New quality of assessment of microbial diversity in arable soils using molecular and biochemical methods

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Summary – Zusammenfassung

New molecular and biomarker approaches allow now a better understanding of the microbial diversity in soils. Examples are presented to demonstrate the new performance of these approaches and the quality of results. Investigations of the diversity of a bacterial indicator genus as well as studies of the whole microbial community structure are presented. Genotypes of *Ochrobactrum* were isolated by immunotrapping and characterized by PCR–fingerprinting. On a follow-up basis no significant changes in the intragenus diversity of this indicator bacterium was observed over a period of one year. In contrast, crop rotation was accompanied by a shift in the dominant genotypes of *Ochrobactrum*, while the genetic potential was not affected.

Phospholipid fatty acid (PLFA)-pattern revealed significant shifts in the structure of the whole microbial community in a soil of a hop plantation as compared to a similar soil with crop rotation. The quantity of anaerobic bacteria, represented by the non-ester linked fatty acids, were lower in soil samples derived from crop rotation as compared to a former hop plantation soil, which was contaminated with copper. In contrast, the ester linked fatty acids which are present in a wide range of Gram-negative and Gram-positive bacteria, exhibited a reverse relation. Soil samples of the hop plantation contained low amounts of saturated ester linked fatty acids branching on the 10° C-atom. This indicates that actinomycetes are present in relative small proportions in the hop plantation soil.

**Key words**: microbial diversity / phospholipid fatty acid analysis / *Ochrobactrum* spp. / genotype fingerprinting

Erfassung der mikrobiellen Diversität in Ackerböden mit Hilfe von modernen molekularbiologischen und biochemischen Methoden


1 Introduction

Biodiversity has become a topic of profound interest and concern not only to the scientific community but also to the public as well as to governmental and non-governmental organizations. Soil microbial diversity is important, because the activity of micro-organisms is essential for geochemical cycles, turnover processes of organic matter, soil fertility and soil quality (Atlas et al., 1990). Communities with a low diversity and only highly adapted micro-organisms can hardly respond to environmental changes (Bianchi and Bianchi, 1995). A major problem for soil microbial analysis is that most of the soil micro-organisms cannot be characterized by classic microbiological cultivation techniques. Estimations calculate about 80–99% of the soil micro-organism as not yet cultured (Aumann et al., 1995).

We characterized the microbial community in soils from different farming management systems using molecular and biochemical approaches: The intragenus diversity of a widely distributed soil bacterium (*Ochrobactrum*) was determined by a genetic fingerprint technique, while the whole microbial community structure was investigated with phospholipid fatty acid analysis (PLFA).

The development of molecular tools for studying microbial populations remarkably progressed in recent years. Torsvik et al. (1990) estimated about 10 000 different species from reassociation kinetics of melted DNA extracted...
from soils. Ward et al. (1990) demonstrated a very high microbial diversity by direct extraction of DNA from soil analysing the genes for the 16S subunit of the rRNA (16S-rDNA). Since that time many researchers used the genes for the rRNA as a marker gene to describe microbial diversity, as these genes are functionally conserved in all organisms and contain conserved, variable and highly variable sequence regions (Ludwig and Schleifer, 1994). More than 5000 sequences for rRNA genes are at present available in databases. Therefore one possibility to analyse microbial diversity is to amplify the rRNA genes by PCR from soil DNA and to sequence the cloned rDNA fragments (Stackebrandt and Liesack, 1993). Using this approach, a new bacterial phylum was discovered recently (Ludwig et al., 1997). This time consuming approach is not suitable for the analysis of large sample numbers. Using fluorescence labeled oligonucleotide probes, which are directed against rRNA sequences (Amann et al., 1995) more samples can be analysed compared to the cloning technique. The quality of the results strongly depends on the activity of the analysed microflora, because a high number of ribosomes per cell is required for this type of analysis. To study population dynamics the DGGE analysis of amplified fragments of the rRNA genes is widely used (Muyzer et al., 1993). But still this analysis is not sufficient for a detailed description of the microbial diversity. This might be possible by studying the diversity of representative microbial populations in detail. For example the intrageneric diversity of the common soil bacterium *Ochrobactrum* could be accurately determined (Schloter et al., 1996). *Ochrobactrum* belongs to the α-2 subclass of proteobacteria (Holmes et al., 1988) and has important properties, e.g. the ability to denitrify and the potential to degrade organic soil contaminants like 2,4 D, atrazine and mineral oil.

