New genus-specific primers for the PCR identification of members of the genera *Pseudonocardia* and *Saccharopolyspora*

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Members of the family *Pseudonocardiaceae* are difficult to identify on the basis of their micromorphology only. The biochemical characterization of each new isolate is a painstaking and time-consuming task which cannot always be undertaken when handling large numbers of strains as is the case in natural product screening programmes. In this study, two sets of genus-specific oligonucleotides were designed which allow rapid detection of members of the genera *Pseudonocardia* and *Saccharopolyspora* by means of PCR-specific amplification. The genus specificity of these primers was validated on a wide range of collection strains and the primers were subsequently used to study a group of 106 wild-type isolates that possessed morphological characteristics of the family. Out of this group, 51 strains could be identified as members of the genus *Pseudonocardia* and only nine isolates could be assigned to the genus *Saccharopolyspora*. The diversity indicated by whole-cell fatty acid profiles of both wild-type and reference strains was compared with that identified using the oligonucleotide primers. The partial 16S rDNA sequencing of representative wild-type strains was used to validate their genus assignment by PCR-specific amplification. This study shows the industrial usefulness of the application of these direct identification tools as well as the complementary use of two sources of data, PCR-specific amplification results and fatty acid composition, to assess the diversity of a microbial population.

Keywords: *Pseudonocardiaceae*, PCR primers, 16S rDNA sequences

INTRODUCTION

Historically, natural product screening programs have focussed on the order *Actinomycetales*, one of the most intensively isolated groups of micro-organisms which, through the years, has efficiently demonstrated its ability to produce a large diversity of new secondary metabolites (Sanglier *et al.*, 1993). Besides the genus *Streptomyces*, which has been extensively exploited, members of other genera are also considered of high interest as potential producers of novel bioactive compounds. However, no specific procedures are normally available for the direct and specific isolation of cultures belonging to these groups. In addition, the wide range of morphologies and phenotypes found

within strains of the same genus, together with the frequent absence of sporulation characteristics, sometimes hinder an initial rapid and reliable morphological identification during routine isolation.

This is the case with the family *Pseudonocardiaceae*, the subject of this study, which includes species that are very well-known producers of the antibiotics erythromycin, vancomycin or rifamycins (Embley, 1992). Initially, this group of actinomycetes contained strains which had the absence of mycolic acids and cell wall chemotype IV (*meso*-diaminopimelic acid, arabinose and galactose) as main chemotaxonomic markers; they were only described as a family in 1988 after phylogenetic analysis (Embley *et al.*, 1988a, b). This family then comprised the genera *Actinopolyspora*, *Amycolatopsis*, *Kibdelosporangium*, *Pseudonocardia*, *Saccharomonospora* and *Saccharopolyspora*. The former genera *Amycolata* and *Pseudonocardia* have been recently combined in the emended genus

Abbreviations: FAMES, fatty acid methyl esters; T_m , melting temperature. The GenBank accession numbers for the sequences reported in this paper are AF061976, AF061977 and AF064968–AF064983.

Table 1. Reference strains used in this study and results obtained from selective PCR amplification

Species	Strain designation	Amplification results*		FAME analysis†
		AMP3/AMP2	SP1/SP3	
<i>Pseudonocardiaceae</i>				
<i>Actinopolyspora</i> :				
<i>Actinopolyspora thermovinacea</i>	ATCC 35862	0	0	+
<i>Amycolatopsis</i> :				
<i>Amycolatopsis azurea</i>	DSM 43854 ^T	0	0	+
<i>Amycolatopsis fastidiosa</i>	ATCC 31181 ^T	0	0	+
<i>Amycolatopsis mediterranei</i>	ATCC 13685 ^T	0	0	
<i>Amycolatopsis methanolica</i>	NCIB 11946 ^T	0	0	+
<i>Amycolatopsis orientalis</i>	NRRL 18098	0	0	+
<i>Amycolatopsis orientalis</i>	NRRL 18099	0	0	+
<i>Amycolatopsis orientalis</i>	NRRL 18100	0	0	+
<i>Amycolatopsis orientalis</i> subsp. <i>orientalis</i>	NRRL 2452	0	0	+
<i>Amycolatopsis orientalis</i> subsp. <i>orientalis</i>	ATCC 21425	0	0	+
<i>Amycolatopsis orientalis</i> subsp. <i>orientalis</i>	DSM 40040 ^T	0	0	+
<i>Amycolatopsis rugosa</i>	ATCC 43014 ^T	0	0	
<i>Amycolatopsis sulphurea</i>	NRRL 2822 ^T	0	0	+
<i>Kibdelosporangium</i> :				
<i>Kibdelosporangium aridum</i> subsp. <i>aridum</i>	ATCC 39323 ^T	0	0	
<i>Kibdelosporangium aridum</i> subsp. <i>largum</i>	ATCC 39922 ^T	0	0	
<i>Pseudonocardia</i> :				
<i>Pseudonocardia autotrophica</i>	ATCC 19727 ^T	0	1	+
<i>Pseudonocardia autotrophica</i>	DSM 43103	0	1	
<i>Pseudonocardia autotrophica</i>	DSM 43098	0	1	+
<i>Pseudonocardia compacta</i>	ATCC 35407 ^T	0	1	+
<i>Pseudonocardia halophobica</i>	DSM 43089 ^T	0	1	+
<i>Pseudonocardia hydrocarbonoxydans</i>	NRRL B-16171 ^T	0	1	+
' <i>Pseudonocardia nitrificans</i> '	DSM 46012	0	1	+
<i>Pseudonocardia petroleophila</i>	DSM 43193 ^T	0	1	+
<i>Pseudonocardia saturnea</i>	NRRL B-16172 ^T	0	1	+
<i>Pseudonocardia thermophila</i>	ATCC 19285 ^T	0	1	+
<i>Saccharomonospora</i> :				
<i>Saccharomonospora azurea</i>	ATCC 43670 ^T	0	0	+
' <i>Saccharomonospora caesia</i> '	ATCC 49227	0	0	+
<i>Saccharomonospora cyanea</i>	ATCC 43724 ^T	0	0	+
<i>Saccharomonospora glauca</i>	DSM 43769 ^T	0	0	
<i>Saccharomonospora internatus</i>	ATCC 33517 ^T	0	0	
<i>Saccharomonospora viridis</i>	ATCC 15345	0	0	+
<i>Saccharomonospora viridis</i>	ATCC 15386 ^T	0	0	
<i>Saccharopolyspora</i> :				
<i>Saccharopolyspora erythraea</i>	NRRL 2338 ^T	1	0	+
<i>Saccharopolyspora gregorii</i>	NCIB 12823 ^T	1	0	+
<i>Saccharopolyspora hirsuta</i> subsp. <i>hirsuta</i>	ATCC 27876	1	0	+
<i>Saccharopolyspora hirsuta</i> subsp. <i>hirsuta</i>	NRRL B5792 ^T	1	0	+
<i>Saccharopolyspora hirsuta</i> subsp. <i>kobensis</i>	ATCC 20501 ^T	1	0	+
<i>Saccharopolyspora hordei</i>	NCIB 12824 ^T	1	0	+
<i>Saccharopolyspora rectivirgula</i>	ATCC 15347	1	0	
<i>Saccharopolyspora rectivirgula</i>	ATCC 33515 ^T	1	0	
<i>Saccharopolyspora spinosa</i>	NRRL 18537	1	0	+
<i>Saccharopolyspora spinosa</i>	ATCC 49460 ^T	1	0	
<i>Saccharopolyspora taberi</i>	NRRL 16173 ^T	1	0	+
<i>Saccharothrix</i> :				
<i>Saccharothrix aerocolonigenes</i>	ATCC 39243	0	0	+

Table 1 (cont.)

