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Taxonomic Utility of PCR-Restriction Pattern Analysis for Rapid Identification of Clinical Isolates of aerobic Actinomycetes to the Genus Level

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Abstract: PCR-restriction pattern analysis (PRA), a previously described method for the identification of aerobic actinomycetes was evaluated for potential use with clinically significant aerobic actinomycetes. In this method, Amplified 16S rDNA of actinomycetes was restricted with selected endonucleases and electrophoresed on agarose gels. The restriction patterns of the unknown isolates were compared to the established patterns. This study included 93 cultures. PRA identification using an amplified 16S rDNA (amplicon) was compared to identification by traditional methods, including growth characteristics, susceptibility patterns and biochemical testing. Microbiological examination of five cultures ruled out aerobic actinomycetes, and they were omitted from the study. The identification method allowed 88 clinical actinomycete isolates to be identified to the genus level. The 88 isolates produced PRA patterns matched to those in the current PRA database which lead to their differentiation into four major genera, *Nocardia, Streptomyces, Gordonia* and *Actinomadura.* Significantly, the genus *Streptomyces* could be differentiated from all other actinomycete clinical isolates by using four restriction endonucleases, *Sau*3AI, *Asn*I, *Kpn*I and *Sph*I. The predominant genus of actinomycetes identified in this study was *Nocardia* which comprised 40 isolate out of 88 (45%). Thirty two isolates (32) out of 88 (36%) were identified as *Streptomyces*, 8 isolates (9%) were identified as *Gordonia* while 8 isolates (9%) were identified as *Actinomadura*. PRA results were reportable within 2 to 5 working days and were accurate, faster and less expensive than those of traditional methods.

Key words: Actinomycetes, identification, PCR, Restriction Pattern Analysis (PRA).

INTRODUCTION

Actinomycetes are widely distributed in terrestrial environments and have long been a source of commercially useful enzymes and therapeutically useful bioactive molecules. Interest in the identification and taxonomy of aerobic actinomycetes, nocardiae and mycobacteria in particular, has been increasing as a result of the increasing number of immunocompromised individuals in the population who are at greater risk for actinomycoses, especially those with advanced human immunodeficiency virus disease (Beaman *et al*., 1995; McNeil *et al*., 1992). Traditional methods for differentiation of species and taxa of aerobic actinomycetes are laborious and time-consuming and frequently require specialized testing that is beyond the capabilities of clinical laboratories (Collins *et al*., 1988; Georghiou and Blacklock, 1988; Wallace *et al*., 1990; Wallace, *et al*., 1991; McNeil *et al*., 1992; Steingrube, *et al*., 1995a; Steingrube *et al*., 1997; Steingrube *et al*., 1995b, 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). The occurrence of clinical isolates of aerobic actinomycetes that are inherently resistant to specific antimicrobials increases the significance of timely and accurate species and taxon recognition (McNeil *et al*., 1992; Steingrube, *et al*., 1995b; Wallace, *et al*., 1995; Wilson *et al*., 1997). Since these bacteria are slowly growing, 2 to 4 weeks is required for genus-level identification and an additional 4 weeks or more is required for species-level identification. Alternative

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methods of identification, including high-performance liquid chromatography (HPLC) and molecular techniques have been applied to this group of bacteria (Bulter *et al*., 1987). HPLC is limited by the inability to determine a species-level identification.

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). Application of this methodology has since been expanded to include 50 commonly encountered pathogenic species and taxa of aerobic actinomycetes comprising the genera *Mycobacterium* (Steingrube *et al*., 1995b; Telenti, *et al*., 1993; Pusterla *et al*., 2007; Zelazny *et al*., 2009); *Nocardia* (Steingrube *et al*., 1995a; Wallace, *et al*., 1995; Wilson *et al*., 1998); and *Actinomadura*, *Gordona*, *Rhodococcus*, *Streptomyces*, and *Tsukamurella* (Steingrube *et al*., 1997; Nakazawa *et al*., 2009). The rapidity and accuracy of PRA prompted the current study (Steingrube *et al*., 1995a; Steingrube *et al*., 1997; Telenti *et al*., 1993; Wallace, *et al*., 1995)

However, 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 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 nonstreptomycetes 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. In this study, we applied a previously described PCR-restriction pattern analysis (PRA) identification method, in which 16S rRNA gene was amplified and restricted by selected endonucleases. The restriction patterns of the unknown isolates were easily compared to the established patterns on the data bases. This method allowed actinomycete clinical isolates to be identified to the genus level in less than a week, following DNA isolation from a pure culture.

