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Analysis of Actinomycete Communities by Specific Amplification of Genes Encoding 16S rRNA and Gel-Electrophoretic Separation in Denaturing Gradients

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A group-specific primer, F243 (positions 226 to 243, *Escherichia coli* numbering), was developed by comparison of sequences of genes encoding 16S rRNA (16S rDNA) for the detection of actinomycetes in the environment with PCR and temperature or denaturing gradient gel electrophoresis (TGGE or DGGE, respectively). The specificity of the forward primer in combination with different reverse ones was tested with genomic DNA from a variety of bacterial strains. Most actinomycetes investigated could be separated by TGGE and DGGE, with both techniques giving similar results. Two strategies were employed to study natural microbial communities. First, we used the selective amplification of actinomycete sequences (*E. coli* positions 226 to 528) for direct analysis of the products in denaturing gradients. Second, a nested PCR providing actinomycete-specific fragments (*E. coli* positions 226 to 1401) was used which served as template for a PCR when conserved primers were used. The products (*E. coli* positions 968 to 1401) of this indirect approach were then separated by use of gradient gels. Both approaches allowed detection of actinomycete communities in soil. The second strategy allowed the estimation of the relative abundance of actinomycetes within the bacterial community. Mixtures of PCR-derived 16S rDNA fragments were used as model communities consisting of five actinomycetes and five other bacterial species. Actinomycete products were obtained over a 100-fold dilution range of the actinomycete DNA in the model community by specific PCR; detection of the diluted actinomycete DNA was not possible when conserved primers were used. The methods tested for detection were applied to monitor actinomycete community changes in potato rhizosphere and to investigate actinomycete diversity in different soils.

Actinomycetes, phylogenetically defined as a number of taxa within the high-G+C subdivision of the gram-positive phylum (11), are involved in important processes in a wide range of habitats (57). They are active in the decomposition of organic materials in soil, including lignin and other recalcitrant polymers, and can degrade agricultural and urban wastes (5, 24). Members of the genus *Frankia* fix nitrogen in nodules of nonleguminous plants. Actinomycetes have been used in the biological control of plant pathogens (6, 10, 22, 38), and a few are known as plant pathogens (50). Mycolic acid-containing actinomycetes are involved in filamentous foaming in activated sludge systems (33). Metabolites from actinomycetes continue to be an important source of antibiotics, enzymes, and bioactive products (2, 14). To overcome the severe limitations of culture-dependent methods in discovering bacterial diversity (13, 52–54), molecular biological techniques have become increasingly popular (for references, see reference 26). Actinomycete groups have been detected and characterized by their 16S rRNA sequences in cases where cultivation has proved unsuccessful (30, 32, 35). Molecular tools have a great potential to assist in isolating yet-uncultured bacteria with known rRNA sequences to further investigate or exploit these microorganisms (19, 51).

One of these molecular tools is the PCR amplification of variable regions of the genes encoding 16S rRNA (16S rDNA) by use of primers homologous to conserved regions of the gene. Subsequent electrophoretic separation of the PCR products in a polyacrylamide matrix over a denaturing gradient is a technique recently introduced in microbial ecology by Muyzer et al. (25). The denaturing gradient can be achieved either chemically with urea and formamide in denaturing gradient gel electrophoresis (DGGE) (28) or physically by temperature in temperature gradient gel electrophoresis (TGGE) (36). Both techniques are reported to be interchangeable, giving comparable fingerprints of microbial communities (17). In contrast to patterns from other fingerprinting methods, TGGE and DGGE patterns offer the possibility to analyze bands of interest in depth by sequencing or probing (25, 27, 51). The banding pattern represents the major constituents of the analyzed community (17); if present as minor constituents, groups such as actinomycetes may not be detected by molecular analysis. Thus, relatively less abundant but very important species may not be visible. This study proposes two strategies to overcome this problem and to allow analysis of selected populations within complex microbial communities. A forward primer to selectively amplify 16S rDNA of actinomycetes by PCR was designed and tested in combination with different reverse primers. In a direct approach, TGGE or DGGE was applied to analyze directly the fragments generated from actinomycete-specific PCR. In an indirect approach, specific PCR was used to generate an actinomycete template for a second bacterium-specific PCR to amplify fragments used in gradient gel analysis.

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MATERIALS AND METHODS

Bacteria. A list of the bacterial strains used to investigate the specificity of actinomycete sequence amplification is given in Table 1. Bacteria were grown in

