DGGE analysis and shot gun cloning detects different actinomycete-communities in soils from fields under conventional and organic management

Karin Hace[1], Ewald B. M. Denner[2], Melanie Kuffner[1], Guadalupe Piñar[2], Michael Handschur[3], Werner Lubitz[2] und Alexander G. Haslberger[2]*

[1] Institute of Ecology and Conservation Biology, University of Vienna, Althanstraße 14, A-1090 Vienna, Austria. [2] Institute of Microbiology and Genetics, University of Vienna, Dr.-Bohr-Gasse 9, A-1030 Vienna, Austria. [3] Institute of Nutritional Science, Althanstrasse 14, A-1090 Vienna, Austria. [4]

* Corresponding Author
Institute of Microbiology and Genetics, University of Vienna
UZA2, Room 2b 522
Althanstrasse 14 , A-1090 Vienna, Austria
Tel: *43 1 4227 51160, FAX: +43 1 4277 54674
Email: alexander.haslberger@univie.ac.at

Key words:

16S rDNA, DGGE, soil, actinomycetes, organic farming.

1 Abstract

2 *Actinomycetes* are involved in many important processes that contribute to soil fertility. We
3 therefore investigated the possibilities of denaturating gel electrophoresis (DGGE) analysis
4 and shot gun cloning to monitor effects of agricultural practices effects on Actinomycete-
5 communities in soils. DNA was directly extracted from soil samples of neighboring fields
6 under conventional or organic farming management and amplified by PCR using 16S rDNA
7 actinomycete- specific primers. The obtained fragments (~ 740 basepairs) were used to create
8 clone libraries for the different fields. A total of 34 different inserts were selected after DGGE
9 profiling and sequenced. The obtained 16S rDNA sequences were mainly affiliated with
10 *Nocardioidaceae* and *Geodermatophilaceae*. *Streptomycetaceae* were only detected in soils
11 from fields under organic culture management. The neighbour-joining tree based on 16S
12 rDNA data indicating the phylogenetic positions of the derived clones shows the complex
13 community structure. Our results suggest that DGGE analysis using group specific primers
14 and subsequent shot gun cloning is an appropriate method to monitor consequences of
15 different agricultural practices on actinomycete- communities in soils.

16

1 # 1 Introduction

2

3 The use of unsustainable agricultural practices may lead to health- or environmental problems
4 such as erosion, groundwater pollution, soil acidification, soil salinization or the spread of
5 antibiotic resistances. (Kennedy and Smith, 1995; Hansen et al., 2001; Nwosu, 2001).
6 Development of organic (or low-input) agriculture as well as integrated pest management is
7 aiming to reduce pesticide uses, preserve the diversity of beneficial organism, keep down
8 weeds and to increase or at least maintain soil fertility (Macilwain, 2004**,** Stolze et al., 2000).
9 For the assessment of soil fertility microbial diversity and community structure are considered
10 to be valid microbiological parameters (Johnsen et al., 2001). The application of eubacterial
11 primers to soil samples and amplification of 16S rDNA results in a complex bandpattern,
12 showing mainly the predominant species. Therefore the use of group-specific primers is a
13 useful approach to investigate also minor constituents like specific *Actinomycetes* (Heuer et
14 al. 1997a).
15 *Actinomycetes* are involved in all processes that contribute to soil fertility such as nutrient
16 cycling, decomposition of various compounds, formation of beneficial soil humus and in the
17 biological control of plant pathogens, insects and weeds (Kennedy, 1999; Heuer et al. 1997a).
18 Furthermore their metabolites are an important source of antibiotics, enzymes and bioactive
19 products (Heuer et al. 1997a). *Actinomycetes* are phylogenetically defined as a taxa within the
20 high-G+C subdivision of the gram positive phylum (Embley et al., 1994), and they comprise
21 more than 30 % of the total population in soil (Kennedy, 1999).
22

23 Conventional cultivation methods are known for their restrictions to investigate the diversity
24 of bacteria in soil, as only ~0,1 – 1% of the total bacterial population can be grown under
25 laboratory conditions (Amann et al. 1995). Cultivation-based limitations can be overcome by
26 analyzing DNA that is directly extracted from soil samples (Smalla et al., 2001; Ranjard et al.
27 2000; Heuer et al. 1997a). The improvement of DNA extraction from soil samples is of graet
28 importance for sensitivity and reproducibility of results. The method should be efficient for all
29 types of microbial cells to obtain high quality and sufficient quantity of DNA to allow further
30 DNA analysis (Schabereiter-Gurtner 2001; Leff 1995).

1 One molecular tool to investigate DNA is the PCR amplification of the 16S rDNA followed
2 by separation of the PCR products over a denaturing gradient formed with urea and
3 formamide (DGGE – denaturing gradient gel electrophoresis, Muyzer et al. 1993). Following
4 the analysis of resulting bandpatterns the bacterial species can be identified by generating
5 clone libraries of the 16S rDNA. Sequencing and comparison of sequences with databases
6 allow a phylogenetic affiliation to cultured as well as uncultured microorganisms (Ranjard et
7 al. 2000; Maidak et al. 1999)
8 These techniques have proved to be very suitable for a comparative fingerprinting of bacterial
9 communities of soils from fields subjected to different agricultural practices (Kuffner et al,
10 submitted). However, it has become evident that soil microbial communities are too complex
11 to be comprehensively fingerprinted as a whole (Ovreas and Torsvik, 1998). The resulting
12 profiles may represent only the major constituents of the analyzed bacterial community
13 (Heuer et al. 1997b) and ecologically relevant microorganisms of low abundance, like specific
14 *Actinomycetes*, may not be detected in such an universal approach.
15 Therefore, in this study a PCR-DGGE approach was combined with the creation of clone
16 libraries of 16S rDNA amplified fragments using Actinomycete-specific primers to get a
17 phylogenetic insight into the diversity of this bacterial group. Furthermore, the Actinomycete
18 populations of soils from fields under different agricultural management (CF; conventional
19 farming and OF; organic farming) were compared.
20

21
2 Materials and Methods

22
2.1 Soil sampling

23 Soil samples were collected from two neighbouring farming sites in Deutsch-Wagram
24 (Marchfeld, Lower Austria) with similar soil characteristis. Both sites were under
25 conventional agricultural management in the past. In 1995 one site was converted into an

1 organic farming field (OF) applying principles standardized and controlled according to
2 Austria conventions for organic farming [VOGL et al, 2004]. The other field remained
3 under conventional farming (CF) practices. Samples were taken from the top 15 cm of the
4 bulk soil on fields prepared for the cultivation of maize after ploughing in spring. To consider
5 spatial heterogeneity samples were taken in triplicates. Each replicate consisted of five 5 cm
6 cores. Samples were kept on ice before freezing and storing at -20°C.
7

8 2.2 DNA extraction and purification

9 DNA was extracted directly from soil by using the Fast DNA SPIN kit for soil (Bio 101). The
10 protocol of the manufacturer was modified as follows: 1 g soil was resuspended in 1 ml
11 sodium phosphate buffer (120 mM; pH 7.6) and incubated on a shaker at 150 rpm for 10 min,
12 to remove extracellular DNA. The slurry was centrifuged at 6000 x *g* for 5 min, and 0.7 g of
13 the soil pellet were placed in the MULTIMIX2 tissue tube. 978 µl sodium phosphate buffer
14 and 122 µl of the MT buffer (Bio 101) were added. The mixture was then homogenized two
15 times for 1 min using a mini-beadbeater (Biospec Products, Howard TM Industries, Mini-
16 Beadbeater-8) with one intervening minute on ice and centrifuged at 14 000 x *g* for 2 min.
17 250 µl PPS reagent and 500 µl binding matrix suspension (Bio 101) were applied to the
18 supernatant. The resulting suspension was transferred to a spin filter and centrifuged at
19 14 000 x *g* for 1 min. DNA was washed twice with 500 µl of the SEWS-M solution (Bio 101)
20 and eluted from the binding matrix with 100 µl DES (Dnase/Pyrogene free water).
21 The extracted DNA was purified with the QIAmp Viral RNA mini kit (Quiagen) and finally
22 eluted from the silica column with 100 µl ddH_2O.

