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Application of nested polymerase chain reaction for the detection of as yet uncultured organisms of the class *Actinobacteria* in environmental samples

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Summary

Different groups of as yet uncultured, phylogenetically deeply rooting organisms of the class *Actinobacteria* have been shown previously by analysis of 16S rDNA clone libraries to be present in different soil and marine environments of worldwide distribution. In order to monitor specifically the occurrence of two of the actinobacterial groups among bacterial DNA extracted from different environments, a nested polymerase chain reaction (PCR) method, aiming at the sensitive detection of 16S rDNA, was used. Analysis of a clone library generated from one of these 16S rDNA-derived, group-specific PCR products of DNA from a forest soil broadened the spectrum of putative taxa within the two phylogenetic groups. Application of this PCR system demonstrated the presence of these organisms in natural environments and in a sample of dried and moistened peat. Fluctuations in the detectability of the target groups in different samples from the same site strongly suggest that the taxon diversity of a 16S rDNA clone library must be regarded as a temporal genomic snapshot taken from this environmental community.

Introduction

Diversity studies of microbial ecosystems by the use of molecular techniques have a well-established place in microbiology (Pace, 1997). Different environments have been investigated by the use of 16S rRNA/rDNA clone libraries to assess the phylogenetic diversity of prokaryotic taxa broadly. In the course of several other environmental 16S rDNA-based studies, a number of deeply clustering clone sequences, originating from organisms of the class *Actinobacteria* (Stackebrandt *et al*., 1997), have been found in different terrestrial and marine environments. They formed fractions of 1–23% of the respective clone libraries (Rheims *et al*., 1996). A member of one of the groups constituted about 6% of the metabolically active part of a Dutch grassland soil community, as shown by ribosomal RNA analysis (Rheims *et al*., 1998). It can be deduced that these groups of organisms play an important role in the soil microbial community of the environments in which they have been detected, but attempts at their cultivation have failed as yet (Rheims *et al*., 1998). This failure is not unexpected, as novel strains have been successfully isolated and described in only a few cases after their presence was predicted by molecular analysis (Huber *et al*., 1998).

In the present study, the occurrence of two of the actinobacterial groups was specifically monitored in different environments. Application of nested PCR, such as the system used for the detection of ammonia-oxidizing bacteria in different environmental samples by Hiorns *et al.* (1995), seems to indicate temporal fluctuations of the population in natural environments and in an artificial peat system. Analysis of a clone library generated from 16S rDNA-derived, group-specific PCR products of DNA from a forest soil broadened the spectrum of putative taxa within the two phylogenetic groups.

Results and discussion

Development and optimization of a nested PCR system

In order to assess 16S rDNA PCR products and clone libraries rapidly for the detection of 16S rDNA sequences of members of phylogenetically coherent clusters (Rheims *et al*., 1996), a nested PCR system was developed. Analysis of the database of 16S rDNA clone sequences of TM groups II and III (TM clones were derived from the peat bog Großes Moor near Gifhorn, Germany; Rheims *et al*., 1996) of hitherto uncultured taxa and relatives of the class *Actinobacteria* revealed the presence of two nucleotide stretches of potential group specificity (positions 126–143 and 405–424; Table 1). Both primers were found to be highly selective according to the CHECK_PROBE program of RDP (Maidak *et al*., 1997). After a first PCR with general bacterial primers, the nested PCR consisted

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Table 1. Correspondence of the primers Abact126f and Abact405f to clones of the actinobacterial groups II and III.

Clone	Member of TM clone group	Abact126f	Abact405f
TM56	II	100%[a]	One mismatch, position 15[b]
TM62	II	One mismatch, position 9	Two mismatches, positions 3, 12
TM81	II	100%	100%
TM208	II	One mismatch, position 9	100%
TM210	II	100%	100%
TM213	II	100%	One mismatch, position 15
TM214	II	100%	100%
TM226	II	Two mismatches, positions 9, 15	Four mismatches, positions 3, 8, 15, 19
TM232	II	100%	100%
DA079	II	100%	100%
TM112	III	100%	100%
TM167	III	100%	100%
TM177	III	100%	100%
TM187	III	100%	One mismatch, position 8
TM262	III	100%	100%

a. Primer completely matches the target sequence.
b. Number of primer mismatches and their relative position in the primer (5′ to 3′ primer direction).

of the general bacterial primer 1100r in combination with one of the two specific primers. Optimization of the PCR conditions was performed with PCR products of TM clones of groups II and III. Under any of the PCR conditions employed (not shown), primer Abact126f resulted in a better yield of PCR product than primer Abact405f, and was therefore chosen. When the former set of primers was applied directly to environmental DNA samples, no amplification products were visualized on ethidium bromide-stained agarose gels. Therefore, the nested PCR approach was used to increase the sensitivity of the assay.

