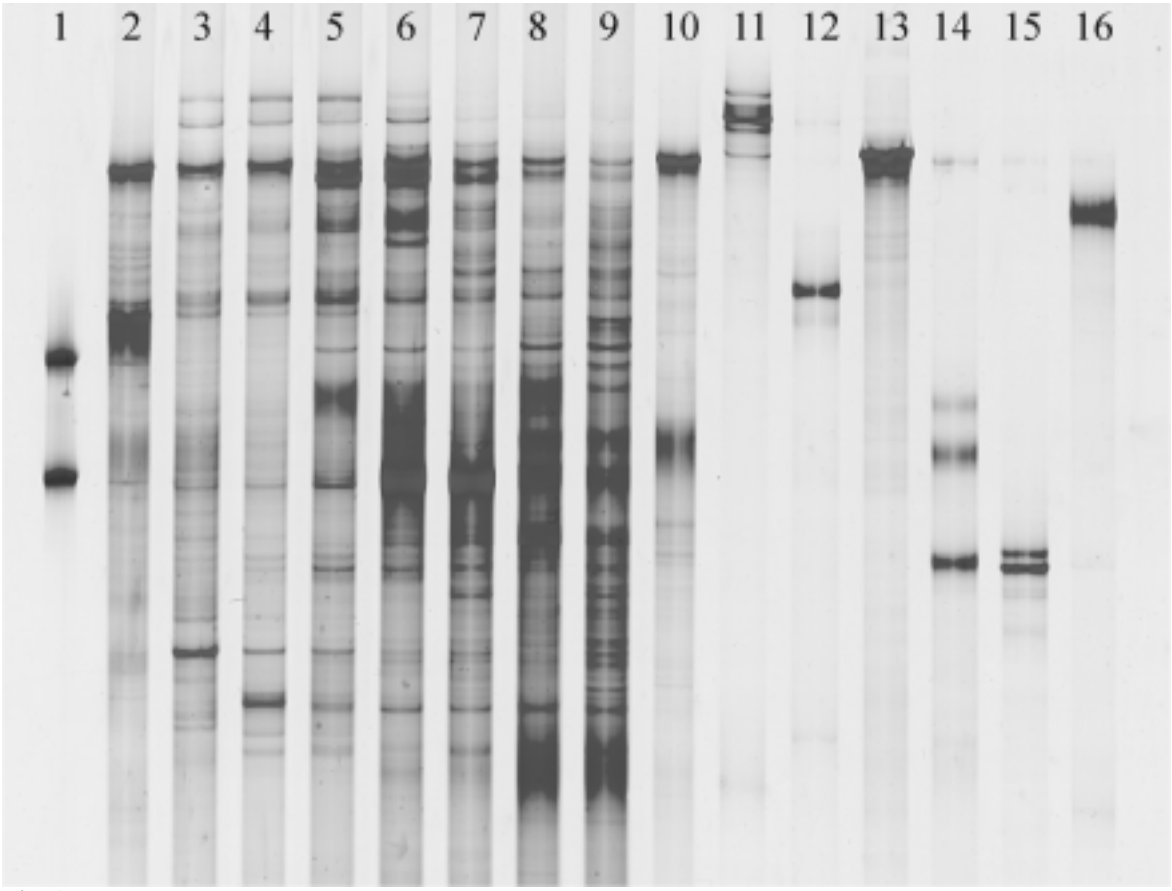

Fig. 2.

Less complex patterns were obtained with PCR primers designated to amplify fungal 18S rRNA genes (Fig. 3). This agrees with general knowledge and recent experimental evidence that during the hot composting stage fungal biomass is reduced [6]. However, two different bands, one detectable from day 1 to day 12 and another detectable from day 13 to 18 indicated that different fungi were present depending on the composting phase. Beside the fungal 18S rRNA genes also there was plant DNA amplified with the selected primers (Fig. 3, lane 19). During the composting process, the 18S gene of corn could only be detected from day 0 to 3. This supported the results obtained from the PCR mediated marker (*pat*) gene detection.

In summary, this study shows that nucleic acid based techniques provide new and valuable tools to characterize both cultivated and non-cultivated members of compost microbial communities. The community based genetic “fingerprinting” techniques offer a