23 In an alternative DNA extraction protocol soil-DNA was extracted with the GL universal
24 DNA Kit (GL Biotech GmbH) following the instructions of the manufacturer for DNA
25 extraction of soils and sediments. The DNA concentration in the purified extracts was

1 estimated by using the Nucleic Dot Metric TM System (Genotech). It ranged between 0.06
2 and 0.11 µg µl^{-1}.
3
4 2.3 PCR amplification of 16S rDNA fragments using eubacterial primers

5 For the analysis of eubacterial bandpatterns of bacterial communities in the soils eubacterial
6 16S rDNA was amplified. In the initial PCR primers 5f (5'-CCT ACG GGA GGC AGC
7 AG-3') (Muyzer et al. 1993) and 985r were added, for the following nested PCR the primer
8 pair 341f GC (sequence like 5f with a GC-clamp (Muyzer et al., 1993) at its 5'-end) and 518r
9 (5'-ATT ACC GCG GCT GCT GG-3') (Neefs et al. 1990) were used. The obtained DNA
10 fragments were applied to DGGE, using a gradient of denaturants from 25 to 60%.
11 Electrophoresis was run at 200 V, 60°C for 3.5 h. Bands were visualized *via*
12 ethidiumbromide-staining and UV illumination.
13

14 2.4 PCR amplification of 16S rDNA fragments using actinomycete-specific primers

15 16S rDNA of *Actinomycetes* was amplified from soil extracts using the primer pair 243f
16 (5'-GGA TGA GCC CGC GGC CTA-3') (Heuer et al. 1997a) and 985r (5'-GTA AGG TTC
17 TTC GCG TT-3') (Heuer et al. 1999). The reaction was carried out in 25 µl volumes
18 containing 12.5 pmol of each primer, 5 pmol of each deoxyribonucleotide triphosphate, 0.5 U
19 of *Taq* polymerase (Roche diagnostics), 2.5 µl 10x PCR buffer (100 mM Tris-HCl, 15 mM
20 $MgCl_2$, 500 mM KCl; pH 8.3; Roche diagnostics), 1.25 µl DMSO (Dimethylsulfoxide;
21 Sigma), 10 µg BSA (bovine serum albumin; Roche diagnostics) and sterile water up to 25 µl.
22 1.5 µl DNA directly extracted from soil were used as template.
23 PCR was performed in a robocycler (Stratagene, La Jolla, CA). After 5 min of denaturation at
24 95°C and 35 thermal cycles of 1 min at 95°C (denaturation), 1 min at 58°C (annealing) and 1

1 min at 72°C (extension), PCR was finished by an extension step at 72°C for 5 min. The
2 products from the first round PCR were too long for complete resolution through DGGE,
3 therefore a second round of PCR (nested PCR) was performed.
4 For the nested PCR the primers 243f and 513r (5'-CGG CCG CGG CTG CTG GCA CGT
5 A-3') (Heuer et al. 1997a) were used. The reaction was carried out in a final volume of 150 µl
6 was, using 12 µl PCR product obtained from the first round of PCR. To the reverse primer a
7 40 bp GC clamp was added as described by Muyzer et al. (1993), which stabilizes the melting
8 behaviour of the DNA fragments. Concentrations of reagents were as in the first round of
9 PCR, but no BSA was added. Amplification was performed using 94°C for 5 min as an initial
10 denaturation step followed by 1 min at 94°C, 1 min at 63°C for primer annealing and 1 min at
11 72°C for primer extension and a final extension step at 72°C for 5 min. To check their size,
12 PCR products of both rounds of PCR were applied on 2% Agarose gels and visualized via
13 ethidiumbromide staining (in a 0.5 µg ml^{-1} solution) and UV-light.
14 Approximately 150 µl PCR product from the nested PCR was precipitated overnight in
15 1 ml 96% EtOH, resuspended in 15 µl ddH_2O and applied to DGGE.

16 2.5 DGGE analysis

17 Electrophoresis was performed as described by Muyzer et al. (1993) with 8% (vol vol^{-1})
18 polyacrylamide gels (acrylamide:bisacrylamide = 37.5:1 [v:v]; BioRad). A denaturing
19 gradient of 45 to 55% denaturants was used. 100% denaturants are defined as 7 M Urea and
20 40% (vol vol^{-1}) of deionized Formamide. Gels were subjected to electrophoreses in 0.5x TAE
21 buffer (20 mM Tris, 10 mM acetate, 0.5 mM Na_2EDTA; pH 7.8) at 60°C and 200 V for 3.5 h
22 using the D Gene system (Bio-Rad). The DGGE band patterns were visualised by ethidium
23 bromide staining and UVP transillumination.
24

1 2.6 Cloning of 16S rDNA fragments

2 5.5 µl PCR product were directly ligated into the pGEM-T easy vector (Promega) following
3 the instructions of the manufacturer. The ligation product was transformed into E. *coli* XL1
4 blue via electroporation (Gene Pulser II, BioRad) . The transformed cells were plated on an
5 indicator BHI (brain heart infusion) agar containing ampicillin (100 mg ml^{-1}), tetracycline
6 (10 mg ml^{-1}), X-Gal (5 bromo-4-chloro-3-indolyl-ß-D-galactopyranoside; 0.1 mM) and IPTG
7 (isopropyl-ß-D-thiogalactopyranoside: 0.2 mM) (Sambrook et al. 1989), which allows the
8 identification of recombinants by their white color.
9

10 2.7 Screening of Clone Libraries by PCR and DGGE

11 48 white colonies were harvested from each soil and resuspended in 40 µl TE buffer (10 mM
12 Tris, 1 mM EDTA, pH = 8). Screening of the clone libraries by PCR and DGGE was
13 performed as described by Schabereiter-Gurtner et al. (2001). To confirm the presence of
14 inserts, cells were lysed by three freeze-thawing cycles. PCR with vector specific primers Sp6
15 (5'-ATT TAG GTG ACA CTA TAG AAT AC-3') and T7 (5'-TAA TAC GAC TCA CTA
16 TAG GG-3') was performed in a volume of 25µl using 2.5 µl DNA extract as a template. For
17 the PCR reactions of the cloned inserts no BSA and DMSO were added. The following
18 thermocycling program was used: 5 min denaturation at 95°C, followed by 35 cycles of 1 min
19 denaturation at 95°C, 40 s annealing at 46°C and 1 min extension and a final extension step of
20 3 min at 72°C. Subsequently products were analyzed by electrophoresis in 2% Agarose gels
21 applying 8 µl of PCR product. Positive clones were identified by their length of
22 approximately 860 bp, containing the 700 bp 16S rDNA insert and the two 80 bp flanking
23 regions of the plasmid.