A possible source for the detection of non-habitat-specific 16S rDNA sequence diversity in clone libraries was described recently as resulting from the introduction of sequences through contaminated reagents (Tanner *et al*., 1998). In the present study, control reactions consisting of a PCR assay containing two universal 16S rDNA primers but omitting template DNA did not result in the formation of a PCR product. A subsequent nested PCR assay was also negative. Owing to the sensitivity of the nested PCR approach in detecting even low copy target sequences, this result suggests that the group-specific products obtained with soil DNA did in fact derive from the investigated samples.

Investigation of a forest soil (WB) clone library

In order to verify that the nested PCR primers exclusively generated PCR fragments of the target groups II and III, a product obtained from DNA of a forest soil (designated WB clones) was cloned. Subsequent analysis of the library was performed by restriction digestion and sequencing of about 10% of the clones obtained.

Restriction analysis

For an initial and rapid verification of the membership of specific PCR inserts, an approach was chosen that detected the presence of a restriction site common among members of groups II and III but absent or located at different positions in unrelated taxa. A restriction enzyme that matches these criteria is *Dra*III. This enzyme cleaves at position 829 of the 16S rDNA insert (*Escherichia coli* nomenclature; Brosius *et al*., 1978) of group II and III TM clones. As the size of the 16S rDNA-containing fragment is defined by the position of the M13 priming sites, it consisted of about 1200 nucleotides. Restriction digestion of this fragment of 33 clones resulted in the formation of two expected fragments of about 790 bp and 410 bp in 30 of the clones (data not shown). Clone WB010 showed two bands that differed from the expected size, although the position of the restriction site was verified by sequence analysis, while the DNA of two clones, WB002 and WB018, was not digested.

Sequence analysis

Membership of cloned fragments to groups II and III was further verified by sequence analysis of 39 cloned nested PCR products. Except for two clones, all sequences belonged to the actinobacterial groups II or III. These two exceptions were those that showed a deviating restriction pattern.