Species	Strain designation	Amplification results*		FAME analysis†
		AMP3/AMP2	SP1/SP3	
<i>Saccharothrix aerocolonigenes</i>	NRRL B3298 ^T	0	0	+
<i>Saccharothrix cryophilis</i>	NRRL B-16238 ^T	0	0	
<i>Saccharothrix flava</i>	DSM 43885 ^T	0	0	+
<i>Saccharothrix longispora</i>	DSM 43749 ^T	0	0	
<i>Saccharothrix mutabilis</i> subsp. <i>capreolus</i>	DSM 40225 ^T	0	0	+
<i>Saccharothrix mutabilis</i> subsp. <i>mutabilis</i>	DSM 43853 ^T	0	0	+
<i>Saccharothrix syringae</i>	DSM 43886 ^T	0	0	+
<i>Saccharothrix waywayandensis</i>	NRRL B-16159 ^T	0	0	+
<i>Actinokineospora</i> :				
<i>Actinokineospora riparia</i>	NRRL B-16433 ^T	0	0	
Other genera				
<i>Actinomadura</i> sp.	MA 6867	0	0	
<i>Actinomadura hibisca</i>	ATCC 53557 ^T	0	0	
<i>Actinomadura pusilla</i>	ATCC 27296 ^T	0	0	
<i>Kitasatospora setalba</i>	ATCC 33774 ^T	0	0	
<i>Micromonospora echinospora</i> subsp. <i>pallida</i>	NRRL 2996 ^T	0	0	
<i>Streptomyces avermitilis</i>	ATCC 31267	0	0	
<i>Streptomyces cattleya</i>	NRRL 8057	0	0	
<i>Streptomyces hygroscopicus</i> subsp. <i>ascomyceticus</i>	MA 6572	0	0	
<i>Streptomyces hygroscopicus</i> subsp. <i>hygroscopicus</i>	ATCC 27438 ^T	0	0	
<i>Streptomyces hygroscopicus</i> subsp. <i>rapamyceticus</i>	NRRL 5491	0	0	
<i>Streptomyces</i> sp.	—	0	0	
<i>Streptomyces tsukubaensis</i>	MA 6492	0	0	
<i>Streptoverticillium</i> sp.	—	0	0	

* Amplification results with primers AMP3/AMP2 and SP1/SP3; '1' represents a band of the expected size (640 or 371 bp, respectively) and '0' represents the absence of amplification products.

† Strains whose fatty acid profiles were included in Fig. 3 are indicated by '+ '.

Pseudonocardia (Warwick *et al.*, 1994). The importance of the presence of arabinose as a chemotaxonomic marker for *Pseudonocardiaceae* has been intensively discussed, given the close phylogenetic relationship found between members of this family and the genera *Saccharothrix*, *Kutzneria* and *Actinosynnema*, as well as the recently described genus *Thermocrispum* (Takeuchi *et al.*, 1992; Embley & Stackebrandt, 1994; Korn-Wendisch *et al.*, 1995), which show cell wall chemotype III rather than chemotype IV and contain rhamnose instead of arabinose. These genera, together with the genus *Actinokineospora*, which shows cell wall chemotype IV but is closely related to the genus *Saccharothrix* (Stackebrandt *et al.*, 1994; Warwick *et al.*, 1994; Yassin *et al.*, 1995), have remained outside the family until recently. The last proposal for a new classification system of the order *Actinomycetales*, based on the phylogenetic relationships established from a 16S rDNA-RNA sequence analysis, includes all these genera within the family *Pseudonocardiaceae* with the exception of the genus *Actinokineospora* (Stackebrandt *et al.*, 1997).

An alternative approach to the traditional isolation and identification methods of members of this family

of *Actinomycetales* should consider not only the need for a better knowledge of their distribution and dependence on the environmental conditions, but the development of new identification techniques based on precise genotypic information that is not influenced by cultivation conditions. Frequently, classical methods have failed to describe completely the natural composition of certain microbial communities from which only the cultivatable micro-organisms can be identified (McVeigh *et al.*, 1996). Recent advances in nucleic acid techniques, based on data derived from the comparative analysis of the 16S rRNA sequences, have allowed the development of new and powerful tools that can be applied to the study of micro-organisms (Fox & Stackebrandt, 1987; Ludwig & Schleifer, 1994). In addition to the phylogenetic analyses derived from the study of the 16S rRNA molecule, the great variation in the regional sequence conservation of this molecule can also be exploited as a reliable tool to develop specific probes that can be applied at different taxonomic levels (Stackebrandt *et al.*, 1991; Stahl & Amann, 1991; McVeigh *et al.*, 1995). The use of specific probes as selective amplification primers offers an alternative approach for the rapid identification of large numbers of strains (Mehling *et al.*, 1995; Yoon *et*

Table 2. Wild-type isolates used in this study

Genus designations of strains were established according to PCR amplification data.

Strain	Country	Source
<i>Saccharopolyspora</i> spp.		
E10*	Bolivia	Soil
C21*	Brazil	Tropical rain forest soil
G3	India	Deciduous forest soil
H3*	Madagascar	Soil
E20*	Puerto Rico	Decomposing plant material
H18	Sri Lanka	Soil
A15*	Taiwan	Soil
H11, H13	West Indies	Decomposing plant material
<i>Amycolatopsis</i> spp.		
B08, EO3	Brazil	Soil
C18	Costa Rica	Sawdust
H4	Costa Rica	Organic material
G11	India	Decomposing plant material
E15	Panama	Tropical rain forest soil
G22	Panama	Mangrove mud
G2, H16	Spain	Soil
G1, G18, G20, G21	Canary Islands, Spain	Soil
C02	Venezuela	Soil
<i>Pseudonocardia</i> spp.		
B07	Brazil	Soil
A09	Costa Rica	Plant material
B04, B09, E09	Costa Rica	Soil
E07, E11	Costa Rica	Decomposing plant material
A04, A05*, A06, B03, B05	Costa Rica	Sawdust
A01, B11, D17, H17	India	Semiarid soil
C10, E08	India	Dense tropical rain forest soil
A03, D01, D02, D07, D08, D14	India	Rain forest soil
B01, D11*, D12	India	Desert soil
A12*, G5, G6, G7*, G13, G14	India	Decomposing plant material
H9*	India	Plant material
C05, C06, C17	Mexico	Volcanic soil
D15	Mexico	Soil
O1*, G23	Panama	Tropical rain forest soil
C07	The Philippines	Soil
A16, E16, E19	Puerto Rico	Decomposing plant material
A02, A14, D16	Sri Lanka	Soil
E13	Taiwan	Rice field soil
H8	Taiwan	Soil
H2	Venezuela	Soil
H10	Costa Rica	Decomposing plant material
Non-identified actinomycetes		
A10	Costa Rica	Plant material
C11	Costa Rica	Soil
A07, A08, G3, G4	Costa Rica	Sawdust
B12, D03	India	Tropical rain forest soil
C03	India	Semitropical forest soil
C01, C12	India	Soil
HH01	India	Arid soil
E12	India	Decomposing plant material
C04	Mexico	Volcanic soil
H14, H15	Philippines	Soil
C19	Puerto Rico	Decomposing plant material

Table 2 (cont.)