1 To screen for different clones nested PCR was carried out in 25 µl reaction volumes adding
2 2 µl of each 860 bp amplicon. Conditions applied are described above. All inserts of clones
3 different to each other in DGGE were sequenced.
4

5 2.8 Creation of an artificial and an original bandpattern as reference in DGGE screening of
6 clones

7 For the artificial bandpattern each sequenced clone was amplified by PCR in a volume of 25
8 µl using 2 µl DNA extract as template. All PCR products were pooled, precipitated overnight
9 in 1.5 µl 96% EtOH and run in a single slot.
10 PCR-amplification of 16S rDNA for the original bandpattern was performed following the
11 instructions in section 2.4.
12

13 2.9 Sequencing of cloned inserts and phylogenetic analysis

14 For sequencing inserts were amplified with primers Sp6 and T7 in a 100 µl PCR mixture
15 adding 10 µl template DNA. The amplicons were subsequently purified and sequenced as
16 described by Schabereiter-Gurtner et al. (2001). Sequencing errors were minimized by
17 combining a short run sequence analysis using the primer Sp6 with a medium run using the
18 primer T7.
19 Sequencing was carried out at the VBC-Genomics Bioscience Research GmbH (Vienna,
20 Austria). Initial 16S rRNA gene sequence database search was done by FASTA analysis
21 (Pearson & Lipman, 1988; Pearson, 1990). Subsequently, relevant nucleotide sequences were
22 retrieved from EMBL and GenBank databases. Phylogenetic analyses were carried out using
23 the services of the Ribosomal Database Project RDP II (Maidak et al., 2001).
24

1 # 3 Results

2 ## 3.1 Characterization of soils

3 The soils from fields managed by conventional and organic methods were derived from the
4 neighbouring sites. Agricultural practices of both, conventional (CF) and organic farming
5 (OF) included tillage, irrigation and crop rotation. Wheat, maize and turnips have been
6 grown on the CF managed field before and included additionally mineral fertilizers and
7 treatment with chemical pesticides. In OF management the nutrient status of the soil was
8 balanced by an elaborate crop rotation system including the cultivation of various cereals, a
9 one-years fallow under legume cover every 5 or 6 years and annual legume-intercropping, to
10 compensate nitrogen depletion. Compost was added once, in fall. Weeds were controlled
11 mechanically by intensified tilling and harrowing. Samples of the different fields did not
12 differ in the pH (pH 7 in distilled water) and the total organic carbon content, measured in an
13 elementary analyser combined with a mass spectroscope, was between 1.1% and 3.4% .
14

15 ## 3.2 Band pattern analysis of eubacterial communities

16 DGGE analysis of eubacterial communities revealed highly complex band patterns. No
17 signifcant differences could be seen between samples from different soils. However, the
18 comparison of the different applied extraction protocols revealed more and clearer bands
19 using the standard optimized protocol compared with the protocol provided by GL Biotech
20 GmbH (Fig. 1).
21

22 ## 3.3 Phylogenetic analysis

23 To analyse the member composition of actinomycete in the soils of the two different
24 agricultural methods clone libraries were done for ACF (conventional farming) and AOF

1 (organic farming), using actinomycete specific primer (Heuer et al., 1997a). Since replicate
2 samples from these two soils showed highly reproducible bandpatterns (data not shown .),
3 only one sample of each soil was subjected to cloning. Length of obtained sequences ranged
4 from 737 to 759 bp.
5 Fig. 2 shows the schematic diagram of the experimental steps used in this study. Cloned 16S
6 rDNA-fragments were PCR amplified and screened by DGGE. Fragments showing different
7 migration behaviour (Fig. 3 and 4) were sequenced (18 inserts of ACF and 16 inserts of
8 AOF) and compared to the EMBL database. Table 1 and 2 show the accession numbers and
9 the tentative phylogenetic affiliation of the ACF- and AOF-clones. Furthermore, a
10 dendrogram was constructed showing the relatedness of clones to each other as well as to
11 typical strains of *Actinomycetales* (Fig. 5).
12
13 *3.3.1 Geodermatophilaceae*
14 Clones ACF12, ACF22, ACF43, ACF44, ACF45, ACF47, AOF3 and AOF 24 were assigned
15 to *Geodermatophilaceae*, showing similarities ranging from 96.3 to 100% to type strain of the
16 *Blastococcus aggregatus* [AJ420193] (Table 1 and 2).
17
18 *3.3.2 Microbacteriaceae*
19 Clone AOF36 showed a similarity of 95.7% to type strain of *Microbacterium lac*ticum
20 [X77441] (Table 2). In the dendrogram (Fig. 5) this clone is clustering near
21 *Microbacteriaceae* indicating the finding of a possible new genus within this family.
22
23 *3.3.3 Nocardioidaceae*
24 Clones ACF1, ACF2, ACF5, ACF21, ACF23, ACF30, ACF41, AOF 13, AOF20, AOF29 and
25 AOF34 were affiliated to this family within the dendrogram (Fig. 5) as well as in the
26 similarity matrix (see Table 1 and 2).

1

2 *3.3.4 Propionibacteriaceae*

3 Clone ACF3 shows 97.1% similarity to strain *Microlunatus* (Table 1) and is matching closest

4 to *Microlunatus phosphovorus* within the dendrogram (Fig. 5).

5

6 *3.3.5 Pseudonocardiaceae*

7 Clone ACF42 was assigned to *Pseudonocardia yunnannensis* at a similarity of 97.4%

8 (Table 1) and therefore belonging to the genus *Pseudonocardia*. This finding is confirmed by

9 the dendrogram (Fig. 5), where this clone was placed to this genus.

10

11 *3.3.6 Streptomycetaceae*

12 Clones ACF37, AOF16, AOF31, AOF41 and AOF42 were affiliated to family

13 *Streptomycetaceae* (Table 1 and 2). In the dendrogram (Fig. 5) clones of the organically

14 managed soil are clustering within or near this family, confirming the findings of the

15 similarity matrix where similarities to genera *Kitasatospora* and *Streptomyces* were ranging

16 from 96 to 99.7%. However, clone ACF37 showed 100% similarity to clone CF44 which was

17 obtained by our group in a former study (Kuffner et al. 2002.), but could only be assigned to

18 type strain *Streptomyces indonesiensis* [AJ391835] at a similarity of 91.3%.

19

20 *3.3.7 Streptosporangiaceae*

21 ACF6 showed 91.7% similarity to *Streptosporangium roseum*, indicating a possible new

22 taxon. This finding is confirmed by the dendrogram (Fig. 5), where this clone was clustering

23 near other clones.

24

1 *3.3.8 Non-Actinomycetales*

2 Clone AOF10 showed 98% similarity to uncultured *Rubrobacteridae* [AY150868], but could
3 be assigned to cultured *Thermoleophilum album* [AJ458462] only at a similarity of 89.1%
4 (Table 2), indicating a possible new taxon.

5

6 Clone AOF47 could be related neither to uncultured (88% similarity; data not shown) nor to
7 cultured species (87.1%; Table 2), indicating the finding of a new family within the class
8 Actinobacteria.

9

10 Clones ACF4 and AOF9 showed highest similarities to *Verrucomicrobia* and clone AOF12
11 was most closely related to *Planctomycetes* in the FASTA-search (data not shown). In an
12 initial calculation of the phylogenetic tree these clones clustered outside of the class
13 *Actinobacteria* (data not shown) and were therefore excluded from further investigations.

