New genus-specific primers for the PCR identification of novel isolates of the genera *Nocardiosis* and *Saccharothrix*

Oscar Salazar, Ignacio González and Olga Genilloud

Centro de Investigación
Básica, Merck Research
Laboratories, Merck Sharp
and Dohme de España SA,
Josefa Valcárcel 38, E-28027
Madrid, Spain

Author for correspondence: Olga Genilloud. Tel: +34 91 3210 568. Fax: +34 91 3210 614.
e-mail: olga_genilloud@merck.com

The taxonomic position of the genera *Saccharothrix* and *Nocardiosis* has evolved in recent years to accommodate an increasing number of actinomycete strains that cannot be clearly distinguished by morphological characters. More recently, the taxonomic reorganization of the genus *Saccharothrix* has determined the creation of new, related genera, increasing the complexity of the identification of this taxon. Nevertheless, today these genera can still only be identified by applying chemotaxonomic and molecular criteria, and no other tools are available for the rapid distinction of members of the two genera. Phylogenetic analysis based on 16S rDNA nucleotide sequences of reference strains has shown that both genera represent complete distinct lineages within the order *Actinomycetales*. Differences in the nucleotide sequences of the 16S rDNAs of reference strains were used to design two pairs of genus-specific primers to identify novel members of the genera *Nocardiosis* and *Saccharothrix* by PCR amplification. The genus specificity of these primers was validated with reference strains as well as with wild-type isolates that exhibited morphological characteristics common to both genera. The diversity and taxonomic position of the isolates identified with these tools is also discussed.

Keywords: *Nocardiosis*, *Saccharothrix*, genus-specific primers, PCR, 16S rDNA sequences

INTRODUCTION

The composition of the genera *Saccharothrix* and *Nocardiosis* has evolved in recent years to accommodate an increasing number of actinomycete strains that, although unrelated phylogenetically, cannot be clearly distinguished by morphological characters and, today, can still only be identified by chemotaxonomic and molecular criteria.

The genus *Nocardiosis* was described by Meyer (1976) on the basis of the morphological characteristics and cell-wall type of the species *Actinomadura dassonvillei*. The genus is currently defined on the basis of chemotaxonomic markers including cell-wall chemotype IIIC

(Lechevalier *et al.*, 1977), phospholipid type III (phosphatidylcholine and phosphatidylmethylethanolamine as diagnostic phospholipids) (Lechevalier *et al.*, 1977), menaquinone MK-10 with variable degrees of saturation (Kroppenstedt, 1992) and fatty acid type 3d (Kroppenstedt, 1985). Other chemotaxonomic features include a lack of mycolic acids, the presence of muramic acid of the acetyl type and DNA G+C contents between 64 and 71 mol% (Labeda *et al.*, 1984; Kroppenstedt, 1985, 1992; Poschner *et al.*, 1985). On the basis of chemotaxonomic characteristics, Grund & Kroppenstedt (1989) transferred five species of this genus (*Nocardiosis coeruleofusca*, *Nocardiosis flava*, *Nocardiosis longispora*, *Nocardiosis mutabilis* and *Nocardiosis syringae*) to the genus *Saccharothrix*. The two invalid species '*Actinomyces alborubidus*' and '*Streptomyces listeri*' were later reclassified as members of *Nocardiosis* on the basis of chemotaxonomic and numerical data (as *Nocardiosis alborubidus* and *Nocardiosis listeri*) (Grund & Kroppenstedt, 1990). More recently, the strain *Nocardiosis* sp. K-252^T

Published online ahead of print on 18 February 2002 as DOI 10.1099/ijs.0.02032-0.

An alignment of the sequences used to evaluate primer specificity is available as supplementary material in IJSEM Online (<http://ijs.sgmjournals.org/>).

(= NRRL 15532^T) was transferred to the genus *Nonomuraea* as *Nonomuraea longicatena* sp. nov. on the basis of chemotaxonomic, morphological, DNA–DNA reassociation and 16S rDNA sequence data (Chiba *et al.*, 1999). The genus *Nocardioopsis* then contained seven validated species: *Nocardioopsis dassonvillei* (Meyer, 1976), *Nocardioopsis alba*, *Nocardioopsis listeri* (Grund & Kroppenstedt, 1990), *Nocardioopsis halophila* (Al-Tai & Ruan, 1994), *Nocardioopsis lucentensis* (Yassin *et al.*, 1993), *Nocardioopsis prasina* and *Nocardioopsis synnemataformans* (Yassin *et al.*, 1997). Recently, the genus has been amended by the addition of four newly described taxa, *Nocardioopsis tropica*, *Nocardioopsis trehalosi* and *N. dassonvillei* subsp. *albirubida* (Evtushenko *et al.*, 2000) and *Nocardioopsis kunsanensis* (Chun *et al.*, 2000). During revision of this work, four further species have been added: *Nocardioopsis exhalans* and *Nocardioopsis umidischolae* (Peltola *et al.*, 2001), *Nocardioopsis halotolerans* (Al-Zarban *et al.*, 2002) and *Nocardioopsis compostus* (Kämpfer *et al.*, 2002). The results of 16S rDNA sequence analysis have shown that the genus *Nocardioopsis* is phylogenetically coherent and represents a distinct lineage within the order *Actinomycetales*, and the novel family *Nocardioopsaceae* was proposed for this genus (Rainey *et al.*, 1996).

