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Rapid identification of filamentous actinomycetes to the genus level using genus-specific 16S rRNA gene restriction fragment patterns

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A rapid method for identifying filamentous actinomycete genera was developed based on 16S rRNA gene restriction fragment patterns. The patterns were generated by using specific restriction endonucleases to perform *in silico* digestions on the 16S rRNA gene sequences of all validly published filamentous actinomycete species. The method was applied to identifying actinomycete isolates from soil. Amplified 16S rDNA of soil actinomycetes was restricted with selected endonucleases and electrophoresed on agarose gels. The restriction fragment patterns of the unknown isolates were easily compared to the established patterns. Significantly, the genus *Streptomyces* could be differentiated from all other actinomycete genera by using only four restriction endonucleases, *Sau*3AI, *Asn*I, *Kpn*I and *Sph*I. This could be achieved in a time period of as little as a week, following PCR-template DNA isolation by a simple method. The identification method allowed unknown, non-*Streptomyces* soil isolates to be identified to a genus or small subgroup of genera. The genera in these subgroups could, in some cases, be distinguished by virtue of colony-morphology differences.

INTRODUCTION

Actinomycetes are widely distributed in terrestrial environments and have long been a source of commercially useful enzymes and therapeutically useful bioactive molecules. Since molecular structure determines molecular function, and molecular diversity underpins the diversity of life on Earth, it follows that identifying biological diversity increases the chances of identifying novel molecules. In the case of bacteria, identifying new species and genera increases the chances that any bioactive molecules produced by such organisms are unknown to science (Lazzarini *et al.*, 2000).

The traditional methods used for the identification of the aerobic filamentous actinomycetes are laborious, time-consuming and often require a series of specialized tests (Steingrube *et al.*, 1995b, 1997; Wilson *et al.*, 1998; Harvey *et al.*, 2001). Chemical criteria, such as the isomer of diaminopimelic acid (DAP) present in the cell wall and the diagnostic sugar(s) present in the whole-cell hydrolysate, have been used to separate the actinomycete genera into broad chemotaxonomic groups. However, determination of these characteristics is time-consuming and, in most cases, cannot identify an isolate to a single genus (Lechevalier, 1989).

PCR-based methods have provided a rapid and accurate way to identify bacteria (Gurtler *et al.*, 1991; Kohler *et al.*, 1991; Beyazova & Lechevalier, 1993; Telenti *et al.*, 1993; Soini *et al.*, 1994; Mehling *et al.*, 1995; Steingrube *et al.*, 1995a, 1997; Wilson *et al.*, 1998; Laurent *et al.*, 1999). In particular, amplified rDNA restriction analysis (ARDRA) has proved to be very useful (Harvey *et al.*, 2001; Alves *et al.*, 2002).

ARDRA has been shown to be useful in differentiating between bacterial species within a genus, for example, *Clostridium* (Gurtler *et al.*, 1991), and in differentiating bacterial strains within a species, for example, *Lactococcus* (Kohler *et al.*, 1991). It has also been shown to be useful in identifying several medically important species of aerobic actinomycetes belonging to the genera *Actinomadura*, *Gordonia*, *Nocardia*, *Rhodococcus*, *Saccharomonospora*, *Saccharopolyspora*, *Streptomyces* and *Tsukamurella* (Steingrube *et al.*, 1997; Wilson *et al.*, 1998; Harvey *et al.*, 2001; Laurent *et al.*, 1999).

By conventional isolation methods, members of the genus *Streptomyces* comprise more than 95 % of the filamentous actinomycete population in soil (Lacey, 1973; Elander, 1987). The streptomycetes produce more antibiotics than any other genus of bacteria and, therefore, have been heavily

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Abbreviation: DAP, diaminopimelic acid.

An interactive Microsoft PowerPoint (version 5.0) presentation of the *in silico* procedure described in this article is available in IJSEM Online.

exploited as a source of novel antimicrobial agents (Watve *et al.*, 2001). The probability of isolating known species of *Streptomyces* from the environment is thus great and the probability of isolating novel antibacterial molecules from such species is very low. The isolation of the rarer, non-*Streptomyces* actinomycetes greatly increases the probability of isolating novel antibacterial molecules (Lazzarini *et al.*, 2000). Therefore, a rapid method to distinguish streptomycetes from other actinomycetes and to identify the non-streptomycetes to the genus level would be extremely useful. This would be of particular value in discerning between streptomycetes and non-streptomycetes, such as *Actinomadura*, *Nocardia* and *Nocardiopsis*, whose colonies may be morphologically similar on agar plates.

We have developed a rapid method to identify filamentous actinomycetes to the genus level in less than a week, following DNA isolation from a pure culture. The method was tested on unknown actinomycetes isolated from soil and can be pursued at moderate cost in any laboratory possessing simple molecular-biology equipment and reagents.