Strain	Country	Source
G8, G10	Taiwan	Garden soil
H7	Taiwan	Soil
H5, H6	Uganda	Soil
HHO2	Costa Rica	Sawdust
D18	Spain	Altitude forest soil
E14, G15, G16, G17, G19	Canary Islands, Spain	Soil
HH03, HH15	Spain	Soil
G9	Taiwan	Rice field soil

*Strains selected to be partially sequenced.

al., 1996). Species-specific probes have already been described for a large number of clinically relevant micro-organisms, but in this case, their application is restricted to identification at the species level. This study is focussed on the search for genus-specific probes that could potentially be applied to the detection and molecular identification of diverse strains belonging to the same genus. The development of new tools that can be used to study the family *Pseudonocardiaceae* is presented. Two sets of genus-specific oligonucleotides have been designed that are specific for the genera *Pseudonocardia* and *Saccharopolyspora* based on the comparative analysis of their 16S rRNA. These pairs of oligonucleotides allow a one-step identification of all strains belonging to these genera by means of selective amplification of partially purified genomic DNA.

METHODS

Bacterial strains. The bacterial strains used as reference organisms in this study are listed in Table 1. The 106 soil actinomycetes (wild-type isolates) included in this study were obtained from the laboratory culture collection. These strains were isolated from soils and plant materials obtained from diverse geographic origins (Table 2). All strains were grown on YMG (0.4% glucose, 0.4% yeast extract and 1.0% malt extract) agar and brain/heart agar (Difco) at 28 °C except for thermophilic strains, which were grown at 50 °C.

Design of oligonucleotide primers. Sequence comparison and analysis were carried out using programs from the University of Wisconsin GCG package (version 7.2, September 1994). The following 16S rRNA-DNA sequences, obtained from GenBank/EMBL, were studied for the design of the primers: X76953, *Actinokineospora riparia* (IFO 14541); X54287, *Actinopolyspora halophila* (ATCC 279); Z22730, *Actinopolyspora mortivallis* (JCM 7550); X76954, *Amycolata alni* (Ac901); X54288, *Amycolata autotrophica* (DSM 43210^T); X76955, *Amycolata hydrocarbonoxydans* (DSM 43281^T); X55609, '*Amycolata nitrificans*' (DSM, IFAM 379); X55608, *Amycolata petroleophila* (DSM, IFAM 78^T); X76956, *Amycolata saturnea* (DSM 43195^T); X53199, *Amycolatopsis azurea* (NRRL 11412^T); X53200, *Amyco-*

latopsis fastidiosa (ATCC 31181^T); X76957, *Amycolatopsis mediterranei* (DSM 13685^T); X54274, *Amycolatopsis methanolica* (NCIB 11946^T); X81573, *Amycolatopsis orientalis* (DSM 43187); X76958, *Amycolatopsis orientalis* subsp. *orientalis* (DSM 40040^T); X53191, *Kibdelosporangium aridum* (ATCC 39323^T); X76959, *Pseudonocardia compacta* (DSM 43592^T); Z14111, *Pseudonocardia halophobica* (DSM 43089^T); X53195, *Pseudonocardia thermophila* (ATCC 19285^T); X76960, '*Saccharomonospora caesia*' (INMI 19125^T); X54286, *Saccharomonospora viridis* (ATCC 15386^T); X76961, *Saccharomonospora* sp. (A1206); X53198, *Saccharopolyspora erythraea* (NRRL 2338^T); X76962, *Saccharopolyspora gregorii* (NCIB 12823^T); X53196, *Saccharopolyspora hirsuta* (ATCC 27875^T); X53197, *Saccharopolyspora hordei* (A54^T); X53194, *Saccharopolyspora rectivirgula* (ATCC 33515^T); X76967, *Saccharopolyspora* sp. (A215); X53193, *Saccharothrix australiensis* (ATCC 31947^T); X76963, *Saccharothrix coerulescens* (DSM 43679^T); X76964, *Saccharothrix longispora* (DSM 43749^T); X76965, *Saccharothrix mutabilis* subsp. *capreolus* (DSM 40225^T); X76966, *Saccharothrix mutabilis* subsp. *mutabilis* (DSM 43853^T); X53163, *Streptomyces albus* (DSM 40313^T); X53162, *Streptomyces brasiliensis* (DSM 43159^T); X53161, *Streptomyces diastaticus* (DSM 40496^T); X53173, *Streptomyces lavendulae* (DSM 2014^T); Y00484, *Streptomyces lividans* (TK21); X53170, *Streptomyces purpureus* (DSM 43460^T); X81574, *Streptomyces* sp. (NRRL 3890); X53168, *Streptovorticillium abikoense* (DSM 40831^T); X53164, *Streptovorticillium baldacii* (DPDU 0819^T); X53165, *Streptovorticillium cinnamomeum* subsp. *azacolatium* (DPDU 0074^T); X53171, *Streptovorticillium cinnamomeum* subsp. *cinnamomeum* (DPDU 0093^T); X53167, *Streptovorticillium ladakanum* var. *ladakanum* (DSM 40587^T); X53172, *Streptovorticillium luteoreticuli* (DPDU 0081); X79323, *Streptovorticillium mashuense* (DSM 40221); X53166, *Streptovorticillium olivoreticuli* subsp. *cellulophilum* (DPDU 0278^T); and X53169, *Streptovorticillium salmonis* (DPDU 0098^T). The genus specificity of oligonucleotides was tested against all DNA sequences available in the EMBL database with the FASTA program. The melting temperature (T_m) was estimated according to Thomas & Dancis (1973) and the Lathe's (1985) formula (Stahl & Amann, 1991). Relative T_m values obtained using 0.3 M as a standard salt concentration were used to design pairs of primers with similarly high T_m . The probabilities of primer-dimer formation and autofolding were also studied to keep them as low as possible.

The designed oligonucleotides, supplied by Pharmacia Biotech, are as follows (M = C or A): SP1, 5' GTGGAACCATCCCCACACC 3'; SP2, 5' GTGGAAMCAGTCCACACACC 3'; SP3, 5' GGTGACGGTAGGTGTAGAAG 3'; AMP3, 5' GCGGCACAGACACCGTGGAAAT 3'; AMP2, 5' GTGGAAAGTTTTTTCGGCTGGGG 3'; AMP4, 5' GCGGCACAGAAACCGTGGAAAT 3'; and AMP5, 5' GTGGAAAGTTTTTTCGGTGGGGG 3'.