The genus *Saccharothrix* was first described to classify actinomycete isolates that were morphologically similar to *Nocardioopsis*, but were differentiated by chemotaxonomic markers (Labeda *et al.*, 1984). The genus *Saccharothrix* is characterized by the presence of cell-wall type III (*meso*-diaminopimelic acid), rhamnose and galactose as diagnostic whole-cell sugars, phospholipid type PII, the presence of MK-9 (Labeda *et al.*, 1984) and fatty acid type 3f (Kroppenstedt, 1985). The DNA G+C contents of *Saccharothrix* species range from 70 to 76 mol % (Labeda & Lechevalier, 1989). On the basis of 16S rDNA sequences, the genus was initially related to the family *Pseudonocardiaceae* (Warwick *et al.*, 1994), but was later proposed to be included in the novel family *Actinosynnemataceae* with the genera *Lentzea*, *Actinosynnema* and *Actinokineospora* (Labeda & Kroppenstedt, 2000). More recently, additional studies have clarified the phylogenetic heterogeneity of the genus *Saccharothrix*, resulting in the taxonomic reorganization of its species. The genus *Saccharothrix* comprises currently nine described species: *Saccharothrix australiensis*, *Saccharothrix coeruleofusca*, *Saccharothrix coeruleoviolacea*, *Saccharothrix espanaensis*, *Saccharothrix longispora*, *Saccharothrix mutabilis*, *Saccharothrix syringae*, *Saccharothrix tangerinus* and *Saccharothrix texasensis* (Labeda, 1986; Labeda & Lyons, 1989). The strains *Saccharothrix aerocolonigenes* NRRL VB-3298^T and *Saccharothrix flava* NRRL B-16131^T have been transferred to the new genus *Lechevalieria* as *Lechevalieria aerocolonigenes* and *Lechevalieria flava*. The genus *Lentzea* has been revived, together with the species *Lentzea albidocapillata*, previously transferred to *Saccharothrix* (Lee *et al.*, 2000). The species *Saccharothrix*

violacea and *Saccharothrix waywayandensis* have been transferred to the revived genus *Lentzea* as *Lentzea violacea* and *Lentzea waywayandensis*, as well as the strain *S. aerocolonigenes* NRRL B-16137, now classified as *Lentzea californiensis* (Labeda *et al.*, 2001). Finally, the genus *Crossiella* has been created to accommodate the species *Saccharothrix cryophilis*, more closely related phylogenetically to the genus *Streptoalloteichus* (Labeda, 2001).

In spite of the clearly different taxonomic positions of the genera *Saccharothrix* and *Nocardioopsis*, no distinction is currently possible, when attempting the identification of novel wild-type isolates that exhibit their morphological characteristics, without an extended chemotaxonomic and molecular analysis. An alternative approach, derived from analysis of the variability of 16S rDNA sequences among different taxonomic groups and already in use for the development of specific probes at different taxonomic levels (Stahl & Amann, 1991; McVeigh *et al.*, 1995), is the design of genus-specific primers for the rapid identification of large numbers of strains. The application and usefulness of these genus-specific primers has already been reported for different members of the family *Pseudonocardiaceae* (Morón *et al.*, 1999; Salazar *et al.*, 2000). In this work, we have focused on the search for genus-specific primers for the genera *Nocardioopsis* and *Saccharothrix*, to be used in one-step molecular identification of strains of the two taxa. In addition, the recent taxonomic reclassification within the genus *Saccharothrix* and the creation of new, related genera necessitates an optimization of these tools to distinguish the genus *Saccharothrix* from these newly created taxa.

METHODS

Bacterial strains. The bacterial reference strains used in this study are listed in Table 1. Wild-type isolates were obtained from the laboratory culture collection. These strains were isolated from soils or decaying organic materials obtained from diverse geographical origins (Table 2). All strains were grown at 28 °C on YMG (0.4 % glucose, 0.4 % yeast extract and 1.0 % malt extract) agar or, in the case of some strains of *Nocardioopsis*, on alkaline yeast extract/malt medium [YMG with 1 % (w/v) Na₂CO₃].