14

15

16 # 4 Discussion

17 DNA extraction is a crucial point if molecular techniques are applied for the analysis of
18 bacterial communities. (Schabereiter-Gurtner, 2001; Leff et al., 1995). Therefore our
19 extraction protocol was optimized for the lysis of cells and the purification of the obtained
20 bacterial DNA. The use of actinomycete-specific primers results in a simplification of the
21 otherwise complex eubacterial bandpatterns in DGGE (Heuer und Smalla, 1997b). The primer
22 243f might not be ideal to amplify all *Actinomycetales* and it matches also some non-
23 actinomycete (Heuer et al. 1997a). However, it aids to enrich 16S rDNA of actinomycete in
24 environmental samples and thus facilitates detecting this group, which otherwise would be
25 outcompeted during PCR (Heuer und Smalla 1997b).

1 Although group specific fingerprinting simplifies DGGE-patterns, the direct excision of
2 single bands for sequencing and phylogenetic identification is usually difficult and laborious.
3 Moreover, short fragments as used for DGGE analysis (200 – 500 bp) are considered
4 insufficient for an accurate phylogenetic placement (Hugenholtz et al., 1998). Creating clone
5 libraries the cutting of bands can be avoided and thus the cloning of longer 16S rDNA
6 fragments is enabled.
7 Electroporation also proved to be very effective transforming E. *coli*. Counting colony-
8 forming-units (CFU) on BHI indicator agar revealed ~1000 clones on each plate containing
9 genes of interest. 54 clones per plate were harvested and screened by DGGE.
10 Assuming that bands with similar migration behaviour in DGGE represent the same species,
11 bands of clones were grouped and a total of 34 inserts were sequenced. Grouping of bands
12 enables the calculation of the contribution of each *Actinomycetals* family to the total of the
13 clone library.
14 The family *Nocardioidaceae* comprise 50% of the community, followed by
15 *Geodermatophilaceae* with 32% and *Streptomycetaceae* with 8%. Due to limited resolution of
16 DGGE such calculations must be handled with caution, because comigration of different
17 sequences (Bruns et al. 1999; Vallaeys et al. 1997) might lead to mistakes in grouping of
18 inserts. This could be proven for clones ACF6 and ACF47 which showed similar migration
19 behaviour in the polyacrylamide gel, but were assigned to *Nocardioidaceae* and
20 *Geodermatophilaceae* respectively.
21 The creation of clone libraries enables the detection of minor constituents of bacterial
22 communities. But if a second library is generated from the same DNA it might happen that
23 other random representatives are obtained (Felske et al, 1999). A statistically relevant
24 reproducibility may be achieved by clone numbers higher than the numbers of present 16S
25 rDNA sequences in the sample (Felske et al., 1999).

1 Although the number of the analyzed clones was certainly too low to reflect the composition
2 of the whole actinomycete-community or to statistically compare communities found in the
3 soil of fields with different farming methods, this approach proved to be useful for a
4 monitoring of communities. The family *Streptomycetaceae*, belonging to Actinobacteria was
5 only found in organically managed soil. Many bioactive metabolites and biologically active
6 compounds have been isolated from streptomyces species. They are known as saprophytic
7 soil bacteria and actively involved in the break down of soil organic matter (McCarthy et al.,
8 1992). Clone AOF41 was affiliated to *Streptomyces flavogriseus*, deriving from soil bacterial
9 isolates decomposing cellulose (Wirth et al, unpubl.). A higher bacterial diversity in soil
10 from low input farming systems has been a matter of discussion for a long time. Workneh
11 and van Bruggen (1994) as well as Clegg et al. (2003) found a positive correlation between
12 organically managed soil and diversity of *Actinomycetes.* Results of our study would support
13 these findings but such conclusions need to be supported by experiments with statistically
14 valid sample sizes.
15 In conclusion, the application of molecular techniques to investigate soil microbial
16 communities may have its methodological limitations (Ranjard et al. 2000; Muyzer et al.
17 1993; Palleroni (1997) but it is an important method to investigate microbial communities
18 which can not be studied otherwise. Many of the sequences obtained in our study could not be
19 assigned to known cultured bacteria and the established dendrogramm adds to the
20 understanding about actinomycete-communities in soils. Moreover, the investigated
21 monitoring approach proves to be appropriate to investigate and monitore consequences of
22 different agricultural practices on microbial communities in the soil.

References

Amann, R.I., Ludwig, W. and Scheidler, K.H. (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. FEMS Microbiol. Rev. 59, 143-169.

Bruns, M.A., Stephen, J.R., Kowalchuk, G.A., Prosser, J.I. and Paul, E.A. (1999) Comparative diversity of ammonia oxidiser 16S rRNA gene sequences in native, tilled and successional soils. Appl. Environ. Microbiol. 65, 2994-3000.

Clegg, C.D., Lovell, R.D.,L., Hobbs, P. J. (2003) The impact of grassland management regime on the community structure of selected bacterial groups in soil. FEMS Microbiol. Ecol. 43, 263-27o

Embley, T.M. and Stackebrandt, E. (1994) The molecular phylogeny and systematics of actinomycetes. Annu. Rev. Microbiol. 48, 257-289.

Felske, A. (1999) Reviewing the DA001-files: a 16S rRNA chase on suspect #X99967, a *Bacillus* and Dutch underground activist. J. Microbiol. Methods 36, 77-93.

Hansen, B., Alrøe, F.H. and Kristensen, E.S. (2001) Approaches to assess the environmental impact of organic farming with particular regard to Denmark. Agric. Ecosyst. Environ. 83, 11-26.

Heuer, H., Krsek, M., Baker, P., Smalla, K. and Wellington, E.M.H. (1997a) Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. Appl. Environ. Microbiol. 63, 3233-3241.

Heuer, H. and Smalla, K. (1997b) Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) for studying soil microbial communities. In: Modern soil microbiology (van Elsas, J.D., Wellington, E.M.H., and Trevors, J.T., Eds.), pp 353-373. Marcel Dekker Inc., New York, N. Y.

1 Heuer, H., Hartung, K., Wieland, G., Kramer, I. and Smalla, K. (1999) Polynucleotide probes
2 that target a hypervariable region of 16S rRNA genes to identify bacterial isolates
3 corresponding to bands of community fingerprints. Appl. Environ. Microbiol. 65,
4 4845-4862.
5 Hugenholtz, P., Goebel, B.M. and Pace, N.R. (1998) Impact of culture-independent studies on
6 the emerging phylogenetic view of bacterial diversity. J. Bacteriol. 180, 4765-4774.
7 Johnsen, K., Jacobsen, C.S., Torsvik, V. and Sørensen, J. (2001) Pesticide effects on bacterial
8 diversity in agricultural soils - a review. Biology and Fertility in Soils 33, 443-453.
9 Kennedy, A.C. (1999) Bacterial diversity in agroecosystems. Agric. Ecosyst. Environ. 74, 65-
10 76.
11 Kennedy, A.C. and Smith, K.L. (1995) Soil microbial diversity and the sustainability of
12 agricultural soils. Plant and Soil 170, 75-86.
13 Kuffner, M., Piñar,G., Hace,K., Handschur, M., Haslberger A.G. (2004). DGGE-
14 Fingerprinting of arable soils shows differences in microbial community structure of
15 conventional and organic farming systems, submitted for publication.
16 Lee, S.D., Kim, E.S. and Hah, Y.C. (2000) Phylogenetic analysis of the genera
17 *Pseudonocardia* and *Actinobispora* based on 16S ribosomal DNA sequences. FEMS
18 Microbiol. Lett. 182, 125-129.
19 Leff, L.G., Dana, J.R., McArthur, J.V. and Shimkets, L.J. (1995) Comparison of methods of
20 DNA extraction from stream sediments. Appl. Environ. Microbiol. 61, 1141-1143.
21 Maidak, B.L., Cole, J.R., Parker, C.T., jr., Garrity, G.M., Larsen, N., Li, B., Lilburn, T.G.,
22 McCaughey, M.J., Olsen, G.J., Overbeek, R., Pramanik, S., Schmidt, T.M., Tiedje,
23 J.M. and Woese, C.R. (1999) A new version of the RDP (Ribosomal Database
24 Project). Nucleic Acids Res. 27, 171-173.

