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Application of temperature-gradient gel electrophoresis in taxonomy of coryneform bacteria

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Strains belonging to the Gram-positive coryneform soil bacteria were screened genotypically by temperature-gradient gel electrophoresis (TGGE). This method allows the sequence-specific separation of amplified fragments of 16S rRNA genes. A total of 115 reference strains representing the majority of the species of the genera *Aeromicrobium*, *Agromyces*, *Arthrobacter*, *Aureobacterium*, *Cellulomonas*, *Curtobacterium*, *Nocardioides* and *Terrabacter* were characterized. Depending on the genus investigated, the resolution limit of the technique appeared to be at the species or genus level or intermediate between the two. Aberrant TGGE profiles of strains within particular taxa revealed genomic heterogeneity and generic misclassification of nine strains studied. Beyond that, indications of 16S rRNA gene heterogeneity were found within the genomes of three *Curtobacterium* strains. The misclassifications revealed by TGGE were confirmed using whole-cell fatty acid methyl ester analysis and subsequent comparison with a database. TGGE has been demonstrated to be a useful tool in bacterial taxonomy.

Keywords: temperature-gradient gel electrophoresis, coryneform bacteria, 16S rDNA gene heterogeneity, fatty acid methyl ester

INTRODUCTION

In recent years, molecular methods have been used increasingly to characterize prokaryotes, because bacterial diversity cannot be described solely by morphological and physiological features, which can be common to bacteria from phylogenetically distant taxa. On the other hand, closely related genera may show a rich morphological and physiological diversity. The coryneform bacteria, on which this paper focuses, and their entire class, *Actinobacteria*, are good examples in this context (Stackebrandt *et al*., 1997). The modern molecular taxonomy of bacteria follows the polyphasic strategy of classification (Colwell, 1970; Vandamme *et al*., 1996), in which different approaches are applied simultaneously. Chemotaxonomic information is collected by analysing the composition of particular cell constituents, such as peptidoglycans, fatty acids and polar lipids. Another popular approach is based on DNA base composition. Analysis of DNA–DNA reassociation kinetics can discriminate closely related species (Stackebrandt & Kandler, 1979) and can be used to determine the boundaries of prokaryote species (Schleifer & Stackebrandt, 1983). In contrast, bacterial phylogeny is nowadays correlated with the phylogeny of their 16S rRNA, as described by Woese & Fox (1977).

Another tool within the arsenal of this polyphasic strategy could be the highly specific, partial analysis of 16S rRNA sequences by temperature-gradient gel electrophoresis (TGGE) (Rosenbaum & Riesner, 1987) or the similar method, denaturing-gradient gel electrophoresis (DGGE) (Fischer & Lerman, 1979). These powerful techniques have been applied particularly to the analysis of 16S rRNA in the area of molecular ecology (Muyzer *et al*., 1993; Ferris *et al*., 1996; Rölleke *et al*., 1996; Teske *et al*., 1996; Felske *et al*., 1997), but not in taxonomy. Buchholz-Cleven *et al*. (1997) found DGGE to be convenient and reliable in the analysis of unidentified isolates before sequencing. Here we report on the application of TGGE for

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Abbreviations: DGGE, denaturing-gradient gel electrophoresis; FAME, fatty acid methyl ester; TGGE, temperature-gradient gel electrophoresis.

Table 1. List of all 115 strains investigated and their TGGE signals (as compiled in Fig. 1)

Bands are indicated only if there was more than one per lane. Bold strains are represented in Fig. 1.

