Molecular biological evidence for the occurrence of uncultured members of the actinomycete line of descent in different environments and geographical locations

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A 16S rDNA based molecular ecological study was performed on a sample taken from a peat bog in Germany. Total DNA was extracted by directly lysing micro-organisms in the peat matrix. The 5' 1400 nucleotides of the bacterial 16S rDNA were amplified using conserved bacterial PCR primers. A clone library was generated by blunt-end cloning and 262 16S rDNA clones were analysed. Of these, 37 were located in the Gram-positive phylum, as determined by hybridization to an oligonucleotide probe specific for Gram-positive bacteria. Analysis of 17 of these clones by sequence analysis and their comparison with published sequences representing all of the main bacterial phyla indicated their membership of the actinomycete line of descent. These peat clones were found to represent three novel lineages, two of which appear to be related to the species *Acidimicrobium ferrooxidans*, and 'Candidatus Microthrix parvicella'. Clone sequences of the third group are phylogenetically related to *Rubrobacter radiotolerans*. Comparison with short 16S rDNA clone sequences obtained from DNA isolated from a geothermally heated soil in New Zealand, and from DNA isolated from soil in Australia, Japan and Finland and marine environments from the Atlantic and the Pacific Oceans, suggests that members of these three groups occur in very different environments across the world.

Keywords: 16S rDNA clone library, peat, bacterial diversity, actinomycete line of descent

INTRODUCTION

Recent analysis of 16S rDNA clone libraries generated from DNA isolated from terrestrial and marine environments indicated the existence of a broad monophyletic group of hitherto uncultured organisms falling within the actinomycete line of descent. Several such 16S rDNA clone sequences, abbreviated MC, were obtained from an Australian forested soil taken from the Mount Coot-tha region in Queensland (Liesack & Stackebrandt, 1992a, b; Stackebrandt *et al.*, 1993). These 16S rDNA clone sequences showed >83% similarity to partial sequences from 16S rDNA clone libraries from a geothermally

heated soil from New Zealand (abbreviated NZ) (Rainey *et al.*, 1993) and a soil sample from Finland (Saano *et al.*, 1995). In addition, short 16S rDNA clone sequences from a paddy field (abbreviated PAD) (Maidak *et al.*, 1994), a soybean field (abbreviated FIE) (Ueda *et al.*, 1995) and from marine environments (Fuhrman *et al.*, 1993) showed significant sequence similarities to clone NZ150 (78–94% partial sequence similarity) and can therefore also be considered members of the MC/NZ 16S rDNA clone sequence cluster.

None of these clone sequences are identical or even highly similar to those of described species. At a lower level of relatedness, this group of environmental sequences appears to be a neighbouring group of the two cultured organisms *Acidimicrobium ferrooxidans* strain TH3 (Clark & Norris, 1996; Lane *et al.*, 1992) and 'Candidatus Microthrix parvicella' (Blackall *et al.*, 1994, 1996), which display a moderate degree of 16S rDNA similarity (87%)

Abbreviation: PVPP, polyvinylpyrrolidone.

The EMBL accession numbers for the sequences reported in this paper are X92694–X92710 for the peat bog sequences, X93010 and X93011 for the clones from New Zealand, and X98372 for *Rubrobacter radiotolerans*.

to one another. The latter two organisms form two remotely related lineages that branch deeply in the actinomycete line of descent. *Acidimicrobium ferrooxidans* (formerly known as strain TH3) was isolated from a mine dump in New Mexico (Brierley, 1978). It is capable of oxidizing ferrous iron at 50 °C, but does not grow at 60 °C. It requires yeast extract and can use either ferrous iron or pyrite as a source of energy. The autotrophic growth of this strain has recently been confirmed (Clark & Norris, 1996). It decreases the pH of the growth medium from 2.4 to 1.9 during 4 d incubation at 50 °C on a pyrite/yeast extract medium, which indicates sulfide oxidation. '*Candidatus* Microthrix parvicella', found in activated sludge sewage treatment plants (Blackall *et al.*, 1994), has *Candidatus* status because of its failure to survive long enough in pure culture to enable determination of the phenotypic properties required for its valid description.

The few common phenotypic data that are available for the two species, such as positive Gram-staining behaviour, formation of filaments and requirement for reduced sulphur compounds for growth on artificial media (Blackall *et al.*, 1994, 1996; Clark & Norris, 1996), are not sufficient to support the apparent phylogenetic relationship of these organisms to one another (Blackall *et al.*, 1994). Consequently, no conclusions can be drawn as to the phenotypic properties of the related MC/NZ clone cluster sequences, of which no more than the partial 16S rDNA sequence is known.