METHODS

Data collection. A database containing 16S rRNA gene sequences of all validly published filamentous actinomycetes (Euzéby, 2002) was compiled from GenBank (http://www.ncbi.nlm.nih.gov). All sequences used were longer than 1400 bp. The sequences were grouped by genus according to Stackebrandt *et al.* (1997) and the NCBI Taxonomy Browser (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi).

***In silico* restriction endonuclease digestions.** Thirty-eight restriction endonucleases were selected. All conformed to the dual requirements of being commercially available and recognizing a specific sequence (4, 6 or 8 bp) in which every nucleotide position is defined. *In silico* digestions were performed on each sequence using DNAMAN (version 4.13; Lynnon Biosoft). The *in silico* analysis allowed for the selection of specific restriction endonucleases that would allow actinomycete genera to be distinguished.

Organisms and culture conditions. Soil actinomycetes were isolated on Czapek Solution Agar (Atlas, 1993), Middlebrook 7H9 Agar (Difco Laboratories) or *Streptomyces* General Defined Medium [GM (800 ml): 0·17 g $Na_2HPO_4.2H_2O$, 0·14 g KH_2PO_4, 0·05 g $MgSO_4.7H_2O$, 0·01 g $FeSO_4.7H_2O$; pH 7·0; autoclaved at 15 p.s.i. (103·5 kPa) for 15 min; after cooling, 100 ml of 100 mM glucose, 50 ml of 50 mM $(NH_4)_2SO_4$ and 50 ml of 50 mM L-glutamic acid, sodium salt, were added)]. All media contained cycloheximide at 50 µg ml^{-1}. Cultures were incubated at 30 °C for 14–28 days. Colony selection was based on the colour of aerial and substrate mycelium, differences in morphology and rate of growth.

DNA extraction. Actinomycete strains were grown in 10 ml International *Streptomyces* Project Medium 1 (ISP 1) (Shirling & Gottlieb, 1966) with agitation at 30 °C for 18–24 h and examined by Gram stain. Cells (4 ml) were harvested by centrifugation (7500 ***g*** for 2 min), washed once with 500 µl of 10 mM Tris-HCl/1 mM EDTA (TE) buffer (pH 7·7) and resuspended in 500 µl TE buffer (pH 7·7). The samples were heated in boiling water for 10 min, allowed to cool for 5 min and centrifuged (7500 ***g*** for 3 min). The supernatant (300 µl) was transferred to a clean tube and stored at 4 °C. If melanin or other pigments were produced during growth in ISP 1, cultures were grown in Middlebrook 7H9 broth, as these pigments interfered with the PCR.

PCR amplification. PCR was carried out in 50 µl volumes containing 2 mM $MgCl_2$, 2 U *Taq* polymerase (JMR Holdings, USA), 150 µM of each dNTP, 0·5 µM of each primer and 2 µl template DNA. Primer F1 (5′-AGAGTTTGATCITGGCTCAG-3′; I = inosine) and primer R5 (5′-ACGGITACCTTGTTACGACTT-3′) were modified from primers fD1 and rP2, respectively, of Weisburg *et al.* (1991). Primer F1 binds to base positions 7–26 and primer R5 to base positions 1496–1476 of the 16S rRNA gene of *Streptomyces ambofaciens* ATCC 23877^T (*rrnD* operon; GenBank accession no. M27245). The primers were used to amplify nearly full-length 16S rDNA sequences. The PCR programme used was an initial denaturation (96 °C for 2 min), 30 cycles of denaturation (96 °C for 45 s), annealing (56 °C for 30 s) and extension (72 °C for 2 min), and a final extension (72 °C for 5 min). The PCR products were electrophoresed on 1 % agarose gels, containing ethidium bromide (10 µg ml^{-1}), to ensure that a fragment of the correct size had been amplified.

Restriction endonuclease digestions and analysis. PCR-amplified DNA for *Sau*3AI digestion was purified using the QIAquick PCR Purification Kit (Qiagen). No pre-treatment of the DNA was required for the other restriction endonucleases. Restriction digestions were incubated at 37 °C for 3–4 h. Samples were electrophoresed on 1·5 % agarose gels containing ethidium bromide (10 µg ml^{-1}). The restriction fragment patterns were compared manually with those from the *in silico* restriction endonuclease digestions.

RESULTS AND DISCUSSION

The rapid identification method

Four hundred and four (404) validly published filamentous actinomycete 16S rRNA gene sequences were downloaded from the GenBank database. Based on the *in silico* results, a set of 13 restriction endonucleases was selected empirically for use in the design of the rapid genus identification method. All sequence sites recognized by these endonucleases occur outside the α, β and γ variable regions of *Streptomyces* 16S rRNA genes (Anderson & Wellington, 2001).