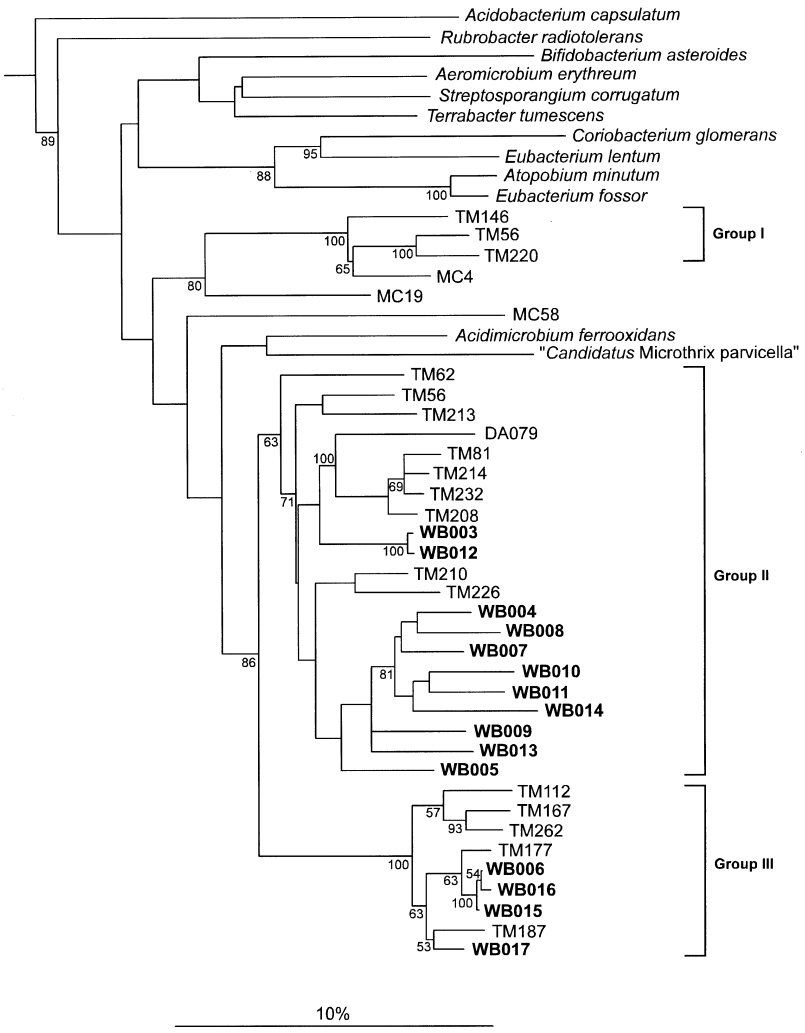

Fig. 1. Phylogenetic dendrogram showing the position of the fully sequenced WB clone inserts within groups II and III of the actinobacterial TM clones. The 16S rRNA sequence of *Bacillus subtilis* served as an outgroup sequence to root the dendrogram. The scale bar indicates 10 nucleotide substitutions per 100 bases. Numbers at the branches indicate the proportional occurrence of the respective nodes in a bootstrap analysis of 500 resamplings. Only values above 50% are shown. On the righthand side, the membership of clones to the previously designated groups I to III is shown. The origin of the clone sequences designated MC, TM and DA is forest soil (Stackebrandt *et al*., 1993), peat bog (Rheims *et al*., 1996) and grassland soil (Felske *et al*., 1997) respectively.

One of these (WB018; sequence not deposited) was unambiguously identified to be a chimerical artifact (CHECK_CHIMERA, RDP; Maidak *et al*., 1997), while the other (WB002) was moderately related to *Actinomadura madurae* (92.7% sequence similarity over the stretch of 966 nucleotides). Whether or not this PCR product is a chimeric structure could not be answered unambiguously by any of the tests used (e.g. chimera, secondary structure or signature nucleotide analyses). Characterization of WB002 as a representative of a new taxonomic lineage could only be achieved if other related RNA sequences or isolates were found in independent studies.

The phylogenetic relationship of the almost completely sequenced clones WB003 to WB017 to previously described clones of clusters II and III (Rheims *et al*., 1996; 1998; Felske *et al*., 1997) is indicated in Fig. 1. The phylogenetic position of the additional 22 partially sequenced clones WB019 to WB040 was also found to be within groups II and III (data not shown).

Inclusion of the fully sequenced WB clones in groups II and III previously defined by TM clone sequences extends the phylogenetic width of the two groups (Fig. 1). The phylogenetic depth of several of the closely related sequence subgroups is as shallow as that of many established actinobacterial genera (e.g. 94–97%), which points towards a rich taxonomic structure of the as yet uncultured organisms. The primer Abact126f was designed to detect a rather narrow spectrum of actinobacterial relatives of the TM clone II and III sequences (Table 1). Indeed, none of the sequences falls outside the radiation of these two groups, which supports the specificity of the forward primer. From an ecological point of view, it is interesting to note that the WB clones are not distributed evenly among the TM clones but concentrate predominantly in one subgroup of group II. In a few cases only, TM and WB clone sequences are highly similar (group III), while the majority of sequences originating from either library form individual sublines and subgroups (defined by similarity values of >96%) that

Table 2. Examination of soil samples from different locations for the occurrence of the actinobacterial clone groups II and III.

Origin of the samples	Time of sampling	pH value of samples	PCR product[a]	Nested PCR product
Peat bog, Germany	June 1996	4.0	++	++
	April 1997	3.9	++	–
Agricultural soil, Germany	January 1997	6.4	++	+
Grassland soil, Germany	January 1997	7.2	++	–
	October 1997	7.8	++	++
Forest soil, Germany	January 1997	4.0	++	++
	April 1997	3.6	++	–
	June 1997	3.7	++	++
	July 1997	4.0	++	+
	October 1997	3.9	++	++
	January 1998	4.2	++	–
	August 1998	4.6	++	++
Garden soil A, Germany	January 1997	6.3	++	+
	October 1997	5.4	++	+
Garden soil B, Germany	July 1997	7.7–8.3[b]	++	++
Garden soil C, Mexico	April 1998	6.9	++	+
Beach sand, Denmark	September 1997	7.9	++	++
Rainforest, Borneo	Summer 1997	4.4	++	–
Activated sludge, Austria	November 1997	6.8	++	–
Leaves, Germany	January 1998	ND	++	++

a. Obtained with universal primers; ++, strong signal; +, weak signal; –, no signal, as judged by agarose gel electrophoresis.
b. Values determined within a depth profile from 0 to 80 cm.
ND, not determined.

are well separated from each other (< 95%). This finding leads us to suggest that each habitat selects for moderately to highly related individual taxa, which are often more closely related to taxa of the other environment than they are related among themselves.