Design of oligonucleotide primers. Sequence comparison and analysis were carried out using programs from the University of Wisconsin GCG package (version 7.2). Alignments of the 16S region were performed using the multiple alignment program CLUSTAL W (Thompson *et al.*, 1994) to determine the regions conserved only among *Nocardioopsis* or *Saccharothrix* species, from which the genus-specific primers were derived. The alignment is available as supplementary material in IJSEM Online (<http://ijs.sgmjournals.org/>). The genus specificity of oligonucleotides was tested against all DNA sequences available in GenBank with the program FASTA. The melting temperature (*T*_m) was estimated using the formulae of Thomas and Dancis and the Lathe formulae (quoted in Stahl & Amann, 1991). Relative *T*_m values obtained using 0.3 M as a standard salt concentration helped to design pairs of primers with similarly high melting temperatures. The probabilities

Table 1. Reference strains used in this study and results obtained from selective PCR amplifications with Stx2/Stx1 and Nsp2/Nsp1

Amplification results: 1, band of the expected size, 0, absence of amplification products; NT, not tested.

Strain	Stx2/Stx1				Nsp2/Nsp1
	61 °C	63 °C	65 °C	66 °C	53 °C
<i>Nocardiopsaceae</i>					
<i>N. dassonvillei</i> subsp. <i>albirubida</i> ATCC 23612 ^T	0	0	0	0	1
<i>N. dassonvillei</i> subsp. <i>dassonvillei</i> ATCC 43517 ^T	0	0	0	0	1
<i>N. lucentensis</i> ATCC 51300 ^T	0	0	0	0	1
<i>N. listeri</i> ATCC 27442 ^T	0	0	0	0	1
<i>N. prasina</i> ATCC 35940 ^T	0	0	0	0	1
<i>Thermobifida alba</i> DSM 43695 ^T	NT	NT	NT	NT	0
<i>Actinosynnemataceae</i>					
<i>S. syringae</i> DSM 43886 ^T	1	1	1	1	0
<i>S. coeruleofusca</i> DSM 43679 ^T	1	1	1	1	0
<i>S. mutabilis</i> subsp. <i>capreolus</i> DSM 40225 ^T	1	1	1	1	0
<i>S. mutabilis</i> subsp. <i>mutabilis</i> DSM 43853 ^T	1	1	1	1	0
<i>S. mutabilis</i> subsp. <i>mutabilis</i> NRRL B-16077 ^T	1	1	1	1	0
<i>S. espanaensis</i> DSM 44229 ^T	1	1	1	1	0
<i>Lechevalieria aerocolonigenes</i> ATCC 39243	1	1	0	0	0
<i>Lechevalieria aerocolonigenes</i> NRRL B-3298 ^T	1	1	0	0	0
<i>Lechevalieria flava</i> DSM 43885 ^T	1	1	0	0	0
<i>Lentzea waywayandensis</i> NRRL B-16159 ^T	1	1	1	0	0
<i>Lentzea albidocapillata</i> ATCC 51859 ^T	1	1	1	0	0
<i>Crossiella cryophila</i> NRRL B-16238 ^T	1	1	1	0	0
<i>Actinosynnema pretiosum</i> ATCC 31281 ^T	1	0	0	0	0
<i>Actinosynnema mirum</i> ATCC 29888 ^T	1	0	0	0	0
<i>Kutzneria viridogrisea</i> ATCC 25242 ^T	1	0	0	0	0
<i>Kutzneria albida</i> ATCC 25243 ^T	1	0	0	0	0
<i>Pseudonocardiaceae</i>					
<i>Saccharomonospora caesia</i> ATCC 49227	0	0	0	0	0
<i>Saccharomonospora azurea</i> ATCC 43670 ^T	0	0	0	0	0
<i>Saccharomonospora cyanea</i> ATCC 43724 ^T	0	0	0	0	0
<i>Saccharomonospora viridis</i> ATCC 15386 ^T	0	0	0	0	0
<i>Amycolatopsis orientalis</i> NRRL 2452	0	0	0	0	0
<i>Amycolatopsis orientalis</i> ATCC 21425	0	0	0	0	0
<i>Amycolatopsis sulphurea</i> NRRL 2282 ^T	0	0	0	0	0
<i>Kibdelosporangium aridum</i> subsp. <i>largum</i> ATCC 39922 ^T	0	0	0	0	0
<i>Micromonosporaceae</i>					
<i>Micromonospora chalcea</i> ATCC 12452 ^T	0	0	0	0	0
<i>Micromonospora carbonacea</i> ATCC 27114 ^T	0	0	0	0	0
<i>Micromonospora echinospora</i> NRRL 2995	0	0	0	0	0
<i>Micromonospora</i> sp. NRRL 3275	0	0	0	0	0
<i>Actinoplanes auranticolor</i> ATCC 15330 ^T	0	0	0	0	0
<i>Actinoplanes philippinensis</i> ATCC 12427 ^T	0	0	0	0	0
<i>Dactylosporangium auranticum</i> ATCC 23491 ^T	0	0	0	0	0
<i>Dactylosporangium vescum</i> ATCC 39499	0	0	0	0	0
<i>Pilimelia anulata</i> ATCC 25604 ^T	0	0	0	0	0
<i>Streptosporangiaceae</i>					
<i>Nonomuraea africana</i> ATCC 35107 ^T	0	0	0	0	0
<i>Streptomycetaceae</i>					
<i>Streptomyces cattleya</i> ATCC 35852	0	0	0	0	0
<i>Streptomyces avermitilis</i> ATCC 31267	0	0	0	0	0
<i>Streptomyces hygroscopicus</i> subsp. <i>hygroscopicus</i> ATCC 27438 ^T	0	0	0	0	0