TABLE 1. Organisms investigated in this study

Species	Strain	Group[b]	Abbreviation	Run[c]
Actinomycete bacteria				
Actinomadura viridis	DSM 43462	*Streptosporangineae*	acma	13
Microtetraspora pusilla	JCM 3144	*Streptosporangineae*	acpu	7
Actinomyces sp.	QM B-814[a]	*Actinomycineae*	amsp	ND[d]
Actinoplanes auranticolor	DSM 43031	*Micromonosporineae*	apau	28
Actinoplanes philippinensis	JCM 3001	*Micromonosporineae*	apph	27
Actinosynnema mirum	DSM 43827	*Pseudonocardineae*	asmi	22
Arthrobacter crystallopoietes	DSM 20117	*Micrococcineae*	arcr	3
Brevibacterium flavum	JCM 1308	*Micrococcineae*	brfl	1
Clavibacter michiganense	DSM 20744	*Corynebacterineae*	clmi	4
Dactylosporangium aurantiacum	ATCC 23491	*Micromonosporineae*	daau	26
Geodermatophilus obscurus	ATCC 25078	*Frankineae*	geob	8
Gordona rubropertinctus	DSM 43197	*Corynebacterineae*	goru	6
Kineosporia aurantiaca	JCM 3230	Uncertain	kicu	18
Microbispora rosea	ATCC 21946	*Streptosporangineae*	mbro	11
Micrococcus luteus	DSM 20030	*Micrococcineae*	mclu	5
Micromonospora citrea	NRRL B-16101	*Micromonosporineae*	mmci	27
Micromonospora chalcea	A2894[a]	*Micromonosporineae*	mmch	9
Micromonospora coerulea	A1999[a]	*Micromonosporineae*	mmco	29
Nocardia asteroides	N3[a]	*Corynebacterineae*	noas	6
Nocardiopsis albus	B5389[a]	*Streptosporangineae*	npal	19
Nocardiopsis atra	ATCC 31511	*Streptosporangineae*	npan	24
Cellulomonas turbata	JCM 3160	*Micrococcineae*	oetu	17
Planobispora longispora	ATCC 23867	*Streptosporangineae*	pblo	10
Planomonospora parontospora	ATCC 23864	*Streptosporangineae*	pmpa	11
Promicromonospora citrea	JCM 3051	*Micrococcineae*	prci	20
Rhodococcus rhodochrous	E120[a]	*Corynebacterineae*	rhrh	2
Saccharothrix waywayandensis	NRLL B-16159	*Pseudonocardineae*	sawa	21
Spirillospora albida	JCM 3041	*Streptosporangineae*	spal	14
Sporichthya polymorpha	JCM 3089	*Frankineae*	sppo	15
Streptomyces abikoense	NRRL B-1516	*Streptomycineae*	svab	25
Streptomyces albus	A1893[a]	*Streptomycineae*	smal	17
Streptomyces aureofaciens	DSM 40127	*Streptomycineae*	smau	ND
Streptomyces violaceoruber	NRRL B-16148	*Streptomycineae*	smli	4
Streptosporangium roseum	CBS 31356	*Streptosporangineae*	ssro	12
Streptosporangium album	A0958[a]	*Streptosporangineae*	ssal	23
Terrabacter tumescens	DSM 20308	*Micrococcineae*	tetu	16
Nonactinomycete bacteria				
Agrobacterium tumefaciens	DSM 30205	α-Proteobacteria	at	
Agrobacterium rhizogenes	DSM 30148	α-Proteobacteria	ar	
Sinorhizobium meliloti	DSM 30135	α-Proteobacteria	rm	
Rhizobium leguminosarum	DSM 30132	α-Proteobacteria	rl	
Burkholderia gladioli	DSM 4285	β-Proteobacteria	bg	
Ralstonia solanacearum	DSM 1993	β-Proteobacteria	bs	
Pseudomonas fluorescens	R2f	γ-Proteobacteria	pf	
Pantoea agglomerans	Isolate	γ-Proteobacteria	pa	
Erwinia carotovora subsp. *atroseptica*	DSM 30168	γ-Proteobacteria	ec	
Clostridium pasteurianum	DSM 525	Low G+C	cp	
Pediococcus pentosaceus	DSM 20336	Low G+C	pp	
Carnobacterium gallinarum	DSM 4847	Low G+C	cg	
Lactobacillus plantarum	DSM 20174	Low G+C	lp	

[a] Culture collection at University of Warwick, Biological Sciences, Coventry, United Kingdom.
[b] Actinomycete groups are defined according to suborders proposed by Stackebrandt et al. (46).
[c] Electrophoretic mobility (rank) of 16S fragment 968–1401 in TGGE.
[d] ND, not determined.

shake flasks with nutrient broth for non-high-G+C groups and with tryptone soy broth for actinomycetes.

Environmental samples. Soils with known actinomycete populations were selected for actinomycete diversity studies. Warwick soil samples were taken from the Cryfield site (56). Cuban soil was from black bean rhizosphere samples, and details of these samples can be obtained directly from Carlos Vallin, Centro de Quimica Farmaceutica, Havana, Cuba.

Potato rhizosphere samples were selected to determine whether previously observed differences in the bacterial community structure of transgenic and control plants were due to differences in actinomycete populations. Samples were obtained from roots with adhering soil of greenhouse-grown potato plants (16). The plants were provided by Klaus Düring (BAZ, Quedlinburg, Germany). The genetic modification of the T4 lysozyme-expressing potato plants (DL4) is described by Düring et al. (9). Nontransgenic control plants were derived from the same cell line as DL4.

Template DNA. Genomic DNA of strains was obtained either (i) by treatment of glycerol cultures with phenol-chloroform-isoamylalcohol (25:24:1) at 60°C for 10 min and extraction with Tris-acetate-EDTA buffer, followed by isopropanol and spermine-HCl precipitation (18), or (ii) by sodium dodecyl sulfate (SDS)-proteinase K lysis, selective precipitation of cell debris and polysaccharides with CTAB (cetyltrimethylammonium bromide), and isopropanol precipitation (58).

The selective amplification of actinomycete sequences in the presence of nonactinomycete targets was demonstrated by the use of model communities composed of PCR-derived fragments of almost the complete 16S rDNA from

TABLE 2. Primers used in PCR experiments

Primer[a]	16S rDNA target (positions)[b]	Sequence (5′→3′)
F243	Actinomycetes (226–243)	GGATGAGCCCGCGGCCTA
R513GC	Actinomycetes and other (513–528)	gc.-CGGCCGCGGCTGCTGGCACGTA
F984GC	Bacteria (968–984)	gc.-AACGCGAAGAACCTTAC
R1378	Bacteria (1378–1401)	CGGTGTGTACAAGGCCCGGGAACG
F27	Bacteria (8–27)	AGAGTTTGATC(A/C)TGGCTCAG
R1492	Bacteria (1492–1513)	TACGG(C/T)TACCTTGTTACGACTT
gc.		5′-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG-3′

[a] F, forward primer; R, reverse primer; GC, G+C-rich sequence (gc.) attached at 5′ end.
[b] *E. coli* numbering (1).

five actinomycetes and five other bacterial strains with primers F27 and R1492, each strain being represented by 0.2 ng of rDNA in the first model community. The actinomycetes were diluted 10 and 100 times compared to the nonactinomycetes in two additional model communities.

Microbial DNA from Warwick soil was extracted by the method of Cresswell et al. (7), modified as follows: a soil sample was subjected to 5 min of bead-beating in Crombach buffer (8) with 0.11-mm glass beads in a Braun (Melsungen, Germany) bead beater, lysozyme lysis (5 mg/ml for 1 h at 37°C) followed by SDS lysis (10% [wt/vol] SDS up to a final concentration of 1% for 30 min at 65°C), and centrifugation (3,000 rpm, 10 min). The supernatant was further purified by precipitation to remove protein after the addition of 1/5 volume of 8 M potassium acetate for 15 min on ice. DNA was precipitated overnight at 4°C with 0.5 volume of 50% (wt/vol) polyethylene glycol and 1/10 volume of 5 M NaCl, purified by phenol-chloroform extraction, and precipitated by isopropanol and finally by spermine-HCl (7). Genomic DNA from potato rhizosphere samples was extracted from bacterial pellets derived by repeated stomacher blending and differential centrifugation (procedure modified from that of reference 15) by the method of Smalla et al. (44).