Genus/lane	Band	Strain
Aeromicrobium		
Lane *Ae*	e1	***Aeromicrobium fastidiosum* LMG 16205^T**
	e2	***Aeromicrobium erythreum* LMG 16472^T**
Agromyces		
Lane *Ag*	g1	***Agromyces cerinus* LMG 16155^T**, LMG 16586, LMG 16587, LMG 16157^T; *Agromyces fucosus* LMG 16156^T, LMG 16588, LMG 16589
	g2	***Agromyces ramosus* LMG 16680^T**
Curtobacterium		
Lane *Cu*		*Curtobacterium albidum* LMG 8759^T; *Curtobacterium citreum* LMG 16240, LMG 8786^T; ***Curtobacterium flaccumfaciens*** LMG 3596^T, LMG 7241, LMG 3645^T, LMG 3650, LMG 3702^T, LMG 3704, LMG 3710, LMG 3715^T; *Curtobacterium luteum* LMG 8787^T; *Curtobacterium pusillum* LMG 8788^T, LMG 8812
Arthrobacter		
Lane *Ar*	r1	*Arthrobacter citreus* LMG 16124, **LMG 16388^T**; *Arthrobacter viscosus* LMG 16186
	r2	***Arthrobacter crystallopoietes* LMG 3819^T**
	r3	*Arthrobacter citreus* LMG 16146; *Arthrobacter mysorens* LMG 16125, **LMG 16219^T**; *Arthrobacter nicotianae* LMG 16126, LMG 16159, LMG 16305^T; *Arthrobacter protophormiae* LMG 16115, LMG 16324^T; *Arthrobacter uratoxydans* LMG 16117, LMG 16129, LMG 16220^T
	r4	***Arthrobacter aurescens* LMG 3815^T**; *Arthrobacter globiformis* LMG 16339, LMG 16340, LMG 3813^T; *Arthrobacter histidinolovorans* LMG 3822^T; *Arthrobacter ilicis* LMG 3659^T, LMG 7254; *Arthrobacter nicotinovorans* LMG 16253^T; *Arthrobacter oxydans* LMG 16254, LMG 3816^T, LMG 3817; *Arthrobacter pascens* LMG 16127, LMG 16255^T; *Arthrobacter polychromogenes* LMG 16306, LMG 16679^T, LMG 3821; *Arthrobacter ramosus* LMG 16185, LMG 16256^T; *Arthrobacter ureafaciens* LMG 16130, LMG 16257, LMG 3812^T
	r5	*Arthrobacter sulfureus* LMG 16116, LMG 16128, **LMG 16694^T**
	r6	***Arthrobacter atrocyaneus* LMG 3814^T**
Lane 1		***Arthrobacter ilicis* LMG 7255**
Lanes 2+3		*Arthrobacter siderocapsulatus* LMG 16118, **LMG 16206^T**
Lane 5		***Arthrobacter picolinophilus* LMG 16262^T**
Lane 6		***Arthrobacter globiformis* LMG 3820**
Terrabacter		
Lane *T*+4	t1	***Terrabacter tumescens* LMG 3818^T**
	t2	***Terrabacter tumescens* LMG 16133**
Nocardioides		
Lane *N*	n1	***Nocardioides jensenii* LMG 16325^T**
	n2	***Nocardioides simplex*** LMG 16261^T
	n3	*Nocardioides albus* LMG 16212, LMG 16213, **LMG 16326^T**
	n4	*Nocardioides luteus* LMG 16160, **LMG 16209^T**
Lane 7		***Nocardioides simplex* LMG 3810**
Cellulomonas		
Lane *Ce*	c1	*Cellulomonas cellulans* LMG 16121, **LMG 16221^T**, LMG 16238, LMG 16259; *Cellulomonas uda* LMG 16123, LMG 16327^T

Table 1 (*cont.*)

Genus/lane	Band	Strain
	c2	*Cellulomonas gelida* LMG 16122, LMG 16697^T; *Cellulomonas flavigena* LMG 16263^T; *C. fimi* LMG 16239, **LMG 16345^T**
	c3	*Cellulomonas biazotea* LMG 16695^T; *Cellulomonas turbata* LMG 16191, **LMG 4072^T**; *Cellulomonas cellasea* LMG 16323^T
Lane 8		***Cellulomonas fimi* LMG 16132**
Lane 9		***Cellulomonas turbata* LMG 16346**
Aureobacterium		
Lane *Au*	u1	***Aureobacterium trichothecenolyticum* LMG 16696^T**
	u2	*Aureobacterium testaceum* LMG 16144, LMG 16237, **LMG 16344^T**
	u3	*Aureobacterium terrae* LMG 16190, **LMG 16471^T**; *Aureobacterium schleiferi* LMG 16189; *Aureobacterium esteraromaticum* LMG 16187, LMG 4020^T
	u4	*Aureobacterium keratanolyticum* LMG 16470^T; *Aureobacterium liquefaciens* LMG 16120, LMG 16188, LMG 16342^T ***Aureobacterium luteolum* LMG 16207^T**; *Aureobacterium saperdae* LMG 16343^T; *Aureobacterium schleiferi* LMG 16153^T
	u5	***Aureobacterium arabinogalactanolyticum* LMG 16469^T**
	u6	*Aureobacterium barkeri* LMG 16142, **LMG 16341^T**

taxonomic classification of 115 coryneform bacterial strains from the BCCM/LMG culture collection. These isolates included the majority of cultured species from the genera *Aeromicrobium*, *Agromyces*, *Arthrobacter*, *Aureobacterium*, *Cellulomonas*, *Curtobacterium*, *Nocardioides* and *Terrabacter*.

TGGE analysis starts with PCR amplification of a variable region of the 16S rDNA. The technique is then used to separate double-stranded PCR products according to the melting behaviour of the molecules. DNA of different sequences migrate different distances. The PCR product generated from a pure bacterial strain normally results in a single band that migrates a specific distance. The detection of additional bands would indicate that more sequences are present in the culture due to contamination of the culture with another organism or the presence of different 16S rDNA sequences in one bacterial genome. Because of the great efficiency and speed with which the technique reveals contamination in pure cultures and 16S rRNA sequence heterogeneity (Nübel *et al.*, 1996), TGGE is a promising tool for taxonomic studies. However, as 16S rRNA sequencing is time-consuming and expensive to perform, it is advisable to determine in advance whether different copies of 16S rRNA are present in the strain to be analysed. It is not known to what extent this phenomenon occurs among prokaryotes. Until now only a few observations of different 16S rDNAs in one strain have been reported (Mylvaganam & Dennis, 1992; Nübel *et al.*, 1996).