Recently a peat bog sample from Northern Germany was the subject of an extensive isolation study (von Wintzingerode, 1994). The majority of isolates obtained in the study were Gram-positive bacteria identified as members of the genus *Bacillus*. Additional isolates were found to be members of the class Proteobacteria and the order *Actinomycetales*. In this communication we present evidence that 16S rDNA sequences similar to those obtained from soil in Australia, Finland, Japan and New Zealand, and from the marine environment, are also found in the low-pH peat bog sample studied by von Wintzingerode (1994). It can thus be speculated that these as yet uncultured organisms are widely distributed in terrestrial and probably in marine samples, where they may contribute to ecologically important processes.

METHODS

Sampling site. A core drill was used to take samples in April 1994 from a peat bog (depth 40 cm) near Gifhorn, Lower Saxony, Germany (10° 33' E, 52° 30' N). The drill was driven horizontally into the wall of a drainage channel for 50 cm. The ends of the peat core were discarded. All instruments were autoclaved or rinsed with ethanol and flamed before use. The peat material was immediately placed in dry ice until storage at -70 °C.

DNA extraction and purification. Genomic DNA was extracted by using a modified direct lysis technique (Ogram *et al.*, 1987). Twenty millilitres of saline/EDTA buffer (0.15 M NaCl, 0.01 M EDTA disodium salt, pH 8.0) were added to 5 g of a thawed peat sample. Following the addition of 2 g PVPP (Sigma), prepared as described by Holben *et al.* (1988), and 150 mg lysozyme (Boehringer), dissolved in 5 ml saline/EDTA

buffer, the suspension was mixed and then homogenized with an Ultra-Turrax (Janke & Kunkel) for 45 s. After incubation at 37 °C for 30 min, 200 µl proteinase K (1%, w/v; Boehringer) and 500 µl SDS (25%, w/v) were added, homogenized by shaking and the homogenate was further incubated at 70 °C for 45 min. Soil debris and PVPP were pelleted by centrifugation at 5800 g for 20 min. The supernatant was transferred to a fresh tube and subsequently extracted with an equal volume of tris/EDTA-saturated phenol, phenol/chloroform (1:1, v:v) and chloroform, each step followed by centrifugation as indicated above. Following the final extraction step, nucleic acids were precipitated for 18 h at -18 °C by addition of 0.1 vol. 3 M sodium acetate (pH 5.2) and 2 vols absolute ethanol. After centrifugation for 30 min at 18000 g, the supernatant was discarded and the pellet washed twice with 10 ml 70% (v/v) ethanol, with centrifugation steps as before. The DNA pellet was dried in a vacuum centrifuge and dissolved in 300 µl sterile water. Further purification was with the Prep-A-Gene DNA Purification Kit (Bio-Rad), using 10 µl of the DNA binding matrix. After this purification procedure, humic acids could not be detected by electrophoresis of a DNA aliquot on a 1% (w/v) agarose gel.

Amplification and cloning of 16S rDNA. This was performed using Ulta DNA polymerase, with the buffer supplied by the manufacturer (Perkin Elmer) and bacterial 16S rDNA oligonucleotide primers 27f and 1385r (Table 1). The PCR protocol is a modification of the one recommended by Perkin Elmer for the use of Ulta polymerase, in that 4 µl of 25 mM MgCl₂ solution was used. PCR was performed on a DNA thermal cycler (model 9600, Perkin Elmer); amplification was for 28 cycles (annealing for 1 min at 52 °C, elongation for 2 min at 72 °C, denaturation for 1 min at 93 °C). PCR products were purified with the Prep-A-Gene DNA Purification Kit. Cloning was performed with the pCR-Script SK(+) Cloning Kit (Stratagene) and Epicurian Coli XL1-BLUE MRF⁺KAN competent cells (Stratagene). Modifications of the protocol given in the Stratagene instruction manual included incubation of the ligation mix for at least 2 h and heat pulsing of the competent cells for 50 s at 42 °C.

Recombinant plasmids were extracted by boiling an inoculation loopful of the bacterial cells in 100 µl water (10 min at 98 °C) and pelleting the debris by centrifugation (1 min at 15000 g). The supernatant was transferred into fresh tubes. The inserts were further amplified using *Taq* polymerase (Boehringer) and M13(-20) and M13rev primers (Table 1) in a reaction volume of 50 µl, and with the PCR cycles as indicated above.

Determination of nucleotide sequences. Sequencing reactions of the 16S rDNA fragments were performed with the *Taq* DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems) and electrophoresed using an automated sequence analyser (model 373A; Applied Biosystems). The length of the analysed stretches of the 17 clones ranged from 1329 to 1373 nucleotides. A shorter stretch comprising about 400 nucleotides was analysed in an additional 20 clones.

