New genus-specific primers for the PCR identification of members of the genus *Saccharomonospora* and evaluation of the microbial diversity of wild-type isolates of *Saccharomonospora* detected from soil DNAs

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Members of the genus *Saccharomonospora* are isolated infrequently, probably due to the low occurrence of these actinomycetes in the environment. Although members of this genus can easily be identified by micromorphological criteria, the extensive chemotaxonomic characterization of each new isolate is a time-consuming task which cannot always be undertaken when handling large numbers of strains as is the case in natural products screening programmes. In this work, the design of one set of genus-specific oligonucleotides which allows rapid detection of members of the genus *Saccharomonospora* by means of PCR-specific amplification is presented. The genus specificity of these primers was validated on a wide range of collection and wild-type strains, and subsequently applied to evaluate the presence of representatives of this taxon directly from soil DNAs. Partial 16S rDNA sequencing of representative wild-type strains was used to validate their genus assignment. Further analyses of PCR fingerprinting patterns and 16S–23S internal transcribed spacer sequences were used to determine the diversity of wild-type isolates obtained from soils. This study shows the usefulness of the application of these primers for the direct identification of members of this genus and in assessment of its occurrence within natural microbial habitats.

Keywords: *Saccharomonospora*, PCR primers, 16S rDNA sequences, ribosomal internal transcribed spacers (ITSs), soil DNA

INTRODUCTION

Historically, natural products screening programmes have focused on the order *Actinomycetales*, one of the most intensively isolated groups of micro-organisms, which has efficiently demonstrated its ability to produce a large diversity of new secondary metabolites

Abbreviations: ITS, internal transcribed spacer; T_m , melting temperature; UPGMA, unweighted pair group method with arithmetic averages.

The EMBL accession numbers for the partial 16S rRNA gene sequences of *Saccharomonospora* sp. isolates 12-82, 02-103, 42-146, 42-147, 42-157, 42-161, 42-162, 42-17, 42-182, 42-187, 42-190, 42-193, 42-27, 42-78, 42-82 and 42-86 are AJ270373–AJ270388, respectively. The EMBL accession numbers for the 16S–23S rDNA internal transcribed spacers of *Saccharomonospora* sp. isolates 42-147, 42-15, 42-157, 42-161, 42-162, 42-166, 42-168, 42-169, 42-17, 42-175, 42-177, 42-187, 42-190, 42-193, 42-206, 42-27 and 42-79 are AJ270389–AJ270405, respectively.

(Sanglier *et al.*, 1993). Besides the genus *Streptomyces*, which has been extensively exploited, members of the other genera are also considered of high interest as potential producers of novel bioactive compounds (Horan, 1994, 1999). Although different procedures have been described for the direct and specific isolation of cultures belonging to many of these groups, certain taxa still remain minor groups among the isolates normally recovered from different environments. 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 of many taxa during routine isolation.

Within the family *Pseudonocardiaceae*, the genus *Saccharomonospora* represents a minor component

which apparently shows little distribution in the environment, according to the numbers of isolates normally obtained with traditional isolation methods, when compared to other taxa (Embley, 1992). The genus *Saccharomonospora* was first described for thermophilic actinomycetes producing a greyish-green aerial mycelium bearing single spores (Nonomura & Ohara, 1971). They share with other *Pseudonocardiaceae* the absence of mycolic acids and have cell wall type IV (meso-diaminopimelic acid, arabinose and galactose) (Embley *et al.*, 1988a, b). There are currently four validly published species, the original type species *Saccharomonospora viridis* (Nonomura & Ohara, 1971), which includes the former species *Saccharomonospora internatus* now considered to be a synonym (Greiner-Mai *et al.*, 1988), and the species *Saccharomonospora azurea* (Runmao, 1987), *Saccharomonospora cyanea* (Runmao *et al.*, 1988) and *Saccharomonospora glauca* (Greiner-Mai *et al.*, 1988). The species '*Saccharomonospora caesia*', previously described as *Micropolyspora caesia*, has not been validly published and has recently been shown to be a synonym of *Saccharomonospora azurea* (Yoon *et al.*, 1999). Other described *Saccharomonospora* strains include the species *Saccharomonospora yunnanensis* and more recently the validly published species *Saccharomonospora xinjiangensis* (Jin *et al.*, 1998). Although thermophilic isolates have been frequently isolated from natural self-heating habitats (compost, mouldy hay, manure and decomposing plant material), mesophilic strains have also been obtained from different soils and stream and lake sediments (Embley, 1992).