The extent to which the TGGE approach yields comparative taxonomic information in the same way as chemotaxonomic fingerprinting techniques, such as cellular fatty acid analysis, or DNA fingerprinting methods, such as restriction fragment length polymorphism/amplified rDNA-restriction analysis (RFLP/ARDRA) (Vaneechoutte *et al.*, 1992) or random amplified polymorphic DNA/arbitrarily primed PCR (RAPD/AP-PCR) (Welsh & McClelland, 1990), remains to be determined. The other DNA methods reveal more or less complex band patterns for one sequence. TGGE, with its 'one sequence–one band' detection, reduces the sequence-specific information and might offer new useful properties.

METHODS

Bacterial strains and cultures. All bacterial cultures were obtained from the BCCM/LMG Culture Collection of the Laboratory of Microbiology, University of Ghent, Belgium. All strains used in this study (listed in Table 1) were cultivated aerobically on trypticase soy agar [TSA; 3% (w/v) trypticase soy broth (BBL) supplemented with 1·5% (w/v) Bacto agar (Difco)] for 24 h at 28 °C.

Isolation and purification of bacterial chromosomal DNA. Genomic DNA was prepared as described by Janssen *et al.* (1996). In short, approximately 30 mg wet weight of cells was scraped off from TSA plates, washed once with 500 µl saline (150 mM NaCl, 10 mM EDTA, pH 8·0) and resuspended in 100 µl TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 7·6). Cells were lysed and DNA was extracted with Sarkosyl/guanidinium thiocyanate (Sigma) as described by Pitcher *et al.* (1989). The DNA was finally dissolved in 100 µl TE buffer and kept overnight at 4 °C. After an RNAse treatment (25 µl stock culture of 0·25 mg ml^{-1} and incubation for 1·5 h at 37 °C), the concentration of all DNA solutions was determined on a Uvikon 940 spectrophotometer (Kontron Instruments) by measuring

A_{260} (1 absorbance unit = 50 μg ml^{-1}). Final DNA preparations were stored at −20 °C.

Amplification of 16S rRNA sequences and separation by TGGE. PCR was performed in an Amplitron II thermocycler (Barnstead/Thermolyne), using 30 cycles of 94 °C for 10 s, 56 °C for 20 s and 68 °C for 40 s. Universal bacterial primers U968-GC and L1401 (Nübel *et al*., 1996) were used for each strain. The primer numbers 968 and 1401 indicate their binding sites according to the 16S rRNA numbering of *Escherichia coli* (Brosius *et al*., 1978). Primer U968-GC contains a GC clamp for directed melting during TGGE (Muyzer *et al*., 1993). The PCR reactions (10 μl) contained 10 mM Tris/HCl (pH 8·3), 50 mM KCl, 3 mM $MgCl_2$, 50 μM each of dATP, dCTP, dGTP and dTTP, 50 pmol U968-GC and L1401, 0·25 U *Taq* DNA polymerase (Life Technologies) and 1 μl template DNA. Six microlitres of amplification product was loaded on the gel.

The Diagen TGGE system was used for sequence-specific separation of PCR products. Electrophoresis took place in a 0·8 mm polyacrylamide gel [6 % (w/v) acrylamide, 0·1 % (w/v) bis-acrylamide, 8 M urea, 20 % (v/v) formamide, 2 % (v/v) glycerol] with 1 × TAE (Sambrook *et al*., 1989) buffer at 9 mA fixed current (about 125 V) for 16 h. A temperature gradient was built up in the electrophoresis direction from 39 to 48 °C. After the run, gels were silver-stained (Sanguinetti *et al*., 1994).

Fatty acid methyl ester (FAME) analysis. A loopful of well-grown cells was harvested, and FAMEs were prepared, separated by GLC and identified using the Microbial Identification System software package version 3.9 as described by Vandamme *et al*. (1992).

RESULTS

TGGE analysis

Analysis of 16S rDNA sequences from 115 different coryneform bacterial strains was performed by partial amplification of the 16S rRNA genes (positions 968–1401) by PCR with bacteria-specific primers. Sequence-specific separation of amplicons by TGGE and visualization by silver staining allowed the comparison of signals (Figs 1–3). Pure cultures usually produce a single band in TGGE analysis. In Fig. 1, all the different TGGE signals obtained in this study are compiled on one gel. As summarized in Table 1, the left-hand side of Fig. 1 represents PCR products of all authentic strains of the eight genera. These signals are considered to be characteristic for each genus because they are produced by at least one authentic type strain of a species in the genus. Signals from lanes 1–8 are considered to be dissident or atypical because they were defined as unique signals within a genus, not represented by an authentic type strain (Table 1). Lanes 3 and 4 contain the type strains of *Arthrobacter picolinophilus* and *Arthrobacter siderocapsulatus*, respectively. However, both taxa are considered to be dissident, because it was further demonstrated that strains of these taxa are generically misclassified (see below). In the course of our studies we observed the appearance of PCR side-products, resulting in faint, additional bands on TGGE. They could be visualized

