Two-dimensional single strand conformation polymorphism (SSCP) of 16S rRNA gene fragments reveals highly dissimilar bacterial communities in an acidic fen

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Abstract

Genetic fingerprinting methods such as denaturing gradient gel electrophoresis (DGGE) and single strand conformation polymorphism (SSCP) are only able to separate about 20 to 40 well distinguishable bands (signals) within each sample. As a result, the diversity of 16S rRNA genes within biological samples may be underestimated, because multiple sequences can migrate at the same rate to form a single band. This study reports a two-dimensional SSCP fingerprinting method that has the capability to resolve hundreds of signals in a single fingerprint by using different gel temperatures in the two dimensions of the separation (20 °C and 30 °C respectively). Unlike previous two-dimensional approaches, the method presented in this study does not rely on DNA products of variable lengths but is able to separate 16S rRNA gene fragments of the same length. To demonstrate the effectiveness of this new method, DNA samples from oxic and anoxic zones of an acidic fen were examined. Whereas one-dimensional SSCP fingerprints indicated high similarity (>93%) between 16S rRNA gene fragments from oxic and anoxic zones of the fen the two-dimensional SSCP approach virtually found no similarities.

Key words: Bacterial diversity / two-dimensional SSCP / fen
1. **Introduction**

Genetic fingerprinting methods such as temperature/denaturing gradient gel electrophoresis (TGGE, DGGE) or single-strand conformation polymorphism (SSCP) are commonly used to characterize bacterial community structures using amplified 16S rRNA gene fragments from terrestrial environments [17]. All these methods can only separate a limited number of distinct signals [17], usually appearing as no more than 40 bands in the gel matrix after DNA-staining, and representing different phylotypes of bacteria. In fact, saturation effects have been identified *in silico* for DGGE and SSCP gels with the result that the number of distinguishable signals is limited to about 35 [14]. To make matters worse, amplification of soil derived bacterial communities with universal primers often result in high levels of background in the DNA profiles (“smears”) and clone libraries of extracted single signals often comprise a high number of different sequences [21]. As a consequence, comparative community analysis using the above mentioned methods frequently underestimates the scale of the bacterial diversity, and differences between samples are likely to be overlooked. Recent studies calculated that a pristine soil sample can harbor up to 50,000 distinct 16S rRNA gene sequences [19] and up to a million different bacterial genomes [7], suggesting that current fingerprinting methods are only scratching at the surface of bacterial diversity. The application of 16S rRNA gene based microarrays is a tempting alternative to study highly diverse communities [8, 26, 5] with up to 300,000 probes per chip [5]. However, the equipment necessary to carry out microarray applications is very expensive and novel sequences can be easily overlooked [5]. A useful alternative is to increase the resolution of the fingerprint. Some reports described an introduction of a second dimension in the DNA based fingerprints to achieve a higher resolution. The ITS (intergenic space) region is a
multiple length target that has been exploited in order to create a second dimension in a DGGE [10] and results showed an improved resolution compared to one-dimensional ARISA (automated ribosomal internal transcribed spacer analysis). For bacterial genome analysis, DGGE has been combined with a non denaturing size discriminating electrophoresis [6, 28, 16]. However, this excludes the exploitation of 16S rRNA genes as a target gene since amplified gene fragments of 16S rRNA genes are highly similar in length. SSCP and RFLP has been combined to obtain a two dimensional fingerprint from bacterial pure cultures [11]. PCR products were digested and fragments of the same length were further characterized by a subsequent SSCP run in the second dimension. Unfortunately, this combination has the disadvantage that the performance enhancing single strand digestion [25] cannot be carried out. As a consequence, heteroduplex DNA (DNA-DNA hybridizations of DNA single strands that are not 100 % complementary) causes additional bands in fingerprints, and this is especially problematic in the study of complex microbial communities. A multi-conditional SSCP approach was patented to study gene mutations by running the same samples in several one-dimensional gels [13]. Our own previous one-dimensional SSCP studies concluded that the specific choice of temperature had a dramatic effect on the migration properties of single signals (Schmalenberger and Tebbe, unpublished).

The aim for this study was to develop a fingerprinting method with a significantly higher resolution compared to the one-dimensional fingerprinting techniques, aiming to achieve a potential resolution of hundreds of unique signals (phylotypes) without encountering the disadvantages and problems of the above mentioned two-dimensional methods. Here we report a two-dimensional SSCP approach to analyze microbial communities, allowing PCR products of the same
length to be separated while DNA-DNA heteroduplex formation was prevented.

Analysis of DNA extracted from peat along a depth gradient in an acidic fen (Fichtelgebirge, Germany) demonstrated that one-dimensional fingerprints with a relatively high similarity can be effectively differentiated with the two-dimensional approach.