MATERIALS AND METHODS

Isolates and Culture Media:

The present study included 93 clinical isolates comprising four taxonomic groups of aerobic actinomycetes. Actinomycete isolates were isolated on Czapek solution agar (Atlas, 1993), Middlebrook 7H9 Agar (Difco Laboratories) or *Streptomyces* General Defined Medium [GM (800 ml): 0.17g $Na_2HPO_4.2H_2O$, 0.14 g KH_2PO_4, 0.05 g $MgSO_4.7H_2O$, 0.01 g $FeSO_4.7H_2O$; pH 7.0; autoclaved 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 ml of 10 mM Tris-HCl/1 mM EDTA (TE) buffer (pH 7.7) and resuspended in 500 ml 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 ml) 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 $MgCl_2$, 2U *Taq* polymerase, 150 mM of each dNTP, 0.5 µM of each primer and 2 µl template DNA. Primer F1 (5`-AGAGTTTGATCITGGCTCAG-3`; I=inosine) and primer R1 (5`-ACGGITACCTTGTTACGACTT-3`). Primer F1 binds to base positions 7–26 and

primer R1 to base positions 1496–1476 of the 16S rRNA gene of *Streptomyces ambofaciens* ATCC 23877T. The primers were used to amplify nearly the 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.

PRA Analysis:

Commercially available restriction endonucleases (New England Biolabs, Beverly, Mass., and Promega, Madison, Wis.) were screened for optimal restriction fragment length polymorphism (RFLP) band patterns by incubation at the appropriate temperatures and by using buffers at the 10X concentration recommended by the manufacturers. Samples were electrophoresed on 1.5% agarose gels containing ethidium bromide (10 µg ml^{-1}). The restriction fragment patterns were compared with those from the in silico restriction endonuclease digestions.

Organisms. On microbiological evaluation, 5 of the cultures submitted to our laboratory for identification and susceptibility testing were not aerobic actinomycetes. These cultures did not yield PCR amplification products (amplicons) and were excluded from further study. The predominant genus of aerobic actinomycetes identified in this study comprised 40 isolate out of 88 (45%) which included *Nocardia*, 32 of 88 (36%), of the isolates *Streptomyces*, *Nocardia*, 40 of 88 (45%) isolates; 8 out of 88 (9%) *Gordonia* and 8 out of 88 (9%) *Actinomadura*

Result:

PCR-Restriction Enzyme Pattern Analysis (PRA):

Based on the published filamentous actinomycete 16S rRNA gene sequences in the GenBank database and the *in silico* analyses results of Cook and Meyers, 2003, a set of different restriction endonucleases was used for the rapid identification of actinomycetes strains. Restriction analysis was applied through a series of dichotomous keys developed by Cook and Meyers, 2003. In these keys, Sau3AI was the most important restriction endonuclease, as it divided the filamentous actinomycetes into three major groups according to the size of the developed band. In group I, Largest DNA fragment developed by digestion with Sau3AI was less than 750bp. In group II, however, two distinct fragments developed between 300–350 bp and 760–980 bp. In group III, largest DNA fragment lies between 990–1300 bp.

All restricted 16S rDNAs of the 88 isolates produced a PRA pattern with *Sau*3AI enzyme similar to those represented in Fig.1 (*i.e.* another 28 strains exhibited a PRA pattern similar to that of strains Az4, Az6, Az7 and Az8, Lanes 6, 8, 9 and 10; another 21 strains like that of strains Az1, Az3 and Az11, lanes 3, 5 and 13; and 28 strains like that of strains Az2, Az5, Az9, AZ10, lanes, 4, 7, 11 and 12). Fig. 1 shows the result of a *Sau*3AI digestion performed on the 16S rDNA of eleven strains which have the same PRA pattern like the rest of the 77 strains (total 88). Lanes 6, 8, 9 and 10 (strains Az4, Az6, Az7 and Az8, respectively). 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 1and therefore are most likely to belong to the genus Streptomyces. Also, strains in lanes 3, 5 and 13 (strains Az1, Az3 and Az11, respectively) show one a single band at less than 750 bp which indicated that these isolates are part of the same group.