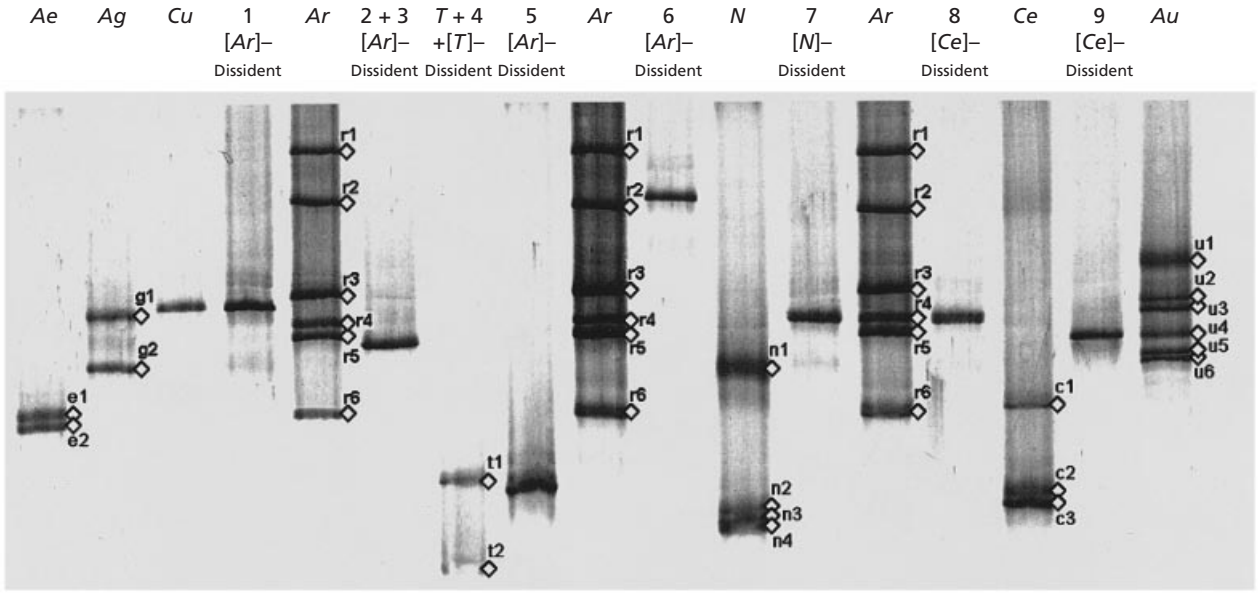

Fig. 1. Silver-stained gel showing TGGE signals of the strains investigated (6 μl 10-fold diluted product per lane). All authentic signals for the studied genera are compiled in the lanes labelled with the genus abbreviation (*Ae*, *Aeromicrobium*; *Ag*, *Agromyces*; *Cu*, *Curtobacterium*; *T*, *Terrabacter* (signal t1); *N*, *Nocardioides*; *Ar*, *Arthrobacter*; *Ce*, *Cellulomonas*; and *Au*, *Aureobacterium*). There are several bands per lane because several PCR products were loaded (labels refer to Table 1). Where several strains form the same signal only one representative is loaded, as indicated in Table 1 (bold strains are loaded on this gel). The nine dissident signals are numbered as follows: 1, *Arthrobacter ilicis* LMG 7255; 2+3, *Arthrobacter siderocapsulatus* LMG 16118, LMG 16206^T; *T*+4, *T. tumescens* LMG 16133 (signal t2); 5, *Ar. picolinophilus* LMG 16262^T; 6, *Arthrobacter globiformis* LMG 3820; 7, *Nocardioides simplex* LMG 3810; 8, *Cellulomonas fimi* LMG 16132; 9, *Cellulomonas turbata* LMG 16346 (see also Fig. 2).