DNA extraction. Total genomic DNAs from the different micro-organisms used in this study were recovered and purified as described previously (Innis *et al.*, 1990).

PCR amplification. DNA preparations were used as template DNA for *Taq* polymerase. Reactions were performed in a final volume of 25 µl containing 0.2 mM each of the four dNTPs (Roche), 0.1 µM each primer, 5 µl extracted DNA (1/100 dilution) and 0.5 U *Taq* polymerase (Appligene) with its appropriate reaction buffer. Controls without bacterial DNA were included for each PCR experiment. Amplifications were performed in a Perkin Elmer Cetus DNA Thermal Cycler 480, according to the following profile: 40 cycles of 30 s at 93 °C, 30 s at 53 °C and 2 min at 72 °C; followed by 10 min at 72 °C. In the case of amplification with AMP4/AMP5, the annealing temperature was 60 °C instead of 53 °C. Amplification products were analysed by electrophoresis (0.25 V cm⁻²) in 1.2% (w/v) agarose gels stained with ethidium bromide.

Direct sequencing of PCR products. The 16S rDNA sequences corresponding to the oligonucleotides SP1/SP2 (nucleotides 801–819) and to SP3 (nucleotides 450–469) were determined as follows for a group of strains of *Saccharopolyspora* (reference and wild-type strains). The sequence between nucleotides 801–809 was determined using two oligonucleotides, SQ1 (5' GCGAACAGGATTAGATACCCTGG 3') and SQ2 (5' CATCTCACGACACGAGCTGACG 3'), which were designed to match the conserved sequences flanking this region. The sequences of these primers correspond to nucleotide positions 750–772 and 1035–1056, respectively. The amplification products obtained with SP3/SQ2 were sequenced directly with the T7 Sequenase v2.0 PCR product sequencing kit (Amersham). The sequence was determined in both strands using SQ1 and SQ2, respectively. Alternatively, a third primer, 685d3 (5' GTAGCGGTGAAATGCGCAGA 3'; Lane, 1991), corresponding to nucleotides 657–676, was also used to sequence one of the strands. The sequence between nucleotides 450–469 was determined using two oligonucleotides, F243 (Heuer *et al.*, 1997) and 685r3 (Lane, 1991), that match conserved sequences flanking this region. The sequences of these primers correspond to nucleotide positions 218–235 and 657–676, respectively. The amplification products obtained with F243/SP1 were sequenced in both strands using F243 and 685r3 (Heuer *et al.*, 1997).

In the case of *Pseudonocardia* strains, the 16S rDNA sequences corresponding to oligonucleotides AMP2 (nucleotides 197–219) and AMP3 (nucleotides 813–833) were determined for a series of wild-type strains as indicated below. The amplification products containing the sequence of AMP2 between nucleotides 197–219 were obtained with the oligonucleotides 27F (Lane, 1991) and AMP3. Both strands were sequenced using the oligonucleotides 27F and 342r as sequencing primers (Lane, 1991). The sequence of AMP3 between nucleotides 813–833 was determined in both strands from an amplification product obtained with the primers AMP2 and SQ2; oligonucleotides 685d3 (complementary to 685r3) and SQ2 were used as sequencing primers.

Whole-cell fatty acid composition. Cultures were grown as confluent patches on YCZ [0.05% yeast extract and 1.17% (w/v) Czapek Dox (Difco)] agar at 28 °C for 10 d. Vegetative growth was then scraped (100–200 mg) and fatty acid methyl esters (FAMES) were prepared using a modified sample preparation (Sasser, 1990). FAME analysis was carried out by capillary GC using a Hewlett-Packard model 5890 GC/MIDI system (Microbial ID) equipped with a phenyl-methylsilicone column (0.2 mm × 25 m). Chromatography conditions were as recommended by the manufacturer. Individual FAME identification and clustering analysis were performed using the MIS software (Microbial ID).

RESULTS AND DISCUSSION

In this work, the design of two sets of genus-specific pairs of primers for the selective amplification of the 16S rDNA region of the genera *Pseudonocardia* and *Saccharopolyspora* is described. The search for conserved sequences within a given genus was based on comparative analysis of variable regions of the known 16S rRNA sequences. The strains listed in the Methods include representatives from different members of the family *Pseudonocardiaceae* as well as the related genera *Saccharothrix* and *Actinokineospora*. Sequences from 16 *Streptomyces* strains were also included in this analysis to rule out any homology of sequences of strains of *Pseudonocardia* and *Saccharopolyspora* with representatives of the genus *Streptomyces*.

The analysis was performed by alignment of all the sequences using the PILEUP function of the GCG software. A detailed study of the aligned sequences allowed the localization of six variable regions in the 16S rDNA sequence corresponding to nucleotide positions 170–230, 430–580, 560–640, 790–830, 960–1020 and 1090–1130 of the *Streptomyces ambofaciens* 16S rDNA gene (Pernodet *et al.*, 1989). Three of these sequences (positions 170–230, 960–1020 and 1090–1120, respectively) corresponded to the alpha, beta and gamma regions previously described for the genus *Streptomyces* (Stackebrandt *et al.*, 1992). The search for genus-specific sequences was focussed on these six variable regions and, from the beginning, the region between nucleotides 790–830 appeared to be the most promising, given the high sequence conservation found within each genus. This homology remained high enough to consider the design of genus-specific primers. Two additional regions, corresponding to positions 197–230 and 430–580, also showed an appropriate degree of conservation within certain genera and were also suitable for this purpose. However, analysis of sequences in the other hypervariable regions showed such a low homology within each genus that these sequences may only be used at the species or even strain level.

Specific primers for the genus *Pseudonocardia*

When the 16S rRNA region between nucleotides 813–833 from different representatives of the genus *Pseudonocardia* was examined in detail, it was observed that the sequence in this region seemed to be unique for

(a)

Pseudonocardia*

Kibdelosporangium aridum (ATCC 39323^T)
Saccharomonospora viridis (ATCC 15386^T)
Saccharothrix australiensis (ATCC 31947^T)
Amycolatopsis orientalis (DSM 40040^T)
Saccharopolyspora hirsuta (ATCC 27875^T)
Streptomyces ambofaciens (ATCC 23877^T)

197 219 813 833

CT CT
TG TG

5' GTGGAAGTTT TTTTCGG TGGG 3' 3' TAAGGTGCCA GACACCGCG 5'

AMP2 → ← AMP3

AMP5 → ← AMP4

(b)

***Saccharopolyspora*†**

Kibdelosporangium aridum (ATCC 39323^T)
Saccharomonospora viridis (ATCC 15386^T)
Saccharothrix australiensis (ATCC 31947^T)
Amycolatopsis orientalis (DSM 40040^T)
Pseudonocardia thermophila (ATCC 19285^T)
Streptomyces ambofaciens (ATCC 23877^T)