Recent phylogenetic analyses of the genus *Saccharomonospora*, based on similarities of ribosomal gene sequences (Kim *et al.*, 1995; Ruan *et al.*, 1994), have shown the homogeneity of this genus and it has been confirmed as a distinct phyletic line of descent within the family *Pseudonocardiaceae*. More recently, study of the interspecific relationships based on the comparison of ribosomal internal transcribed spacers (ITSs) has shown the species diversity that can be found among this apparently homogeneous taxon and the usefulness of 16S–23S and 23S–5S sequences for classification and identification of new *Saccharomonospora* strains (Yoon *et al.*, 1997a).

An alternative approach to the traditional isolation and identification methods of members of the genus *Saccharomonospora* would require not only a better knowledge of their distribution and dependence on their occurrence according to the environmental conditions, but the development of new detection and 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 cultivable micro-organisms can be identified (McVeigh *et al.*, 1995). The advances in nucleic acid techniques, based on data derived from the comparative analysis

of the ribosomal gene sequences, have allowed the development of powerful tools that can be applied to the study of micro-organisms (Fox & Stackebrandt, 1987; Ludwig & Schleifer, 1994; Kim *et al.*, 1995; Yoon *et al.*, 1996a, 1997b). 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 to 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 to the rapid identification of large numbers of strains under study (Mehling *et al.*, 1995; Yoon *et al.*, 1996b). Although species-specific probes have also been described for a large number of relevant micro-organisms, their use is restricted to identification at the species level. The application and usefulness of genus-specific primers for the rapid identification of members of two frequently occurring genera of the family have already been shown (Morón *et al.*, 1999). This work has focused on the search for genus-specific probes for the genus *Saccharomonospora* which can then be applied to the direct detection and to one-step molecular identification of strains of this taxon.

METHODS

Bacterial strains. The bacterial reference strains used in this study are listed in Table 1; 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 the thermophilic strains, which were grown at 50 °C.

Wild-type *Saccharomonospora* isolates were obtained following standard dilution platings of soils on R8 agar medium at 50 °C (Amner *et al.*, 1989). Soils tested were selected from diverse geographical origins and habitats (Table 2).

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, were studied for the design of the primers: X76953, *Actinokineospora riparia* IFO 14541^T; X54287, *Actinopolyspora halophila* ATCC 27976^T; Z22730, *Actinopolyspora mortivallis* JCM 7550^T; X53199, *Amycolatopsis azurea* NRRL 11412^T; X53200, *Amycolatopsis fastidiosa* ATCC 31181^T; X76957, *Amycolatopsis mediterranei* ATCC 13685^T; X54274, *Amycolatopsis methanolica* NCIB 11946^T; X81573, *Amycolatopsis orientalis* DSM 43187; X76958, *Amycolatopsis orientalis* subsp. *orientalis* DSM 40040^T; X53191, *Kibdelosporangium aridum* subsp. *aridum* ATCC 39323^T; X76954, *Pseudonocardia alni* Ac-901; X54288, *Pseudonocardia autotrophica* DSM 535^T; X76955, *Pseudonocardia hydrocarbonoxydans* DSM 43281^T; X55609, '*Pseudonocardia nitrificans*' DSM 46012 = IFAM 379; X55608, *Pseudonocardia petroleophila* DSM 655^T = IFAM 78^T; X76956, *Pseudonocardia saturnea* DSM 43195^T; X76959, *Pseudonocardia compacta* DSM 43592^T; Z14111, *Pseudonocardia halophobica* DSM 43089^T; X53195, *Pseudonocardia thermophila* ATCC 19285^T; Z38017, *Saccharomonospora azurea* Na-128^T; X76960, '*Saccharomonospora caesia*' INMI 19125^T; Z30819, '*Saccharomonospora caesia*' DSM 43068; Z38018, *Saccharomonospora cyanea* K168^T;