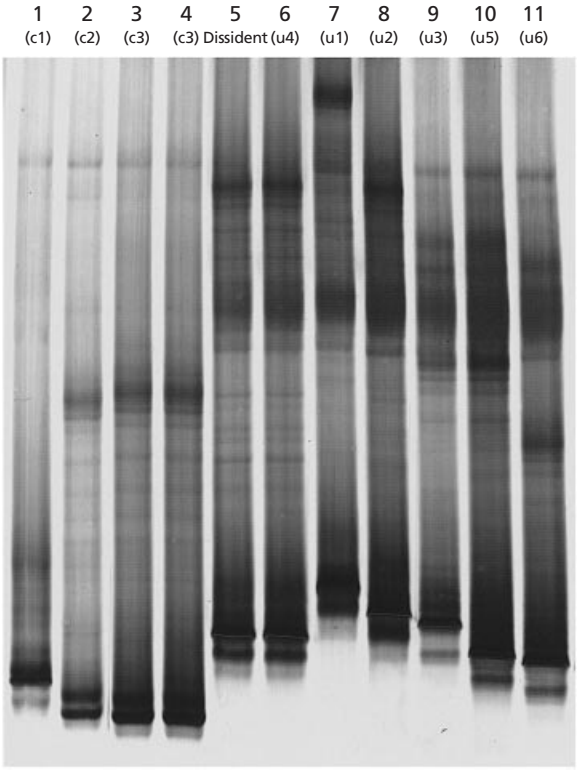

Fig. 2. TGGE signals and genus-specific side-product patterns (6 μl PCR product per lane) of four authentic *Cellulomonas* strains (lanes: 1, *Cellulomonas cellulans* LMG 16221^T; 2, *Cellulomonas fimi* LMG 16345^T; 3, *Cellulomonas turbata* LMG 16191; 4, *Cellulomonas turbata* LMG 4072^T); six authentic *Aureobacterium* strains (lanes: 6, *Aureobacterium luteolum* LMG 16207^T; 7, *Aureobacterium trichothecenolyticum* LMG 16696^T; 8, *Aureobacterium testaceum* LMG 16344^T; 9, *Aureobacterium terrae* LMG 16471^T; 10, *Aureobacterium arabinogalactanolyticum* LMG 16469^T; and 11, *Aureobacterium barkeri* LMG 16341^T) and one misclassified strain *Cellulomonas turbata* LMG 16346 (lane 5).

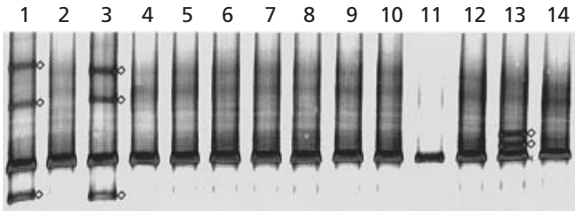

Fig. 3. TGGE signals (6 μl PCR product per lane) of 14 investigated *Curtobacterium* strains. Lanes: 1, *Curtobacterium albidum* LMG 8759^T; 2, *Curtobacterium citreum* LMG 16240; 3, *Curtobacterium citreum* LMG 8786^T; 4, *Curtobacterium flaccumfaciens* LMG 3596; 5, *Curtobacterium flaccumfaciens* LMG 7241; 6, *Curtobacterium flaccumfaciens* LMG 3645^T; 7, *Curtobacterium flaccumfaciens* LMG 3650; 8, *Curtobacterium flaccumfaciens* LMG 3702; 9, *Curtobacterium flaccumfaciens* LMG 3704; 10, *Curtobacterium flaccumfaciens* LMG 3710; 11, *Curtobacterium flaccumfaciens* LMG 3715; 12, *Curtobacterium luteum* LMG 8787^T; 13, *Curtobacterium pusillum* LMG 8788^T; 14, *Curtobacterium pusillum*, LMG 8812. 16S rRNA sequence heterogeneities are revealed and marked in lanes 1, 3 and 13.

by loading 6 μl original PCR product (Figs 2 and 3) instead of 6 μl 10-fold diluted PCR product (Fig. 1).

Signals of the type strains of both *Aeromicrobium* species, *Aeromicrobium erythreum* and *Aeromicrobium fastidiosum*, could be separated on TGGE, and their migration distances are quite similar (Fig. 1, lane *Ae*).

Eight strains of three different *Agromyces* species were investigated (Table 1). PCR products of *Agromyces cerinus* and *Agromyces fucosus* migrated to the same position. *Agromyces ramosus* could be clearly separated from the other species (Fig. 1, lane *Ag*).

The 45 investigated *Arthrobacter* strains, assigned to 21 different species, showed 10 different band positions (Table 1; Fig. 1, lanes *Ar* and 1–4). Three of the signals in lane *Ar* each seem to be characteristic for a single species, i.e. *Arthrobacter crystallopoietes* (band r2, one strain), *Arthrobacter sulfureus* (band r5, three strains) and *Arthrobacter atrocyaneus* (band r6, one strain). Bands r1, r3 and r4 were formed by strains of various species. The same band position (band r1) was obtained for two strains of *Arthrobacter citreus* and one strain of *Arthrobacter viscosus* (not the type strain). Band r3 contained signals of one strain of *Arthrobacter citreus* (not the type strain) and all strains studied of the species *Arthrobacter mysorens*, *Arthrobacter nicotianae*, *Arthrobacter protophormiae* and *Arthrobacter uratoxydans*. Band r4 comprised the signals of at least the type strain, and in some cases one or two additional strains, of the species *Arthrobacter aurescens*, *Arthrobacter globiformis*, *Arthrobacter histidinolovorans*, *Arthrobacter ilicis*, *Arthrobacter nicotinovorans*, *Arthrobacter oxydans*, *Arthrobacter pascens*, *Arthrobacter polychromogenes*, *Arthrobacter ramosus* and *Arthrobacter ureafaciens*. Four different bands were produced by the dissident strains *Arthrobacter globiformis* LMG 3820, *Arthrobacter ilicis* LMG 7255, *Arthrobacter picolinophilus* LMG 16262^T and two investigated strains of *Arthrobacter siderocapsulatus* (Fig. 1, lanes 1, 2+3, 5 and 6).