Detection of the target groups in different terrestrial samples

Members of the targeted clone groups II and III were detectable using the nested PCR system in samples taken from peat bog (Gifhorn, Germany), forest soil (Braunschweig), garden soil (Wolfenbüttel, Germany; designated garden soil B), beach sand (Vejers, Denmark), grassland soil (Braunschweig) and fallen beech leaves (Braunschweig) in different months of 1996–98 (Table 2). In garden soil B, strong PCR signals were detected throughout a profile from the top down to a depth of 80 cm in samples taken every 20 cm. Weaker signals were obtained with DNA from an agricultural soil (Braunschweig) and two different garden soils (Braunschweig, designated garden soil A; Mexico City, Mexico, designated garden soil C). No signals were obtained from samples taken from a rainforest soil (Borneo) and an activated sludge (Salzburg, Austria). In order to exclude the occurrence of false-negative results because of the relatively small amount of sample used (0.4 g), analysis of negatively tested environments was repeated with DNA extracted from new subsamples. In no case was it possible to detect the target organisms in these samples.

Detailed chemical analyses of samples allowing the recognition of a putative common denominator for the occurrence of the target taxa are not available. These organisms occur in different environments, such as a mainly mineral beach sand, forest soil and litter, and peat with pH values ranging between 3.5 and 8.3 (Table 2). The range from acidic to even slightly basic pH values provides a broad spectrum of growth conditions, which are not usually found to be represented in members of a single prokaryotic taxon. Most probably, however, a terrestrial sample of about 50 g should not be considered a homogeneous environmental niche (Paul and Clark, 1989). Possibly, occurring microniches with very defined pH values and other growth-determining gradients would have been destroyed through the sampling process.

Occasionally, the molecular detection of members of the two actinobacterial groups failed in samples taken in different months and seasons from the same habitat. In samples taken from different sites within the same month, e.g. January, signals were detected in some, but not all, samples (Table 2). A similar effect was observed when the target group was monitored in an artificial peat system. While bacterial 16S rDNA products were detected daily over a period of 4 weeks, target nested PCR products were only obtained from day 1 to day 14, with a maximum signal strength from days 6–10 (Fig. 2). After the first 4 week experiment, an additional cycle of drying and moistening of the same peat sample resulted in the reappearance of the specific signal (not shown). Several factors, none of which could be investigated more closely, may influence the dynamic of occurrence of the target strains, such as periodic release of nutrients from the organic matter, grazing by lower eukaryotes or changes in the structure of the cell wall of the target organisms. The applied

nested PCR method should be sensitive enough to detect even low amounts of target DNA if nucleic acids are released from cells. However, under the mild enzymatic lysis procedure used, cell lysis and subsequent release of amplifiable amounts of DNA may fail with cells that have undergone significant changes in morphology, size and structure of the cell wall. Such changes are known to occur in many terrestrial prokaryotic species, e.g. bacilli, and actinomycetes during sporulation, but whether these modifications occur in the target strains is not known.

Application of the nested PCR technique allowed rapid and specific monitoring of DNA extracts from different habitats for the occurrence of two phylogenetic groups of as yet uncultured organisms. The combination of the nested PCR approach and characterization of cloned products by restriction with a single enzyme enabled their unambiguous affiliation to the target groups and demonstrated their occurrence in environments in which these groups had not been reported previously. This methodology should be transferable to any other bacterial DNA extract, offering the possibility of a specific and relatively fast screening for the occurrence of taxa of interest.