*Sau*3AI was the first restriction endonuclease used, as it divided the filamentous actinomycetes into three major groups (Fig. 1). Most genera were placed in a single *Sau*3AI group. However, the genera *Gordonia*, *Microbispora*, *Nocardia* and *Nonomuraea* were represented in Groups 1 and 3; *Nocardiopsis*, *Saccharomonospora*, *Saccharopolyspora*, *Streptosporangium* and *Thermomonospora* were represented in Groups 2 and 3. Members of the genera *Nocardioides* and *Pseudonocardia* were distributed across all three groups. The distribution of genera across two or all three *Sau*3AI groups arose as a result of mutations that created a new *Sau*3AI recognition site or destroyed a genus-characteristic *Sau*3AI site. Whether this reflects true sequence differences or sequencing error is not known. The *Sau*3AI group in which the majority of species of a genus are placed is considered characteristic of that genus.

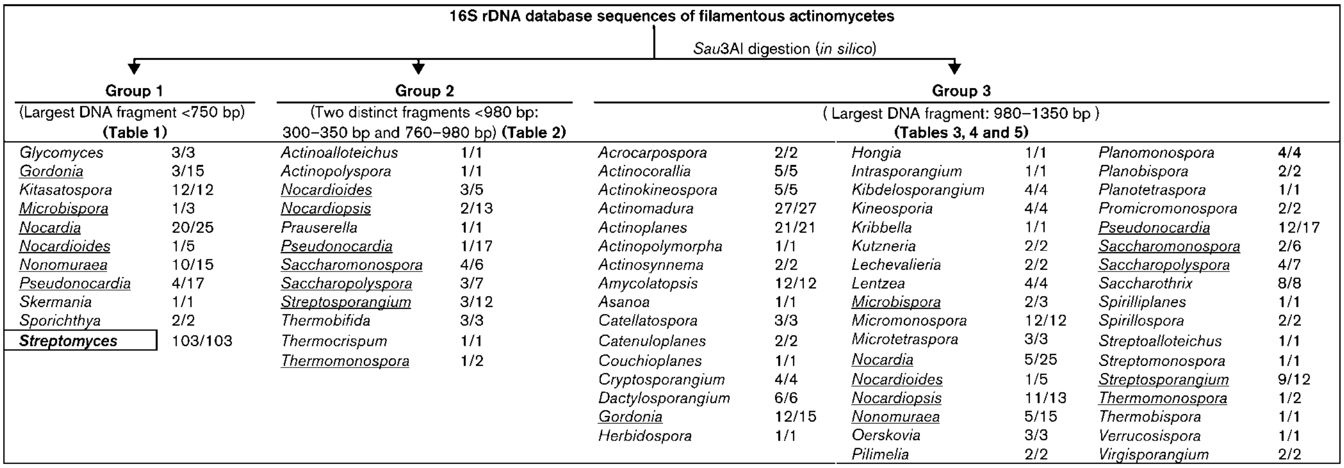

Fig. 1. Chart showing the three groups of actinomycete genera formed after the *Sau*3AI *in silico* digestion. The numbers after each genus indicate how many species of that genus exhibited that banding pattern. For example, in Group 1, three *Gordonia* species, out of 15 analysed, produced a large band of less than 750 bp. Underlined names indicate genera represented in more than one *Sau*3AI group.

A series of dichotomous keys was developed to identify rapidly the genera within the three *Sau*3AI groups. Table 1 allows the identification of the actinomycete genera in *Sau*3AI Group 1. An unknown *Streptomyces* isolate could be identified by the sequential use of four restriction endonucleases: *Sau*3AI, *Asn*I, *Kpn*I and *Sph*I (Table 1). Although the genus *Streptomyces* could not be differentiated from the extremely rare genus *Sporichthya* based on the 16S rRNA gene restriction analysis (Table 1, 4b), the morphology of *Sporichthya* clearly distinguishes it from the genus *Streptomyces* (Tamura *et al.*, 1999). The genus *Skermania* and some members of the genera *Gordonia* and *Nocardia* formed a subgroup (Table 1, 6b), which could not be resolved. The genus *Nocardia* belongs to the family

Table 1. Identification of the genera in *Sau*3AI Group 1

The number in parentheses after each genus name indicates how many species of that genus follow that specific pattern. In most cases, DNA fragments smaller than 300 bp were not considered.