1 Maidak, B.L., Cole, J.R., Liburn, T.G., Parker, C.T.j., Saxman, P.R., Farris, R.J., Garrity,
2 G.M., Olsen, G.J., Schmidt, T.M. and Tiedje, J.M. (2001) The RDP II (Ribosomal
3 Database Project). Nucl. Acids Res. 29, 173-174.
4 Macilwain , C. (2004) Organic, is it the future of farming? Nature 428, 793
5 McCarthy A.J., Williams, S.T. (1992) Actinomycetes as agents of biodegradation in the
6 environment- a review. Gene 115, 189-192
7 Muyzer, G., de Waal, E.C. and Uitterlinden, A.G. (1993) Profiling of complex microbial
8 populations by denaturing gradient gel electrophoresis analysis of polymerase chain
9 reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. 59, 695-
10 700.
11 Neefs, J.M., van de Peer, Y., Hendriks, L. and de Wachter, R. (1990) Compilation of small
12 ribosomal subunit RNA sequences. Nucleic Acids Res. 18, 2237-2317.
13 Nwosu, V.C. (2001) Antibiotic resistance with particular reference to soil microorganisms.
14 Res. Microbiol. 152, 421-430.
15 Omura, S., Takahashi, Y., Iwai, Y. and Tanaka, H. (1982) *Kitasatosporia*, a new genus of the
16 order *Actinomycetales*. J. Antibiot. 35, 1013-1019.
17 Øvreås, L. and Torsvik, V. (1998) Microbial diversity and community structure in two
18 different agricultural soil communities. Microb. Ecol. 36, 303-315.
19 Palleroni, N.J. (1997) Prokaryotic diversity and the importance of culturing. Antonie van
20 Leeuwenhoek 72, 3-19.
21 Pearson, W.R. and Lipman, D.J. (1988) Improved tools for biological sequence comparison.
22 Proc. Natl. Acad. Sci. USA 85, 2444-2448.
23 Pearson, W.R. (1990) pid and Sensitive Sequence Comparison with FASTP and FASTA.
24 Methods Enzymol. 183,
25 Rainey, F.A., Weiss, N., Prauser, H. and Stackebrandt, E. (1994) Further evidence for the
26 phylogenetic coherence of actinomycetes with group B-peptidoglycan and evidence

1 for the phylogenetic intermixing of the genera *Microbacterium* and *Aureobacterium* as
2 determined by 16S rDNA analysis. FEMS Microbiol. Lett. 118, 135-140.
3 Ranjard, L., Poly, F. and Nazaret, S. (2000) Monitoring complex bacterial communities using
4 culture-independent molecular techniques: application to soil environment. Res.
5 Microbiol. 151, 167-177.
6 Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual,
7 2nd edition. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.
8 Schabereiter-Gurtner, C., Piñar, G., Lubitz, W. and Rölleke, S. (2001) An advanced molecular
9 strategy to identify bacterial communities on art objects. J. Microbiol. Methods 45, 77-
10 87.
11 Schumann, P., Prauser, H., Rainey, F.A., Stackebrandt, E. and Hirsch, P. (1997)
12 *Friedmanniella antarctica* gen. nov., sp. nov., an LL-diaminopimelic acid-containing
13 actinomycete from Antarctic sandstone. Int. J. Syst. Bacteriol. 47, 278-283.
14 Sembiring, L., Ward, A.C. and Goodfellow, M. (2000) Selective isolation and
15 characterisation of members of the *Streptomyces violaceusniger* clade associated with
16 the roots of *Paraserianthes falcataria.* Antonie van Leeuwenhoek 78, 353-366.
17 Smalla, K., Wieland, G., Buchner, A., Zock, A., Parzy, J., Kaiser, S., Roskot, N., Heuer, H.
18 and Berg, G. (2001) Bulk and rhizosphere soil bacterial communities studied by
19 denaturing gradient gel electrophoresis: plant-dependent enrichment and seasonal
20 shifts revealed. Appl. Environ. Microbiol. 67, 4742-4751.
21 Stolze, M., Piorr, A., Häring, A. and Dabbert, S. (2000) The environmental impact of organic
22 farming in Europe. In: Organic Farming in Europe: Economics and Policy, Vol. 6,
23 University of Hohenheim, Hohenheim, Germany.
24 Tamura, T., Hayakawa, M. and Hatano, K. (1997) A new genus of the order *Actinomycetales*,
25 *Spirilliplanes* gen. nov., with description of *Spirilliplanes yamanshiensis* sp. nov. Int.
26 J. Syst. Bacteriol. 47, 97-102.

1 Urzi, C., Salamone, P., Schumann, P., Rohde, M. and Stackebrandt, E. (2004) *Blastococcus*
2 *saxobsidens* sp. nov., and emended descriptions of the genus *Blastococcus* Ahrens and
3 Moll 1970 and *Blastococcus aggregatus* Ahrens and Moll 1970. Int. J. Syst. Evol.
4 Microbiol. 54, 253-259.
5 Urzi, C., Salamone, P., Schumann, P. and Stackebrandt, E. (2000) *Marmoricola aurantiacus*
6 gen. nov., sp. nov., a coccoid member of the family *Nocardioidaceae* isolated from a
7 marble statue. Int. J. Syst. Evol. Microbiol. 50, 529-536.
8 Vallaeys, T., Topp, E. and Muyzer, G. (1997) Evaluation of denaturing gradient gel
9 electrophoresis in the detection of 16S rDNA sequence variation in rhizobia and
10 methanotrophs. FEMS Microbiol. Ecol. 24, 279-285.
11 Vogel C.R., Birbauer S., Darnhofer I. 2003. Das Bio-Kontrollsystem in Österreich. Ökologie
12 und Landbau 127, 3/2003
13 Ward-Rainey, N.L., Rainey, F.A. and Stackebrandt, E. (1996) The phylogenetic structure of
14 the genus *Streptosporangium.* Syst. Appl. Microbiol. 19, 50-55.
15 Wirth S., Ulrich A. Cellulose -degrading potentials and phylogenetic classification of
16 caboxymethyl -cellulose decomposing soil bacterial isolates. unpublished, EMBL.
17 Workneh,F., vanBruggen,A.H.C. (1994) microbial density, composition and diversity in
18 organically and conventionally managed rhizosphere soil in relation to suppression of
19 corky roots of tomatoes. Allp. Soil Ecol. 1, 219-230
20 Yakimov, M.M., Lünsdorf, H. and Golyshin, P.N. (2003) *Thermoleophilum album* and
21 *Thermoleophilum minutum* are culturable representatives of group 2 of the
22 *Rubrobacteridae* (*Actinobacteria*). Int. J. Syst. Evol. Microbiol. 53, 377-380.
23 Yamaguchi, T. and Saburi, Y. (1955) Studies on the anti-trichomonal actinomycetes and their
24 classification. J. Gen. Appl. Microbiol. 1, 201-235.