450 469 801 819

TA TA
CG CG

5' GGTGACGGTAGGTG GAAG 3' 3' CCACACCCCT C AAGGTG 5'

SP3 → ← SP2

SP1

Fig. 1. Comparison of the 16S rRNA-DNA sequences of representatives of different genera of the family *Pseudonocardiaceae* and *Streptomyces ambofaciens* in the regions where the specific oligonucleotides are located. (a) Sequence of the nucleotide regions 197–219 and 813–833; *, consensus sequences for the genus *Pseudonocardia* from which the oligonucleotides AMP2/AMP5 and AMP3/AMP4 were designed, respectively (N = any nucleotide). (b) Sequence of the regions 450–469 and 801–820; †, consensus sequences for the genus *Saccharopolyspora* from which SP3 and SP2/SP1 were designed (degenerate position M = C or A).

this genus (Fig. 1a). However, based on a single base change in this region (nucleotide position 823), strains were divided into two different groups: one formed by the strains *Pseudonocardia alni*, *Pseudonocardia hydrocarbonoxydans*, *Pseudonocardia petroleophila*, *Pseudonocardia saturnea*, *Pseudonocardia halophobica*, *Pseudonocardia compacta* and *Pseudonocardia thermophila*; and a smaller group comprising *Pseudonocardia autotrophica* and '*Pseudonocardia nitrificans*'. Although the sequences of both groups of strains differed by just one nucleotide in this region, analysis of the whole 16S rRNA showed how the sequences of these strains usually divide into the same two groups in the variable regions of these molecules, which could indicate a phylogenetic clustering within the genus. In order to assess this possibility, two different reverse primers were designed that differ in this single nucleotide: AMP3 (5' GCGGCACAGACCGTGGAAAT 3'), which is specific for the largest group of sequences; and AMP4 (5' GCGGCACAGAAACCGTGGAAAT

3'), which matches the strains of *Pseudonocardia autotrophica* and '*Pseudonocardia nitrificans*' (Fig. 1a).

Another conserved region observed in the members of this genus was also found around nucleotide positions 197–219 and this sequence was selected for the design of the forward primer (Fig. 1a). Again, two differences in the sequences in this region (nucleotide positions 214 and 215) divided the species of this genus into the same two groups mentioned above. Therefore, two forward primers were constructed: AMP2 (5' GTGG-AAAGTTTTTTCGGCTGGGG 3'), which is specific for the same group of sequences as AMP3; and AMP5 (5' GTGGAAAGTTTTTTCGGTGGGGG 3'), to be used in the amplification assays in combination with AMP4. According to the sequence, the expected size of the amplification product using both pairs of oligonucleotides, AMP3/AMP2 or AMP4/AMP5, is 640 bp (Fig. 1a).

To test the genus specificity of these two pairs of

Fig. 2. Agarose gel electrophoresis of PCR products. (a) Selective amplification of the 640 bp fragment using primers AMP2/AMP3 specific for the genus *Pseudonocardia*. Lanes: 1, control reaction without DNA; 2, *Pseudonocardia autotrophica*; 3, *Pseudonocardia compacta*; 4, *Pseudonocardia halophobica*; 5, *Pseudonocardia hydrocarbonoxydans*; 6, '*Pseudonocardia nitrificans*'; 7, *Pseudonocardia petroleophila*; 8, *Pseudonocardia saturnea*; 9, *Pseudonocardia thermophila*; 10, *Saccharopolyspora hirsuta* subsp. *hirsuta*; 11, *Amycolatopsis orientalis* subsp. *orientalis*; 12, *Saccharomonospora viridis*; 13, *Kibdelosporangium aridum*; 14, *Saccharothrix aerocolonigenes*; 15, *Actinomadura pusilla*; and 16, *Streptomyces cattleya*. (b) Selective amplification of the 371 bp fragment using primers SP1/SP3 specific for the genus *Saccharopolyspora*. Lanes: 1, control reaction without DNA; 2, *Saccharopolyspora erythraea*; 3, *Saccharopolyspora gregorii*; 4, *Saccharopolyspora hirsuta* subsp. *hirsuta*; 5, *Saccharopolyspora hirsuta* subsp. *kobensis*; 6, *Saccharopolyspora hordei*; 7, *Saccharopolyspora rectivirgula*; 8, *Saccharopolyspora spinosa*; 9, *Saccharopolyspora taberi*; 10, *Pseudonocardia compacta*; 11, *Amycolatopsis orientalis* subsp. *orientalis*; 12, *Saccharomonospora viridis*; 13, *Kibdelosporangium aridum*; 14, *Actinomadura pusilla*; and 15, *Streptomyces cattleya*. In (a) and (b) lane L, molecular mass marker (100 bp ladder; Pharmacia Biotech).

oligonucleotides, PCR assays were performed using template DNAs isolated from a series of reference strains of 11 different genera (Table 1). Using the AMP3/AMP2 primer pair and an annealing temperature of 53 °C, the specific amplification product of 640 bp was obtained from all strains of the eight different species of *Pseudonocardia* tested [*Pseudonocardia autotrophica* (three strains), *Pseudonocardia compacta*, *Pseudonocardia halophobica*, *Pseudonocardia hydrocarbonoxydans*, '*Pseudonocardia nitrificans*', *Pseudonocardia petroleophila*, *Pseudonocardia saturnea* and *Pseudonocardia thermophila*]. It should

be noted that this pair of primers is specific for all strains of *Pseudonocardia* tested, in spite of their sequence differences. No amplification products were detected in control experiments performed with DNAs from any of the other bacterial strains analysed (Table 1; Fig. 2a). To confirm that this lack of amplification with the reference strains other than *Pseudonocardia* was not due to the potentially poor condition of the DNA extracts, amplification reactions were performed with previously published primers, designed specifically for actinomycetes (F243) and for Gram-positive bacteria (R513), under the conditions recommended

by Heuer *et al.* (1997). The expected amplification product of 271 bp was observed in all actinomycete strains tested.

When the specificity of the AMP4/AMP5 primer pair was tested under the same reaction conditions with this group of strains, the annealing temperature of 53 °C proved to be too low and non-specific bands were observed in the DNA amplification products from several strains belonging to other genera. Experiments were performed at different annealing temperatures and it was found that increasing this temperature to 60 °C rendered the pair of primers specific for the genus *Pseudonocardia*. At 60 °C, a single PCR amplification product of 640 bp was obtained from all strains of *Pseudonocardia* tested. Again, no amplification products were detected when template DNAs from all the other reference strains were used. The specificity of the AMP2/AMP3 pair was also confirmed at 60 °C with all the reference strains of *Pseudonocardia* tested.