Fig. 2 shows the results of a series of further restriction endonuclease digestions of the 16S rDNA of strains Az4; Az6, Az7 and Az8 (lanes 6, 8, 9 and 10 in Fig.1). *Asn*I did not restrict 16 rDNAs of strains Az4; Az6, Az7 and Az8 (Fig.2a. lanes 3-6, respectively). *Kpn*I restricted their 16 rDNA producing two bands between 410–470 bp and 1000–1100 bp (Fig.2b. lanes 3-6, respectively). *Sph*I restricted the 16S rDNA producing two bands (390–450 bp & 1050–1110 bp) in case strain Az4 and Az7 while the same enzyme was gave no bands in case of strain Az6 and Az8 (Fig.2c, lanes 3-6, respectively). Based on the morphological and the biochemical analysis (data not shown), PRA patterns in Fig. 1and 2, and the dichotomous keys of (Cook and Meyers, 2003), strains Az4; Az6, Az7 and Az8 belongs to the genus S*treptomyces*. Restricted 16 rDNAs of strains Az3; Az5 and Az1 (lanes 3, 5 and 13 in fig. 1) by *Sau*3A developed a single DNA band at less than 750bp. Therefore, they considered as a member of group I.

They were identified by the sequential use of three restriction endonucleases: *Sau3A*I, *Asn*I, *Sca*I. These strains produced PRA patterns that matched species or taxon-specific patterns in the database and the *in silico* analysis they gave and resulted in an identification that correlated with the identification by traditional methods.

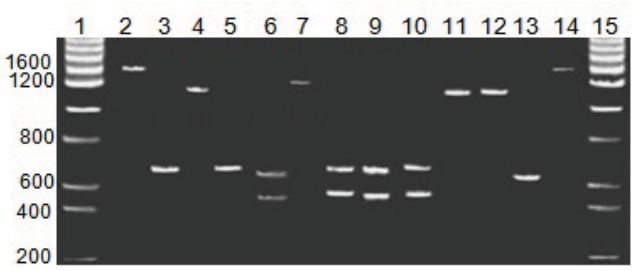

Fig. 1: Restriction analysis of eleven unknown actinomycete strains by *Sau*3AI endonuclease . Lanes: 1 and 15, DNA fragments of 200bp DNA ladder; 2 and 14 uncut 16S rDNA (1.5 kb); 3, strain Az1; 4, strain Az2; 5, strain Az3; 6, strain Az4; 7, strain Az5; 8, strain Az6; 9, strain Az7; 10, strain Az8; 11, strain Az9; 2, strain Az10; 13, strain Az11.

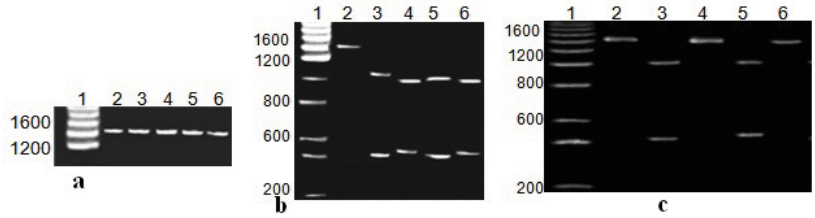

Fig. 2: a. Restriction analysis of the 16 rDNA of strains Az4; Az6, Az7 and Az8 by *Asn*I. Lanes: 1, DNA fragments of 200bp DNA ladder; 2, uncut 16S rDNA (1.5 kb); 3-6, strain Az4; Az6-8. b and c. Restriction analysis of the 16 rDNA of the same strains represented in figure a by *Kpn*I *Sph*I, respectively.

*Asn*I restricted the 16S rDNA producing two bands at (470–590 bp & 900–960 bp) in case strain Az1 and Az3 and Az11. The same strains produced different pattern upon restriction with *Sca*I where they developed two bands at (570–630 bp & 850–900 bp) as given in (Fig. 3).

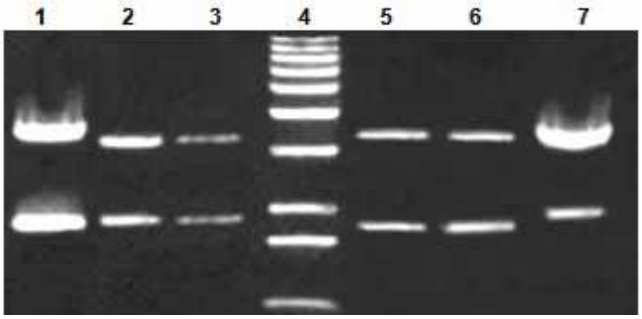

Fig. 3: Restriction analysis of the 16 rDNA of strains Az1; Az3 and Az11 by *Asn*I and *Sca*I. Lanes: 1, 2 and 3 restricted 16 rDNAs of strains Az11; Az3 and Az1, by *Asn*I, respectively. Lane 4, DNA fragments of 200bp DNA ladder. Lanes 5, 6 and 7 restricted 16 rDNAs of strains Az1; Az3 and Az11, by *Sca*I, respectively.