Z38003, *Saccharomonospora glauca* DSM 43769^T; Z38007, *Saccharomonospora viridis* NCIB 9602; X54286, *Saccharomonospora viridis* ATCC 15386^T; Z38021, *Saccharomonospora viridis*, E. Greiner-Mai, R25; X76961, *Saccharomonospora* sp. A1206; Z38004, *Saccharomonospora* sp., J. S. Ruan, 350; Z38022, *Saccharomonospora* sp., A. J. McCarthy; Z38023, *Saccharomonospora* sp., S. B. Kim; Z38017, *Saccharomonospora* sp., S. B. Kim; Z38005, *Saccharomonospora* sp., S. B. Kim; Z38006, *Saccharomonospora* sp., S. B. Kim; Z38024, *Saccharomonospora* sp., S. B. Kim; AF0056710, *Saccharomonospora xinjiangensis* XJ-54^T; X53198, *Saccharopolyspora erythraea* NRRL 2338^T; X76962, *Saccharopolyspora gregorii* NCIB 12823^T; X53196, *Saccharopolyspora hirsuta* subsp. *hirsuta* ATCC 27875^T; X53197, *Saccharopolyspora hordei* A54^T; X53194, *Saccharopolyspora rectivirgula* ATCC 33515^T; X76967, *Saccharopolyspora* sp. A215; X53193, *Saccharothrix australiensis* ATCC 31497^T; X76963, *Saccharothrix coeruleofusca* 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* subsp. *albus* DSM 40313^T; X53162, *Streptomyces brasiliensis* DSM 43159^T; X53161, *Streptomyces diastaticus* subsp. *diastaticus* DSM 40496^T; X53173, *Streptomyces lavendulae* subsp. *lavendulae* DSM 2014^T; Y00484, '*Streptomyces lividans*' TK21; X53170, *Streptomyces purpureus* DSM 43460^T; X81574, *Streptomyces* sp. NRRL 3890; X53168, *Streptomyces abikoensis* DSM 40831^T; X53164, *Streptomyces baldaccii* DPDU 0819^T; X53165, *Streptomyces cinnamoneus* subsp. *azacoluta* DPDU 0074^T; X53171, *Streptomyces cinnamoneus* subsp. *cinnamoneus* DPDU 0093^T; X53167, *Streptomyces ladakanum* var. *ladakanum* DSM 40587^T; X53172, '*Streptomyces luteoreticuli*' DPDU 0081; X79323, *Streptomyces mashuensis* DSM 40221^T; X53166, *Streptomyces olivoreticuli* subsp. *cellulophilus* DPDU 0278^T; and X53169, *Streptomyces salmonis* DPDU 0098^T. The genus specificity of oligonucleotides was tested against all DNA sequences available in GenBank with the FASTA program. The melting temperature (T_m) was estimated using the formulae of Thomas & Dancis and Lathe (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 of primer-dimer formation and autofolding were also studied in order to keep them as low as possible. The designed oligonucleotides were supplied by Pharmacia Biotech.

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

Soil DNA extraction. Total DNA extraction from soils was adapted from a previously described method (Volossiouk *et al.*, 1995). Soil samples (250 mg) were frozen in liquid nitrogen, ground and suspended in 0.5 ml 0.4% (w/v) skim milk solution (Difco). Soil particles were sedimented by centrifugation for 10 min at 17 500 g. Supernatants (300 µl) were mixed with 200 µl extraction buffer (0.66% SDS, 0.31 M NaCl, 110 mM potassium acetate pH 5.1), vortexed and added to 500 µl water-saturated phenol. After extraction, supernatants (400 µl) were recovered by centrifugation (15 min, 17 500 g) and total DNA was precipitated overnight at -20 °C by addition of 1 ml ethanol. After a 15 min centrifugation at 17 500 g, DNA was washed with 75% cold ethanol and finally dissolved in 100 µl sterile distilled water.