Digested with	Relevant feature(s)	Go to:
(1a) *Sau*3AI	Largest DNA fragment <750 bp *Sau*3AI (Group 1)	2
(1b) *Sau*3AI	Largest DNA fragment >950 bp *Sau*3AI (Groups 2 and 3)	Table 2
(2a) *Asn*I	Not cut	3
(2b) *Asn*I	Cut (470–590 bp and 900–960 bp)	6
(3a) *Kpn*I	Cut (410–470 bp and 1000–1100 bp)	4
(3b) *Kpn*I	Not cut	8
(4a) *Sph*I	Cut	5
(4b) *Sph*I	Not cut: ***Streptomyces*** (56/103); *Sporichthya* (2/2)	
(5a) *Sph*I	Cut (170–260 bp and 1010–1350 bp): *Kitasatospora* (12/12)	
(5b) *Sph*I	Cut (390–450 bp and 1050–1110 bp): ***Streptomyces*** (47/103)	
(6a) *Sca*I	Not cut	7
(6b) *Sca*I	Cut (570–630 bp and 850–900 bp): *Gordonia* (3/15); *Nocardia* (20/25); *Skermania* (1/1)	
(7a) *Sph*I	Cut (280–310 bp and 890–920 bp): *Glycomyces* (3/3); *Nocardioides* (1/5)	
(7b) *Sph*I	Not cut: *Pseudonocardia* (4/17)	
(8a) *Pst*I	Cut (400–430 bp and 1000–1050 bp): *Nonomuraea* (10/15)	
(8b) *Pst*I	Not cut: *Microbispora* (1/3)	

Table 2. Identification of the genera in *Sau*3AI Group 2

The number in parentheses after each genus name indicates how many species of that genus follow that specific pattern. In most cases, DNA fragments smaller than 300 bp were not considered.

Digested with	Relevant feature(s)	Go to:
(1a) *Sau*3AI	Cut (300–350 bp and 760–980 bp): Group 2	2
(1b) *Sau*3AI	Cut (990–1300 bp): Group 3	Table 3
(2a) *Asn*I	Cut (470–590 bp and 900–960 bp)	3
(2b) *Asn*I	Not cut	6
(3a) *Kpn*I	Cut (410–470 bp and 1000–1100 bp)	4
(3b) *Kpn*I	Not cut	5
(4a) *Hin*dIII	Cut (440 bp and 1070 bp): *Pseudonocardia* (1/17)	
(4b) *Hin*dIII	Not cut: *Nocardioides* (1/5); *Prauserella* (1/1); *Saccharomonospora* (4/6)	
(5a) *Sph*I	Cut (270–280 bp and 900 bp): *Nocardioides* (2/5)	
(5b) *Sph*I	Not cut: *Actinoalloteichus* (1/1); *Actinopolyspora* (1/1) *Saccharopolyspora* (3/7); *Thermocrispum* (1/1)	
(6a) *Hin*dIII	Cut (400–550 bp and 900–920 bp): *Streptosporangium* (3/12)	
(6b) *Hin*dIII	Not cut	7
(7a) *Sph*I	Cut (595–615 bp and 860–900 bp): *Nocardiopsis* (2/13)	
(7b) *Sph*I	Not cut	8
(8a) *Pst*I	Not cut: *Thermobifida* (3/3)	
(8b) *Pst*I	Cut (415–460 bp and 995–1100 bp): *Thermomonospora* (1/2)	

Table 3. Identification of the genera in *Sau*3AI Group 3

The 16S rDNA of these genera is cut by *Asn*I **and** *Sph*I.

Digested with	Relevant feature(s)	Go to:
(1a) *Asn*I	Cut (470–590 bp and 900–960 bp)	2
(1b) *Asn*I	Not cut	Table 5
(2a) *Sph*I	Cut (280–310 bp and 890–920 bp)	3
(2b) *Sph*I	Not cut	Table 4
(3a) *Sna*BI	Not cut	4
(3b) *Sna*BI	Cut (470–510 bp and 970–1000 bp)	6
(4a) *Sal*I	Not cut	5
(4b) *Sal*I	Cut (540–560 bp and 900–1040 bp): *Actinoplanes* (7/21); *Micromonospora* (12/12); *Couchioplanes* (1/1); *Pilimelia* (2/2); *Spirilliplanes* (1/1); *Verrucosispora* (1/1); *Virgisporangium* (2/2)	
(5a) *Pvu*II	Cut (560 bp and 970–980 bp): *Dactylosporangium* (6/6)	
(5b) *Pvu*II	Not cut: *Actinoplanes* (14/21); *Asanoa* (1/1); *Catellatospora* (3/3); *Catenuloplanes* (2/2)	
(6a) *Sal*I	Cut (235–265 bp and 1215–1245 bp)	7
(6b) *Sal*I	Not cut	9
(7a) *Age*I	Cut (660–680 bp and 910–930 bp)	8
(7b) *Age*I	Not cut: *Promicromonospora* (2/2)	
(8a) *Sst*I	Cut (585 bp and 930 bp): *Hongia* (1/1)	
(8b) *Sst*I	Not cut: *Kribbella* (1/1); *Nocardioides* (1/5)	
(9a) *Age*I	Cut	10
(9b) *Age*I	Not cut: *Oerskovia* (3/3); *Saccharopolyspora* (1/7)	
(10a) *Age*I	Produces two bands between 660 bp and 910 bp	11
(10b) *Age*I	Cut (230 bp and 1240 bp): *Intrasporangium* (1/1)	
(11a) *Age*I	670–680 bp and 750–830 bp: *Kineosporia* (4/4)	
(11b) *Age*I	660 bp and 910 bp: *Nocardioides* (1/5)	