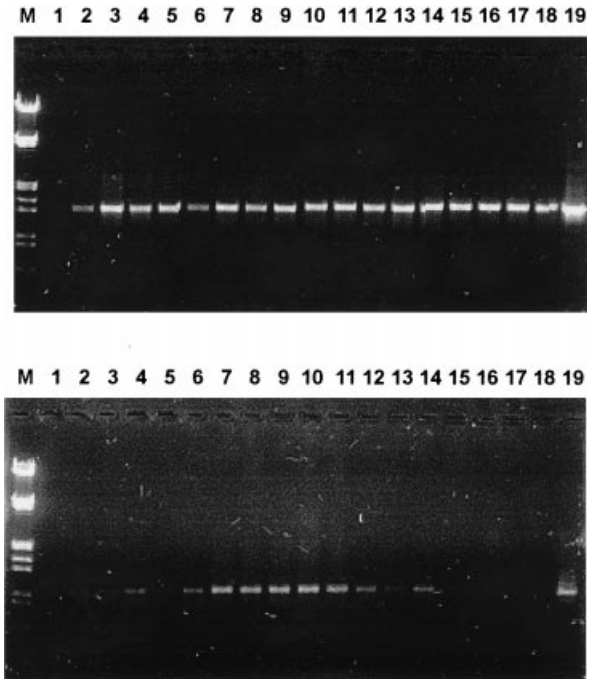

Fig. 2. Ethidium bromide-stained 1% (w/v) agarose gels containing amplified DNA fragments from PCR/nested PCR carried out from DNA obtained from an artificial peat system. Bacterial PCR products of about 1.4 kb are shown on the upper gel, specific nested PCR products derived from these (about 960 bp) on the lower gel. The lane designated M contains the length standard (Boehringer molecular weight marker III; Boehringer Mannheim). Lanes 1–18 contain the products from day 0 (sample taken immediately after moistening of the peat) to day 17 respectively. Lane 19 contains the PCR positive control (upper gel, *Escherichia coli* 16S rDNA; lower gel, specific product from clone TM262).

Experimental procedures

Sampling sites

Samples of about 50 g were taken from the top layer of different soils. Forest soil samples were taken from a beech forest soil near Braunschweig, Germany. Samples used for the generation of the WB (= Waldboden, forest soil) clone library were taken in the autumn. Further samples were taken from peat bog (Gifhorn, Germany), garden soil (Braunschweig; Wolfenbüttel, Germany; Mexico City, Mexico), beach sand (Vejers, Denmark), agricultural soil (Braunschweig), grassland soil (Braunschweig), rainforest soil (Borneo), activated sludge (Salzburg, Austria) and fallen beech leaves (Braunschweig) at different times (see Table 2 for details).

Extraction of genomic DNA and bacteria-specific PCR

Subsamples of 0.4 g of the investigated soils were lysed enzymatically according to a modification of a protocol described previously (Rheims *et al*., 1996). An equal volume of acid-washed PVPP (Sigma-Aldrich) and 1300 μl of saline–EDTA buffer (Rainey *et al*., 1996) were added to the sample. After the addition of 20 μl of lysozyme (10 mg ml^{-1}) and incubation at 37°C for 30 min, 20 μl of 1% (w/v) proteinase K and 40 μl of 25% (w/v) SDS were added, and samples were incubated at 65°C for 45 min. Purification of the genomic DNA was carried out as described by Rainey *et al.* (1996). DNA solutions that showed a brownish colour were treated with an equal volume of PVPP and 350 μl of saline–EDTA and placed at 65°C for 30 min. The supernatant was recovered by centrifugation. Purification and PCR-mediated amplification of the 16S rDNA were carried out as described by Rainey *et al.* (1996), using the primer set 27f (5′-GAGTTTGATCCTGGCTCAG-3′; Lane, 1991) and 1385r (5′-CGGTGTGT[A/G]CAAGGCCC-3′; Stackebrandt and Liesack, 1993). PCR products were purified using a QIAquick PCR purification kit (Qiagen).

The complete extraction protocol was also performed without the addition of soil to confirm the absence of PCR products originating from possible contamination of the reagents applied. Although no PCR products could be visualized on ethidium bromide-stained agarose gels, the purified reactions were also subjected to nested PCR.

Preparation and investigation of an artificial peat system

A peat sample was air dried (dry weight 85 g) in a Petri dish for 6 weeks. After moistening with 300 ml of deionized water, the dish was stored at room temperature for 4 weeks. Samples of 1 ml of the aqueous supernatant were taken daily, centrifuged at 15 000 × *g* for 5 min, and the pellet dissolved in 400 μl of saline–EDTA and subjected to enzymatic lysis (Rainey *et al*., 1996). Purified DNA suspension (2 μl) served as the template for PCR amplification of 16S rDNA

as indicated above. The detection of group II and III specific signals was carried out with the primer pair Abact126f and 1100r as described below.