PCR amplification. DNA preparations were used as template DNA for the *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 (Applied Biosystems) 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. Amplification products were analysed by electrophoresis (0.25 V cm⁻²) in 1.2% (w/v) agarose gels stained with ethidium bromide.

PCR fingerprinting. The high-resolution PCR fingerprinting primers that were used were described by Jensen *et al.* (1993). The primers L1 (5'-CAAGGCATCCACCGT-3') and G1 (5'-GAAGTCGTAACAACG-3') are complementary to conserved regions of the 16S and 23S rRNA genes and PCR reactions using both primers result in the amplification of the transcribed spacer sequences between the genes. Amplification conditions were as follow: 30 cycles of 0.5 min at 94 °C, 0.5 min at 58 °C and 2 min at 72 °C, followed by a final 10 min incubation at 72 °C. Negative controls without template DNA were routinely run for all amplifications. PCR reaction products (10 µl) were analysed in 4–20% acrylamide gradient minigels (Novex). Band patterns were compared with the GELCOMP software package version 4.1 (Applied Maths). A similarity matrix was generated using the Pearson product-moment and cluster analysis was performed using the unweighted pair group method with arithmetic averages (UPGMA) (Roberts *et al.*, 1998; Vauterin *et al.*, 1996).

DNA sequencing. PCR and purification procedures were as described previously (Yoon *et al.*, 1997a) with 50:1 molar ratio between the primers, obtaining a major amplification product of single-strand DNA directly used for sequencing. The PCR primers 530f and 907r (Lane, 1991) were used for the amplification of DNA fragments containing the hybridization region of primers SM1 and SM2. The 16S–23S intergenic spacers were amplified using as PCR primers the oligonucleotides 1497f (5'-GGACGAAGTCGTAACAAGGT-3') and 115r (5'-GGGTTTCCCCATTTCGG-3') selected from conserved sequences at the end and beginning of the 16S and 23S rDNAs, respectively (Lane, 1991). Sequencing was performed using Cy 5'-labelled primers and the Cy Thermo sequenase Dye terminator kit. Sequencing reactions were electrophoresed in an ALFexpress DNA sequencing instrument (Amersham Pharmacia Biotech). All samples were sequenced at least twice in both directions.

Data analysis. Sequences were assembled using the GCG Fragment Assembly System (Program Manual for the Wisconsin Package, version 8). Alignments of the 16S–23S spacer region were performed using the multiple alignment program CLUSTAL W (Thompson *et al.*, 1994; IntelliGenetics). The percentage of sequence homology was calculated with DISTANCES from the GCG package.

The phylogenetic analysis was completed with 16S–23S ITS sequences of *Saccharomonospora* spp. type strains previously reported (Yoon *et al.*, 1997a). These 16S–23S ITS sequences were: *Saccharomonospora cyanea* (U73402), *Saccharomonospora azurea* (U73397), '*Saccharomonospora caesia*' (U73428, U73400, U73410, U73422, U73430, U73432, U73440), *Saccharomonospora* sp. (U73408), *Saccharomonospora viridis* (U73426, U73412, U73414, U73420, U73434, U73436) and *Saccharomonospora glauca* (U73404, U73406, U73416, U73418, U73424, U73438).

Phylogenetic analysis of the aligned 16S–23S ITS sequences was performed using the maximum-parsimony analysis with

Table 1. Reference strains used in this study and results obtained from selective PCR amplification with SM1/SM2