Taking colony morphology into account, chemotaxonomic analysis (DAP isomer and whole-cell sugar pattern) strains Az1; Az3 could have been assigned to any of the genera *Amycolata, Amycolatopsis, Nocardia, Pseudonocardia or Saccharopolyspora*. Amplicon patterns produced by these strains (Az1 and Az3 and Az11) upon the analysis of the fragment patterns in Fig. 3 and the dichotomous keys of the *in silico* analysis stra ins Az1, Az3 and Az11 matched taxon-specific patterns in the database and resulted in an identification belongs to the genus *Nocardia and Gordonia*, respectively.

*Sau3AI, Asn*I, *Sph*I and *Kpn*I endonucleases produced digests with amplicons from of strains Az2; Az5; Az9 and Az10 that comprised three general categories: (i) enzymes for which there were no recognition sites

included *Sph*I and *Kpn*I (ii) enzymes that produced generally large-band PRA patterns with considerable intraspecies polymorphism included *Sau3A*I (iii) enzymes that gave two band PRA pattern with 3 strains and no bands with one strains included *Asn*I, (fig.4a-c).

Fig. 4 shows the results of a series of restriction endonuclease digestions of the 16S rDNA of strains Az2; Az5; Az9 and Az10. *Sau*3AI restricted the DNA, producing one band greater than 980 bp and less than 1350bp (lane 4, 7, 11 and 12 in fig 1). *Asn*I did not restrict the DNA of strain 5 (Fig.4a, lane 4) while restricted the DNA of strains Az2, Az9 and Az10 producing two bands, one of which was in the size range 470–590 bp and the other ranged from 900-960bp. *Kpn*I (Fig 4b, lanes 3-6) and *Sph*I (fig 4b, lanes 7-10) could not recoginze the 16S rDNAs of the four strains. However, *Pst*I could not recoginze the 16S rDNA of strain Az5 (data not shown). On the other hand, *Hin*dIII restricted the DNA producing two bands, one of which was in the size range 550–570 bp and the other at 890-990bp (Fig 4c, lane 3, 5, 6) could not recognizes the 16S rDNA of strain Az5 (fig 4c, lane 4).

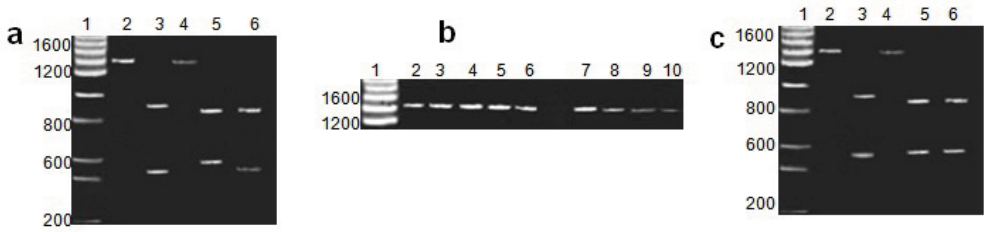

Fig. 4: Restriction analysis of the 16 rDNAs of strains Az2; Az5; Az9 and Az10 by *Asn*I in fig a; *Sph*I and *Kpn*I in fig. b and by *Hin*dIII in fig. c, respectively. Lanes: 1, DNA fragments of 200bp DNA ladder; 2, uncut 16S rDNA (1.5 kb); 3-6, 16 rDNAs of strains Az2; Az5; Az9 and Az10, respectively.

Based on the analysis of the fragment patterns in Fig. 4 and the dichotomous keys strain Az5 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 Az5 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 Az5 belongs *Actinomadura*. At the same time, PRA pattern produced by strains (Az2 and Az5 and Az10) upon the restriction analysis of their 16S rDNA (fig. 4) and the dichotomous keys of the *in silico* analysis matched taxon-specific patterns in the database and resulted in an identification belongs to the genus *Nocardia*

Discussion:

Traditional differentiation of genera and species comprising the aerobic actinomycetes has involved the examination of staining characteristics and colonial morphology and the evaluation of various biochemical reactions. These methods often require a series of specialized tests (Steingrube *et al*., 1995a&b, 1997; Wilson *et al*., 1998; Harvey *et al*., 2001) and have not always been reproducible and have been inadequate for the identification of recently characterized *Nocardia* species (e.g., *Nocardia farcinica* and *Nocardia nova*), for which additional antibiogram and specialized biochemical data are required for differentiation (Wallace, *et al*., 1990, 1991&1995).