Recently many publications have been published using phospholipid fatty acids (PLFA) for the assessment of soil microbial biomass and the composition of the microbial community in soils. Phospholipids are essential components of membranes of all living cells and are not present in storage products of micro-organisms or in dead cells. Under the conditions expected in natural communities the bacteria contain a relatively constant proportion of their biomass as phospholipids (White et al., 1979a). Viable microbes have an intact membrane which contains fatty acids as components of its phospholipids. After cell lysis, cellular enzymes hydrolyze the phospholipids and release the phosphate group within minutes to hours (White et al., 1979b). Therefore, PLFA-analysis is suitable for detecting rapid changes of living populations. A number of sub-fractions of PLFAs can be used as signature or marker molecules for specific groups of micro-organisms (Tunlid and White, 1992). This biomarker approach has recently been applied to characterize the soil microflora and showed considerable promise to differentiate related groups of microorganisms (e.g Zak et al., 1996; Zelles et al., 1994, 1997; Zogg et al., 1997).

## 2 Material and Methods

### 2.1 Soil samples

The sampling area is part of the research farm at Scheeheim (FAM), 40 km north of Munich, Germany. The main goal of FAM is to develop productive and environmental saving agricultural land usage systems. The soils are all derived from loess and molasse sediments. Soil DP2 and GT has been under crop rotation. Soil Hop from a former hop plantation contained a high amount of copper (200 mg kg⁻¹) due to the prior extensive use of copper fungicides in this soil. S30 was a fallow land. DP2, GT and Hop were cropped with maize in the year of sampling. The physical and chemical properties of the four soils are listed in Tab. 1. All soil samples were taken from the top soil horizon (0–10 cm) and sieved (2 mm).

### 2.2 Intrageneric diversity of *Ochrobactrum*

Bacteria were extracted from the soil according to Hopkins et al. (1991). With the use of genus- (or strain-) specific monoclonal antibodies (Schloter et al., 1995) bacteria belonging to the whole genus or a certain strain of *Ochrobactrum* were enriched from different soil samples by immunotraping without selective plating (Schloter et al., 1996). The genotypes of the isolates (50 per soil sample) were characterized by ERIC – PCR pattern analysis Endts et al. (1993). The diversity patterns were calculated using the statistic program SPSS (SPSS, Munich, Germany).

### 2.3 PLFA analysis

The extraction of lipids and the separation of the PLFA have been described in detail by Zelles (1996). Briefly, a modified one-phase extraction procedure was used for extraction of phospholipids and separation of fatty acids. The lipid material was fractionated into neutral lipids, glycolipids and polar phospholipids by a silica-bonded phase column. The phospholipid fraction was subjected to mild alkaline hydrolysis in order to liberate the ester-linked (EL) fatty acids. The products of hydrolysis were separated into unsubstituted fatty acids, hydroxy-substituted fatty acids (POH), and the remaining unsaponifiable lipids. Unsubstituted fatty acids were further separated into saturated (SATFA), monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) by silver ion chromatography. The unsaponifiable lipid fraction was subjected to a one-step acid hydrolysis. The liberated fatty acids of the nonester-linked phospholipid fatty acids (NEL-PLFA) were separated into unsubstituted fatty acids (UNSAFA) and hydroxy substituted fatty acids (UNOH) by an aminopropyl-bonded phase column.