Phylogenetic analysis. The sequencing data files were manually aligned with ESEE (the Eyeball Sequence Editor) (Cabot & Beckenbach, 1989) and then included in the alignment editor ae2 (Maidak *et al.*, 1994). The presence of possible chimeric structures was investigated using the CHECK_CHIMERA program (Maidak *et al.*, 1994). The 16S rDNA clone sequences were compared to sequences representing major lines of descent. Similarity values were transformed into phylogenetic distance values that compensate for multiple substitutions at any given site in the sequence (Jukes & Cantor, 1969). The least-squares distance method of DeSoete (1983) as well as the neighbour-joining method contained in the PHYLIP package (Felsenstein,

Table 1. Sequences of oligonucleotide primers and probes used

Primer/probe	Sequence (5'-3')	Reference/source
27f	GAG TTT GAT CCT GGC TCA G	Lane (1991)
1385r	CGG TGT GT(A/G) CAA GGC CC	Stackebrandt & Liesack (1993)
519r	G(T/A)A TTA CCG CGG C(T/G)G CTG	Stackebrandt & Liesack (1993)
M13(-20)	GTA AAA CGA CGG CCA GT	Stratagene (1994)
M13rev	GGA AAC AGC TAT GAC CAT G	Stratagene (1994)
<i>Gram-positive</i>	TCA TCA TGC CCC TTA TG	This work
<i>mycoides</i>	TAA TAT TTT GAA CTG CAT AG	von Wintzingerode (1994)
TH3	CAG CTC GCT GGG ACG	This work

1993) were used in the construction of phylogenetic dendrograms. For calculation of bootstrap values, 100 trees were analysed using the programs NJFIND and NJBOOT.

DNA blotting and hybridization with oligonucleotide probes.

The PCR products of the 16S rDNA inserts were purified with the Prep-A-Gene DNA purification kit and eluted in 50 µl water. Three microlitre samples of each of the 16S rDNA PCR products were then denatured and applied to a nylon membrane (Hybond N, Amersham), using a dot-blot apparatus (Bio-Rad) and fixed by UV (260 nm) for 100 s. Hybridization and detection were performed according to the manufacturer's instructions using the DIG Luminescent Detection Kit (Boehringer). Oligonucleotide probes were synthesized by the Pharmacia Biotech Oligo-Synthesis-Service (Pharmacia) and labelled using the DIG Oligonucleotide 3'-End labelling kit (Boehringer). The 16S rDNA PCR products of the controls and the peat clones were fixed onto a total of three membranes. All three membranes were processed simultaneously in the same roller tube to ensure reproducible hybridization and detection conditions. Hybridization was carried out at room temperature for 4 h. The temperature of the stringency wash in 6× SSC, 0.1% SDS buffer was 37 °C. Even after washing at more stringent temperatures, unspecific binding was not observed. Exposure to an X-ray film (Fuji 51209) was for 2 h.

RESULTS

Isolation of DNA

Comparison of the isolated DNA by agarose gel electrophoresis with and without treatment with PVPP showed a significant difference in the amount of humic acids present in the extracts. Without PVPP treatment, humic acids appeared under UV light as a bright band migrating faster than DNA in the agarose gel. Using DNA prepared without PVPP treatment as PCR template resulted in no 16S rDNA PCR product being obtained. As judged from the UV-visualized gel, the amount of humic acids was significantly reduced by PVPP treatment. When used as a PCR template, DNA which had been PVPP treated and Prep-A-Gene purified gave a 16S rDNA amplification product.

Analysis of the clone library

The generated 16S rDNA clone library comprised a total of 262 clones, designated TM (for 'Torf, mittlere Schicht' = peat, middle layer). The reamplified PCR products of

the 16S rDNA clone inserts, together with reference 16S rDNA PCR products of 26 strains of known phylogenetic affiliation, were blotted onto a membrane filter. The reference 16S rDNA represented 13 different bacterial genera, seven isolates from the same peat sample (von Wintzingerode, 1994) and two plant chloroplasts (Table 2). In order to check the availability of target DNA for oligonucleotide probing, the filters were hybridized with a universal bacterial oligonucleotide probe (519r, see Table 1). The reference 16S rDNAs and all but three of the 262 peat clones gave positive signals when hybridized with this probe (data not shown).

As a previous analysis of cultured strains isolated from the same location indicated the presence of mainly Gram-positive bacteria (von Wintzingerode, 1994), their presence in the clone library was checked with a probe designed to specifically detect 16S rDNA of Gram-positive bacteria (*Gram-positive*, Table 1). This probe hybridized with all of the Gram-positive control rDNAs and with 37 of the 16S rDNA clones (15% of the total) (Fig. 1). In contrast to the isolation study, which indicated the presence of a substantial number of *Bacillus mycoides* strains in the peat sample, 16S rDNA of this taxon was not present in the clone library as indicated by the absence of signals with a *B. mycoides*-specific oligonucleotide probe (*mycoides*, Table 1). As only the DNAs of the *Bacillus* reference strains (listed in Table 2) gave a positive signal under the conditions used with this probe, it was concluded that clones originating from *B. mycoides* were absent in the 262 16S rDNA clones investigated.