These results show that both pairs of primers, AMP3/AMP2 and AMP4/AMP5, allow identification of all the tested strains belonging to the genus, in spite of the differences observed in their sequences. The low-stringency conditions used in the PCR reaction were not selective enough to distinguish between sequences which differ in just one nucleotide. On the other hand, the AMP2/AMP3 pair is genus-specific at a lower annealing temperature (53 °C), providing a much more accurate tool for the detection and identification of members of this genus. In fact, comparative analysis of AMP3 and AMP4, in which both sequences correspond to the nucleotide region 813–833, with sequences from other representative type species showed that the single base change makes AMP3 more genus-specific than AMP4. In the case of the oligonucleotides AMP2 and AMP5, there is a difference at two positions. These nucleotides in AMP2 (CT) do not occur in any of the tested reference strains, whereas the corresponding nucleotides in AMP5 (TG) are also shared by other genera and make this primer less specific (Fig. 1a). In consequence, the AMP2/AMP3 pair was finally selected to be used in the subsequent amplification experiments.

To rule out possible PCR amplification with primers AMP2/AMP3 of 16S rDNA sequences other than those of the genera already tested, FASTA analysis was carried out against all the DNA sequences available in GenBank. It was found that, although oligonucleotide AMP2 showed total homology with sequences of two unidentified isolates (U27857 and D50066) and a strain of *Microsphaera multipartita* (Y08541), no homology was observed with any of these sequences with AMP3, and therefore no amplification could be expected in these cases. High homology of AMP2 was also obtained with a series of sequences of different species of the genera *Microbispora* (U48984 and D86936), *Microtetraspora* (D85490 and U48976), *Planobispora* (D85494), *Planotetraspora* (D85496) and *Strepto-*

sporangium (X89934, X89937, X89939, X89943, X89944, U48976, X89953, X89955 and X89958) among others. All these sequences showed a single nucleotide difference at position 214. Although effective binding of AMP2 to these sequences could be expected, these strains showed at least three, as in the case of *Microbispora rosea* (D86936), or four different bases in their sequence corresponding to the other primer AMP3; therefore, amplification should not be expected using this pair of primers. To experimentally verify this hypothesis, the DNA of representative reference strains [*Microbispora aerata* ATCC 1544^T (U48984), *Microbispora rosea* ATCC 12950^T (D86936), *Microtetraspora glauca* ATCC 23057^T (D85490), *Microtetraspora niveoalba* ATCC 27301^T (U48976), *Streptosporangium roseum* ATCC 12428^T (U48976) and *Streptosporangium vulgare* ATCC 33329^T (X89955)] was tested with the AMP2/AMP3 pair. No amplification was obtained at 53 °C with any of these strains except for *Microbispora rosea* ATCC 12950^T, which positively amplified with these primers. When the DNA from this strain was tested at an annealing temperature of 60 °C, no amplification product was obtained, confirming the specificity of these primers for sequences of the genus *Pseudonocardia* at this temperature.

The only complete homology found for the primer AMP3 corresponded to the sequence of *Actinobispora yunnanensis* IFO15681 (D85472), a member of the *Pseudonocardiaceae*. The primer AMP2 did not perfectly match the corresponding sequence of this strain which presented one deletion in one of the series of six T bases in the sequence.

Genus-specific primers for *Saccharopolyspora*

The same strategy was used in the design of oligonucleotides specific for the identification of members of the genus *Saccharopolyspora*. After a close examination of the conservation of the 16S rDNA region around nucleotides 801–819 of the sequenced strains of *Saccharopolyspora*, two groups of sequences could be defined (Fig. 1b). One group included strains of *Saccharopolyspora hirsuta* subsp. *hirsuta*, *Saccharopolyspora hordei* and *Saccharopolyspora* sp. (A215), whereas the second group comprised strains of *Saccharopolyspora erythraea*, *Saccharopolyspora gregorii* and *Saccharopolyspora rectivirgula* (Table 1). According to the differences in their sequence, two reverse oligonucleotides, SP1 (5' GTGGAACCC-ATCCCCACACC 3') and SP2 (5' GTGGAAMC-AGTCCCCACACC 3'), were constructed that were directed to each of the groups mentioned above. The lack of complete conservation within the second group of sequences required the use of a degenerate nucleotide within SP2 (Fig. 1b).

To define a specific oligonucleotide to be used as a forward PCR primer, the remaining upstream variable sequences were studied and the region included within positions 450–469 was selected. In this case, identical

sequences were found for different strains of *Saccharopolyspora* (*Saccharopolyspora hirsuta* subsp. *hirsuta*, *Saccharopolyspora hordei*, *Saccharopolyspora erythraea* and *Saccharopolyspora gregorii*). The exception was a strain of *Saccharopolyspora rectivirgula* ATCC 33515, which differed at only two positions (nucleotides 464 and 465) and the *Saccharopolyspora* sp. A215 strain which differed at positions 460 and 461. This region was used to design the forward oligonucleotide SP3 (5' GGTGACGGTAGGTGTAGAAG 3'). The expected size of the PCR product using either SP1/SP3 or SP2/SP3 as amplification primers, was 371 bp (Fig. 1b).

To examine the genus specificity of both primer combinations, they were tested in PCR reactions at an annealing temperature of 53 °C with chromosomal DNAs extracted from all the bacterial strains listed in Table 1. In spite of the sequence differences between SP1 and SP2, both primers were able to hybridize to all the *Saccharopolyspora* sequences. No amplification products were observed with the other reference strains, whereas in all these strains, a PCR fragment was obtained with the pair of primers F243/R513 for actinomycetes.

The same amplification product of 371 bp was obtained with both pairs of primers, SP1/SP3 and SP2/SP3, from all the *Saccharopolyspora* strains tested [*Saccharopolyspora erythraea*, *Saccharopolyspora gregorii*, *Saccharopolyspora hirsuta* subsp. *hirsuta* (two strains), *Saccharopolyspora hirsuta kobensis*, *Saccharopolyspora hordei*, *Saccharopolyspora rectivirgula* (two strains), *Saccharopolyspora spinosa* (two strains) and *Saccharopolyspora taberi*]. In fact, two strains of *Saccharopolyspora rectivirgula* which were positively identified with the primers, contained sequences around positions 450–469 that do not perfectly match with SP3 (nucleotide positions 464 and 465), but still produce the 371 bp amplification fragment. The effect of this divergence was only observed when the annealing temperature was increased by at least 4 °C. In this case, experiments done at annealing temperatures of 57 °C or higher failed to produce amplification fragments. In the case of the strain of *Saccharopolyspora* A215, positive amplification with the primers SP1/SP3 would be expected, taking into account the results obtained with *Saccharopolyspora rectivirgula*. Nevertheless, these data will need to be confirmed experimentally.

At the time when these experiments were performed, the sequence of this region was not available for two strains of *Saccharopolyspora* (*Saccharopolyspora spinosa* and *Saccharopolyspora rectivirgula*), which also produce the expected amplification fragment. To confirm their sequence homology, either to SP1 or SP2, both strands of the region at positions 801–819 were sequenced from two of these strains. As expected, the sequence from *Saccharopolyspora spinosa* NRRL 18537 (AF061977) perfectly matched the sequence of primer SP1, whereas that of *Saccharopolyspora rectivirgula* ATCC 15347 (AF061976) matched SP2.