## 3 Results and Discussion

### 3.1 Intrageneric diversity of *Ochrobactrum*

#### 3.1.1 Time dependent analysis in a fallow soil

Bacteria of the genus *Ochrobactrum* were enriched from top soil samples, taken from a fallow land (S30) at 4 different times during the years 1995/1996. Among 50 isolates seven different genotypes (S30-1 to S30-7, as defined by PCR-fingerprinting) were present in April, October and January, while only five could be detected in July (S30-2 to S30-6) (Fig. 1). Genotypes S30-2 and S30-4 were dominant at each sampling date, while the frequencies of the genotypes S30-1, S30-5, S30-6, and S30-7 were clearly lower (Fig. 1). Studying the relationship between the isolates,
Table 1: Physical and chemical properties of the soils.
Tabelle 1: Physikalische und chemische Eigenschaften der Böden.

<table>
<thead>
<tr>
<th>Soil</th>
<th>DP2 Cropping formerly</th>
<th>DP2 Cropping at sampling time</th>
<th>Hop Cropping formerly</th>
<th>Hop Cropping at sampling time</th>
<th>GT Cropping formerly</th>
<th>GT Cropping at sampling time</th>
<th>S30 Cropping formerly</th>
<th>S30 Cropping at sampling time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Crop rotation</td>
<td>Hop plantation</td>
<td>Crop rotation</td>
<td>Hop plantation</td>
<td>Crop rotation</td>
<td>Hop plantation</td>
<td>Crop rotation</td>
<td>Hop plantation</td>
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<tr>
<td></td>
<td>Maize</td>
<td>Maize</td>
<td>Maize</td>
<td>Maize</td>
<td>Maize</td>
<td>Maize</td>
<td>Maize</td>
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</tr>
<tr>
<td>Clay (%)</td>
<td>18</td>
<td>13</td>
<td>20</td>
<td>22</td>
<td>58</td>
<td>60</td>
<td>60</td>
<td>64</td>
</tr>
<tr>
<td>Silt (%)</td>
<td>38</td>
<td>36</td>
<td>58</td>
<td>60</td>
<td>22</td>
<td>19</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td>Sand (%)</td>
<td>44</td>
<td>51</td>
<td>59</td>
<td>64</td>
<td>1.90</td>
<td>1.41</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>pH (CaCl₂)</td>
<td>6.0</td>
<td>6.1</td>
<td>0.20</td>
<td>0.16</td>
<td>10.18</td>
<td>9.50</td>
<td>7.13</td>
<td></td>
</tr>
<tr>
<td>C₆H₁₂O₆ (%)</td>
<td>1.73</td>
<td>1.71</td>
<td>1.90</td>
<td>1.41</td>
<td>0.17</td>
<td>0.18</td>
<td>0.16</td>
<td></td>
</tr>
<tr>
<td>C/N</td>
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<td>9.50</td>
<td>9.50</td>
<td>7.13</td>
<td>7.13</td>
<td></td>
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</tr>
</tbody>
</table>

Figure 1: Annual distribution of the genotypes (S30-1–S30-7) of *Ochrobactrum* from a fallow land (S30). 50 isolates per soil sample were analysed by ERIC-PCR, which defined 7 genotypes.

Abbildung 1: Häufigkeit der Genotypen (S30-1 bis S30-7) von *Ochrobactrum* in einem brachliegenden Boden (S30) im jahreszeitlichen Verlauf. Pro Probennahme wurden 50 Isolate mit ERIC-PCR analysiert, welche 7 Genotypen definierte.

Two main clusters could be identified: The pattern of Cluster S30-1/S30-3 and cluster S30-4/S30-7 had a less than 90% homology (Fig. 2). Each cluster represents about 50% of the *Ochrobactrum* population. The stability of occurrence of certain genotypes in fallow land during the course of one year could be due to the fact, that no organic material with altered quality was introduced into the soil.