The 19 strains of 11 different *Aureobacterium* species formed six different bands (Table 1; Fig. 1, lane *Au*). As all positions represented at least one type strain, all signals are considered to be characteristic for the genus. Separate signals are observed for the species *Aureobacterium trichothecenolyticum* (band u1), *Aureobacterium testaceum* (band u2), *Aureobacterium arabinogalactanolyticum* (band u5) and *Aureobacterium barkeri* (band u6). One band (u3) is formed by all investigated strains of *Aureobacterium keratanolyticum*, *Aureobacterium liquefaciens*, *Aureobacterium luteolum*, *Aureobacterium saperdae* and the type strain of *Aureobacterium schleiferi*, and another band (u4) is produced by both investigated strains of *Aureobacterium esteraromaticum* and *Aureobacterium terrae* and one strain (not the type strain) of *Aureobacterium schleiferi*. The genus *Aureobacterium* apparently forms a very clear, specific side-product band pattern (Fig. 2) that seems to be constant for all strains studied.

Table 2. FAME identification of strains with atypical TGGE signals using the commercial MIS database (version 3.9)

Species name as received	Strain no.	FAME identification (similarity of first choice)
Arthrobacter globiformis	LMG 3820	*Nocardia asteroides* (0·068)
Arthrobacter ilicis	LMG 7255	*Curtobacterium flaccumfaciens* pv. *oortii* (0·291)
Arthrobacter picolinophilus	LMG 16262^T	*Rhodococcus erythropolis* (0·657)
Arthrobacter siderocapsulatus	LMG 16118	*Pseudomonas putida* biotype A (0·846)
Arthrobacter siderocapsulatus	LMG 16206^T	*Pseudomonas putida* biotype A (0·650)
Cellulomonas fimi	LMG 16132	*Arthrobacter pascens* (0·476)
Cellulomonas turbata	LMG 16346	*Aureobacterium esteroaromaticum* (0·845)
Nocardioides simplex	LMG 3810	*Arthrobacter oxydans* (0·524)
Terrabacter tumescens	LMG 16133	*Rhodococcus equi* (0·586)

Five bands were formed by 17 strains of eight different *Cellulomonas* species (Table 1). Three of them were formed by strains of at least two species, including their type strain, and are considered to be characteristic for the genus (Fig. 1, lane *Ce*). Band c1 was formed by strains of *Cellulomonas cellulans* and *Cellulomonas uda*, band c2 contained signals of two strains of *Cellulomonas fimi* (including the type strain), both studied strains of *Cellulomonas gelida* and the type strain of *Cellulomonas flavigena*, and band c3 was formed by the species *Cellulomonas biazotae*, *Cellulomonas cellasea* and two of the three strains of *Cellulomonas turbata* (including the type strain). Two other bands were formed by single dissident strains: *Cellulomonas turbata* LMG 16346 (Fig. 1, lane 9) and *Cellulomonas fimi* LMG 16132 (Fig. 1, lane 8).

All 14 investigated *Curtobacterium* strains gave the same main TGGE band (Fig. 1, lane *Cu*) and also the same side-product pattern type (Fig. 3). This might indicate that the five different species contain an (almost) identical 16S rRNA sequence. Three strains, *Curtobacterium albidum* LMG 8759^T, *Curtobacterium citreum* LMG 8786^T and *Curtobacterium pusillum* LMG 8788^T, are not presented in Fig. 1 because they showed two or three more bands, indicating 16S rRNA gene heterogeneity (Fig. 3, lanes 1, 3 and 13).

Eight strains of four *Nocardioides* species were investigated. Each of the four bands that characterize the genus was formed by a single species, i.e. *Nocardioides jensenii*, *Nocardioides simplex*, *Nocardioides albus* and *Nocardioides luteus* (Fig. 1, lane *N*; Table 1). In addition, the genus *Nocardioides* formed a prominent, specific side-product band pattern (data not shown). The dissident strain *Nocardioides simplex* LMG 3810 gave a separate signal (Fig. 1, lane 7).

Two strains assigned to the species *Terrabacter tumescens* were investigated. Different bands were formed by the type strain (Fig. 1, lane $T+4$, band t1) and one aberrant strain (Fig. 1, lane $T+4$, band t2).