Primers. Primers for PCR were designed to be specific for actinomycete or bacterial 16S rDNA targets. 16S rRNA sequences of the Ribosomal Database Project (RDP) database (23) were imported into VSM program version 4.0 (Richard Christen, CNRS and Université Paris 6, Villefranche-sur-mer, France) if not already present in the VSM database. For a number of bacterial taxa, one sequence of each species was selected from the database, but for broader groups (more than five genera), up to five sequences per genus were selected. Sequences of 30, 60, and 90% consensus were constructed from each group. The alignment of the consensus sequences revealed different regions with various levels of conservation. A region was used as the 3′ end of a group-specific primer which is conserved for the high-G+C subdivision and whose sequence is different from the sequences of other bacterial taxa. Primers were optimized with Oligo program version 4.0 (39). A GC-rich sequence is attached to one primer of a pair; therefore, the PCR products have a GC clamp to prevent complete melting during separation in the denaturing gradient (28). Primers used in this study are shown in Table 2. Primer F243 was designed for specific amplification of actinomycete 16S rDNA templates in combination with reverse primer R513GC or R1378. Primer R513GC anneals to a universally conserved domain (59), but due to a base A at the 3′ end, it preferentially anneals to target sequences in gram-positive bacteria and β-proteobacteria. Primer F984GC (31) in combination with primer R1378 amplifies the bacterial 16S rDNA fragment at positions 968 to 1401 (fragment 968–1401; *E. coli* numbering [1]). F984GC and R513GC attach a GC clamp for TGGE or DGGE analysis. Primers F27 and R1492 (55) were used to amplify almost the complete 16S rDNA sequence for model community studies.

PCR amplifications. PCR (41) was performed with a DNA thermal cycler 480 (Perkin-Elmer Cetus, Norwalk, Conn.). For the specific amplification of 16S rDNA fragments of actinomycetes, the reaction mixture was as follows: 1 μl of template DNA (ca. 20 ng), 10 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.2 mM deoxynucleoside triphosphates, 2.5 mM $MgCl_2$, 5% (vol/vol) dimethyl sulfoxide, 100 nM F243, and 100 nM R1378 or R513GC. Dimethyl sulfoxide was added to the reaction mixture to facilitate the denaturation of double-stranded DNA and to circumvent the formation of secondary structures. For amplification of environmental DNA, 0.1 μg of bovine serum albumin per ml was used to prevent inhibition (37). A 23-μl volume of the mixture was added to a 0.5-ml reaction tube and overlaid with a drop of mineral oil. After 5 min of denaturation at 94°C, 2 μl (1.25 U) of diluted AmpliTaq Stoffel fragment was added at a temperature of 80°C. This hot start was necessary to prevent nonspecific annealing of the primers to nontarget DNA (4). Amplification was performed by use of 35 cycles of 1 min of denaturation at 94°C, 1 min at 63°C for primer annealing, and 2 min at 72°C for primer extension, followed by a final step at 72°C for 10 min and cooling to 4°C. Products were first analyzed by electrophoresis in 1.5% (wt/vol) agarose gels and ethidium bromide staining (42). If bovine serum albumin was used, PCR products were purified by PCR purification spin columns (QIAquick; Qiagen GmbH, Hilden, Germany).

Two strategies were employed to analyze community DNA (Fig. 1). First, fragment 226–528 amplified with primer pair F243-R513GC was analyzed in TGGE or DGGE. Second, the products from primer pair F243-R1378 were excised from the agarose gel, extracted and recovered by spin columns (QIAquick gel extraction kit; Qiagen Germany), and used as a template for a second PCR with primer pair F984GC-R1378 to obtain fragment 968–1401 suitable for gradient gel analysis. For comparison with the whole bacterial community profile, soil DNA was used as a template for PCR with primer pair F984GC-R1378. PCR conditions differed from those described above in the use of $MgCl_2$ (3.75 mM), an annealing temperature of 60°C and a final step of 10 cycles of 0.5 min at 60°C and 1 min at 72°C and then cooling the mixture to 4°C. A hot start was not necessary.

TGGE and DGGE. The TGGE (Qiagen) and DGGE (D GENE System; Bio-Rad, Inc., Hercules, Calif.) systems were used as described in the instructions of the manufacturers. The majority of the results presented here were achieved by TGGE and repeated with DGGE in a collaborative study. No discrepant results were obtained, showing the interchangeability of both techniques. Natural community studies in the respective laboratories were done by either DGGE (Warwick) or TGGE (Braunschweig).

For TGGE, polyacrylamide gels were composed of 0.17% (vol/vol) TEMED (*N*,*N*,*N*′,*N*′-tetramethylenediamine), 0.03% (wt/vol) ammonium persulfate, acrylamide–*N*,*N*′-methylenebisacrylamide (60:1), 1× TAE buffer (42), 2% glycerol, 8 M urea, and 20% (vol/vol) formamide, deionized with AG501-X8 mixed-bed resin (Bio-Rad, Munich, Germany). Gels were polymerized on a gel support film (FMC, Vallensbaek Strand, Denmark). Horizontal electrophoresis was performed with 1× TAE buffer as the running buffer at a constant voltage of 100 V overnight (15 h) on a temperature gradient of 38 to 52°C or 45 to 58°C for fragments 968–1401 and 226–528, respectively.

The gel composition for DGGE was as described for TGGE except for the urea and formamide, which were changed as follows: denaturing gradients of 30 to 60% or 40 to 60% of denaturant (100% denaturant corresponds to 7 M urea plus 40% [vol/vol] of deionized formamide) were used for fragments 968–1401 and 226–528, respectively. Gradients were created with a MSE (Loughborough, Leicestershire, United Kingdom) gradient maker, and gels were allowed to polymerize overnight. DGGE was performed in 0.5× TAE buffer at 60°C at a constant voltage of 150 V for 6 h (fragment 968–1401) and 4 h (fragment 226–528), depending on the PCR products.