1 Yoon, J.H., Lee, S.T. and Park, Y.H. (1998) Inter- and intraspecific phylogenetic analysis of
2 the genus *Nocardioides* and related taxa based on 16S rDNA sequences. Int. J. Syst.
3 Bacteriol. 48, 187-194.

4

5

1 **Legends**
2
3 **Fig 1:** Eubacterial bandpatterns: Positive image of ethidium bromide stained 16S rDNA of
4 *Eubacteria*. Lanes 1 to 3 show profiles obtained from soils using the conventional DNA
5 extraction method based on the fast DNA SPIN kit for soil based . Lanes 4 to 5 show
6 patterns of sample aliquots obtained with the GL universal DNA Kit.
7
8 **Fig 2:** Schematic diagram of the experimental steps used in this study.
9
10 **Fig 3:** Negative image of ethidium bromide stained 16S rDNA clone library from
11 conventionally managed soil. Lanes OB: original fingerprint of *Actinomycetes* in
12 conventionally managed soil; lane AB: artificial bandpattern of sequenced and pooled clones;
13 remaining lanes are named with the number of the clone where the prefix ACF
14 (Actinomycetes clone library of Conventionally Farmed soil) has to be added.
15
16 **Fig. 4**. Negative image of ethidium bromide stained 16S rDNA clone library from organically
17 managed soil. Lanes OB: original fingerprint of *Actinomycetes* in organically managed soil;
18 lane AB: artificial bandpattern of sequenced and pooled clones; remaining lanes are named
19 with the number of the clone where the prefix AOF (Actinomycetes clone library of
20 Organically Farmed soil) has to be added.
21
22 **Fig. 5.** Neighbour-joining tree based on 16S rDNA data showing the phylogenetic positions
23 of the individual clones obtained from agricultural soils (ACF – conventionally managed soil;
24 AOF – organically managed soil).

Table 1. Phylogenetic affinities of partial 16S rRNA coding sequences detected in conventionally managed agricultural soil using actinomycete-specific primers.

Clone **[Accessio no. [a]]**	**Most closely related type strain** **[Accession no.[b]]**	**Similarity (%)**	**Reference**
ACF1 [AJ555194]	*Nocardioides jensenii* KCTC 9134^T [AF005006]	98,0	[29] Yoon et al., 1998
ACF2 [AJ555195]	*Aeromicrobium erythreum* NRRL B-3381^T [AF005021]	98,3	[29] Yoon et al., 1998
ACF3 [AJ555196]	*Microlunatus phosphovorus* DSM 10555^T [Z78207]	97,1	[30] Schumann et al., 1997
ACF5 [AJ555198]	*Aeromicrobium erythreum* NRRL B-3381^T [AF005021]	98,3	[29] Yoon et al., 1998
ACF6 [AJ555199]	*Streptosporangium roseum* DSM 43021^T [X89947]	91,7	[31] Ward-Rainey et al., 1996
ACF12 [AJ555200]	*Blastococcus aggregatus* DSM 4725^T [AJ430193]	100	[32] Urzi et al. 2004
ACF21 [AJ555201]	*Aeromicrobium erythreum* NRRL B-3381^T [AF005021]	97,7	[29] Yoon et al., 1998
ACF22 [AJ555202]	*Blastococcus aggregatus* DSM 4725^T [AJ430193]	99,2	[32] Urzi et al. 2004
ACF23 [AJ555203]	*Nocardioides jensenii* KCTC 9134^T [AF005006]	97,1	[29] Yoon et al., 1998
ACF30 [AJ555204]	*Marmoricola aurantiacus* BC361^T [Y18629]	97,7	[33] Urzi et al., 2000
ACF37 [AJ555205]	*Streptomyces indonesiensis* A4R2^T [AJ391835]	91,3	[34] Sembiring et al., 2000
ACF41 [AJ555206]	*Marmoricola aurantiacus* BC361^T [Y18629]	96,0	[33] Urzi et al., 2000
ACF42 [AJ555207]	*Pseudonocardia yunnanensis* IMSNU 22019^T [AJ252822]	97,4	[35] Lee et al., 2000
ACF43 [AJ555208]	*Blastococcus aggregatus* DSM 4725^T [AJ430193]	96,8	[32] Urzi et al. 2004
ACF44 [AJ555209]	*Blastococcus aggregatus* DSM 4725^T [AJ430193]	98,9	[32] Urzi et al. 2004
ACF45 [AJ555210]	*Blastococcus aggregatus* DSM 4725^T [AJ430193]	98,9	[32] Urzi et al. 2004
ACF47 [AJ555211]	*Blastococcus aggregatus* DSM 4725^T [AJ430193]	99,2	[32] Urzi et al. 2004

[a] EMBL sequence accession number
[b] EMBL sequence accession number of most closely related bacterial species

Table 2. Phylogenetic affinities of partial 16S rRNA coding sequences detected in organically managed agricultural soil using actinomycete-specific primers.

Clone **[Accessio no. [a]]**	**Most closely related type strain** **[Accession no.[b]]**	**Similarity (%)**	**Reference**
AOF3 [AJ555216]	*Blastococcus aggregatus* DSM 4725^T [AJ430193]	98,9	[32] Urzi et al. 2004
AOF4 [AJ555217]	*Streptoalloteichus hindustanus* IFO 15115^T [D85497]	87,5[c]	[36] Tamura et al., 1997
AOF10 [AJ555219]	*Thermoleophilum album* ATCC 35263^T [AJ458462]	89,1[c]	[37] Yakimov et al., 2003
AOF13 [AJ555221]	*Aeromicrobium erythreum* NRRL B-3381^T [AF005021]	96,5	[29] Yoon et al., 1998
AOF16 [AJ555222]	*Streptomyces griseoruber* ICSSB 1013^T [AY094585]	97,1	[38] Yamaguchi et al. 1955
AOF20 [AJ555223]	*Marmoricola aurantiacus* BC361^T [Y18629]	95,7	[33] Urzi et al., 2000
AOF24 [AJ555224]	*Blastococcus aggregatus* DSM 4725^T [AJ430193]	96,3	[32] Urzi et al. 2004
AOF29 [AJ555225]	*Marmoricola aurantiacus* BC361^T [Y18629]	95,4	[33] Urzi et al., 2000
AOF31 [AJ555226]	*Streptomyces indonesiensis* A4R2^T [AJ391835]	96,0	[34] Sembiring et al., 2000
AOF34 [AJ555227]	*Aeromicrobium erythreum* NRRL B-3381^T [AF005021]	95,4	[29] Yoon et al., 1998
AOF36 [AJ555228]	*Microbacterium lacticum* DSM 20427^T [X77441]	95,7	[39] Rainey et al., 1994
AOF41 [AJ555229]	*Kitasatospora setae* DSM 43861^T [M55220]	97,4	[40] Omura et al. 1982
AOF42 [AJ555230]	*Streptomyces griseoruber* ICSSB 1013^T [AY094585]	99,7	[38] Yamaguchi et al. 1955
AOF47 [AJ555231]	*Thermoleophilum album* ATCC 35263^T [AJ458462]	87,1[d]	[37] Yakimov et al., 2003

[a] EMBL sequence accession number
[b] EMBL sequence accession number of most closely related bacterial species
[c] higher similarity to uncultured strain
[d] no relation to cultured or uncultured strain