Classification of dissident strains

All strains of the present study were also investigated by cellular fatty acid analysis (data not shown). This revealed that only strains producing dissident TGGE signals phylogenetically belong to a different genus from the one in which they are currently classified. Profiles of dissident strains were identified with the commercial MIS database (version 3.9). The results are summarized in Table 2. Four of the *Arthrobacter* strains were classified by FAME analysis in different genera (Table 2). *Arthrobacter globiformis* LMG 3820 was identified with a low similarity as *Nocardia asteroides*. *Arthrobacter ilicis* LMG 7255 was identified as *Curtobacterium flaccumfaciens*. The genus *Curtobacterium* was included in our study, and it was indeed confirmed that *Arthrobacter ilicis* LMG 7255 produced a signal characteristic for the genus *Curtobacterium* (Fig. 1, lane 1, shows *Arthrobacter ilicis* LMG 7255; directly next to it on the left-hand side in lane *Cu*). *Arthrobacter picolinophilus* LMG 16262^T was identified with a high identification score as *Rhodococcus erythropolis* and confirmed the data of Koch *et al.* (1995a), who reclassified the organism in the latter taxon. Both investigated strains of *Arthrobacter siderocapsulatus* (lane 4) were identified as *Pseudomonas putida* using FAME analysis. Concerning aberrant *Cellulomonas* strains, *Cellulomonas fimi*, LMG 16132 was identified by FAME analysis as *Arthrobacter pascens* and *Cellulomonas turbata* LMG 16346 as *Aureobacterium esteroaromaticum* (Table 2). In each case, the strain identifications revealed by FAME analysis were confirmed by TGGE analysis; the band positions were r4 and u4, respectively (Fig. 1; Table 1). Additionally, *Cellulomonas turbata* LMG 16346 demonstrated a side-band pattern identical to that found for *Aureobacterium* reference strains (Fig. 2). One dissident strain of the genus *Nocardioides*, *Nocardioides simplex* LMG 3810, was identified as *Arthrobacter oxydans* using FAME analysis and indeed produced a TGGE signal at the same migration distance as band r4 of *Arthrobacter* (Fig. 1, lane 7, shows *Nocardioides simplex* LMG 3810; directly next to it on the right-hand side lane *Ar*). Strain *Terrabacter tumescens* LMG 16133, identified as *Rhodococcus equi* using FAME analysis, produced a different signal (Fig. 1, lane $T+4$, band t2) from that of the type strain (Fig. 1, lane $T+4$, band t1).

DISCUSSION

Taxonomic significance of TGGE analysis

The present study demonstrates that TGGE may be a convenient and alternative screening method in polyphasic taxonomy that allows reliable taxonomic conclusions to be drawn. The resolution limit of this technique appears to be somewhere between the genus and species level. The 16S rDNA fragment amplified here (primers U968-GC and L1401) could be separated by only 1 bp sequence difference (Nübel *et al*., 1996). Depending on the genus investigated, signals were species-specific (e.g. *Nocardioides*), specific for several species of the genus (e.g. *Cellulomonas*) or genus-specific (e.g. *Curtobacterium*). In all cases, aberrant TGGE signals of strains of a single species indicated misclassifications.

When comparing TGGE banding patterns with available 16S rRNA sequence data of reference strains, some preliminary conclusions concerning the applicability of TGGE in phylogenetic studies may be drawn. Within the genus *Arthrobacter*, the TGGE signals r1–r6 (Fig. 1, lanes *Ar*) were created by strains of one or more authentic species of the genus and were compared with the phylogenetic distances of the 16S rRNA sequences of these species (Koch *et al*., 1995b). In both approaches, the species *Arthrobacter atrocyaneus* and *Arthrobacter crystallopoietes* occupy a clearly separate position. Furthermore, *Arthrobacter nicotianae*, *Arthrobacter protophormiae* and *Arthrobacter uratoxydans*, which constitute band r3, are all classified into rRNA group II (species with peptidoglycan type A4α), and species *Arthrobacter aurescens*, *Arthrobacter globiformis*, *Arthrobacter histidinolovorans*, *Arthrobacter ilicis*, *Arthrobacter nicotinovorans*, *Arthrobacter oxydans*, *Arthrobacter polychromogenes*, *Arthrobacter ramosus* and *Arthrobacter ureafaciens* are all classified in rRNA group I (peptidoglycan type A3α). When comparing sequencing data of *Cellulomonas* species (Funke *et al*., 1995) with data from the present study, less analogy is observed. Species that are phylogenetically more closely related do not always exhibit a similar TGGE signal. Within the genus *Agromyces*, a more distant relationship of the species *Agromyces ramosus* is also supported by the 16S rRNA phylogeny (Suzuki *et al*., 1996). However, it should be remembered that, with TGGE, the migration distance of amplicons, i.e. the distance between two different bands, is not correlated with the degree of nucleobase difference between the sequences. The migration of amplicons in TGGE depends on the melting behaviour of the molecule, and a nucleobase exchange might have quite different effects, depending on its position within the sequence.