Species	Strain designation	Amplification result*
<i>Actinopolyspora</i>		
' <i>Actinopolyspora thermovinacea</i> '	ATCC 35862	0
<i>Amycolatopsis</i>		
<i>Amycolatopsis azurea</i>	DSM 43854 ^T	0
<i>Amycolatopsis fastidiosus</i>	ATCC 31181 ^T	0
<i>Amycolatopsis mediterranei</i>	ATCC 13685 ^T	0
<i>Amycolatopsis methanolica</i>	NCIB 11946 ^T	0
<i>Amycolatopsis orientalis</i>	NRRL 18098	0
<i>Amycolatopsis orientalis</i>	NRRL 18099	0
<i>Amycolatopsis orientalis</i>	NRRL 18100	0
<i>Amycolatopsis orientalis</i> subsp. <i>orientalis</i>	NRRL 2452	0
<i>Amycolatopsis orientalis</i> subsp. <i>orientalis</i>	ATCC 21425	0
<i>Amycolatopsis orientalis</i> subsp. <i>orientalis</i>	DSM 40040 ^T	0
<i>Amycolatopsis rugosa</i>	ATCC 43014 ^T	0
<i>Amycolatopsis sulphurea</i>	NRRL 2822 ^T	0
<i>Kibdelosporangium</i>		
<i>Kibdelosporangium aridum</i> subsp. <i>aridum</i>	ATCC 39323 ^T	0
<i>Kibdelosporangium aridum</i> subsp. <i>largum</i>	ATCC 39922 ^T	0
<i>Prauserella</i>		
<i>Prauserella rugosa</i>	ATCC 43014 ^T	0
<i>Pseudonocardia</i>		
<i>Pseudonocardia autotrophica</i>	ATCC 19727 ^T	0
<i>Pseudonocardia autotrophica</i>	DSM 43103	0
<i>Pseudonocardia autotrophica</i>	DSM 43098	0
<i>Pseudonocardia compacta</i>	ATCC 35407 ^T	0
<i>Pseudonocardia halophobica</i>	DSM 43089 ^T	0
<i>Pseudonocardia hydrocarbonoxydans</i>	NRRL B-16171 ^T	0
' <i>Pseudonocardia nitrificans</i> '	DSM 46012	0
<i>Pseudonocardia petroleophila</i>	DSM 43193 ^T	0
<i>Pseudonocardia saturnea</i>	NRRL B-16172 ^T	0
<i>Pseudonocardia thermophila</i>	ATCC 19285 ^T	0
<i>Saccharomonospora</i>		
<i>Saccharomonospora azurea</i>	ATCC 43670 ^T	1
' <i>Saccharomonospora caesia</i> '	ATCC 49227	1
<i>Saccharomonospora cyanea</i>	ATCC 43724 ^T	1
<i>Saccharomonospora glauca</i>	DSM 43769 ^T	1
' <i>Saccharomonospora internatus</i> '	ATCC 33517	1
<i>Saccharomonospora viridis</i>	ATCC 15345	1
<i>Saccharomonospora viridis</i>	ATCC 15386 ^T	1
<i>Saccharopolyspora</i>		
<i>Saccharopolyspora erythraea</i>	NRRL 2338 ^T	0
<i>Saccharopolyspora gregorii</i>	NCIB 12823 ^T	0
<i>Saccharopolyspora hirsuta</i> subsp. <i>hirsuta</i>	ATCC 27876	0
<i>Saccharopolyspora hirsuta</i> subsp. <i>hirsuta</i>	NRRL B5792 ^T	0
<i>Saccharopolyspora hirsuta</i> subsp. <i>kobensis</i>	ATCC 20501 ^T	0
<i>Saccharopolyspora hordei</i>	NCIB 12824 ^T	0
<i>Saccharopolyspora rectivirgula</i>	ATCC 15347	0
<i>Saccharopolyspora rectivirgula</i>	ATCC 33515 ^T	0
<i>Saccharopolyspora spinosa</i>	NRRL 18537	0
<i>Saccharopolyspora spinosa</i>	ATCC 49460 ^T	0
<i>Saccharopolyspora taberi</i>	NRRL 16173 ^T	0
<i>Saccharothrix</i>		
<i>Saccharothrix aerocolonigenes</i>	ATCC 39243	0
<i>Saccharothrix aerocolonigenes</i>	NRRL B3298 ^T	0
<i>Saccharothrix cryophilis</i>	NRRL B-16238 ^T	0
<i>Saccharothrix flava</i>	DSM 43885 ^T	0

Table 1 (cont.)