Identification of clinical isolates of aerobic actinomycetes by PCR-restriction enzyme pattern analysis (PRA) performed on a continuing daily basis common to the routine of clinical laboratories provided final identification results within 2 to 5 working days, compared to traditional identification methods that required from 2 to 6 weeks for final results. Prompt and accurate identification of pathogenic aerobic actinomycete isolates is particularly important when invasive species such as *Nocardia pseudobrasiliensis* (Ruimy, *et al*., 1996; Wallace *et al*., 1995) are encountered or when innately drug-resistant species such as those of the *N. transvalensis* complex, which are resistant to all aminoglycosides (Steingrube, et al., 1995a&b, Wallace *et al*., 1988; Wilson *et al*., 1997), and *Nocardia farcinica*, which is resistant to all extended-spectrum cephalosporins (Steingrube, *et al*., 1993; Wallace *et al*., 1990), are involved. This is particularly significant when immunocompromised patients such as those with advanced human immunodeficiency virus disease are infected with these organisms (McNeil *et al*., 1992; Steingrube *et al*., 1995a&b; Wallace *et al*., 1995; Wilson *et al*., 1997).

In this study, we used PRA analysis of 16S rRNA gene and compared it to the profiles obtained by morphological, biochemical analysis, and drug susceptibility testing for identification of isolates of the aerobic

actinomycetes. In general, we found that sequence differences correspond to the differences obtained by traditional identification methods. For example, *Nocardia* strains that had the same biochemical pattern were found to have unique PRA patterns, and all had the same 16S rRNA gene sequence. A series of dichotomous keys developed by (Cook and Meyers, 2003) to identify rapidly the genera within the three *Sau3*AI groups was used. *Sau*3AI was the first and the most important restriction endonuclease used, as it divided the filamentous actinomycetes into three major groups. Most genera were placed in a single *Sau*3AI group, according to the *in silico* analysis of Cook and Meyers, 2003.

In *Sau*3AI group I of these keys, an unknown *Streptomyces* isolate could be identified by the sequential use of four restriction endonucleases: *Sau*3AI, *Asn*I, *Kpn*I and *Sph*I. Although the genus *Streptomyces* can not be differentiated from the extremely rare genus *Sporichthya* based on the 16S rRNA gene restriction analysis, the morphology of *Sporichthya* clearly distinguishes it from the genus *Streptomyces* (Tamura *et al*., 1999). 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 *Sau3*AI 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 (Cook and Meyers, 2003). This finding is consistent with those in a recent report by Roth et al. 2003, who compared the full 16S rRNA gene sequences (1,500 bp) of 74 *Nocardia* isolates, and a report by Cloud et al. 2004, who compared the 500-bp 16S rRNA gene sequences of 28 reference *Nocardia* strains.

As with Group 1, PRA analysis could not resolve every genus which lead to the generation of two two small subgroups. The first small subgroup comprised *Nocardioides jensenii*, *Prauserella rugosa* and four members of the genus Saccharomonospora. 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*. Their morphological characteristics are sufficiently different to permit differentiation (Cook and Meyers, 2003). 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). PRA method could identify rapidly environmental *Streptomyces* isolates using only four restriction 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). Online version of this method (Cook and Meyers, 2003) method (http://ijs.sgmjournals.org) provides access to an interactive Microsoft PowerPoint (version 5.0) version of this procedure.

Screening of 30 restriction endonucleases resulted in the selection of *Msp*I for initial digestion and then digestion with *Bsa*HI, a procedure which produced complete separation of all of the commonly encountered species and unnamed taxa of the genus *Nocardia* by RFLP band patterns (Steingrube, et al., 1995a&b). Successful application of molecular biological methodology to the development of protocols for rapid differentiation of mycobacterial species was demonstrated by Telenti et al. in 1993. These authors used PCR-restriction enzyme pattern analysis (PRA) of an amplified 439-bp segment of the 65-kDa heat shock protein (*hsp-65*) gene and introduced the abbreviation PRA for this method that has now gained wide acceptance (Wallace *et al*., 1995). PRA was cost-effective, with the expenses of specialized equipment and reagents being more than compensated for by savings in time and labor, and could be economically incorporated into the clinical laboratory setting. This methodology has proven both practical and cost-effective as a rapid, efficient, and highly accurate identification system for use in identifying clinically significant species and taxa of aerobic actinomycetes.

As PRA analysis of an amplified 439-bp segment of the 65-kDa heat shock protein (*hsp-65*) gene gained wide acceptance, it will be useful to test both protocols of (Wallace *et al*., 1995) and (Cook & Myers 2003) and extend this study in a trial to resolve the genera in the various subgroups and complete the actinomycetes identification to the species level.

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