Table 2. Wild-type isolates used in PCR experiments with genus-specific primers Nsp2/Nsp1 and Stx2/Stx1

Amplification results: 1, band of the expected size; 0, absence of amplification products.

Strain	Country	Source	Nsp1/Nsp2	Stx2/Stx1
<i>Nocardiosis</i> spp.				
98-52-146A*†, 98-52-148A*†, 98-52-149A*†, 98-52-204A*†	Australia	Decaying leaves	1	0
98-52-173A†	Australia	Soil	1	0
99-20-007A*	Belize	Soil	1	0
97-38-018A*	Equatorial Guinea	Soil	1	0
99-21-444A*†	Madagascar	Soil	1	0
98-08-290A*, 99-08-186A*†, 99-20-430A	Mexico	Soil	1	0
98-29-191A*	South Africa	Soil	1	0
98-10-097A*†	Spain	Decomposing organic material	1	0
<i>Saccharothrix</i> spp.				
98-05-051A*†	Costa Rica	Soil	0	1
99-08-200A†, 99-08-201A†, 99-08-211A*†, 99-08-219A†, 99-08-221A*†, 99-08-222A*†, 99-08-236A*†, 99-08-243A*†, 99-08-286A†	Mexico	Soil	0	1
99-15-236A†, 99-19-550A*†	Senegal	Soil	0	1
98-10-099A*	Spain	Decomposing organic material	0	1
98-41-143A*, 98-41-281A*, 98-41-338A*, 98-48-079A†	South Africa	Soil	0	1
<i>Streptomyces</i> spp.				
99-08-217A†, 99-08-218A†	Mexico	Soil	0	0
<i>Nocardia</i> spp.				
98-37-068A*†	French Guyana	Soil	0	0
99-08-244A*†	Mexico	Soil	0	0
97-21-103A*†	West Indies	Soil	0	0
Unidentified actinomycetes				
97-39-041A	Costa Rica	Soil	0	0
99-08-103A	Niger	Soil	0	0
98-29-235A, 98-31-122A†	South Africa	Soil	0	0
98-10-149A	Spain	Decomposing organic material	0	0
98-21-170A	Thailand	Soil	0	0

* Sequenced strain.

† Strain subjected to fatty acid analysis.

of primer-dimer formation and autofolding were also studied in order to keep them as low as possible. The oligonucleotides designed were supplied by Pharmacia Biotech.

DNA extraction. Total genomic DNA from the different micro-organisms used in this study was recovered and purified as described previously (Lee & Taylor, 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 of each of the four dNTPs (Roche), 0.1 µM of each primer, 5 µl of the 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 55 °C (primers Nsp2/Nsp1) or 61–66 °C (primers Stx2/Stx1) and 2 min at 72 °C, followed by 10 min at 72 °C. Amplification products were

analysed by electrophoresis (5 V cm⁻¹) in standard 1.2% (w/v) agarose gels stained with ethidium bromide or using a Ready-to-Run Separation Unit (Amersham Pharmacia Biotech).

DNA sequencing. The PCR primers 27f and 1525r (Lane, 1991) were used for amplification of the 16S rDNA. The 1500 bp PCR products were purified and used as templates in sequencing reactions using the primers 27f, 357f, 530f, 926f, 1392f, 1525r, 1110r, 685r and 357r (Lane, 1991). Amplified products were sequenced using an ABI PRISM Dye Terminator cycle sequencing kit (Perkin Elmer). The GenBank accession numbers for the 16S rDNA sequences obtained in this study are as follows: *Nocardiosis* sp. isolates 98-38-018A, 98-10-097A, 98-29-191A, 98-52-146A, 98-52-148A, 98-52-149A, 98-52-204A, 98-08-290A, 99-21-444A, 99-20-007A and 99-08-186A, respectively AF328401–AF328410 and AF361322; *Saccharothrix* sp. isolates 97-38-018A, 98-05-051A, 99-08-221A, 99-08-222A, 99-08-236A, 98-10-099A, 98-41-143A, 98-41-338A, 98-41-

281A, 99-08-211A, 99-08-143A and 99-19-550A, respectively AF328411–AF328418 and AF361323–AF361325; *Nocardia* sp. isolates 99-37-68A, 99-08-244A and 97-21-103A, AF421563–AF421565.