Under the low-stringency conditions used, both pairs of primers (SP1/SP3 and SP2/SP3) showed the same specificity for all the strains of *Saccharopolyspora* tested, including those whose sequences did not match perfectly. As mentioned above, the sequence specificity of the oligonucleotides showed only when the annealing temperature in the PCR reaction was above 57 °C. In fact, at annealing temperatures of 57 °C *Saccharopolyspora rectivirgula* did not always present an amplification product and when the annealing temperature was increased to 60 °C, PCR reactions with SP1/SP3 did not produce a band with strains from the group which matches with SP2 and vice versa.

Identification of wild-type isolates

Once the genus specificity of the oligonucleotides had been verified on the reference strains from culture collections, the probes were tested as identification tools for potential members of the genera *Pseudonocardia* and *Saccharopolyspora*. A group of 106 wild-type actinomycetes, obtained in the course of a screening programme from a large diversity of geographic origins (Table 2), was tested. These strains had been tentatively assigned to the family *Pseudonocardiaceae* on the basis of their microscopic morphological characters. According to the results presented above, the AMP3/AMP2 primer pair, which allows genus-specific amplification under low-stringency conditions, was selected to identify those members of the genus *Pseudonocardia*. In the case of the genus *Saccharopolyspora*, the SP1/SP3 primer pair was used. Under the conditions used in the PCR assay, the same information could be obtained with the other two pairs of oligonucleotides, AMP4/AMP5 and SP2/SP3, respectively. DNA was extracted from each of the 106 wild-type strains and was amplified in two parallel PCR assays with AMP3/AMP2 and SP1/SP3. Of the 106 isolates, 51 produced a 640 bp amplification product with AMP3/AMP2, whereas no PCR products were obtained with SP1/SP3. According to these results, the 51 strains were tentatively assigned to the genus *Pseudonocardia*. On the other hand, only nine wild-type strains gave an amplification product of 371 bp with SP1/SP3 and no amplification was observed with the oligonucleotides specific for *Pseudonocardia*. These cultures were thus identified as strains of the genus *Saccharopolyspora*. The remaining isolates gave no amplification products with either pair of oligonucleotides. Again, the quality of these DNA extracts was tested with primers F243/R513 and, in all cases, an amplification product of the expected size for actinomycetes was observed. These strains probably correspond to members of the other related genera of the family.

Diversity of the strains identified with the probes

To determine the diversity of the isolates that could be detected within a genus with these oligonucleotides, the existing relationship of the isolates to the reference strains used in this study was examined by FAME

Fig. 3. Dendrogram showing the diversity of the wild-type (wt) strains identified with probes specific for the genera *Pseudonocardia* and *Saccharopolyspora* and their relationship to the reference strains of the family *Pseudonocardaceae*. Reference strains are those included in Table 1. Cultures marked (a) to (l) correspond to strains of the same species: (a), *Amycolatopsis orientalis* subsp. *orientalis* ATCC 21425; (b, c, d), *Amycolatopsis orientalis* NRRL 18098, NRRL 18099 and NRRL 18100, respectively; (e), *Amycolatopsis orientalis* subsp. *orientalis* DSM 40040; (f), *Amycolatopsis orientalis* subsp. *orientalis* NRRL 2452; (g), *Saccharopolyspora hirsuta* subsp. *hirsuta* NRRL B5792^T; (h), *Saccharopolyspora hirsuta* subsp. *hirsuta* ATCC 27876; (i, j), *Pseudonocardia autotrophica* ATCC 19727^T and DSM 43098, respectively; (k, l), *Saccharothrix aerocolonigenes* ATCC 39243 and NRRL B3298^T, respectively. Strains referred to as *Amycolatopsis* sp. correspond to wild-type strains identified as members of the genus *Amycolatopsis* by means of a pair of PCR primers designed specifically for several species of this genus (unpublished data).

composition analysis (Embley *et al.*, 1987; Embley & Wait, 1994). The fatty acid composition of a group of 121 strains was analysed, including 39 reference strains of the genera *Amycolatopsis* (ten strains), *Saccharopolyspora* (eight strains), *Saccharomonospora* (four strains), *Pseudonocardia* (nine strains) and *Saccharothrix* (seven strains) (Table 1), and a subset of 82 wild-type isolates selected from the group of 106 actino-

mycetes previously mentioned, which contained strains that were positively identified to the genus level by PCR. Cluster analysis of the strains was based on their fatty acid profiles and their relationship was established in a dendrogram generated by treating the Euclidian distances of the fatty acids with the unweighted pair group method with arithmetic mean algorithm (Fig. 3).

Table 3. Partial 16S rDNA sequences of the wild-type isolates assigned to the genus *Saccharopolyspora*

Strain	Nucleotide sequence region		GenBank no.
	450–469	801–819	
	SP3		SP1*
			SP2*
CIBE A15	GGTGACGGTAGGTGTAGAAG	GGTGTGGGGATGGGTTCAC	AF064978
CIBE C21	GGTGACGGTAGGTGTAGAAG	GGTGTGGGGACTGTTCCAC	AF064979
CIBE E10	GGTGACGGTAGGTGTAGAAG	GGTGTGGGGATGGGTTCAC	AF064980
CIBE H3	GTGACGGTAGGTGTAGAAG	GGTGTGNGGATGGGTTCNN	AF064981
CIBE E20	GGTGACGGTAGGTGTAGAAG	GGTGTGGGGACTGTTCCAC	AF064982, AF064983

* For the best comparison with the sequenced regions, complementary sequences are shown for reverse primers SP1 and SP2.

Strains of the genus *Saccharopolyspora* clustered in a homogeneous group defined at a Euclidian distance below 21.0. Within this cluster, six of the eight reference *Saccharopolyspora* strains used in this study (*Saccharopolyspora erythraea*, *Saccharopolyspora gregorii*, *Saccharopolyspora hirsuta* subsp. *hirsuta*, *Saccharopolyspora hordei*, *Saccharopolyspora spinosa* and *Saccharopolyspora taberi*) and eight wild-type strains that had been positively assigned to this genus by PCR were included. The only exceptions were two strains of *Saccharopolyspora hirsuta* (*Saccharopolyspora hirsuta* subsp. *hirsuta* and *Saccharopolyspora hirsuta* subsp. *kobensis*) that were found in a separate branch of the dendrogram (Fig. 3). It is interesting to note that the phenotypic homogeneity observed for the group of wild-type strains of *Saccharopolyspora* contrasts with the diversity in the geographic source of the isolates (Table 2).

In contrast to the genus *Saccharopolyspora*, strains of *Pseudonocardia* tested (reference strains and wild-type isolates) that produced a specific PCR amplification product with the oligonucleotides designed for this genus were distributed in 13 different branches of the dendrogram defined at a Euclidian distance below 21.0. The largest group of wild-type strains assigned by PCR to this genus (37 out of 46 isolates) was contained within a single cluster. These strains, although closely related on the basis of their fatty acid profiles, correspond to different isolates and were obtained from quite different environmental substrates and geographic origins (Table 2). This group also included four reference strains of the genus *Pseudonocardia* (*Pseudonocardia nitrificans* DSM 46012, *Pseudonocardia autotrophica* ATCC 19727^T and DSM 43098, and *Pseudonocardia saturnea* NRRL B-16172^T). The other nine wild-type isolates, as well as five strains of the species *Pseudonocardia petroleophila*, *Pseudonocardia thermophila*, *Pseudonocardia halophobica*, *Pseudonocardia compacta* and *Pseudonocardia hydrocarbonoxydans*, used in the study appeared on unique branches of the dendrogram or clustered with other members of the family (Fig. 3).