Aliquots of the PCR samples (2 to 4 μl) were applied directly to 6 or 8% (wt/vol) polyacrylamide gels for fragments 968–1401 and 226–528, respectively.

Prediction of relative running distances in a denaturing gradient. To assist in the choice of 16S rDNA fragments for separation by gradient gel analysis, the electrophoretic mobilities of the fragments were determined by their melting behavior calculated with the Poland program (48). One representative sequence for each of the genera *Arthrobacter*, *Frankia*, *Nocardia*, *Saccharopolyspora*, and *Streptomyces* was constructed from aligned database sequences, taking the most common base at each position. Electrophoretic mobilities of the partially melted fragments 968–1401 and 226–528 with the attached GC clamp were calculated relative to the mobilities of the complete double-stranded fragments for a range of temperatures with the Poland program. The plots of mobility versus temperature were used to calculate the plots of running distance versus time to predict the resolution of the fragments in TGGE.

Silver staining. A routine silver staining protocol was used for detection of DNA in TGGE and DGGE gels (36). The solutions used were 10% (vol/vol) ethanol plus 0.5% acetic acid for fixation, 0.1% (wt/vol) silver nitrate for staining, freshly prepared developing solution containing 0.01% (wt/vol) sodium borohydride, 0.15% formaldehyde, 1.5% (wt/vol) NaOH, and, finally, 0.75% (wt/vol) sodium carbonate solution to stop the development. Gels were dried and documented with a video system (Bio Image Products, Ann Arbor, Mich.).

RESULTS

Specificity of primer F243 in database comparisons. The designed primer F243 might not be the ideal primer for all studies of actinomycetes because (i) it does not match the 16S rDNA of all actinomycetes and (ii) it matches the 16S rDNA of

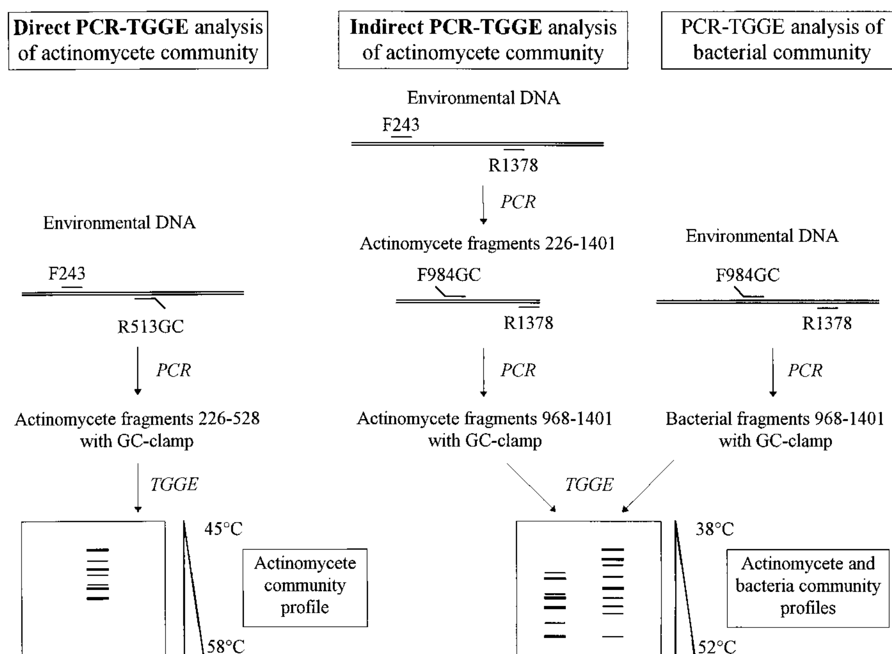

FIG. 1. Scheme of the two approaches applied in this study, direct and indirect analysis of the diversity of actinomycetes in environmental samples. In the indirect approach, the actinomycete profiles can be compared to bacterial profiles of the same sample. Separation of PCR products can be done either by TGGE or DGGE.

a few nonactinomycetes. Nevertheless, the primer is of interest for an enrichment of actinomycete 16S rDNA to improve the detection of this group from environmental samples. The problems are discussed comprehensively, so that the potential user of this PCR can estimate the limitations. Matching of the primer sequence to consensus sequences at positions 226 to 243 of various taxa is given in Fig. 2. The taxa with a good fit to the primer's 3′ terminus, which determines its specificity, belonged to the phylogenetic group of actinomycetes (11). Within the actinomycetes, only 10 of 364 sequences had a mismatch to the first 3 bases at the 3′ end of the primer, and 5 of these 10 belonged to the *Propionibacterium* group (names are used as in the RDP database). The primer had no mismatches in the first 6 bases at the 3′ end for the following groups: *Arthrobacter*, *Catenuloplanes*, *Frankia*, *Kineococcus* (only one sequence), *Mycobacterium*, and *Streptosporangium*. Other groups showed only a few exceptions: the *Nocardia* assemblage (at position 239 in one of two sequences of *Gordona terrae*), *Streptomyces* and relatives (*Streptomyces* species strain NRRL 3890, with a deletion at position 240), the *Actinomyces* subgroup (*Actinomyces neuii*, having a T at position 241), and some members of the *Saccharopolyspora* group. All sequences of the *Bifidobacterium* subgroup had a T at position 239. In the *Atopodium* group, which belongs to the high-G+C subdivision but not to the actinomycetes, none of the sequences matched the primer at the 3′ end.

Of the 2,270 16S rRNA nonactinomycete sequences in the database, 2,257 did not match the primer sequence at positions 239 to 243. The nonactinomycetes whose sequences matched the primer sequence were *Desulfurella acetivorans*, *Thermomicrobium roseum*, *Treponema pectinovorum*, four strains of "*Serpula*," and six environmental clone sequences. The sequence of strain TH3, representing a group within the high-G+C subdivision phylogenetically similar to the actinomycetes (11), also fit the primer F243 sequence. In addition, some actinomycetes had mismatches to the primer at its 3′ end. A recently described group of actinomycetes isolated from nodules and phylogenetically related to *Frankia* had a T instead of a C at position 240 (30).