Data analysis. Sequences were assembled using the GCG Fragment Assembly system. Alignments of the 16S rDNA sequences were performed using the multiple alignment program CLUSTAL W (Thompson *et al.*, 1994; Intelli-Genetics).

The phylogenetic analysis was completed with 16S rDNA sequences of *Nocardiopsis* spp. and *Saccharothrix* spp. Phylogenetic analysis of the aligned sequences was performed using maximum-parsimony analysis with the branch-and-bound algorithm of PAUP version 4.0 (Swofford, 1993), with gaps treated as missing data. Data were resampled with 1000 bootstrap replicates (Felsenstein, 1985) by using the heuristic search option of PAUP. The percentage of bootstrap replicates that yielded each grouping was used as a measure of statistical confidence. A grouping found on 95% of bootstrap replicates was considered statistically significant.

Whole-cell fatty acid composition. Cultures were grown as confluent patches on TSB agar (BBL) at 28 °C for 4 days. Vegetative growth was then scraped (100–200 mg) and fatty acid methyl esters (FAMES) were prepared according to Miller (1982). Analysis of FAMES was carried out by capillary gas chromatography using a Hewlett Packard model 5890 gas chromatograph/MIDI system (Microbial ID) equipped with a phenyl methyl silicone column (0.2 mm × 25 m). Chromatography conditions were as recommended by the manufacturer. Individual FAME identification was achieved using the MIS software (Microbial ID) and clustering analysis was performed using the statistical software package S-Plus 2000 (Mathsoft).

RESULTS AND DISCUSSION

Design of genus-specific primers

The design of two sets of genus-specific pairs of primers for the selective amplification of the 16S rDNA regions of the genera *Nocardiopsis* and *Saccharothrix* has been based on the search for conserved sequences within each genus after a comparative analysis of the variable regions of known 16S rRNA sequences. An alignment of the GenBank 16S rRNA/DNA sequences that were used for the design of the primers is available as supplementary material in IJSEM Online (<http://ijs.sgmjournals.org/>).

In the case of the genus *Nocardiopsis*, two regions, covering nucleotides 440–465 and 995–1020, corresponding to nucleotide positions of the *Streptomyces ambofaciens* 16S rDNA (Pernodet *et al.*, 1989), were found to be highly conserved among the different species of the genus. In contrast, the highly conserved ribosomal sequences among members of the genus *Saccharothrix* were found at the beginning of the gene, around positions 80–100 and 590–615. These sequence homologies were high enough to consider the design of genus-specific primers for the two genera. The two genus-specific primers Nsp2 (5'-TCTCTTGGGGT-GACGGTAG-3') and Nsp1 (5'-TAAATGACCTCA-CATCTCT-3') that were designed for the genus

Nocardiopsis nested respectively at positions 445–464 and 996–1014 of the 16S rDNA, whereas the two genus-specific primers for the genus *Saccharothrix*, Stx2 (5'-AAGGCCCTTCGGGGTACACGAG-3') and Stx1 (5'-TCGACCGCAGGCTCCACG-3'), respectively hybridized at positions 80–101 and 594–611.

Specificity of the primers for the genus *Nocardiopsis*

The sequence alignment with primer Nsp1 showed total identity to the sequences of *N. dassonvillei* subsp. *albirubida*, *N. dassonvillei* subsp. *dassonvillei*, *N. lucentensis* and *N. kunsanensis* only (see supplementary material). In the other species studied, one to six non-matched positions were observed within the annealing region. The alignment with *Thermobifida fusca* and *Thermobifida alba*, also members of the family *Nocardiopsaceae*, showed five non-matched positions with the primer sequence. This lack of identity increased when the primer was aligned with the sequences of species of the genera *Saccharothrix*, *Lentzea*, *Lechevalieria*, *Crossiella*, *Actinosynnema* and *Kutzneria*, where up to eight non-matching positions were observed. The sequence identity of the primer decreased even more when the same region was considered in other actinomycetes, such as the genera *Saccharomonospora*, *Amycolatopsis*, *Nonomuraea*, *Actinomadura*, *Micromonospora* and *Streptomyces*.