Further phylogenetic analyses concentrated on sequence comparison of 26 randomly selected 16S rDNA clones which had hybridized with the *Gram-positive* probe. The CHECK_CHIMERA analysis (Maidak *et al.*, 1994) of 17 almost complete sequences indicated that none of them were chimeric structures. Phylogenetic analysis of these 17 sequences, based on the comparison of stretches of 1329 to 1373 nucleotides, revealed the presence of three groups I–III, see Fig. 2), of which groups II and III were more closely related (88–91% 16S rDNA sequence similarity) to each other than either was to clone sequences of group I (82–86% 16S rDNA sequence similarity). For groups I and III the intragroup relatedness was > 94%, while within group II the relatedness was > 92%. 16S rDNA sequences of clones from group I could be distinguished

Table 2. 16S rDNA PCR products used for dot-blot hybridization with different oligonucleotide probes

Position on membrane*	Designation/identity	Source/strain†
A1	<i>Bacillus cereus</i>	DSM 31 ^T
A2	<i>Bacillus mycoides</i>	DSM 307
A3	<i>Bacillus mycoides</i>	DSM 2048 ^T
A4	<i>Bacillus thuringiensis</i>	DSM 2046 ^T
A5	<i>Bacillus subtilis</i>	DSM 10 ^T
A6	Isolate 1F8o2‡	99.5 % <i>Bacillus mycoides</i> §
A7	Isolate 3Gym7‡	96 % <i>Methylobacterium fujisawaense</i> §
A8	Isolate 3SCo6‡	100 % <i>Streptomyces murinus</i> §
A9	Isolate 2Miu8‡	99.2 % <i>Mycobacterium diernhoferi</i> §
A10	Isolate 2Gyu7‡	98.3 % <i>Mycobacterium sphagnum</i> §
A11	Isolate 2Gyo9‡	97.8 % <i>Bacillus circulans</i> §
A12	Isolate 3Mio8‡	96.1 % <i>Paenibacillus polymyxa</i> §
B1	<i>Comamonas acidovorans</i>	DSM 39 ^T
B2	<i>Janthinobacterium lividum</i>	DSM 1522 ^T
B3	<i>Streptomyces albidoflavus</i>	DSM 40455 ^T
B4	<i>Methylobacterium fujisawaense</i>	DSM 5686 ^T
B5	<i>Rhodococcus rhodochrous</i>	DSM 43241 ^T
B6	<i>Desulfotomaculum orientis</i>	DSM 765 ^T
B7	<i>Desulfovibrio africanus</i>	DSM 2603 ^T
B8	<i>Spirochaeta africana</i>	DSM 8902 ^T
B9	<i>Arthrobacter globiformis</i>	DSM 20124 ^T
B10	<i>Escherichia coli</i>	DSM 498
B11	<i>Planctomyces limnophilus</i>	DSM 3776 ^T
B12	<i>Gemmata obscuriglobus</i>	DSM 5831 ^T
C1	<i>Equisetum giganteum</i>	DSM PC 592
C2	<i>Marchantia polymorpha</i>	DSM PC 713

* The letter and the number indicate the row and column on the membrane, blot I in Fig. 1.

† T, type strain.

‡ Strain designation of isolates obtained from F. von Wintzingerode.

§ Values indicate 16S rDNA similarity values between the isolate and the closest phylogenetic neighbour, based on partial sequence comparison (von Wintzingerode, 1994).

from those of groups II and III by a short hairpin loop present in the secondary structure from nucleotide positions 198–219 (*Escherichia coli* nomenclature). Comparison of the analysed Gram-positive TM 16S rDNA clone sequences and 16S rDNA/RNA reference sequences revealed that all of the TM sequences were most closely related to three 16S rDNA sequences from organisms that are phylogenetic members of the actinomycete subphylum of the Gram-positive bacteria (Embley & Stackebrandt, 1994), i.e. *Rubrobacter radiotolerans* (sequence determined in this study), *Acidimicrobium ferrooxidans* (Clark & Norris, 1996) and ‘*Candidatus Microthrix parvicella*’ (Blackall *et al.*, 1994). The 16S rDNA similarity values found between clone sequences of cluster I and *Rubrobacter radiotolerans* were about 85 %, while those of groups II and III ranged between 84 and 91 % to *A. ferrooxidans* and 83 and 89 % to ‘*Candidatus M. parvicella*’.