Nocardiaceae and the genera *Gordonia* and *Skermania* belong to the family *Gordoniaceae*. Both families are members of the suborder *Corynebacterineae* (Stackebrandt *et al.*, 1997). The genus *Glycomyces* and *Nocardioides luteus* also formed a subgroup (Table 1, 7a), which could not be resolved. However, members of the genus *Glycomyces* do not exhibit mycelial fragmentation (Labeda *et al.*, 1985), whereas fragmentation does occur in the members of the genus *Nocardioides* (Prauser, 1976), thus providing a simple way to distinguish between the genera in this subgroup.

Table 2 refers to the identification of the genera within *Sau*3AI Group 2. As with Group 1, not every genus was resolved (two small subgroups were generated). The first small subgroup comprised *Nocardioides jensenii*, *Prauserella rugosa* and four members of the genus *Saccharomonospora* (Table 2, 4b). Morphologically, these three genera are indistinguishable (*Nocardioides jensenii* is a member of the family *Nocardioidaceae* and *Prauserella rugosa* and *Saccharomonospora* are members of the family *Pseudonocardiaceae* (Stackebrandt *et al.*, 1997). The second small subgroup comprised *Actinoalloteichus cyanogriseus*, *Actinopolyspora halophila*, *Thermocrispum municipale* and three members of the genus *Saccharopolyspora* (Table 2, 5b). Their morphological characteristics are sufficiently different to permit differentiation. The genus *Actinoalloteichus* forms aerial and substrate mycelium, both of which exhibit fragmentation (Tamura *et al.*, 2000). The genus *Actinopolyspora* contains one species, a halophilic actinomycete, which forms aerial mycelium and produces a black pigment (Gochnauer *et al.*, 1975). The genus *Saccharopolyspora* forms an extensive substrate mycelium that fragments and an aerial mycelium that does not fragment (Lu *et al.*, 2001). The genus *Thermocrispum* forms a white aerial mycelium and yellow to light-brown vegetative mycelium (Korn-Wendisch *et al.*, 1995).

*Sau*3AI Group 3 comprised the largest group of filamentous actinomycete genera. This group is so large that the dichotomous key has been divided into three tables (Tables 3, 4 and 5). Table 3 shows the first part of the key, based on restriction by *Asn*I and *Sph*I. Not every genus could be resolved. In most cases, an unknown isolate could, however, be placed in one of a small group of genera. Table 4 shows those genera, the 16S rDNA of which is restricted by *Asn*I, but not *Sph*I. Although not every genus could be

Table 4. Identification of the genera in *Sau*3AI Group 3

The 16S rDNA of the genera listed in this table is cut by *Asn*I, but not *Sph*I.

Digested with	Relevant feature(s)	Go to:
(1a) *Kpn*I	Cut (410–470 bp and 990–1100 bp)	5
(1b) *Kpn*I	Not cut	2
(2a) *Hin*dIII	Cut	3
(2b) *Hin*dIII	Not cut: *Actinosynnema* (2/2); *Pseudonocardia* (3/17); *Saccharopolyspora* (2/7)	
(3a) *Hin*dIII	170–200 bp and 1270–1290 bp: *Gordonia* (1/15)	
(3b) *Hin*dIII	Two bands (460–570 bp, doublet) OR (460–570 bp and 890–990 bp)	4
(4a) *Hin*dIII	460 bp and 990 bp: *Kibdelosporangium* (1/1)	
(4b) *Hin*dIII	550–570 bp and 890–990 bp: *Nocardia* (5/25)	
(5a) *Hin*dIII	Cut	6
(5b) *Hin*dIII	Not cut	8
(6a) *Hin*dIII	Produces at least one band <250 bp	7
(6b) *Hin*dIII	470–600 bp and 910–970 bp: *Lentzea* (3/4)	
(7a) *Sca*I	Cut (570–630 bp and 850–900 bp): *Gordonia* (8/15)	
(7b) *Sca*I	Not cut: *Pseudonocardia* (1/17); *Saccharomonospora* (1/6)	
(8a) *Sal*I	Cut (540–560 bp and 950–990 bp)	9
(8b) *Sal*I	Not cut	10
(9a) *Psp*1406I	Cut (440 bp and 1030 bp): *Streptoalloteichus* (1/1)	
(9b) *Psp*1406I	Not cut: *Kutzneria* (2/2)	
(10a) *Psp*1406I	Cut	11
(10b) *Psp*1406I	Not cut: *Actinokineospora* (5/5); *Lechevalieria* (2/2); *Lentzea* (1/4); *Pseudonocardia* (4/17); *Saccharothrix* (7/7)	
(11a) *Psp*1406I	Produces one band of 780 bp: *Saccharomonospora* (1/6)	
(11b) *Psp*1406I	600 bp and 800 bp: *Amycolatopsis* (11/12); *Pseudonocardia* (4/17); *Saccharopolyspora* (1/7)	