The primer Nsp2 showed only one or two mismatching positions in the *Nocardiopsis* species studied, with the exception of *N. trehalosi*, where four mismatching positions were observed. A similar number, three mismatching positions, was found in members of the genus *Thermobifida*. The primer showed no homology with *Saccharothrix* spp. and other related genera, with up to 10 mismatching positions (see supplementary material).

When the primer pair Nsp2/Nsp1 was tested in PCR using an annealing temperature of 53 °C, defined as optimal for them, we obtained the expected amplification product of 575 bp from all the type strains of the genus *Nocardiopsis* tested (*N. dassonvillei* subsp. *albirubida* ATCC 23612^T, *N. dassonvillei* subsp. *dassonvillei* ATCC 43517^T, *N. lucentensis* ATCC 51300^T, *N. listeri* ATCC 27442^T and *N. prasina* ATCC 35940^T) (Fig. 1). No amplification product was obtained with DNA from different *Saccharothrix* species or from other actinomycetes taxa, including the closely related genus *Thermobifida* (Table 1). The same results were obtained when amplifications were performed at different annealing temperatures (51, 55 and 57 °C), indicating that the genus specificity of the primers was not affected over a wide range of temperatures, of 51–57 °C.

Specificity of the primers for the genus *Saccharothrix*

When comparing the sequence alignments with the primers specific for the genus *Saccharothrix* (see supplementary material), we observed total identity or

Fig. 1. Agarose gel electrophoresis of PCR products from DNA. (a) Selective amplification at 53 °C of a 575 bp fragment using primers Nsp2/Nsp1 specific for the genus *Nocardiopsis*. (b) Selective amplification at 66 °C of a 545 bp fragment using primers Stx2/Stx1 specific for the genus *Saccharothrix*. Lanes: L, DNA size ladder; 1, *N. dassonvillei* subsp. *albirubida* ATCC 23612^T; 2, *N. dassonvillei* subsp. *dassonvillei* ATCC 43517^T; 3, *N. lucentensis* ATCC 51300^T; 4, *N. listeri* ATCC 27442^T; 5, *N. prasina* ATCC 35940^T; 6, *S. syringae* DSM 43886^T; 7, *S. coeruleofusca* DSM 43679^T; 8, *S. mutabilis* subsp. *mutabilis* DSM 43853^T; 9, *S. espanaensis* DSM 44229^T; 10, *Lentzea waywayandensis* NRRL B-16159^T; 11, *Lentzea albidocapillata* ATCC 51859^T; 12, *Crossiella cryophila* NRRL B-16238^T; 13, *Lechevalieria aerocolonigenes* ATCC 39243^T; 14, *Lechevalieria flava* DSM 43885^T; 15, *Actinosynnema pretiosum* ATCC 31281^T; 16, *Kutzneria viridogrisea* ATCC 25242^T; 17, *Amycolatopsis orientalis* NRRL 2452; 18, *Kibdelosporangium aridum* subsp. *largum* ATCC 39922^T; 19, *Micromonospora carbonacea* ATCC 27114^T; 20, control reaction without DNA.

a single mismatch in the members of this genus in the region used to design the forward primer Stx2. The same sequence was also contained in members of the related genera *Lentzea*, *Lechevalieria*, *Crossiella*, *Kutzneria* and *Actinosynnema*, as well as in members of the genus *Nocardiopsis*, and it was less well conserved in the rest of the actinomycete taxa studied.

In contrast, the reverse primer Stx1 showed total identity to all members of the genus *Saccharothrix*, with the exception of two species, *S. texasensis* and *S. tangerinus*, where one and five non-matching positions, respectively, were observed. In the new related genera *Lechevalieria* and *Crossiella*, as well as in the genera *Lentzea*, *Kutzneria* and *Actinosynnema*, three to five non-matching positions were also observed in the extending end of the primer. This lack of homology increased to nine non-matching positions with different sequences of the genus *Nocardiopsis*, which ensured that the amplification reaction with the pair of primers Stx2/Stx1 was specific to sequences from *Saccharothrix* and other related taxa. The primer Stx2 had been designed originally to match the rDNA sequences of former *Saccharothrix* species recently transferred to the genera *Lentzea*, *Lechevalieria* and

Fig. 2. Effect of annealing temperature on selective amplification with primers Stx2/Stx1 of members of the family *Actinosynnemataceae*. Lanes: 1, *S. syringae* DSM 43886^T; 2, *S. coeruleofusca* DSM 43679^T; 3, *S. espanaensis* DSM 44229^T; 4, *S. mutabilis* subsp. *capreolus* DSM 40225^T; 5, *S. mutabilis* subsp. *mutabilis* DSM 43853^T; 6, *Lentzea waywayandensis* NRRL B-16159^T; 7, *Lentzea albidocapillata* ATCC 51859^T; 8, *Crossiella cryophila* NRRL B-16238^T; 9, *Lechevalieria aerocolonigenes* NRRL B-3298^T; 10, *Lechevalieria flava* DSM 43885^T; 11, *Actinosynnema pretiosum* ATCC 31281^T; 12, *Kutzneria viridogrisea* ATCC 25242^T.