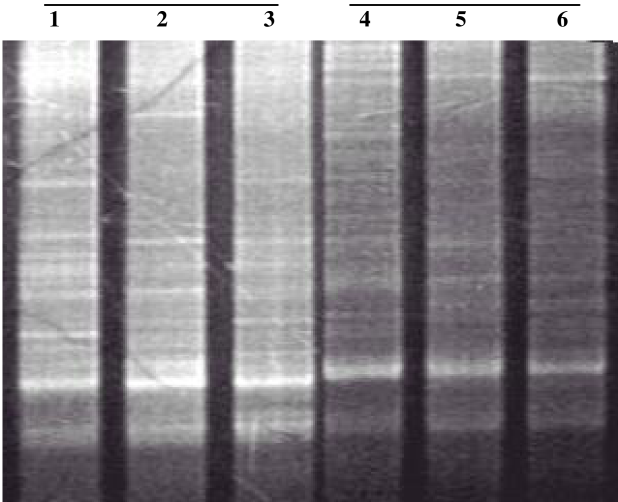
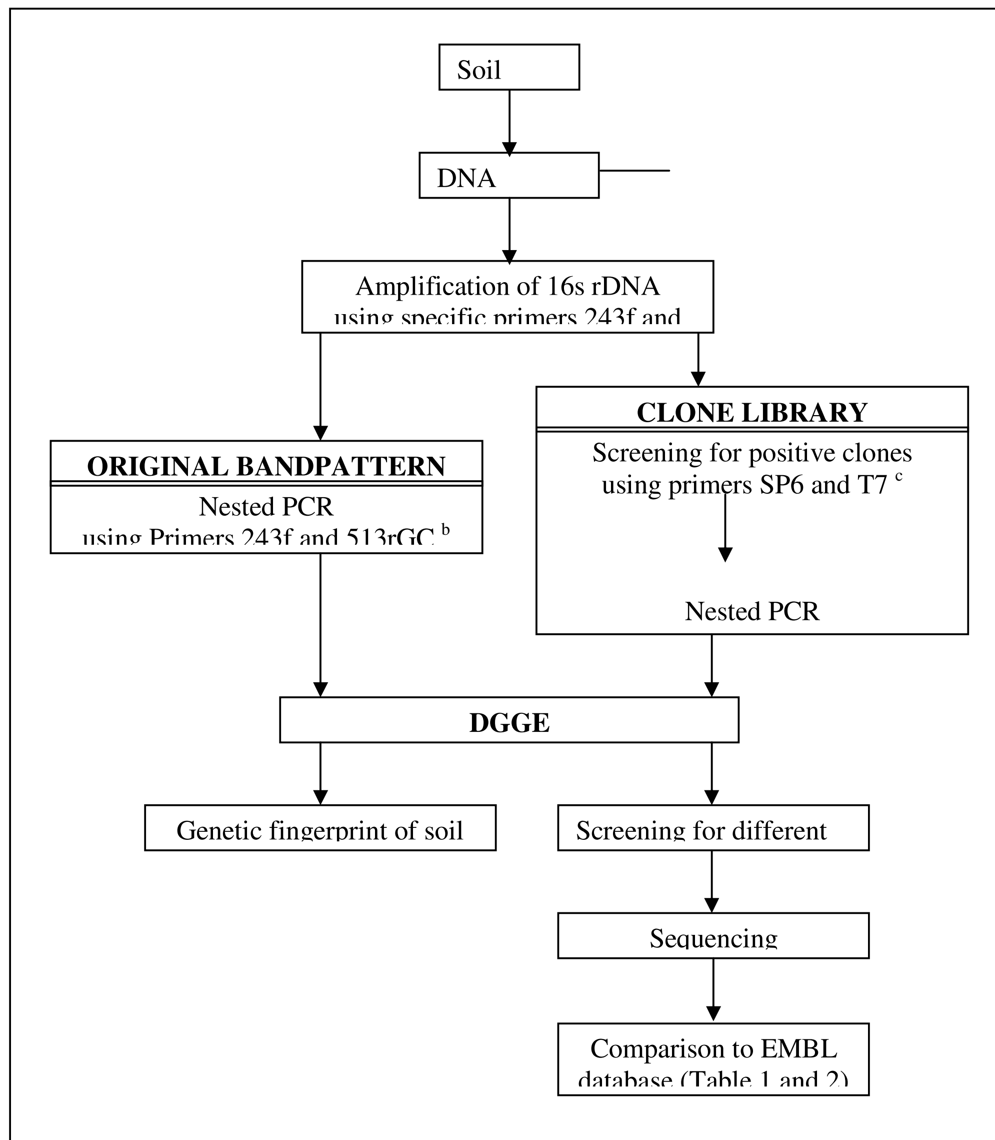


Fig1


Soil
DNA
Amplification of 16s rDNA
using specific primers 243f and
CLONE LIBRARY
Screening for positive clones
using primers SP6 and T7 c
Nested PCR
ORIGINAL BANDPATTERN
Nested PCR
using Primers 243f and 513rGC b
DGGE
Genetic fingerprint of soil
Screening for different
Sequencing
Comparison to EMBL
database (Table 1 and 2)