Specificity of primer F243 checked with pure-culture DNA as template. The specificity of primer F243 in combination with reverse primers R513GC and R1401 was analyzed by PCR. Products of the appropriate size were formed with all of the actinomycetes tested but not with any of the control strains of the other bacterial taxa (Table 1). An annealing temperature of 63°C, which is slightly above the calculated optimal annealing temperature of 60°C for both primer pairs, was used (40). The annealing temperature could be increased to 68°C with both primer pairs to improve stringency, but the product yield then varied significantly between different target sequences. This differential amplification would bias community analysis. To examine whether one mismatch to the primer at the 3′ end is sufficient to significantly reduce primer extension, three nonactinomycete strains with homologous sequences to primer F243 but mismatches at position 241 (additionally in positions 232 and 231) were selected, i.e., *Carnobacterium gallinarum*, *Pediococcus pentosaceus*, and *Lactobacillus plantarum*. Generally, the three strains failed to give a product except under conditions of highly concentrated target (ca. 200 ng) in a PCR with primers F243 and R1378. The product was very weak, so that an amplification in the presence of actinomycete sequences would be negligible. The template DNA of all strains tested was amplifiable in PCR with primers F984GC and R1378.

Electrophoretic separation of fragments 226–528 and 968–1401. Primer F243 in combination with R513GC was used to specifically amplify 16S rDNA fragments of actinomycetes which could be analyzed directly by TGGE or DGGE. The separation of fragment 226–528 of some actinomycetes by TGGE is shown in Fig. 3. There was sufficient separation to differentiate between most of the strains analyzed. Two species of *Nocardiopsis* were clearly separated, but *Nocardiopsis atra*

Taxonomic group	F243	5′	GGATGAGCCCGCGGCCTA	3′
Streptomyces (A, HGC)	60 %	GUGAA	------------------	TCAGC
	90 %	--..-	------.-----------	-----
Frankia (A, HGC)	60 %	-CT.G	-----G------------	-----
	90 %	-CT.G	-----G------------	-----
Saccharopolyspora (A, HGC)	60 %	--..G	-----..-----------	-----
	90 %	-....	-----..-.-------.-	-----
Nocardia (A, HGC)	60 %	--.C.	-----G------------	-----
	90 %	--...	.----.--.---------	-----
Mycobacteria (A, HGC)	60 %	---TG	-----G------------	-----
	90 %	---TG	-----..-----------	-----
Actinomyces (A, HGC)	60 %	--.GG	-----G--T---------	-----
	90 %	-...G	-----G--T-.-------	-----
Arthrobacter (A, HGC)	60 %	--T.G	-----G.-T---------	-----
	90 %	.-...	-----G.-T---------	----
Propionibacterium (A, HGC)	60 %	--...	-----..-T---------	-----
	90 %	-....	----...-T----.----	-----
Bifidobacterium (A, HGC)	60 %	-.ATG	-----G-GT----T----	-----
	90 %	-.ATG	-----G-GT----T----	-----
Atopodium (HGC)	60 %	-A-A.	-----G-----------G	-T---
	90 %	-.--.	-----G-..-------..	-.--.
α-Proteobacteria	60 %	...-.	.----.-------T..G-	-T---
	90 %	.....	.----.-------T..G-	-T--.
β-Proteobacteria	60 %	C-ATT	---GCG---GAT.T-.G-	-T---
	90 %	.-...	.--GCG---GAT..-.G-	-T--.
γ-Proteobacteria	60 %	C..T.	.--------.A.-T..G-	-T---
	90 %	C....	.---...--....T..G-	-T--.
δ-Proteobacteria	60 %	C....	.------T-----..-C-	-T---
	90 %	.....	.----.-...---..-C-	-.---
ϵ-Proteobacteria	60 %	.-A..	----.--.-TA-AT.T--	-----
	90 %	.-...	.---..-.-T.-...T--	----.
FBC-group	60 %	--..-	A----G--AT---T...-	-T--.
	90 %	.....	.---.....T---T...-	-T--.
Low G+C gram+	60 %	C-...	.----G.------.-GC-	-T---
	90 %	.-...	.--.-G...----....-	-T--.
Cyanobacteria	60 %	CCTG-	-----G--T----T-TG-	-T---
	90 %	CC...	.--.-G--..---T-.G-	-T---

FIG. 2. Alignment of primer F243 and 16S rDNA sequences of actinomycete (A) and nonactinomycete groups of the RDP database at *E. coli* positions 226 to 243 at 60 and 90% consensus levels. Dashes indicate bases identical to those in the sequence of primer F243. Dots indicate positions without a common base at the given consensus level. HGC, high-G+C gram-positive bacteria.

showed similar electrophoretic mobilities to those of *Streptosporangium roseum* and *Actinomadura viridis*. The bands of *Dactylosporangium aurantiacum*, *Actinoplanes philippinensis*, and *Planobispora longispora* were barely resolved. Analysis of streptomycete sequence data indicated that several species groups could be distinguished by use of fragment 226–528 (data not shown). Attempts to further narrow the temperature gradient did not result in a better separation. Separation comparable to that shown in Fig. 3 by TGGE was achieved by DGGE (data not shown). The band common to all lanes in Fig. 3 was an artifact representing single-stranded DNA (ssDNA) which was not influenced differentially by the temperature gradient. It was stained by silver (much weaker by ethidium bromide) but could be identified by its reddish color.

An alternative approach enabled the comparison of actinomycete profiles to total bacterial community profiles by gradient gel analysis. For indirect detection, primer F243 was used in combination with primer R1378 in a first PCR. The products served as a template for a second PCR with bacterial primers F984GC and R1378 to give the GC-clamped fragment 968–

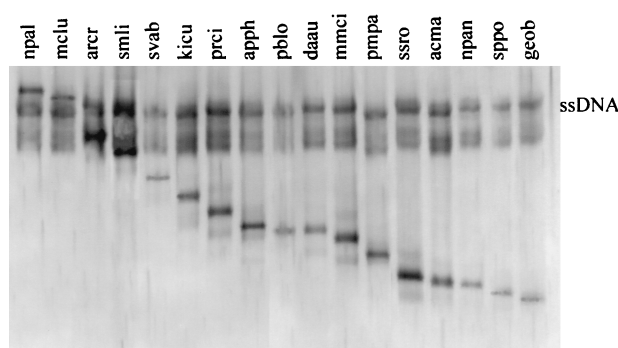

FIG. 3. Separation of 16S rDNA fragments of actinomycetes spanning the region of positions 226 to 528 (*E. coli* positions) joined to a GC clamp in TGGE; the temperature gradient was 45 to 58°C. The common band represents ssDNA. For abbreviations of species names, see Table 1.