In order to determine whether any of the re-amplified TM clone inserts hybridized with a probe originally designed to detect *A. ferrooxidans* strain TH3, the filters were

hybridized with the TH3 probe (Table 1). As neither strain TH3, nor its DNA, was available to us, a 16S rDNA product of this strain could not be included in the hybridization experiments as a positive control. The probe gave positive signals with five clones, of which four were subsequently sequenced (TM36, TM146/TM160, TM220). As revealed by 16S rDNA sequence analysis, the four clone sequences were found to be members of clone group I. Apparently, the 15-mer oligonucleotide probe TH3, as originally designed, is not exclusively specific to strain TH3.

The results of phylogenetic analysis using two different distance methods and the Maximum Likelihood method gave identical branching patterns; differences were only found in the length of the branches. A tree based on the distance method of DeSoete (1983) is shown in Fig. 2, in which the 16S rDNA sequences of *Bacillus subtilis* as well as *Acidobacterium capsulatum* (Kishimoto *et al.*, 1991; Hiraishi *et al.*, 1995) and several related clone sequences from Mount Coot-tha soil served as outgroup sequences.

Fig. 1. Hybridization of the TM clones with probe *Gram-positive*. Each membrane (designated Blot I, Blot II and Blot III) carried 96 16S rDNA PCR products. These are: for Blot I, controls 1 to 26 (see Table 2 for details) from position A1 to C2 and clones TM1 to TM70 from position C3 to H12; for Blot II, clones TM71 to TM166 from position A1 to H12; for Blot III, clones TM167 to TM262 from position A1 to H12. Clones showing a positive signal with the probe are: TM36, 56, 62; TM75, 77, 81, 90, 93, 97, 100, 108, 109, 112, 114, 146, 154, 156, 160; TM167, 177, 185, 187, 192, 202, 208, 210, 213, 214, 216, 219, 220, 223, 226, 232, 243, 259, 262.

The latter sequences were included because they were previously found to cluster deeply with *Atopobium* (*Lactobacillus*) *minutum* at the base of the actinomycete line of descent (Stackebrandt *et al.*, 1993). The inclusion of the 16S rDNA sequence of *Acidobacterium capsulatum* (Hiraishi *et al.*, 1995) in the database revealed the affiliation of these MC 16S rDNA sequences to this species, which constitutes a novel main line of descent within the domain Bacteria. The three TM 16S rDNA clone groups represent distinct lineages, of which groups II and III appear to represent phylogenetic neighbouring taxa. Although some of the bootstrap values are as high as 70% the relationships of the TM clones are not supported by statistical analysis.

Comparison with published environmental clone sequences

Comparison of the 16S rDNA sequences of actinomycetes and the TM clone sequences with around 150 environmental clone sequences listed in the Ribosomal Database Project (Maidak *et al.*, 1994) constitutes a problem. Many of these environmental sequences cannot be compared as

Fig. 2. Dendrogram based upon the distance method of DeSoete (1983) showing the phylogenetic position of TM groups I-III and related MC clone sequences within the actinomycete line of descent. Analysis is based upon the 5' 1000 nucleotides of the 16S rDNA sequences. Numbers indicate the percentages of occurrence in 100 bootstrapped trees. Only values above 65% are shown. The bar represents 10 nucleotide substitutions per 100 nucleotides. The number of partially sequenced TM clones with identical sequence (based on a comparison of about 500 nucleotides) is indicated next to the full sequence clone designation.

they are of different lengths and the stretches originate from different parts of the 16S rDNA molecule. Comparison with the environmental 16S rDNA clone sequences for which a common stretch of about 500 nucleotides is available indicated that three MC 16S rDNA clone sequences from an Australian soil (Stackebrandt *et al.*, 1993) exhibit a close (MC4) to moderate (MC19 and MC87) degree of relatedness to members of Group I (clone MC87 is not shown in Fig. 2, because its sequence comprises less than 500 nucleotides). MC clone clusters MC19 and MC87 appear to share a common origin with members of TM group I (bootstrap value of 94%). Additional sequence information from a poster presentation (Saano *et al.*, 1995) indicates that two short, identical 16S rDNA clone sequences obtained from a Finnish forest soil are about 92% similar to the Australian 16S rDNA clone sequences MC19 and MC87.

Although the common sequence stretch comprises only 260 nucleotides, moderately high sequence similarity exists between two sequences from a paddy field (PAD, Maidak *et al.*, 1994), three sequences from a soybean field in Japan (FIE, Ueda *et al.*, 1995) and certain TM 16S rDNA clones, indicating that they are derived from phylogenetically closely related organisms. This is shown for the environmental clone sequence env. PAD57, which shares about 94.4% 16S rDNA similarity to clones TM210 and TM226 (group III), and for clone sequences env. PAD13 and the three sequences of the env. FIE2 cluster, showing 16S rDNA sequence similarities between 88.4 and 90.9% to representatives of group I. In each case the phylogenetic analyses support the monophyletic origin of members of different environments of groups I and II.