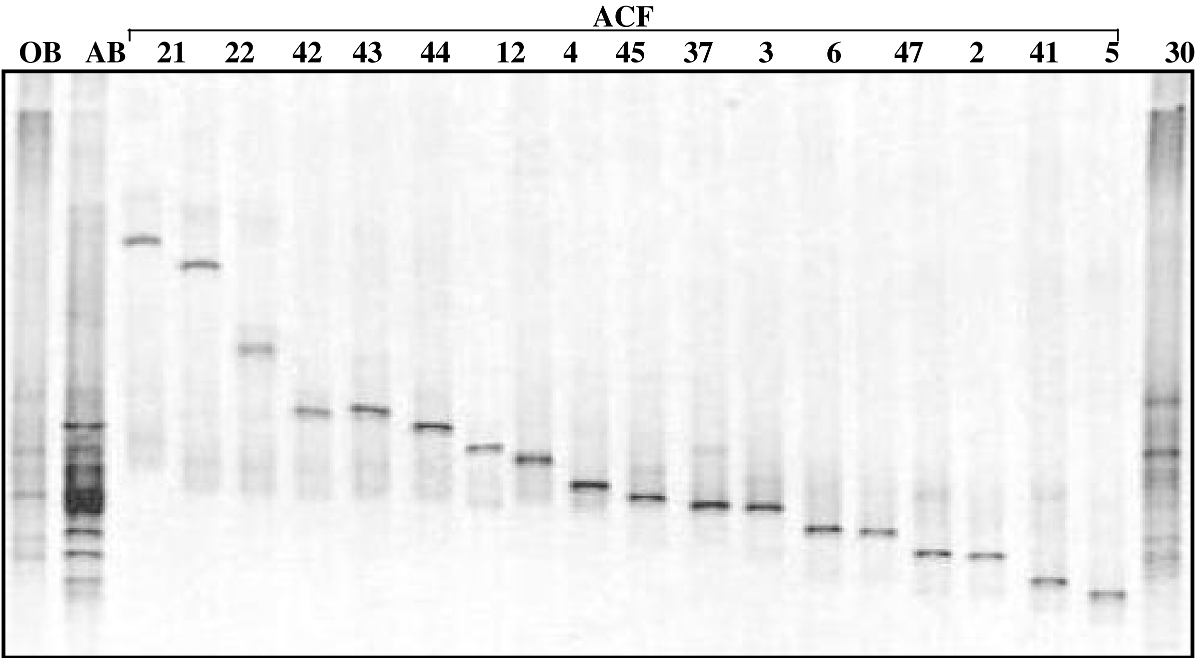


Fig 3

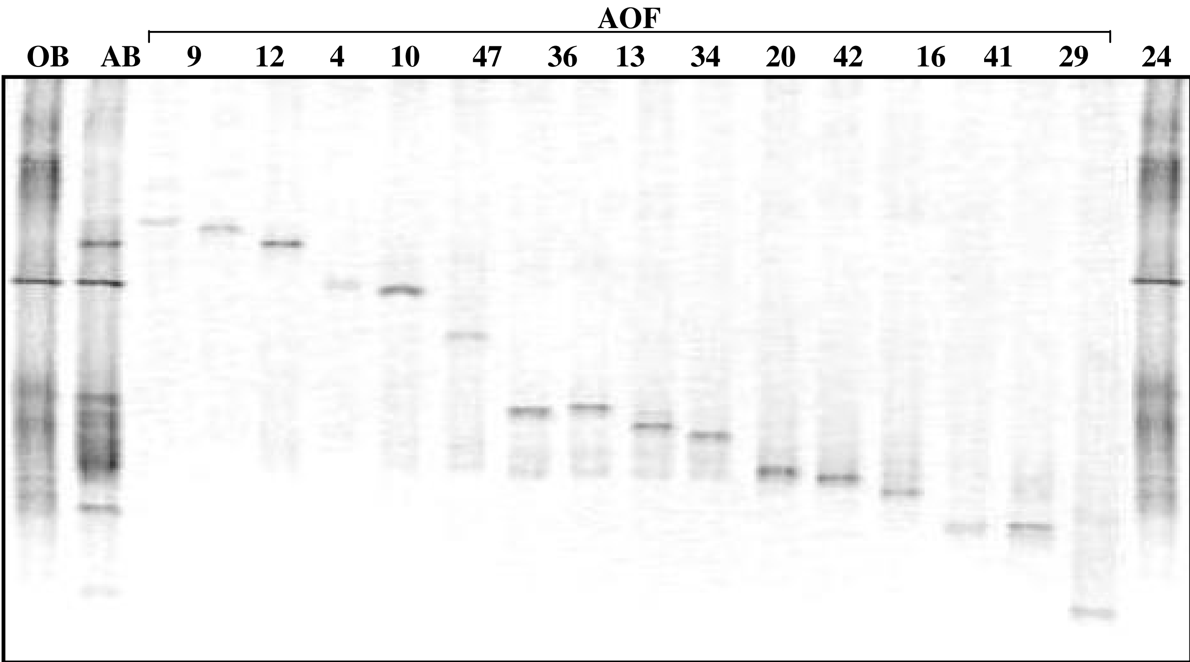


Fig 4

Uncultured actinobacterium clone P2-1H
AOF4
Candidatus Microthrix parvicella DAN1-3
Uncultured *Rubrobacteridae* bacterium clone glen99_21
AOF10
Conexibacter woesei DSM 14684^{T}
Solirubrobacter pauli B33D1^{T}
Thermoleophilum album ATCC 35263^{T}
Acidimicrobium ferrooxidans DSM 10331^{T}
Coriobacterium glomerans DSM 20642^{T}
AOF47
Streptomyces sp. 91-Sy-13
AOF42
Streptomyces griseoruber ICSSB 1013^{T}
Kitasatospora setae DSM 43861^{T}
Streptomyces flavogriseus DSM 40323^{T}
Streptacidiphilus albus DSM 41753^{T}
Streptomyces indonesiensis A4R2^{T}
Streptomyces albus DSM 40313^{T}
AOF31
AOF41
AOF16
ACF42
Pseudonocardia thermophila ATCC 19285^{T}
Pseudonocardia yunnanensis IMSNU 22019^{T}
Saccharopolyspora hirsuta subsp. *hirsuta* ATCC 27875^{T}
Thermocrispum municipale DSM 44069^{T}
Amycolatopsis orientalis subsp. *orientalis* DSM 40040^{T}
Prauserella rugosa DSM 43194^{T}
Saccharomonospora viridis ATCC 15386^{T}
Kibdelosporangium aridum subsp. *aridum* DSM 43828^{T}
Actinoalloteichus cyanogriseus IFO 14455^{T}
Kutzneria viridogrisea JCM 3282^{T}
Crossiella cryophila NRRL B-16238^{T}
Streptoalloteichus hindustanus IFO 15115^{T}
Actinosynnema mirum IFO 14064^{T}
Sporichthya polymorpha IFO 12702^{T}
Frankia sp. Cn nod
Acidothermus cellulolyticus ATCC 43068^{T}
Geodermatophilus obscurus subsp. *obscurus* DSM 43160^{T}
Modestobacter multiseptatus AA826^{T}
ACF44
ACF12
Blastococcus aggregatus DSM 4725^{T}
Blastococcus sp. BC521
ACF47
ACF43
AOF24
ACF22
ACF45
AOF3
Blastococcus sp. BC412
Bogoriella caseilytica HKI 0088^{T}
Micrococcus luteus DSM 20030^{T}
Cellulomonas flavigena DSM 20109^{T}
Agreia bicolorata VKM Ac-1804^{T}
Subtercola boreus DSM 13056^{T}
Frigoribacterium faeni 801^{T}
Salinibacterium amurskyense KMM 3673^{T}
Rhodoglobus vestalii LV3^{T}
Agrococcus jenensis DSM 9580^{T}
Cryobacterium psychrophilum JCM 1463^{T}
Mycetocola saprophilus CM-01^{T}
Clavibacter michiganensis DSM 46364^{T}
Microbacterium lacticum DSM 20427^{T}
Okibacterium fritillariae VKM Ac-2059^{T}
Rathayibacter rathayi DSM 7485^{T}
Leucobacter komagatae JCM 9414^{T}
Agromyces ramosus DSM 43045^{T}
Leifsonia aquatica DSM 20146^{T}
Curtobacterium citreum DSM 20528^{T}
Plantibacter flavus DSM 14012^{T}
AOF36
Dermatophilus congolensis DSM 44180^{T}
Corynebacterium diphtheriae CIP 100721^{T}
ACF1
Nocardioides sp. KP7
Nocardioides jensenii KCTC 9134^{T}
Pimelobacter simplex DSM 20130^{T}
Nocardioides albus DSM 43109^{T}
ACF2
ACF5
Uncultured soil bacterium clone 528-1
ACF21
Nocardioides sp. LMG 20237
ACF30
Marmoricola aurantiacus BC361^{T}
ACF23
ACF41
AOF20
Aeromicrobium erythreum NRRL B-3381^{T}
AOF34
AOF13
AOF29
ACF3
Microlunatus phosphovorus DSM 10555^{T}
Luteococcus japonicus IFO 12422^{T}
Propionibacterium freudenreichii subsp. *freudenreichii* DSM 20271^{T}
Propionimicrobium lymphophilum DSM 4903^{T}
Tessaracoccus bendigoensis ACM 5119^{T}
Propioniferax innocua ATCC 49929^{T}
Friedmanniella antarctica DSM 11053^{T}
Micropruina glycogenica Lg2^{T}
Actinopolymorpha singaporensis IM7744^{T}
Kribbella flavida IFO 14399^{T}
ACF6
ACF37
Uncultured actinobacterium clone CF44
Streptosporangium roseum DSM 43021^{T}
Thermomonospora curvata IFO 15933^{T}
Nocardiopsis dassonvillei DSM 43111^{T}
Actinopolyspora halophila ATCC 27976^{T}
Thermobispora bispora ATCC 19993^{T}
Bacillus subtilis ATCC 6051^{T}

Scale:
0.1

Fig 5