1401. The potential of this fragment to differentiate actinomycetes was investigated by using pure-culture DNA. A TGGE gel with fragment 968–1401 from selected actinomycetes is shown in Fig. 4, and relative electrophoretic mobilities of other actinomycetes are given in Table 1. The upper bands, representing fragments with lower melting temperatures, belonged to species related to *Arthrobacter* and *Nocardia*, the central bands were mainly from species related to *Streptomyces* and *Streptosporangium*, and the lower bands, representing fragments with a high melting temperature, corresponded to species related to *Actinoplanes*. Most strains separated well. Species of the same genus could be distinguished, e.g., species of *Micromonospora* or *Streptomyces* (Table 1), but the running distance is not directly related to the phylogenetic affiliation. A few species could not be resolved under the conditions applied, e.g., *Micromonospora citrea* and *Actinoplanes philippinensis*. The PCR product of *Saccharothrix waywayandensis* was separated by TGGE into two distinct bands probably due to multiple *rrn* operons. The DGGE separation of various actinomycetes corresponded to that in TGGE, showing the interchangeability of these two methods (data not shown).

The overall separation distance was larger with fragment 968–1401 than with fragment 226–528, and that of the former could be further increased by using narrower gradients. For the purposes of this study, a gradient of 38 to 52°C for TGGE or 30 to 60% of denaturant for DGGE was chosen since this was optimal to allow a comparison between bacterial and actino-

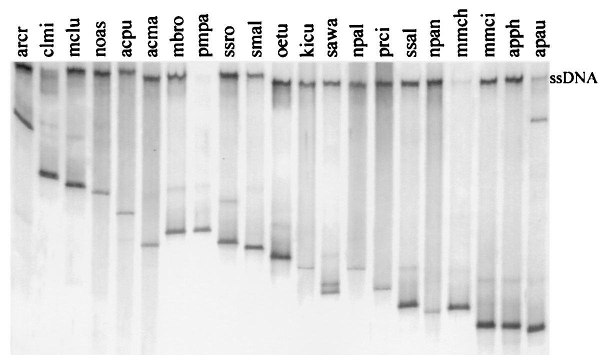

FIG. 4. Separation of 16S rDNA fragments of actinomycete strains spanning the region of positions 968 to 1401 (*E. coli* positions) joined to a GC clamp in TGGE; the temperature gradient was 38 to 52°C. The common band represents ssDNA. For abbreviations of species names, see Table 1.

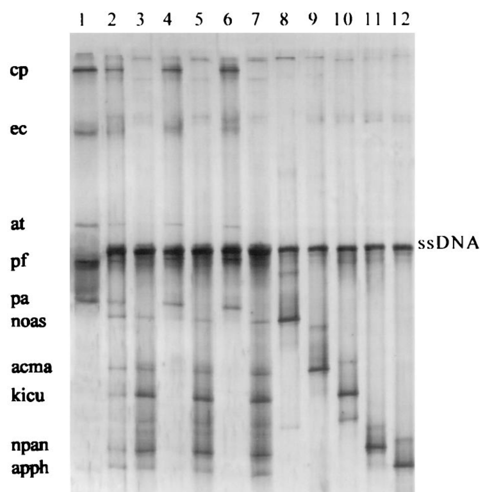

FIG. 5. Analysis of model communities composed of PCR-derived 16S rDNA templates from five actinomycetes and five species of other bacterial taxa. Either amounts of template in the PCR were equal for all strains (lanes 2 and 3) or the actinomycete templates were diluted 10 times (lanes 4 and 5) or 100 times (lanes 6 and 7). The GC-clamped fragment 968–1401 was amplified either directly for bacterial community patterns (lanes 2, 4, and 6) or from the products of a preceding actinomycete-specific PCR (lanes 3, 5, and 7). Community constituents were the nonactinomycetes *Clostridium pasteurianum*, *Erwinia carotovora*, *Agrobacterium tumefaciens*, *Pseudomonas fluorescens*, *Pantoea agglomerans* (all lane 1), and the actinomycetes *Nocardia asteroides* (lane 8), *Actinomadura viridis* (lane 9), *Kineosporia aurantiaca* (lane 10), *Nocardiopsis atra* (lane 11), and *Actinoplanes philippiensis* (lane 12). For abbreviations of species names, see Table 1.

mycete profiles for the whole bacterial community pattern. The calculation of running distances of sequences representative for the actinomycete groups analyzed predicted a better resolution for fragment 968–1401 than for 226–528 (data not shown).

Selective amplification of actinomycetes from synthetic model communities, rhizosphere, and soil. Three model communities comprised 16S rDNA fragments from five actinomycetes and five other strains. They were constructed with (i) equal amounts of template 16S rDNA, (ii) actinomycetes being 10-fold diluted, and (iii) actinomycetes being 100-fold diluted. The DNA mixtures were analyzed either directly by PCR-TGGE with primers F984GC and R1378 to obtain a whole community pattern or indirectly by an initial PCR with primers F243 and R1378, PCR product from which served as a template for a second PCR with primers F984GC and R1378 (Fig. 1). This product was subjected to TGGE analysis to obtain the actinomycete profile. The pattern derived from the indirect approach consisted only of the actinomycete bands even if the nonactinomycete targets were present in 100-fold excess (Fig. 5, lanes 3, 5, and 7). This showed the specificity of primer F243 in a PCR with competing templates. The whole community pattern resulting from the direct use of fragment 968–1401, without the initial PCR with specific primers F243 and R1378, reflected the ratios of target rDNA (Fig. 5, lanes 2, 4, and 6). No bands were obtained from actinomycetes if they were diluted 100-fold (lane 6), and only weak bands were obtained if they were diluted 10-fold (faintly visible in lane 4).