Table 5. Identification of the genera in *Sau*3AI Group 3

The 16S rDNA of the genera listed in this table is not cut by *Asn*I.

Digested with	Relevant feature(s)	Go to:
(1a) *Kpn*I	Cut (410–470 bp and 1000–1100 bp)	2
(1b) *Kpn*I	Not cut	5
(2a) *Sph*I	Cut (540 bp and 910–930 bp): *Cryptosporangium* (4/4)	
(2b) *Sph*I	Not cut	3
(3a) *Pst*I	Not cut	4
(3b) *Pst*I	Cut, producing one band between 400 bp and 465 bp: *Actinocorallia* (5/5); *Actinomadura* (21/27); *Saccharothrix* (1/8); *Spirillospora* (2/2)	
(4a) *Sna*B1	Cut (500 bp and 930 bp): *Amycolatopsis* (1/12)	
(4b) *Sna*B1	Not cut: *Actinopolymorpha* (1/1)	
(5a) *Sph*I	Cut (595–630 bp and 840–900 bp)	6
(5b) *Sph*I	Not cut	7
(6a) *Pst*I	Cut (500–580 bp and 860–900 bp): *Nocardiopsis* (7/13)	
(6b) *Pst*I	Not cut: *Nocardiopsis* (3/13); *Streptomonospora* (1/1)	
(7a) *Pst*I	Not cut	8
(7b) *Pst*I	Cut (430–590 bp and 870–1040 bp): *Acrocarpospora* (1/2); *Actinomadura* (2/27); *Nocardiopsis* (1/13); *Nonomuraea* (5/15); *Planomonospora* (4/4); *Planobispora* (2/2); *Streptosporangium* (8/9); *Thermomonospora* (1/2); *Thermobispora* (1/1)	
(8a) *Hind*III	Cut (520–540 bp and 890–900 bp): *Microtetraspora* (3/3)	
(8b) *Hind*III	Not cut: *Acrocarpospora* (1/2); *Actinomadura* (4/27); *Herbidospora* (1/1); *Planotetraspora* (1/1); *Microbispora* (2/3); *Streptosporangium* (1/9)	

individually identified, an isolate could be placed in one of a small group of genera. Table 5 shows the genera an isolate could belong to if its 16S rDNA is not restricted by *Asn*I.

Although the method described here is intended to identify an unknown actinomycete to the genus level, in some cases it allows identification to the species level. Thus, *Microbispora rosea* (Table 1, 8b), *Pseudonocardia kongjuensis* (Table 2, 4a), *Thermomonospora chromogena* (Table 2, 8b), *Hongia koreensis* (Table 3, 8a), *Intrasporangium calvum* (Table 3, 10b), *Nocardioides albus* (Table 3, 11b), *Gordonia rhizosphera* (Table 4, 3a), *Kibdelosporangium aridum* (Table 4, 4a), *Streptoalloteichus hindustanus* (Table 4, 9a), *Saccharomonospora xinjiangensis* (Table 4, 11a), *Amycolatopsis mediterranei* (Table 5, 4a) and *Actinopolymorpha singaporensis* (Table 5, 4b) can be identified. *Kribbella sandramycini* and *Nocardioides nitrophenolicus* (Table 3, 8b) cannot be differentiated based on morphological differences (Park *et al.*, 1999); both are members of the family *Pseudonocardiaceae* (Stackebrandt *et al.*, 1997). *Pseudonocardia sulfidoxydans* and *Saccharomonospora viridis* (Table 4, 7b) also cannot be differentiated morphologically (Reichert *et al.*, 1998; Runmao *et al.*, 1988). Both genera are members of the family *Nocardiaceae* (Stackebrandt *et al.*, 1997).

We investigated the possibility that the method could be used to group phylogenetically related *Streptomyces* species, but were unable to identify any significant groupings below the genus level.