Primers and PCR conditions used for the specific detection of members of clone groups II and III

Nested PCR reactions were set up with 1 μl of purified 16S rDNA PCR product. The specific nested primers were derived from TM clones obtained from a previous environmental study (Rheims *et al*., 1996). Use of the specific primer Abact126f, 5′-AGCAACCTGCCCCGAAGA-3′ (binding from nucleotide position 126–143, *Escherichia coli* numbering system; Brosius *et al*., 1978) or Abact405f, 5′-TGAGGGACGAAGG-CTTTCTG-3′ (positions 405–424) in combination with the general bacterial primer 1100r 5′-GGGTTGCGCTCGT-TGC-3′ (positions 1099–1114; Lane, 1991), resulted in PCR products of about 960 bp and 680 bp in length respectively. The specificity of the primers was confirmed by the CHECK_PROBE program of the RDP (Maidak *et al*., 1997). The actinobacterial clones of groups II and III and the degree of match with the specific primers are indicated in Table 1.

The reactions were carried out in a Perkin-Elmer model PE9600 DNA thermal cycler under the following conditions: primary denaturation for 4 min at 94°C was followed by a two-step PCR of 40 cycles of 2 min at 74°C and 30 s at 93°C. The final extension step consisted of 5 min at 74°C. The PCR product of clone TM262 served as a positive control in the nested PCR reactions.

Cloning of the specific nested PCR product

Three nested PCR reactions were set up from a PCR product obtained from forest soil. Products were purified, combined, and 2 μl of this solution was used for ligation into the pGEM-T vector system (Promega), performed as recommended by the manufacturer. Transformation was carried out with JM109 high-efficiency competent cells (Promega). Of about 400 transformants, 74% showed a white colour after incubation. Cells were picked, grown overnight and plasmid DNA was prepared from 70 of the white transformants by boiling an inoculation loop of cell material in 100 μl of water for 10 min, followed by centrifugation at 15 000 × *g* for 1 min. The supernatant (1 μl) was used for PCR amplification of the insert with the M13 primer set according to a previously described protocol (Rheims *et al*., 1996). The correct insert size was verified by electrophoresis in 1% (w/v) agarose gels stained with ethidium bromide.

Restriction digestion of the WB clones

The 16S rDNA nucleotide sequences of the TM peat clones (Rheims *et al*., 1996) were subjected to a computational restriction enzyme digest using the program DIGEST (Nakisa, 1992) to find rarely cutting enzymes potentially useful for fast molecular characterization of the nested PCR clones. The enzyme *Dra*III was found to be suitable, as its recognition site (5′ CACNNN′GUG 3′) was present from position 824–832 in all members of the clone groups II and III, resulting in two fragments of about 680 bp and 280 bp in length. For analysis of the WB clones, restriction digestions of amplified PCR inserts using *Dra*III were set up (2 h at 37°C) according to the manufacturer's instructions (New England Biolabs). The cleaved fragments were visualized by electrophoresis in 2% (w/v) agarose gels stained with ethidium bromide.

Determination of the WB clone nucleotide sequences and test for the occurrence of chimerical artifacts

Sequencing reactions were performed with the ABI Prism dye terminator cycle sequencing ready reaction kit (Perkin-Elmer) and electrophoresed using an automated sequence analyser (model 373A; Applied Biosystems). The length of the analysed stretch of 17 of the fully sequenced clones was about 960 nucleotides. A shorter stretch comprising about 350 nucleotides was analysed for an additional 22 clones. The sequences were subjected to the CHECK_CHIMERA program of the RDP (Maidak *et al*., 1997) for the detection of chimerical PCR artifacts.

Determination of the phylogenetic branching order of the forest soil clone sequences

The 16S rDNA WB clone sequences were compared with the actinobacterial peat bog clone groups I to III (as defined by Rheims *et al*., 1996) and sequences from related clones and cultivated organisms from the class *Actinobacteria* (Stackebrandt *et al*., 1997) using the alignment editor ae2 (Maidak *et al*., 1997). Sequences were obtained from the EMBL (Stoesser *et al*., 1998) and RDP (Maidak *et al*., 1997) nucleotide sequence databases. The neighbour joining method contained in the PHYLIP package (Felsenstein, 1993) was used in the construction of phylogenetic dendrograms. Bootstrap analysis was performed to evaluate the tree topology of the neighbour-joining data by calculating 500 resamplings (Felsenstein, 1985).

Nucleotide sequences

The sequence data of the forest soil clones WB002 to WB017 have been submitted to the EMBL database under accession numbers AJ232680 to AJ232695; and those of clones WB019 to WB040 under accession numbers AJ232696 to AJ232717.

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