An example of the application of this approach in the analysis of natural microbial communities is given in Fig. 6 for the PCR-TGGE pattern of potato rhizosphere communities. Actinomycete and bacterial community patterns of rhizosphere samples from transgenic plants and wild-type plants were compared. The transgenic plants produced T4 lysozyme against bacterial infection. Actinomycetes were especially interesting, since they are reported to be more susceptible to T4 lysozyme than gram-negative bacteria are. The actinomycete patterns (Fig. 6, lanes 1 to 4) consisted of 13 to 15 bands with long running distances according to their high G+C content. The bacterial pattern consisted of 18 to 22 bands. Two bands were hidden under the ssDNA but were visible due to their different color. There were no apparent differences in the actinomycete community patterns of the rhizosphere samples from transgenic and wild-type potatoes, whereas in the bacterial community pattern, at least one band appeared to reflect a difference in the relative abundance of the corresponding species (Fig. 6, lanes 7 and 8, below marker band labeled bg). This was confirmed by samples of eight replicate plants (unpublished data). Most of the actinomycete bands also appeared in the bacterial pattern; some of them were very weak.

Community DNA from soil indicated a considerable range of bands, and the comparison of two soil types clearly showed pronounced differences in the actinomycete diversity when either fragment 226–528 (Fig. 7, lanes C3 and W3) or 968–1401 (Fig. 7, lanes C1, C2, W1, and W2) was used. A greater range and diversity of bands was resolved from the Cuban soil DNA than from the Warwick soil. Comparison of DGGE banding patterns by the direct and indirect approaches for soil actinomycete community analysis showed a high number of putative actinomycete bands resolved by the indirect approach which are not visible in the bacterial community pattern (Fig. 7, lanes C1, C2, W1, and W2). This indicates that the diverse actinomycete groups were outcompeted as targets for the nonspecific PCR probably due to low population size.

DISCUSSION

The structure of natural microbial communities of terrestrial habitats is often highly complex (52) and therefore difficult to characterize. It is possible by the use of PCR-TGGE or -DGGE analysis to compare, at the community level, a large number of environmental samples and evaluate impacts of

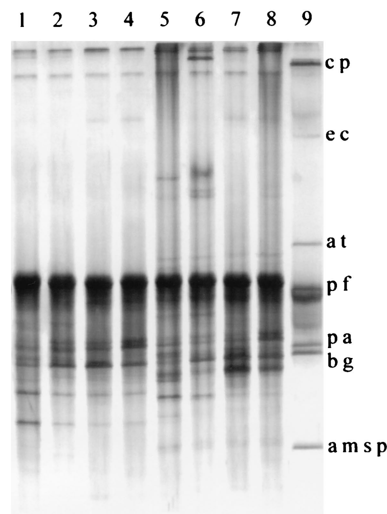

FIG. 6. Comparison of potato rhizosphere communities by PCR-TGGE analysis of 16S rDNA fragment 968–1401. Actinomycete patterns of T4 lysozyme-expressing potatoes (lanes 1 and 2) and wild-type potatoes (lanes 3 and 4) were derived by the indirect approach. Corresponding bacterial community patterns were derived by amplification of fragment 968–1401 from total community DNA (lanes 5 and 8). Lane 9 contains a mixture of fragments 968–1401 from the indicated strains (see Table 1 for abbreviations).

different treatments on soil microbial community structure (17, 26). Total soil microbial community patterns may be difficult to resolve if species are evenly abundant (12), and differences in relative abundance may result in the inability to detect certain groups due to competition during PCR (17). This problem occurs in the molecular detection of actinomycetes colonizing soil since the group is often present in low numbers, necessitating the use of group-specific primers (17). In addition, the relatively high G+C content of the actinomycete DNA might reduce the competitiveness of their DNA targets in PCR (34, 49).

The specificity of a primer to anneal to a unique sequence is achieved by homology to the target site, primer length, and G+C content. Once annealing has occurred, primer extension to form product is the next step sensitive to primer-target mismatches. In contrast to probes, primer-template mismatches are most critical at the 3′ terminus of the primer, mainly the first 3 bases at the 3′ end (20, 45), although not all mismatch types reduce the PCR product yield equally (21). A moderate number of mismatches near the 5′ end will not affect amplification since the annealing temperature is well below the melting temperature of the primer and thus annealing can still occur. This need for a perfect match at the 3′ end was used as a criterion to design primer F243. The target site at positions 226 to 243 is fairly conserved within the high-G+C subdivision, and bases 239 to 242 (bases 2 to 5 from the 3′ end of the primer) are different for sequences of the low-G+C subdivision, proteobacteria, cyanobacteria, and the flavobacterium-bacteroides-cytophaga group. Primer F243 in combination with conserved-sequence reverse primers selectively amplified 16S rDNA fragments of actinomycetes during PCR. It must be noted that a DNA polymerase like the Stoffel fragment, which lacks 3′-5′ exonuclease activity, had to be applied (3). In the absence of actinomycetes, weak nonspecific products were formed in the PCR from high amounts of genomic DNA from nonactinomycete strains. This will not bias the application to community analysis such as studies of terrestrial environments if actinomycetes are present, because their DNA will outcompete other targets with a lower affinity to primer F243 in the PCR. This was demonstrated by use of the model community in which the presence of actinomycete targets suppressed the amplification of nonactinomycete DNA, which was 100-fold more abundant. A more general problem in the application of group-specific primers for the analysis of natural microbial communities is that the prediction of specificity relies on the selection of tested strains and known sequences from databases which may not adequately reflect the occurrence of 16S rDNA sequences in nature. In addition, a few known sequences of nonactinomycetes also matched the primer sequence quite well, and some known actinomycete sequences did not match. However, the results presented here indicate that primer F243 is specific for the majority of actinomycetes, giving good evidence that 16S rDNA fragments of actinomycetes will be enriched in PCR from environmental samples.