Identification of environmental isolates

The most important restriction endonuclease used in this rapid identification method is *Sau*3AI. Fig. 2 shows the result of a *Sau*3AI digestion performed on the 16S rDNA of seven environmental isolates. Lanes 3, 6, 8 and 9 show a doublet band in the size range 540–650 bp. The size of the doublet band indicates that the isolates represented in these lanes are part of *Sau*3AI Group 1 (Fig. 1) and therefore are

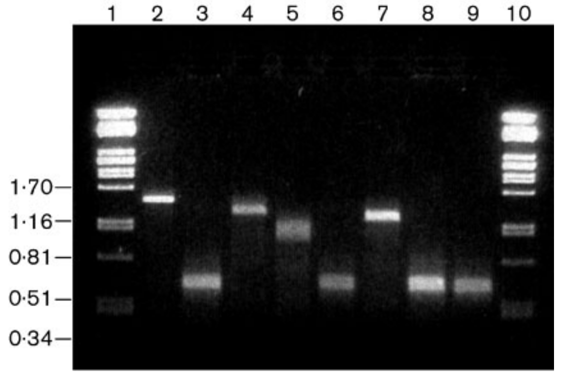

Fig. 2. *Sau*3AI endonuclease restriction analysis of seven unknown actinomycete strains. Lanes: 1 and 10, molecular size marker, λ DNA digested with *Pst*I (sizes shown in kb); 2, uncut 16S rDNA (1·5 kb); 3, strain 5; 4, strain N.CZ.8; 5, strain MTCT; 6, strain 17; 7, strain 23; 8, strain 30; 9, strain 37.

most likely to belong to the genus *Streptomyces*. However, the isolates in lanes 4, 5 and 7 (strains N.CZ.8, MTCT and 23, respectively) show one band greater than 980 bp in size. These isolates are non-*Streptomyces* species belonging to *Sau*3AI Group 3 (Fig. 1) and were investigated further.

Fig. 3(a) shows the results of a series of restriction endonuclease digestions of the 16S rDNA of strain MTCT (Fig. 2, lane 5). *Sau*3AI restricted the DNA, producing one band greater than 980 bp (lane 3). *Asn*I restricted the DNA producing two bands, 470–590 bp and 900–960 bp (lane 4). *Sph*I and *Kpn*I did not restrict the DNA (lanes 5 and 6, respectively). *Hin*dIII restricted the DNA producing two bands, 550–570 bp and 890–990 bp in size (lane 8). Based on the analysis of the fragment patterns in Fig. 3(a) and the dichotomous keys in Tables 1, 2, 3 and 4, strain MTCT belongs to the genus *Nocardia*. If strain MTCT had been characterized by chemotaxonomic analysis (DAP isomer and whole-cell sugar pattern), it could have been assigned to any of the genera *Amycolata*, *Amycolatopsis*, *Nocardia*, *Pseudonocardia* or *Saccharopolyspora* (taking colony morphology into account).

Fig. 3(b) shows the results of a series of restriction endonuclease digestions of the 16S rDNA of strain N.CZ.8 (Fig. 2, lane 4). *Sau*3AI restricted the DNA, producing one band greater than 980 bp (lane 3). *Asn*I and *Kpn*I did not restrict the DNA (lanes 4 and 6, respectively). *Sph*I restricted the DNA producing two bands, 595–630 bp and 840–900 bp (lane 5). *Pst*I restricted the DNA producing two bands, 500–580 bp and 860–900 bp (lane 9). Based on the analysis of the fragment patterns in Fig. 3(b) and the dichotomous keys in Tables 1, 2 and 5, strain N.CZ.8 belongs to the genus *Nocardiopsis*. If DAP-isomer and whole-cell sugar pattern analyses had been carried out, strain N.CZ.8 could have been assigned to the genus *Nocardiopsis* or *Thermoactinomyces* (taking colony characteristics into consideration).

Fig. 3(c) shows the results of a series of restriction endonuclease digestions of the 16S rDNA of strain 23 (Fig. 2, lane 7). *Sau*3AI restricted the DNA, producing one band greater than 980 bp (lane 3). *Asn*I did not restrict the DNA (lane 4). *Kpn*I restricted the DNA producing two bands of 410–470 bp and 1000–1100 bp (lane 6). *Sph*I did not restrict the DNA (lane 5). *Pst*I restricted the DNA producing two bands, one of which was in the size range 400–465 bp (lane 9). Based on the analysis of the fragment patterns in Fig. 3(c) and the dichotomous keys in Tables 1, 2 and 5, strain 23 could belong to one of the following genera: *Actinocorallia*, *Actinomadura*, *Saccharothrix* or *Spirillospora*. Based on an examination of colony morphology using a light microscope, the non-fragmenting, asporangiate strain 23 cannot belong to the genus *Saccharothrix*, which exhibits aerial and substrate mycelium fragmentation (Labeda *et al.*, 1984), nor the sporangiate genus *Spirillospora* (Vobis & Kothe, 1989). Therefore, strain 23 belongs to either the genus *Actinocorallia* or the genus *Actinomadura*. In this case, a similar result would have been obtained by DAP-isomer and whole-cell sugar pattern analyses.