Crossiella. The use of low annealing temperatures (61 °C) ensured the amplification of sequences from species of the genus *Saccharothrix*, as well as members of other genera of the family *Actinosynnemataceae*, but no amplification was detected from strains of the genus *Nocardiopsis* or members of other actinomycete families (Table 1). The taxonomic reorganization of the genus *Saccharothrix* has required an optimization of the amplification conditions and, therefore, different annealing temperatures ranging from 61 to 66 °C were tested to increase the specificity of the amplification reaction (Fig. 2). When PCRs were performed at 66 °C, a specific amplification was obtained only with members of the genus *Saccharothrix* (Figs 1b and 2).

These data show that the two pairs of primers Nsp2/Nsp1 and Stx2/Stx1 allow the specific identification of all the tested strains belonging to these genera, in spite of the small differences observed in their sequences. The high specificity for the targeted sequences of at least one of the oligonucleotides in each pair of primers, together with the low-stringency conditions defined for the annealing reactions, ensures amplifications not as selective as to distinguish between sequences of the same genus, which differ in a few nucleotides, but specific enough to identify members of these genera selectively.

Identification of wild-type isolates and evaluation of their diversity

One of the purposes of the design of these primers was the rapid detection of members of the two genera among the large numbers of environmental isolates that share morphological traits with the genera *Nocar-*

Fig. 3. Diversity of wild-type strains identified with primers specific for the genera *Nocardiopsis* and *Saccharothrix* and their relationships to reference strains of the two genera. The dendrogram was generated by treating the Euclidian distances of the fatty acids with UPGMA. Reference strains are those included in Table 1.

diopsis and *Saccharothrix*. We selected from our culture collection a group of 41 wild-type isolates with these morphological characteristics that, in the absence of the required extended chemotaxonomic study, had never been definitively assigned to any of these genera. DNA was extracted from each of the 41 wild-type isolates and tested with both pairs of genus-specific primers, in the case of primers Stx1/Stx2, using two different temperatures (61 or 66 °C) for the annealing conditions. A positive specific amplification was only obtained in 30 isolates, of which 13 and 17 strains, respectively, under all conditions, were amplified with the primers for *Nocardiopsis* and *Saccharothrix* (Table

2). These strains, although they were obtained from quite diverse geographical origins and using a wide range of isolation conditions, also shared, in some cases, their origin and morphological characteristics with other strains that did not amplify and probably correspond to other related taxa.

In an attempt to determine the diversity of our wild-type isolates, identified to the genus level with the specific primers, and evaluate their relationships with reference strains of the two genera, we determined the fatty acid composition of a representative group of strains. Among other chemotaxonomic markers, the

Fig. 4. For legend see facing page.

genera *Nocardiothrix* and *Saccharothrix* can be distinguished by their fatty acid composition. The genus *Nocardiothrix*, with a 3d fatty acid type, contains substantial amounts of tuberculostearic acid (18:0-10Me) and oleic acid (18:1-*cis*9), which are not normally present in the 3f type of the genus *Saccharothrix*, where 2-hydroxylated iso- and anteiso-branched fatty acids are detected (Grund & Kroppenstedt, 1990; Kroppenstedt, 1985, 1992). Clustering analysis of the strains was based on their fatty acid patterns and the relationship among the strains was established in a dendrogram generated by treating the Euclidian distances of the fatty acids with the unweighted pair group method (UPGMA) (Fig. 3). Almost all the reference strains from the genera *Nocardiothrix*, *Saccharothrix* and other related taxa appeared to be clearly separated in different branches and clustered tightly, with the exception of *N. lucentensis* ATCC 51300^T, which appeared as a single branch on the dendrogram. Among the wild-type strains, we found that most of

the strains that were amplified positively with the primers for *Nocardiothrix* clustered with reference strains of *Nocardiothrix*. We also observed some exceptions, of strains clustering with members of *Lechevalieria* (isolate 99-21-444A), with other wild-type strains not amplified by the primers (isolate 99-08-186A) or not related to any of the strains in the study (isolate 98-52-173A). In the case of the strains identified with the primers as *Saccharothrix*, most of them appeared to be grouped in separate clusters not related to the branch defined by the fatty composition of most reference strains of this genus. Isolate 99-19-550A, identified as *Saccharothrix* by amplification at 66 °C, appeared as a single branch, closely related to *Crossiella cryophila*.