Two different strategies were applied to analyze actinomycetes within natural microbial communities. In the first approach, the specific PCR products (positions 226 to 528) were used for direct analysis in TGGE or DGGE. This approach is straightforward, allowing the routine analysis of many samples in parallel and thereby enabling investigation of community dynamics. Fragment 226–528 did not allow complete separation of all actinomycete species tested, but the resolution of bands was sufficient to separate species of the same genus (Fig. 3). It could be useful to quantitatively estimate specific groups, to detect the presence of actinomycetes, and to compare patterns from soil communities. This was shown clearly in the

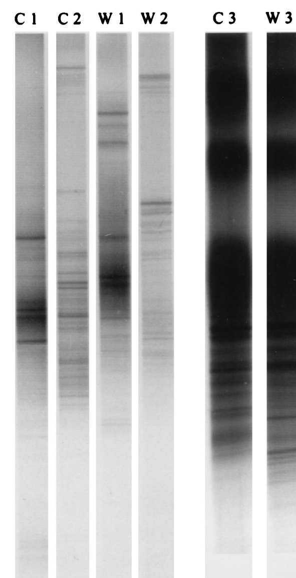

FIG. 7. Analysis of two soil communities, Warwick (W) and Cuban (C), by DGGE separation of 16S rDNA fragments with actinomycete-specific and bacterial primers. Lanes: C1 and W1, community pattern obtained by the indirect method with actinomycete-specific primers F243-R1378 and F984GC-R1378 for DGGE analysis; C2 and W2, total community profiles obtained with primers F984GC-R1378; C3 and W3, community pattern obtained by the direct method with actinomycete-specific primers F243-R513GC for DGGE analysis.

community analysis of two soil types, the Cuban and Warwick soils (Fig. 7). In both soils, the culturable actinomycetes exceeded 10^6 CFU/g of soil (7, 19a), but the patterns were distinctly different, showing only a few common bands. The greater number of high-G+C bands observed in the Cuban soil sample may indicate a higher diversity of actinomycete genera, particularly the nonstreptomycete groups. Comparison of running distances for species of the *Streptomyces* genus indicated that species groups could be well separated by use of fragment 226–528, which may prove to be useful in diversity studies of this important genus.

The second (indirect) approach, using nested PCR with primers F243 and R1378 followed by a second PCR with primers F984GC and R1378, showed a higher resolution with DNA of both strains and soil communities, probably due to a higher variability within the 16S rDNA fragment 968–1401. This was confirmed by calculations of the running distances of different actinomycete genera. Nearly all strains tested could be separated, with some belonging to the same genus. The running distance was not directly correlated with the phylogenetic affiliation of the strains since most of the sequence variation of fragment 968–1401 is located in hypervariable regions, including the variable regions V6 to V9 (29). The high variability of the fragment was also used in a recent study to detect sequence heterogeneities of the *rrn* operons of *Paenibacillus polymyxa* in TGGE (31). Probably for the same reason, the PCR product of *Saccharothrix waywayandensis* was resolved into two bands in TGGE (Fig. 4). The technique is sensitive enough to detect nearly all single base substitutions in fragments of 500 bp with a GC clamp (28, 43).

Fragment 968–1401 has proved useful in TGGE and DGGE analysis of bacterial communities (12, 16, 17). The V6 region (positions 986–1043) was previously used to generate species- or genus-specific probes by PCR which can assist in the analysis of complex community patterns (16, 17). The fragment also includes two of the three regions of the 16S rDNA which are

reported to be suited for species discrimination of streptomycetes (47). Another advantage of the second approach is that the group-specific pattern of the actinomycetes from one sample can be directly compared to the bacterial community pattern of the same sample because the same fragment is analyzed. Thus, the actinomycetes of rhizosphere and soil samples could be analyzed in the context of the bacterial community. For the potato rhizosphere, most of the actinomycete bands corresponded to a weaker band of the same running distance in one or more of the bacterial community patterns (Fig. 6), indicating a high proportion of this group in the whole community. The variability of the actinomycete patterns between replicate plants was low, and no difference between actinomycete communities of transgenic and wild-type plants was evident. This comparison was not possible when the bacterial pattern was used because actinomycete bands were too weak to estimate relative abundances. The obvious difference in at least one of the major bands in the bacterial pattern between rhizosphere samples from transgenic and wild-type plants (Fig. 6, lane 5 to 8) could not be attributed to differences in the actinomycete communities (Fig. 6, lane 1 to 4). In soil samples, several actinomycete bands did not appear in the corresponding bacterial pattern (Fig. 7, lanes C1, C2, W1, and W2). This indicates that nonactinomycete species were more abundant in these soil types but also that the high diversity of actinomycetes could have resulted in too-little PCR product per species to give visible bands. The appearance of new bands when group-specific primers were used demonstrates that diversity reflected by TGGE or DGGE analysis is more related to the evenness of species abundance than to the number of different species in the environmental sample. Previous studies have shown that the presence of a few highly dominant species will produce simpler patterns, and species of less than 1% of the analyzed community were not represented in the community pattern (17, 25). In the current study, soil patterns were much more complex than the rhizosphere pattern, indicating a higher bacterial and actinomycete diversity, i.e., evenness of the most abundant species. In a recent investigation by PCR-DGGE of community changes following the introduction of a strain into a groundwater aquifer for bioremediation, the high density of the inoculum in the beginning of the experiment interfered with community analysis by PCR-DGGE with bacterial primers F968GC and R1378. The number of bands was largely reduced, hiding the natural community and thus creating a false pattern of low diversity (unpublished results). To overcome this problem, group-specific primers excluding the introduced strain were needed to investigate dynamics of natural populations and sample diversity.

The proposed approach can also be applied with other group-specific forward primers, avoiding the laborious optimization of the denaturing gradient and the electrophoretic conditions which are necessary for new fragments. Thus, primers specific for α- or β-proteobacteria were designed and are currently under investigation to define their specificity range. A possible hazard for the indirect approach is the introduction of a bias resulting from two successive PCRs due to preferential amplification (49). Differences in the target concentrations tend to be equalized in the ongoing PCR since reannealing of frequently occurring products competes with primer annealing; therefore, the numbers of cycles in the first PCR should be as low as possible. However, it has been shown that the intensity of bands in TGGE corresponded semiquantitatively with the abundance of species, and the bias of preferential amplification may be overestimated (17).

According to the recently proposed hierarchic classification system of the actinomycete bacteria and relatives based on 16S rDNA sequences (46), the approaches presented here with primer F243 mainly target species of the order *Actinomycetales*, which has the signature nucleotide G at position 239. It is, together with the order *Bifidobacteriales*, part of the subclass *Actinobacteridae* and *Actinobacteria* classis nov.

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