Two further 16S rDNA sequences from a clone library generated from DNA isolated from a geothermally heated soil in New Zealand are monophyletically related to members of TM group II. These clones are NZ44 (93.0% similarity) and NZ150 (89.2% similarity). As the longest of the two clone sequences contains only 890 nucleotides (NZ150), they are not included in Fig. 2.

In the case of the marine clone sequences, only one of nine (BDA1-5) can be used in phylogenetic analysis as it comprises more than 600 nucleotides (Fuhrman *et al.* 1993). Its phylogenetic position is within group I of the TM clones. The remaining sequences from Bermuda and northeastern Pacific Sargasso Sea comprise only about 100 nucleotides of the 3' half of the 16S rDNA. This stretch of sequence is sufficient to link them to clone sequence BDA1-5 on the basis of similarity.

DISCUSSION

The outcome of the molecular investigation of a peat bog sample is the presence of a clone sequence cluster that has previously been described to be present in geographically widely separated soil environments, i.e. Australia, Finland, Japan, New Zealand, and in the Atlantic and Pacific oceans. The cultured phylogenetic relatives of the uncultured strains represented by the 16S rDNA clone

sequences are *Acidimicrobium ferrooxidans*, '*Candidatus* Microthrix parvicella' and *Rubrobacter radiotolerans*, which originate from New Mexico, Australia, and Japan, respectively. It is also interesting that these uncultured taxa originate from habitats of very different physico-chemical composition, such as forested soil, paddy fields, soybean field, geothermally heated soil, and peat, while the cultured strains were isolated from a bioleach environment and from activated sludge. The properties of the environments from which these taxa and their cultured relatives originate have little in common with respect to the matrix composition. However, five of the environments – forested soils from Australia and Finland, peat, the geothermally heated soil from New Zealand, and the bioleach sample – are moderately to highly acidic habitats (Liesack & Stackebrandt, 1992a; Rainey *et al.*, 1993), while rice paddy fields, soybean field and sewage sludge tend to be in the neutral to alkaline pH range (Neue, 1993).

In this peat bog study, of the 259 16S rDNA clones investigated by dot-blot hybridization, 37 clones (15% of the clones investigated) reacted with the Gram-positive probe. Of these, at least 26 (those for which 16S rDNA sequences were determined) are monophyletically related to either the *Acidimicrobium ferrooxidans* or *Rubrobacter radiotolerans* lineages, representing at least 10% of the total 16S rDNA clones investigated. These values are comparable to the percentage of members of this uncultured taxon detected in the paddy field study (15 clones out of 64, 23%), Finnish forest soil (2 clones out of 11, 18%), the soybean field study (3 clones out of 20, 15%), and the marine environments (9 clones out of 61, 15%), while those from Australian soil were less abundant (8 out of 113, 7%). The percentage of clones belonging to this uncultured taxon detected in the geothermally heated soil sample from New Zealand was low, i.e. 1% (2 out of 165 clones). This habitat has a similar pH to the peat environment, but its extreme temperature conditions (75 °C) makes it less comparable to the other soil samples.

Previous concerns have been raised with respect to the influence of biases in the current methodologies available

Table 3. Experimental procedures used in the generation of 16S rDNA clone libraries from environmental samples from which TM cluster sequences have been recovered

Habitat	Cell lysis procedure	PCR primers	DNA polymerase	Cloning system/method	No. of clones analysed or reported	Percentage of clones investigated found to be related to the TM clone clusters I, II and III*	Reference
Peat bog (Germany)	Enzymic	27f-1385r	<i>Ultma</i>	pCR-Script, blunt-end	259	15	This study
Soybean field (Japan)	Freeze-thaw, enzymic	1114f-1385r	Not indicated	pT7 Blue T, A-T ligation	20	15	Ueda <i>et al.</i> (1995)
Forest soil (Finland)	Not indicated	66f-343r	Not indicated	pGEM-T, not indicated	11	18	Saano <i>et al.</i> (1995)
Forest soil (Australia)	Bead beater, enzymic	27f-1224r	Perkin-Elmer	M13, restriction site ligation	113	7	Liesack & Stackebrandt (1992a)
Geothermal soil (New Zealand)	Sonication, enzymic	536f-1492r	Boehringer	Blue Script, restriction site ligation	165	1	Rainey <i>et al.</i> (1993)
Paddy field soil	Not indicated	Not indicated	Not indicated	Not indicated	64	23	Maidak <i>et al.</i> (1994)
Marine environment (Atlantic and Pacific oceans)	Boiling in 1% SDS	536f-1385r	Perkin-Elmer	M13, restriction site ligation	61	15	Fuhrman <i>et al.</i> (1993)

* Clusters as defined in Fig. 2.

for molecular ecological studies and the inability of the molecular approach to estimate the degree of microbial diversity (Reysenbach *et al.*, 1992; Farrelly *et al.*, 1995; Kopzynski *et al.*, 1994; Rainey *et al.*, 1996). Although these biases undoubtedly influence the taxon composition of the clone libraries obtained, it is noteworthy that the uncultured taxa of the *Acidimicrobium* and *Rubrobacter* lines of descent were found in the different clone libraries even though significantly different experimental procedures were employed (Table 3).