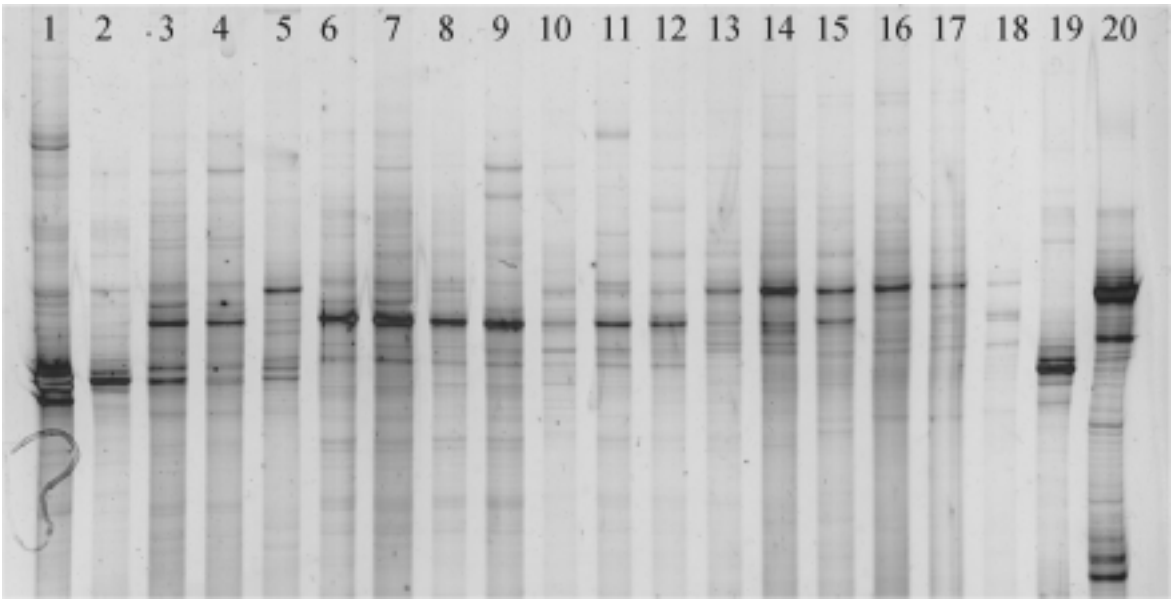

Fig. 3.

high potential to directly and quickly characterize the microbial diversity in compost samples. The patterns, generated with these techniques still need more detailed analysis to understand their ecological relevance. Also such techniques cannot directly distinguish between active and dormant cells, e.g. spores. Therefore, new developments concerning the detection of microbial gene expression in composts would be desirable. It is likely, that techniques for purposes will also be a nucleic acid based.

Acknowledgements

We thank Evelin Schmmer and Katja Lübke for their excellent assistance. The work was supported by the Federal Environmental Agency of Germany (grant 11201032).

References

1. Altschul, SF, Madden TL, Schäffer AA, Zhang J, Zahng Z, Miller W, Lipman DJ (1998) Gapped BLAST and PSIBLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389-3402
2. Bassam BJ, Caetano-Anolles G, Gresshoff PM (1991) Fast and sensitive silver staining of DNA in polyacrylamide gels. Anal Biochem 80:81-84.
3. Blanc M, Marilley L., Beffa T, Aragno M (1997) Rapid identification of heterotrophic, thermophilic, sporeforming bacteria isolated from hot composts. Internat J Sys Bacteriol 47:1246-1248
4. Droffner ML, Brinton WF (1995) Survival of *E. coli* and *Salmonella* populations in aerobic thermophilic composts as measured with DNA gene probes. Zbl Hyg 197:387-397
5. Hoffmann A, Thimm T, Dröge M, Moore ERB, Munch JC, Tebbe CC (1998) Intergeneric transfer of conjugative and mobilizable plasmids harbored by *Escherichia coli* in the gut of the soil microarthropod *Folsomia candida* (Collembola). Appl Environ Microbiol 64:2652-2659

6. Klamer M, Bååth E (1998) Microbial community dynamics during composting of straw material studied using phospholipid fatty acid analysis. FEMS Microbiol Ecol 27:9-20
7. Lee DH, Zo Y-G, Kim S-J (1996) Nonradioactive method to study genetic profiles of natural bacterial communities by PCR-single-strand-conformation polymorphism. Appl Environ Microbiol 62:3112-3120
8. Liu WT, Marsh TL, Cheng H, Forney LJ (1997) Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. Appl Environ Microbiol 63:4516-4522
9. Muyzer G, de Waal EC, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reactionamplified genes coding for 16S rRNA. Appl Environ Microbiol 59:695-700
10. Pfaller SL, Vesper SJ, Moreno H (1994) The use of PCR to detect a pathogen in compost. Compost Science & Utilization 2:48-54
11. Schwieger F, Tebbe CC (1997) Efficient and accurate PCR ampflification and detection of a recombinant gene in DNA directly extracted from soil using the Expand® High Fidelity PCR system and T4 gene 32 protein. In: Ziebolz B (ed.) Biochemica 1997, Vol. 2. Boehringer Mannheim, Mannheim, Germany (ISSN 0946-1310) p 21-23
12. Schwieger F, Tebbe CC (1998) A new approach to utilize PCR-single-strand-conformation polymorphism for 16S rRNA based microbial community analysis. Appl Environ Microbiol 63:(issue 12)
13. Tebbe CC, Vahjen W (1993) Interference of humic acids and DNA extracted directly from soil in detection and transformation of recombinant DNA from bacteria and a yeast. Appl Environ Microbiol 59:2657-2665
14. White TJ, Bruns T, Lee S, Tayler J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols. A guide to methods and applications. pp.315-322. Academic Press, San Diego, USA.
15. Wolf JM van der, Beckhoven JRCM van, Vries PM de, Raaijmakers JM, Bakker PAHM, Bertheau Y, Vuurde JWL van (1995) Polymerase chain reaction for verification of fluorescent colonies of *Erwinia chrysanthemi* and *Pseudomonas putida* WCS358 by immunofluorescence colony staining. J Appl Bacteriol 9:567-577