2. Materials and methods

2.1 Sampling and DNA extraction

Peat samples were taken from depths of 5 to 50 cm of an acidic fen, located in the Lehstenbach catchment of the Fichtelgebirge (Spruce Mountains, Germany, 50°08’14” N, 11°53’07” E) as described previously [24]. The site was dominated by Sphagnum mosses. Soils were classified as Fibric Histosol. Typically these soils were water saturated except in extreme hot summer months when the upper fen soil dried. Total DNA was extracted from peat samples taken in duplicates (A and B) from 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 cm depth in September 2003 with the Fast DNA spin kit for soil (Q-Biogene/Bio101) as recommended by the manufacturer.

2.2 Amplification and SSCP-fingerprinting

PCR amplification was carried out in a thermal cycler T-gradient (Biometra, Germany) in a final volume of 50 µl, using 1.5 U HotMaster-Taq-Polymerase, 1X PCR buffer with 2,5 mM MgCl₂, 1X PCR Enhancer, 0.5 µM primers, and each dNTP at 200 µM (all Eppendorf, Germany). Primers Com1 and Com2-Ph were used as “universal” primers, and have been reported elsewhere [25]. PCR was performed under the following conditions: 94°C for 3 min, followed by 28 cycles of 1 min at 94°C, 50°C for 1 min, 72°C for 1 min and finally 72°C for 10min. Two independently generated PCR products originating from the same DNA template were pooled and
purified with the Qiagen PCR purification kit as recommended by the manufacturer.

Subsequently, a single strand digestion with lambda exonuclease was carried out with 10 U per 100 µl of PCR product at 37°C for 45 min. The single strand DNA molecules (ssDNA) were purified with the Qiagen mini elute PCR purification kit, eluted in 8 µl and mixed with 8 µl of loading dye (95% w/v formamide, 10 mM NaOH, 0.25% w/v bromphenol blue and xylene xyanol). For two-dimensional SSCP a mixture of duplicates A and B (from 5, 20 and 50 cm respectively) was applied and the total volume of the sample was reduced from 16 µl to a total of 8 µl in a vacuum-concentrator (RCV 2-18, Christ, Germany) to allow the complete loading of the ssDNA products. DNA was heated to 95°C for 2 min and transferred immediately on ice for 3 min before loading. The electrophoresis was carried out in a LKB 2010 manual sequencing chamber. The gel (20 x 20 cm) contained a SSCP specific 0.6 fold MDE acryl-amide concentration as recommended by the manufacturer (Lonza Group Ltd., Switzerland) in 1 fold TBE buffer [23] and was conducted on a hydrophilic GelBond film for acrylamide gels by casting the gel on top of the hydrophilic surface of the film as recommended by the manufacturer (Lonza Group Ltd.). Electrophoresis was carried out at 9 mA and 350 V for 17 h at 20°C. For the two-dimensional SSCP, a second electrophoresis was carried out by using a gel where the first electrophoresis has been already completed (with only one sample loaded into a single well at the left side of the gel). The complete gel was removed from the LKB chamber while the SSCP gel was firmly attached to the GelBond film. In order to establish a second dimension, the acrylamide gel was rotated clockwise by 90° and placed onto the horizontal TGGE maxi system from Biometra. The originally loaded well was now positioned at the right hand side of the minus electrode so that DNA fragments, separated by the first gel run were positioned close to the minus electrode and in
position to migrate to the plus electrode during the second run. Electrophoresis was then carried out at 30°C for 18 h at 10 mA and 250 V. DNA fragments were visualized by silver staining as published elsewhere [27]. One-dimensional SSCP profiles were compared using Ward’s cluster analysis with the GelCompar II program package (Applied Maths, Belgium).

2.3 Re-amplification, cloning and sequencing of representative signals

Single bands were cut out of the gel-matrices and DNA was extracted as described previously [20]. Re-amplification was carried out using the above mentioned PCR protocol and products were cloned and sequenced as described previously [20]. Obtained DNA sequences were truncated to remove vector and primer sequence information using the Vector NTI 8 software (Invitrogen). Similarity of the 16S DNA sequences were identified via BLAST [1]. The DNA sequences of this study were deposited in GenBank (http://www.ncbi.nlm.nih.gov/Genbank/) under the following accession numbers: AM889384 to AM889404 (from one-dimensional SSCP gel) and FM177182 to FM177189 (from two-dimensional SSCP gel).