The information in Table 3 regarding the primers used and their corresponding target sites raises the problem of the lack of comparability between uncultured taxa. In the case of the soybean and the Finnish soil studies, the regions sequenced are totally different, with no overlap available for phylogenetic analysis. It is impossible to compare the sequence information obtained from these two environments, and the affiliation of their sequences to a known taxon can only be evaluated by separate analysis against a reference sequence that covers both regions. As pointed out recently in the literature (Stackebrandt & Rainey, 1995; Ueda *et al.*, 1995), the region amplified by the primer pair should be comparable with the majority of reference sequences and uncultured taxon sequences currently available. In order to be able to carry out more precise phylogenetic analysis, especially the determination of the branching points of novel lineages, we recommend the amplification of the almost complete 16S rDNA primary structure.

Little can be said about the possible taxonomic structure of groups I, II and III. Any relationship at the species and genus level can be excluded for any member of the three taxa and the two cultured relatives. Considering the current taxonomic structure of the order *Actinomycetales* (Embley & Stackebrandt, 1994), even a relationship at the family level seems unlikely. Although the 16S rDNA clone sequences of the uncultured organisms fall into three phylogenetic groups, showing detailed internal structure, it is unwise to speculate on their taxonomic rank. The fact that the 16S rDNA sequences of the three groups form phylogenetically coherent clusters does not imply that the organisms from which the sequences were derived will be phenotypically or physiologically similar. For example the phylogenetic depth of group II is as large as that found between the anaerobic rod-shaped bifidobacteria and the aerobic mycelium-forming streptoporangia (Fig. 2). In addition, as long as only a single or a few strains are described for *Acidimicrobium ferrooxidans* and *Rubrobacter radiotolerans*, nothing can be said about the range of physiological properties of these taxa. The shallower phylogenetic structure of groups I and III could be indicative of a more coherent phenotype for its members. Having identified this group of organisms by phylogenetic analysis, the emphasis should now be on the detection, isolation and characterization of strains, with the aim of elucidating their function in the ecosystem.

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REFERENCES

- Blackall, L. L., Seviour, E. M., Cunningham, M. A., Seviour, R. J. & Hugenholtz, P. (1994). '*Microthrix parvicella*' is a novel, deep branching member of the *Actinomyces* subphylum. *Syst Appl Microbiol* **17**, 513–518.
- Blackall, L. L., Stratton, H., Bradford, D., Del Dot, T., Sjörup, C., Seviour, E. M. & Seviour, R. J. (1996). '*Candidatus* *Microthrix parvicella*', a filamentous bacterium from activated sludge sewage treatment plants. *Int J Syst Bacteriol* **46**, 344–346.
- Brierley, J. A. (1978). Thermophilic iron-oxidizing bacteria found in copper leaching dumps. *Appl Environ Microbiol* **36**, 523–525.
- Cabot, E. L. & Beckenbach, A. T. (1989). Simultaneous editing of multiple nucleic acid and protein sequences with ESEE. *Comput Appl Biosci* **5**, 233–234.
- Clark, D. A. & Norris, P. R. (1996). *Acidimicrobium ferrooxidans* gen. nov., sp. nov.: mixed culture ferrous iron oxidation with *Sulfo- bacillus* species. *Microbiology* **142**, 785–790.
- DeSoete, G. (1983). A least squares algorithm for fitting additive trees to proximity data. *Psychometrika* **48**, 621–626.
- Embley, T. M. & Stackebrandt, E. (1994). The molecular phylogeny and systematics of the Actinomycetes. *Annu Rev Microbiol* **48**, 257–289.
- Farrelly, V., Rainey, F. A. & Stackebrandt, E. (1995). Effect of genome size and *rrn* gene copy number on PCR amplification of 16S rRNA genes from a mixture of bacterial species. *Appl Environ Microbiol* **61**, 2798–2801.
- Felsenstein, J. (1993). PHYLIP (Phylogeny Inference Package), version 3.5.1. Seattle: Department of Genetics, University of Washington.
- Fuhrman, J. A., McCallum, K. & Davis, A. A. (1993). Phylogenetic diversity of subsurface marine microbial communities from the Atlantic and Pacific oceans. *Appl Environ Microbiol* **59**, 1294–1302.
- Hiraishi, A., Kishimoto, N., Kosako, Y., Wakao, N. & Tano, T. (1995). Phylogenetic position of the menaquinone-containing acidophilic chemo-organotroph *Acidobacterium capsulatum*. *FEMS Microbiol Lett* **132**, 91–94.
- Holben, W. E., Jansson, J. K., Chelm, B. K. & Tiedje, J. M. (1988). DNA probe method for the detection of specific microorganisms in the soil bacterial community. *Appl Environ Microbiol* **54**, 703–711.
- Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.
- Kishimoto, N., Kosako, Y. & Tano, T. (1991). *Acidobacterium capsulatum* gen. nov., spec. nov.: an acidophilic chemoorganotrophic bacterium containing menaquinones from acidic mineral environment. *Curr Microbiol* **22**, 1–7.
- Kopzynski, E. D., Bateson, M. M. & Ward, D. M. (1994). Recognition of chimeric small-subunit ribosomal DNAs composed of genes from uncultivated microorganisms. *Appl Environ Microbiol* **60**, 746–748.
- Lane, D. J. (1991). 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*, pp. 115–175. Edited by E. Stackebrandt & M. Goodfellow. Chichester: Wiley.
- Lane, D. J., Harrison, A. P., Jr, Stahl, D., Pace, B., Giovannoni, S. J., Olsen, G. J. & Pace, N. R. (1992). Evolutionary relationships among sulfur- and iron-oxidizing eubacteria. *J Bacteriol* **174**, 269–278.
- Liesack, W. & Stackebrandt, E. (1992a). Occurrence of novel groups of the domain Bacteria as revealed by analysis of genetic material isolated from an Australian terrestrial environment. *J Bacteriol* **174**, 5072–5078.
- Liesack, W. & Stackebrandt, E. (1992b). Unculturable microbes