3.1.2 Intragenus diversity and landuse

When the *Ochrobactrum* diversity from a fallow land (S30) was compared to the diversity in agriculturally used soil (maize cultivation; GT), the dominant genotype patterns were similar but not identical. Fig. 3 shows the relationship between isolates from soil S30 (S30-1 to S30-7) as compared to soil GT (GT1 to GT8). Three main clusters were identified: Cluster 1 with the genotypes S30-1 to S30-3 and GT1 to GT4, cluster 2 with the genotypes S30-4–S30-7 and GT5–GT7 and cluster 3 with only one genotype (GT8). Although some genotypes from S30 and GT are quite closely related (e.g. S30-1–GT1) no identical genotypes were identified among the dominant *Ochrobactrum* populations in the two soils. Two dominant genotypes...
could also be found in soil derived from fallow land (S30). The ERIC-PCR patterns from both types of isolates enriched with a strain specific antibody were identical (Fig. 5). This demonstrates that genotype GT8 is also present in the soil S30 but does not belong to the dominant Ochrobactrum population. These results indicate, that a change in land use does not change the genetic pool of a soil, but causes a shift in the dominantly occurring genotypes. This is in contrast to highly contaminated soil sites, which showed a reduction in genetic diversity (Atlas et al., 1991; Schloter et al., 1996; Torsvik et al., 1990).

3.2 PLFA distribution

The average amount of single fatty acids (amount/numbers) in EL-PLFA and in most of their sub-fractions (SATFA, MUFA and PUFA) was larger in DP2 soil than in Hop soil (Fig. 6). The largest differences between both soils were obtained in the SATFA fraction. A great deal of the SATFAs contain branched chain fatty acids (Zelles et al., 1997). Ester-linked branched chain fatty acids are characteristic for Gram-positive, sulphate-reducing Gram-negative bacteria and for the genera Cytophaga and Flavobacterium (Haack et al., 1994; see Wilkinson, 1988 for an overview).

The NEL-PLFA exhibited larger amounts in Hop than in DP2 soils. Fatty acids linked to lipids by chemical bonds other than ester (non ester-linked, NEL-PLFA) exist in e.g. sphingolipids, ornithine lipids, plasmalogens and other aminolipids, which are also regarded as phospholipids (Lechevalier and Lechevalier, 1988). In the Bacteroides/Flavobacterium branch sphingolipids have been found (Shah, 1992), while in Clostridia mainly plasmalogens are present (Tunlid and White, 1992). Anaerobic bacteria contain relatively large amounts of plasmalogens which have both ester and ether linkages. Only very few aerobic or facultative anaerobic bacteria contain plasmalogens (Harwood and Russel, 1984).

In the DP2 samples alpha-hydroxy and in Hop mid-chain hydroxy fatty acids were the dominant sub-fraction of the PLOH. Both soils contained alpha-hydroxy fatty acids as the largest subfraction of UNOH (Fig. 7). In the MUFA fraction the Δ9 and Δ11 fatty acid were the most dominating ones, whereas in the PUFA fraction the fatty acids with a chain length of 18 C atoms were prevalent (Fig. 7). No clear differences were observed in the soil samples of both sites. After the exclusion of these generally occurring fatty acids from the evaluation the characteristic fatty acids in lower concentrations became important, which differ remarkably in the investigated soil samples. This demonstrates, that many fatty acids occurring in extremely high concentration are rather ubiquitously distributed among the organisms and consequently do not have signature character. Many Gram-negative bacteria harbouing the anaerobic-desaturase pathway contain MUFA, especially cis-vaccenic acids as signature fatty acid.
-Isolates from S30-  

-Isolates from GT-

Figure 5: Comparison of ERIC PCR fingerprints of 5 *Ochrobactrum* isolates per soil sample (sites S30 and GT) enriched with a strain specific antibody for genotype GT.