3. Results

3.1 One-dimensional profiling of partial 16S rRNA genes

Biogeochemical parameters measured in the fen soil solution characterize the upper 5–10 cm as oxic and periodically dry, whereas oxygen could penetrate down to a depth of 25 cm in the water-saturated fen [24]. Permanently anoxic conditions occur below 30 cm depth. Comparison of one-dimensional SSCP fingerprints with the naked eye identified variations in the bacterial community structures (Fig. 1). While signals like the ones at position 9 seemed to occur in all SSCP profiles, other signals like those at position 1 and 2 (Fig. 1) seemed to occur in several but not all profiles, independently of the oxic/anoxic zones in the fen (5-25 cm and 30-50 cm...
respectively). Other signals, like the one at position 6, appeared to be more abundant in profiles from 25 to 50 cm and in 5 cm than in 10 to 20 cm.

Cluster analysis (Ward) of these profiles revealed high similarity values between the single replicates and a moderate separation of the profiles from the upper zones and lower depths zones (Fig. 2). Two major clusters were identified, which only partially reflected the biogeochemical gradients [24]. Cluster I contained profiles from 5 to 20 cm and 40 to 45 cm and cluster II profiles of 25 to 35 cm and 45 to 50 cm (samples from 45 cm were found in both clusters). The similarity of the profiles or the identical position of the signals varied in most cases from 98% (replicate level) to 96% (major cluster level). Clusters I and II had a similarity of about 93%, suggesting an overall high similarity of the samples (Fig. 2).

Sequencing of 21 clones retrieved from a total of 14 single signals (370bp, excluding primer sequence) identified a high number of \textit{Proteobacteria} (10 out of 21 sequences in signals 1, 3, 4, 6, 7, 8, 32, 41) and \textit{Acidobacteria} (4 out of 21 sequences in signals 2, 3, 6, 51). Furthermore, two sequences belonging to Gram positive bacteria (one \textit{Actinobacterium}, sequence in signal 51; one \textit{Firmicutes}, sequence in signal 9) were retrieved and sequences belonging to \textit{Chloroflexus} (sequence in signal 52) and \textit{Bacteroidetes} (sequence in signal 31) were identified once each. In three cases, the sequence information did not lead to a phylogenetic identification (in signals 12, 52) due to low levels of similarity (below 90%).

3.2 Two-dimensional profiling of partial 16S rRNA genes

Despite the biogeochemical differences described between the upper and lower zones in the fen [24], only moderate levels of dissimilarity (> 93% similarity, see above) were found by one-dimensional SSCP profiles along the depths. The similarity of the bacterial communities was therefore analyzed in more detail in three different
depths representing the oxic, oxic/anoxic inter-zone and anoxic zone of the fen (5, 20 and 50 cm, respectively), by two-dimensional SSCP profiling (Fig. 3A, 3B, 3C).

Separation of the signals in two dimensions allowed the identification of about 100 distinct signals compared to approximately 50 signals that were visible with the one-dimensional fingerprint. Comparison of the two-dimensional fingerprints revealed that almost all signals detected in the 50 cm zone did not occur in the 5 cm zone and vice versa, suggesting that almost no similarity existed between the fingerprints from the oxic and anoxic zone. Likewise, a comparison of all three gels identified a very low level of similarity. Only a few signals could be identified in both the 5 cm and the 20 cm zone (such as 4a and 4b in Fig. 3A and 3B). Thus, the two-dimensional separation of the signals differentiated between several signals that had been superimposed on one another to form a single signal in the one-dimensional fingerprints (Fig. 3) such as signal 4 in the 5 and 20 cm zone and signal 9 in the 50 cm zone (Fig. 1 and 3).

The selection of the temperature as a discriminating factor was very efficient, because several signals in the two-dimensional fingerprint had a low migration rate at 20°C but a high migration rate at 30°C (Fig. 3, see upper right corner of the two-dimensional gels). Likewise, several signals migrated quickly through the gel at 20°C but only slowly at 30°C (Fig. 3, see lower left corner of the two-dimensional gels). Overall, the two-dimensional fingerprint showed signals in almost all sectors of the two-dimensional gel which indicated a good distribution of the signals over the chosen array.

Signals 9a and 9b were isolated from the 50 cm gel (Fig. 3C), re-amplified and 4 clones were sequenced, respectively. The range of sequences obtained from each signal allowed clear differentiation between the signals. They nonetheless revealed a substantial diversity within each signal, with signal 9a containing sequences from
Archaea, Actinobacteria and Proteobacteria, while sequence fragments from signal 9b were affiliated with Archaea, Bacteroidetes, Firmicutes and Verrucomicrobia.