In order to confirm the genus assignment of our isolates and to discard the possibility of a lack of specificity of the primers as the source of the phenotypic heterogeneity among our strains revealed by the

Fig. 4. Phylogenetic tree showing the position of wild-type strains of the genera *Nocardiopsis* and *Saccharothrix* derived from 16S rDNA analysis using the maximum-parsimony method in PAUP 4.0. Numbers above branches indicate bootstrap percentages from 1000 replicates, which were used as a measure of statistical confidence.

fatty acid composition, we determined the almost complete nucleotide sequence of the 16S rDNAs of a representative group of 25 wild-type strains selected from different origins, including 11 isolates identified as *Saccharothrix*, 11 strains as *Nocardiopsis* and three strains that did not amplify with the primers (Table 2). In all cases, we found that the annealing sequence of each pair of primers was highly conserved among the strains that were amplified positively, confirming the specificity of each pair of primers. Among the strains assigned to the genus *Nocardiopsis*, the level of sequence similarity for the complete 16S rDNA sequence ranged from 100 to 99.6%, indicating the high sequence similarity within this group of strains in spite of their different origin. A close relationship was observed for all these isolates with the species *N. trehalosi* (sequence similarity 99.8–100%). In fact, the

nucleotide sequence of the annealing region of primer Nsp2 in these strains exactly matched the sequence of *N. trehalosi*, which presents four mismatches with Nsp2 in this region. The isolates identified as *Saccharothrix* presented greater sequence heterogeneity, but the similarity levels among the strains still remained very high, ranging from 99.3 to 98.5%. The closest relationship of these strains with reference species of this genus was found with *S. australiensis*, where the levels of sequence similarity were 99.5–97.4%. The three strains sequenced that did not amplify with any of the primers showed 98–99% sequence similarity to different species of the genus *Nocardia*.

A phylogenetic tree based on these 16S rDNA sequences was built using the maximum-parsimony method (Fig. 4), showing the inter- and intraspecific

relationships of the wild-type strains to reference strains of the genera *Saccharothrix* and *Nocardiopsis* and other members of the order *Actinomycetales*. The topology of the tree confirms previous studies that have determined the taxonomic reorganization of the genus *Saccharothrix*, which contrasts with the monophyletic lineage of the genus *Nocardiopsis* (Labeda & Kroppenstedt, 2000; Evtushenko *et al.*, 2000; Labeda *et al.*, 2001; Labeda, 2001). Almost all the wild-type strains of the genus *Saccharothrix* appear associated in closely related branches, in spite of the observed differences in their fatty acid compositions revealed in the clustering analysis. Their closest relationship to members of the species *S. australiensis* is not highly supported by the bootstrapping values (53%) and, from the available data, none of these wild-type isolates can be associated with any of the species included in the study. The existence of this cluster suggests the existence of novel species within the genus *Saccharothrix*. In contrast, isolate 99-19-550A, confirmed as *Saccharothrix* by amplification at high temperature, was placed in a separate branch, clustering tightly with *Crossiella cryophila*, and must represent another strain of the same species (bootstrapping value of 100%). Surprisingly, strain 99-19-550A, which presents 99% 16S rDNA sequence similarity to *Crossiella cryophila*, contains five non-matching nucleotides with the sequence of *Crossiella* in the region that corresponds exactly to the hybridization sequence of primer Stx1, which explains the amplification of this isolate with primers Stx2/Stx1.

In contrast, all the wild-type isolates of the genus *Nocardiopsis* clustered tightly with *N. trehalosi*, in spite of their diverse origin. In this case, this single cluster was strongly supported by bootstrapping analysis (bootstrapping value of 100%) and was clearly separated from other reference strains, as previously observed for *N. trehalosi* (Evtushenko *et al.*, 2000).

In this work, we have presented the application of two new pairs of genus-specific primers as a simple method for the rapid identification of novel members of the genera *Nocardiopsis* and *Saccharothrix* from the large numbers of strains that are normally obtained in industrial isolation programmes. Our results with reference strains, as well as the high degree of conservation observed among the sequences of the wild-type isolates, validate the specificity of these new pairs of primers and support the usefulness of these tools for rapid distinction between the two genera and the tentative genus assignment of novel isolates of these taxa. Nevertheless, the phenotypic diversity of the wild-type strains obtained from quite different habitats contrasts with the high degree of conservation observed at the sequence level, especially in the case of members of the genus *Nocardiopsis*. This result can be only partially explained by their common exhibition of similar micromorphological characters, which were used as a selection criterion for this work. Further studies with these primers are required to evaluate the presence of novel members of these genera among

isolates in our culture collection not suspected to belong to these taxa.

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