We have developed a method to identify rapidly environmental *Streptomyces* isolates using only four restriction

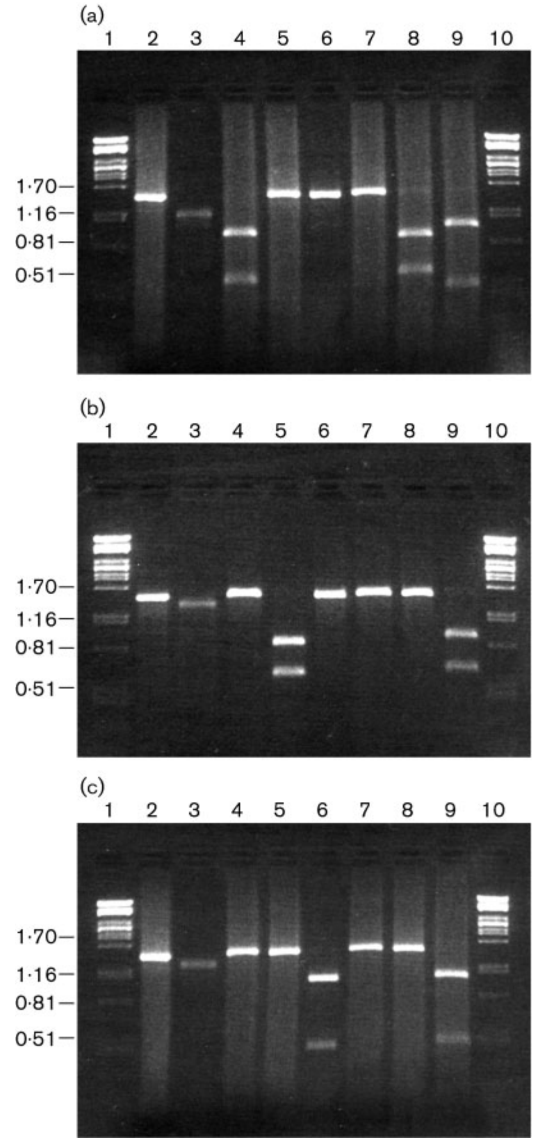

Fig. 3. (a) Restriction analysis of the 16S rDNA of strain MTCT. Lanes: 1 and 10, molecular size marker, λ DNA digested with *Pst*I (sizes shown in kb); 2, uncut MTCT 16S rDNA (1·5 kb); 3, *Sau*3AI; 4, *Asn*I; 5, *Sph*I; 6, *Kpn*I; 7, *Sal*I; 8, *Hin*dIII; 9, *Pst*I. (b) Restriction analysis of the 16S rDNA of strain N.CZ.8. Lanes 1 and 10, molecular size marker, λ DNA digested with *Pst*I (sizes shown in kb); 2, uncut N.CZ.8 16S rDNA (1·5 kb); 3, *Sau*3AI; 4, *Asn*I; 5, *Sph*I; 6, *Kpn*I; 7, *Sal*I; 8, *Hin*dIII; 9, *Pst*I. (c) Restriction analysis of the 16S rDNA of strain 23. Lanes: 1 and 10, molecular size marker, λ DNA digested with *Pst*I (sizes shown in kb); 2, uncut strain 23 16S rDNA (1·5 kb); 3, *Sau*3AI; 4, *Asn*I; 5, *Sph*I; 6, *Kpn*I; 7, *Sal*I; 8, *Hin*dIII; 9, *Pst*I.

endonucleases. Non-*Streptomyces* species were identified rapidly to a specific genus or a small subgroup of genera (in which case, other readily available information, such as colony morphology, was sufficient to restrict further the number of genus possibilities). The online version of this article (http://ijs.sgmjournals.org) provides access to an interactive Microsoft PowerPoint (version 5.0) version of this method.

It would be logical to extend this method to the analysis of 23S rDNA sequences to try and resolve the genera in the various subgroups shown in Tables 1–5. However, at present, this approach would be hampered by the paucity of 23S rDNA sequence data in the public databases. Thus, it will be impossible to expand this method until many more 23S rDNA sequences are made available. Nevertheless, as presented, the method allows the determination of sufficient information about an environmental isolate to decide whether it is worth pursuing as a research culture.

In laboratories in developed countries, a partial 16S rDNA sequence of a new actinomycete isolate can be obtained quickly and at low cost to give an unambiguous identification of the genus to which the isolate belongs. This is certainly not the case in developing countries, such as South Africa, where high sequencing costs and possible restricted access to sequencing facilities preclude the use of 16S rDNA sequencing as a routine genus-identification tool.

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