4. Discussion

The two-dimensional SSCP method developed here demonstrated its high potential for screening complex mixtures of DNA strands of the same length, especially when diverse bacterial community structures are compared at the 16S rRNA gene fragment level. It has a clear advantage over the classical one-dimensional fingerprint. Previous studies that detected little or no variation in bacterial communities in rhizosphere and soil after treatments with herbicides or introduction of transgenic plant hosts [9, 20, 22] might have overestimated the apparent similarity. Such samples can now be distinguished by two-dimensional SSCP to elucidate and compare the actual diversity without requiring the generation of extensive clone libraries and high throughput sequencing, the latter still very cost intensive in comparison to genetic profiling methods like SSCP. Unlike other two-dimensional techniques using a target with variable length such as the ribosomal intergenic spacer region [10], the approach shown here has been used to retrieve the 16S rRNA gene information for identification purpose. The two-dimensional SSCP approach would be highly useful not only to distinguish between highly diverse 16S rRNA gene PCR products but also between diverse 18S rRNA gene products in order to analyze fungal communities, for instance. Whenever a highly diverse target gene is analyzed, the two-dimensional SSCP approach should have an advantage over the one-dimensional SSCP. However, the numbers of samples that can be analyzed within a short period of time using the two-dimensional SSCP approach is much lower than the traditional one-dimensional approach and this limits the applicability of this method.
Nonetheless, microbial patterns of two habitats can be compared within three days employing the two-dimensional SSCP. The use of differentially fluorescently labeled PCR products should enhance and simplify the capability of the two-dimensional SSCP for differential display analysis, similarly to previous approaches to improve the quality of one-dimensional DGGE [2, 3, 18], but this has not yet been explored.

The number of signals that have been retrieved with the two-dimensional SSCP approach in this study was not as high as initially expected (one hundred instead of several hundreds). One probable explanation for the limited number of signals is the limitation of the silver staining. The signals have over ten times more space to spread out across the gel. A possible alternative would be to use fluorescently labeled primers that can be detected by a laser-scanning device [24] in order to increase sensitivity. Furthermore, not all sequences in SSCP gels have a stable conformation and consequently do not form a single signal. Signals that can be found with the two-dimensional SSCP approach need to have a stable conformation at two different temperatures, in this case at 20 and 30ºC. Signals 9a and 9b from the 50 cm zone were composed of several distinct sequences showing that even after a two-dimensional separation, at least some signals still contained a mixture of phylogenetically distinct sequences.

The acidic fen analyzed in this study as a paradigm has been investigated previously for the presence of sulphate-reducing bacteria (SRB) [15, 24]. Multiplex (T-)RFLP and clone libraries of the functional key gene dsrAB revealed the presence of different SRB communities in oxic and anoxic zones of the fen [24]. However, SRBs appeared not to dominate the microbial community, because sequencing of single SSCP signals in this study identified mostly Proteobacteria (not related to SRBs) and Acidobacteria (identified in 4 signals of the one-dimensional SSCP gel,
Fig.1; and clone 9a4 from signal 9a in the two-dimensional SSCP gel, Fig. 3c). The low pH value (4 to 5) in the fen might favor the presence of acidophilic or moderate acidophilic *Acidobacteria* which made up a significant part of the total bacterial community. The results confirm previous studies, which have also identified these types of bacteria in fens and peat lands [4, 12].

In conclusion, the two-dimensional SSCP profiling applied here was very effective in differentiating apparently similar bacterial communities, leading to recognition of diversity that had been overlooked by one-dimensional fingerprints. Unlike other two-dimensional DNA fingerprinting techniques, the technique chosen in this study does not rely on a size specific separation and no gel slabs or bands have to be excised and transposed in order to establish a second dimension. Although no commercial equipment is currently available for two-dimensional SSCP, modification of existing apparatus for this purpose is technically not very demanding.

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References


Figure Legends

Figure 1. One-dimensional SSCP of 16S rRNA gene fragments from the bacterial community, amplified from a depth gradient (5, 10, 15, 20, 25, 30, 35, 40, 45, 50 cm) of an acidic fen (Schloepnerbrunnen I, Fichtelgebirge, Germany). Replicates are indicated as A and B. Sequence information from selected signals (boxed) was obtained through cloning and sequencing (clone number next to boxed signals). Marker lanes flanking the community profiles contained 16S rRNA gene sequence fragments retrieved from (top to bottom) Bacillus licheniformis, Rhizobium trifolii, Flavobacterium johnsonae, and Agrobacterium tumefaciens (the double band is due to two operons).

Figure 2. Similarity analysis (Ward) of one-dimensional SSCP profiles of 16S rRNA gene fragments as seen in Fig. 1 using GelCompar II. Bacterial community extracts were amplified from a depth gradient (depth indicated in cm) in replicates A and B. Cophenetic values inside the tree indicate the probability of a correct branching.

Figure 3. Two-dimensional SSCP profiles of 16S rRNA gene fragments, amplified from 5 (A), 20 (B) and 50 cm (C) depth of an acidic fen (Schloepnerbrunnen I, Fichtelgebirge, Germany). Signals 4 and 9 identified in the one-dimensional SSCP profiles from Fig. 1 are indicated (boxed) in the two-dimensional gels (4a, 4b, 9a, 9b). Signals 9a and 9b were identified through cloning and sequencing.