With regard to the other genera included in the study,

the reference strains of the genera *Amycolatopsis* and *Saccharothrix* were also distributed in different branches of the dendrogram. One large cluster included six strains of *Amycolatopsis* (four strains of *Amycolatopsis orientalis*, *Amycolatopsis sulphurea* and *Amycolatopsis azurea*) and five strains of *Saccharothrix* (three strains of *Saccharothrix mutabilis*, one of *Saccharothrix flava* and one of *Saccharothrix syringae*). Three of the four strains of *Saccharomonospora* clustered together, whereas the strain of *Saccharomonospora azurea* was found in another branch related to *Pseudonocardia* isolates.

Clustering analysis based on fatty acid composition was only used to show the large diversity of new isolates that could be identified to the genus level by means of selective PCR amplification with the specific primers. It was observed that although a large number of wild-type isolates identified with these primers and assigned to the genus *Pseudonocardia* are associated with the same clusters, many strains also appeared on separate branches of the dendrogram and did not match any of the clusters defined by the fatty acid composition of reference strains. Such a large diversity was not detected with the probes among the strains identified as *Saccharopolyspora*. In this case, all the strains consistently clustered with the reference strains.

To determine that the strains positively identified with the pairs of primers effectively corresponded to these genera and to discard the possibility of a lack of specificity of the primers as the source of the inconsistencies observed in the clustering, the nucleotide sequences of the 16S rDNA regions corresponding to SP1/SP3 and AMP3/AMP2 were determined for a representative subset of wild-type isolates included in the analysis. From the group of wild-type *Saccharopolyspora* strains, the sequences of five isolates were determined (strains CIBE A15, CIBE C21, CIBE E10, CIBE E20 and CIBE H3) in the regions corresponding to primers SP1/SP3 (Table 3). In all strains except CIBE E20, the sequence between positions 801–819 perfectly matched the sequence of SP1. In strain CIBE E20, the sequence corresponded to that of SP2. Analysis of the sequences between positions 450–469

Table 4. Partial 16S rDNA sequences of the wild-type isolates assigned to the genus *Pseudonocardia*

Dots indicate the deletion of one of the six T bases found in wild-type strains.

Strain		Nucleotide sequence region		GenBank no.
		197–219	813–833	
	AMP2	GTGGAAAGTTTTTTCGG CT GGGG	ATTCCACGGT CT CTGTGCCGC	AMP3*
	AMP5	GTGGAAAGTTTTTTCGG TG GGGG	ATTCCACGGT TT CTGTGCCGC	AMP4*
CIBE G7		GTGGAAAG .TTTTTCGG TG GGGG	ATTCCACGGT CT CTGTGCCGC	AF064968, AF064969
CIBE D11		GTGGAAAG .TTTTTCGG CT GGGG	ATTCCACGGT CT CTGTGCCGC	AF064970
CIBE O1		GTGGAAAG .TTTTTCGG TG GGGG	ATTCCACGGT TT CTGTGCCGC	AF064971, AF064972
CIBE H9		GTGGAAAG .TTTTTCGG TG GGGG	ATTCCACGGT CT CTGTGCCGC	AF064973, AF064975
CIBE A12		GTGGAAAGTTTT G TCGG TG GGGG	ATTCCACGG GT TC CG TGCCGC	AF064976
CIBE A5		GTGGAAAG .TTTTTCGG CCT GGG	ATTCCACGG GT TC CG TGCCGC	AF064977

* For the best comparison with the sequenced regions, complementary sequences are shown for reverse primers AMP3 and AMP4.

(primer SP3) showed that all the strains shared the same sequence as SP3 with the exception of CIBE H3, which presented a single base change (G to T) at position 451. The high degree of conservation of the sequences analysed from the wild-type isolates supports the specificity of the primers used for the identification of the strains.

From the group of *Pseudonocardia* strains, six isolates were selected (strains CIBE A5, CIBE A12, CIBE D11, CIBE G1, CIBE G7 and CIBE H9) from the group of wild-type strains assigned to this genus from different representative clusters and the nucleotide regions corresponding to the primers AMP3 and AMP2 were sequenced (Table 4). Within the region between positions 197–219, all strains except for CIBE A5 almost exactly matched either the sequence of AMP2 or AMP5, presenting one single difference in their sequences: a deletion of one of the six T bases in the case of strains CIBE G7, D11, O1 and H9, and a base change (T to G) at position 208 for strain CIBE A12. In the region between positions 813–833 corresponding to the other pair of primers, the four strains CIBE G7, D11, O1 and H9 showed a perfect match with AMP3 or AMP4. In the case of strain CIBE A12, two substitutions were found at positions 822 and 826 that did not seem to interfere with annealing of the primer. In fact, the DNA from this strain, as well as the DNAs from the other four isolates previously mentioned, positively amplified with the pair of primers even when more restrictive annealing conditions (60 °C) were used. The sequence of strain CIBE A5 was identical to that of strain CIBE A12 in the region 813–833, but in the region 197–219 it showed, in addition to the deletion of one of the six T bases that was already observed in the other strains, three substitutions close to the 3' end of the primer that should affect primer annealing. Surprisingly, the DNA of this strain positively amplified with AMP2/AMP3 at 60 °C. Further analysis will be necessary to evaluate the degree of variability of these sequences that can be found among wild-type isolates of this taxon. Such analysis may also

detect similarities in the sequences with other genera, as observed in the case of the wild-type isolate CIBE D11 and *Actinobispora yunnanensis* IFO 15681. Both strains share the same sequence in the regions corresponding to both primers AMP2 and AMP3 and therefore positive amplification should also be expected in the case of the *Actinobispora* strain.

Results obtained here with reference strains as well as the high degree of conservation observed in most of the sequences of the analysed wild-type isolates, validate the specificity of these new primer pairs for 16S rDNA sequences of members of the genera *Pseudonocardia* and *Saccharopolyspora*. These data support the usefulness of these primers for the tentative assignment of new isolates to this genera from the large numbers of strains which are normally obtained from the environment. In fact, amplification with these primers does not even require the isolation of chromosomal DNA. The same results can be obtained by scraping some mycelium, even from the original colony, and using whole cells directly in the PCR reaction (data not shown). Nevertheless, these tools have their limitations, as some of the exceptions mentioned above have shown, given that they are based on the current available sequence data which are constantly being renewed. Additional applications of these probes, directed to the future *in situ* identification of the presence of members of these genera in the environment, are being tested, although such kinds of approach would require further optimization of the probes.

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