*Abbildung 5:* Vergleich der ERIC-PCR Muster von 5 *Ochrobactrum* Isolaten je Standort (S30 und GT), die mit einem stammspezifischen Antikörper für Genotyp GT8 angereichert wurden.

(Dobbs and Guckert, 1988). Additionally, specific groups of microbes, e.g. *Mycobacterium*, often contain unusual lipids particularly mycolic acids (Yano et al., 1978).

The relative amount of 10-methyl branched fatty acids, in terms of nmol per gram soil and mol%, was only half in soil Hop as compared to soil DP2 (Fig. 8). Since branching at the 10\(^{th}\) C-atom in certain SATFAs is exclusively produced in actinomycetes, these fatty acids are commonly used as signature molecules for this group of bacteria. Low concentrations of this biomarker indicates a decrease of actinomycetes in former hop plantation soil. This observation is confirmed by studies which demonstrate actinomycetes as markedly sensitive against heavy metal contamination (Hiroki, 1992). Although in many cases a definite assignment of one single fatty acid fraction to one specific group of micro-organisms is critical (Zelles, 1997) and may be affected by diverse environmental factors (Petersen and Klug, 1995), the use of marker fatty acids provides an appropriate tool to obtain information about complex soil microbial diversity.

The fractionation of fatty acids into chemically relevant groups before their final separation on GC-MS yields accurate information concerning the taxonomic relevance of the fatty acid molecules. The methodology used here enabled a large number of fatty acids to be differentiated. However, lipid profiles can provide additional insights into microbial community composition, because the relative abundance of certain fatty acids differs considerably among specific groups of micro-organisms.

These results demonstrate a considerable influence of agricultural soil management on qualitative and quantitative soil microbial properties.
4 Conclusion

The application of molecular and biochemical approaches to investigate the microbial diversity of soils at two different levels of complexity confirmed the validity of this type of soil microbial community analysis, but also revealed limitations of each method. The intragenus diversity describes in detail variations in only a small sector of the community, while the PLFA-profiles represent the whole spectrum of the soil inhabiting micro-organisms in a coarse manner. Both approaches demonstrate, that differences in soil management cause significant shifts in the actually active microbial community. Nevertheless, the total genetic pool of microbes in soil ecosystems seems stable at normal agricultural practice. The consequences of shifts in microbial population structure for the ecosystem functions need to be investigated in more detail.

Figure 7: Distribution of ester-linked hydroxy substituted fatty acids (PLOH) (A) and non ester-linked hydroxy substituted fatty acids (UNOH) (B). Distribution of subclasses of monounsaturated fatty acids (MUFA) (C) and polyunsaturated fatty acids (PUFA) (E) according to the position of unsaturation and chain length, respectively. The distribution was either presenting all fatty acids (C, E), or excluding the dominant fatty acids (D, F), respectively.

Abbildung 7: Verteilung der Subklassen der estergebundenen, hydroxy substituierten Fettsäuren (PLOH) (A), der nicht estergebundenen hydroxy substituierten Fettsäuren (UNOH) (B), jeweils bezogen auf die Position der Hydroxygruppe.

Verteilung der monounsättigten Fettsäuren (MUFA) (C) und der polyunsättigten Fettsäuren (PUFA) (E) in Bezug auf die Position der Doppelbindung bzw. Kettenlänge aus Proben der Oberböden von DP2 und Hop. Es wurden entweder alle entsprechenden Fettsäuren (C, E) oder die Fettsäuren nach Ausschluß der Dominanten (D, F) dargestellt.

Figure 8: Concentrations of fatty acid fractions (nmol per g soil and mol%) used as biomarker for actinomycetes (branching on the 10th C-atom) originating from DP2 and Hop top soils.

Abbildung 8: Konzentration (nmol pro g Boden und mol%) der Fettsäurefraktion, die als Biomarker für Actinomyceten gilt (verzweigt am 10. C-Atom) aus den Oberböden von DP2 und Hop.
Acknowledgement
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References