The application of TGGE for taxonomic characterization is most effective in the fast screening of large numbers of pure cultures. For example, dozens of environmental isolates identified by classic microscopical and biochemical analyses as *Arthrobacter* species could be subjected to further analysis. A typical procedure would be to reactivate the strains by transferring cell material from frozen or lyophilized storage samples to fresh culture media. As bacterial cells multiply exponentially, this is a crucial step. For instance, one particular strain may have suffered during sample preparation or storage, resulting in poor viability and leaving only a few surviving cells to grow with an extended lag phase. In this situation, fast-growing contaminants might easily outcompete the original strains during culture. As an increasing number of strains are investigated, the 'human factor' also becomes more and more important. During handling, samples could be confused and the pressure to increase the speed of even 'routine' processing might result in less efficient sterilization of equipment. TGGE is helpful in this respect in that it can readily establish the authenticity of batch cultures, both by confirming the original identification obtained by classical methods and by enabling subsequent cultivation procedures that might be affected by contamination to be monitored. After DNA extraction from batch culture samples and subsequent PCR, the strains could be compared with the six *Arthrobacter* signals identified in this study (Fig. 1, lane *Ar*). Mismatches would be considered as suspicious, possibly caused by a wrong previous identification or contamination of the original strain. The latter could be verified by comparison with a PCR product from the original storage sample. However, it would not be possible to exclude the possibility that some novel members of the genus *Arthrobacter* might result in a seventh (or more) band position. The aberrant signals on TGGE can reliably indicate bias during strain processing or novel sequences in the culture collection concerned.

16S rRNA gene heterogeneity

TGGE is the optimal tool to unveil 16S rRNA gene heterogeneity. Of the 115 strains investigated, additional strong signals were detected in three *Curtobacterium* strains, indicating 16S rRNA gene heterogeneity (Fig. 3, lanes 1, 3 and 13). Amplicons from pure cultures show one band if all copies of the 16S rDNA in the bacterial genome have an identical sequence. The appearance of more than one strong band indicates heterogeneity of 16S rRNA genes in the genome. Although only about the last third of the 16S rRNA (including variable regions V6–V8) was studied, our screens of 115 strains are the most comprehensive performed to date. The finding of only three positive *Curtobacterium* strains indicates that 16S rRNA sequence heterogeneity is not very common within the coryneform bacteria. This phenomenon has been reported previously for *Paenibacillus polymyxa* (Nübel *et al*., 1996). Therefore, studies involving *Curtobacterium* or *Paenibacillus* strains should consider possible 16S rRNA gene heterogeneity, which might confuse 16S rRNA analysis.

It should be remembered that additional bands can also represent contamination of the original culture. In the case of gene heterogeneity, bands are often arranged close to each other (Nübel *et al*., 1996), at more or less constant intervals ('ladder-like'). Additional bands of identical intensity or whose intensity is identical to that of the 'main band' also indicate gene heterogeneity (sequences that are present in the same copy number yield products of identical intensity). Additional bands that vary greatly in their intensity are more likely to represent contamination of the original culture than 16S rRNA sequence heterogeneity. Also, signals due to gene heterogeneity within a pure culture will remain stable during different subculture, whereas a mixed culture will probably shift its composition and consequently change its TGGE band pattern upon subculturing.

In addition to culture-specific contamination and species-specific signal heterogeneity, a third group of apparently genus-specific additional bands might be observed. We found that the main bands, attributable to the 16S rDNA amplicons, were often accompanied by faint, but reproducible, band patterns of variable complexity (Figs 2 and 3). These became increasingly visible when the gels were loaded with increasing amounts of PCR product. Most likely these signals represent sequence-specific DNA polymerization artefacts. Those side-products showed genus-specific patterns and varied between different species only by position. Side-product formation could be an additional indicator of sequence identity. One example has been demonstrated: *Cellulomonas* strain LMG 16346 produced a banding pattern that was similar to that of the genus *Aureobacterium*, an identification that was confirmed using other approaches (see above; Fig. 2, lanes 4 and 5).

Conclusions

TGGE and the similar technique DGGE are attractive methods for the analysis of mixed cultures or environmental bacterial communities. After separation of the 16S rRNA sequence mixture in electrophoretic fingerprints (Muyzer *et al*., 1993), the single signals in such a band pattern could be identified by comparison with pure culture signals (Rölleke *et al*., 1996) or clone libraries (Felske *et al*., 1997). The present study shows that this technique could also be used to assess the identity of pure cultures. The resolution limit for the taxonomic TGGE analysis appears to be between the genus and species level. Dissident signals reliably revealed misclassified strains and by chance also pointed to the real identity of the misfits. TGGE is also a fast and powerful method to reveal 16S rDNA gene heterogeneity in bacterial strains. This is especially important for subsequent sequencing of the 16S rRNA genes. Comparative TGGE has been demonstrated to be a useful tool for taxonomic studies, particularly where large sets of bacterial cultures are screened.

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