detected by molecular sequences and probes. *Biodivers Conserv* **1**, 250–262.

Maidak, B. L., Larsen, N., McCaughey, M. J., Overbeck, R., Olsen, G. J., Fogel, K., Blandy, J. & Woese, C. R. (1994). The Ribosomal Database Project. *Nucleic Acids Res* **22**, 3483–3487.

Neue, H.-U. (1993). Methane emission from rice fields. *BioScience* **43**, 466–473.

Ogram, A., Saylor, G. S. & Barkay, T. (1987). The extraction and purification of microbial DNA from sediments. *J Microbiol Methods* **7**, 57–66.

Rainey, F. A., Ward, N. L. & Stackebrandt, E. (1993). Molecular ecology study of a New Zealand acidothermal soil. In *Thermophiles '93, Hamilton, New Zealand*, Abstract A7. Hamilton: University of Waikato.

Rainey, F. A., Ward-Rainey, N. L., Janssen, P. H., Hippe, H. & Stackebrandt, E. (1996). *Clostridium paradoxum* DSM 7308^T contains multiple 16S rRNA genes with heterogeneous intervening sequences. *Microbiology* **142**, 2087–2095.

Reysenbach, A., Giver, L. J., Wickham, G. S. & Pace, N. R. (1992). Differential amplification of 16S rRNA genes by the polymerase chain reaction. *Appl Environ Microbiol* **58**, 3417–3418.

Saano, A., Lindström, K. & Van Elsas, J. D. (1995). Eubacterial diversity in Finnish forest soil. In *7th International Symposium on*

Microbial Ecology, Santos, Brazil, Abstract P1-3.9. Santos: Brazilian Society for Microbiology.

Stackebrandt, E. & Liesack, W. (1993). Nucleic acids and classification. In *Handbook of New Bacterial Systematics*, pp. 151–194. Edited by M. Goodfellow & A. O'Donnell. New York: Academic Press.

Stackebrandt, E. & Rainey, F. A. (1995). Partial and complete 16S rDNA sequences, their use in generation of 16S rDNA phylogenetic trees and their implications in molecular ecological studies. In *Molecular Microbial Ecology Manual*, pp. 3.1.1: 1–17. Edited by A. D. L. Akkermans, J. D. van Elsas & F. J. de Bruijn. Dordrecht: Kluwer.

Stackebrandt, E., Liesack, W. & Goebel, B. M. (1993). Bacterial diversity in a soil sample from a subtropical Australian environment as determined by 16S rDNA analysis. *FASEB* **7**, 232–236.

Ueda, T., Suga, Y. & Matsuguchi, T. (1995). Molecular phylogenetic analysis of a soil microbial community in a soybean field. *Eur J Soil Sci* **46**, 415–421.

von Wintzingerode, F. (1994). *Phylogenetische und chemotaxonomische Hinweise auf die Anwesenheit neuer Bakterienarten in einer Torflagerstätte in Ost-Niedersachsen*. Diploma thesis, Braunschweig Technical University, Germany.

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