Species	Strain designation	Amplification result*
<i>Saccharothrix longispora</i>	DSM 43749 ^T	0
<i>Saccharothrix mutabilis</i> subsp. <i>capreolus</i>	DSM 40225 ^T	0
<i>Saccharothrix mutabilis</i> subsp. <i>mutabilis</i>	DSM 43853 ^T	0
<i>Saccharothrix syringae</i>	DSM 43886 ^T	0
<i>Saccharothrix waywayandensis</i>	NRRL B-16159 ^T	0
<i>Actinokineospora</i>		
<i>Actinokineospora riparia</i>	NRRL B-16432 ^T	0
Other non-related genera		
<i>Actinomadura</i> sp.	MA 6867	0
<i>Actinomadura hibisca</i>	ATCC 53557 ^T	0
<i>Actinomadura pusilla</i>	ATCC 27296 ^T	0
<i>Kitasatosporia setalba</i>	ATCC 33774 ^T	0
<i>Micromonospora echinospora</i> subsp. <i>pallida</i>	NRRL 2996 ^T	0
<i>Streptomyces avermitilis</i>	ATCC 31267	0
<i>Streptomyces cattleya</i>	NRRL 8057	0
' <i>Streptomyces hygroscopicus</i> subsp. <i>ascomyceticus</i> '	MA 6572	0
<i>Streptomyces hygroscopicus</i> subsp. <i>hygroscopicus</i>	ATCC 27438 ^T	0
' <i>Streptomyces hygroscopicus</i> subsp. <i>rapamyceticus</i> '	NRRL 5491	0
<i>Streptomyces tsukubaensis</i>	MA 6492	0

* Amplification results are as follows: (1) represents a band of the expected size (266 bp); and (0) represents the absence of amplification products.

Table 2. Wild-type isolates of *Saccharomonospora* sp. used in this study

Strain no.	Country	Substrate and origin
42-14, 42-15†, 42-78*, 42-79†, 42-159, 42-160, 42-161*†, 42-162*†, 42-163, 42-164, 42-165, 42-166†, 42-167, 42-168†, 42-169†, 42-170	Sri Lanka	Soil 1, this study
42-17*†, 42-86*	Sri Lanka	Soil 2, this study
42-82, 42-83	Sri Lanka	Soil 3, this study
42-27*†, 42-96	Senegal	Soil 4, this study
42-106, 42-188, 42-189, 42-190*†	Mexico	Soil 5, this study
42-147*†	Mexico	Soil 6, this study
42-157*†, 42-187†	Mexico	Soil 7, this study
42-146*, 42-171, 42-172, 42-173, 42-174, 42-175†, 42-176, 42-177†, 42-191, 42-192, 42-193*†, 42-194, 42-195, 42-196, 42-197*, 42-198, 42-199, 42-200, 42-201, 42-202, 42-205, 42-206†	Mexico	Soil 8, this study
42-178, 42-179, 42-180, 42-181*, 42-182, 42-183	Costa Rica	Soil 9, this study
12-82*	Venezuela	Soil, laboratory collection
02-103*	Spain	Dry seaweed, laboratory collection
10-150	Spain	Decomposing barley, laboratory collection

* Strains partially sequenced in the 16S rDNA annealing region of primers SM1/SM2.

† Strains sequenced in their 16S–23S rDNA ITS region.

the heuristic search algorithm of the phylogenetic analysis using parsimony (PAUP) program 3.1.1 (Swofford, 1993), with gaps treated as missing data. The heuristic-search algorithm provides an efficient approach to find all the most parsimonious trees. Data were resampled with 1000 boot-

strap 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% bootstrap replicates was considered to be statistically significant.

RESULTS AND DISCUSSION

In this work, the design of a pair of genus-specific primers for the selective amplification of the 16S rDNA region of the genus *Saccharomonospora* and their use for the detection of members of the genus in environmental samples are described. The search for conserved sequences within this genus was based on the comparative analysis of the variable regions of the known 16S rRNA sequences. The strains listed in Methods include representatives from different members of the family *Pseudonocardiaceae* as well as the related genera *Saccharothrix* and *Actinokineospora*. Sequences from *Streptomyces* strains were also included in the analysis in order to rule out any homology between sequences of strains of *Saccharomonospora* and representatives of the genus *Streptomyces*.

The analysis was performed by means of alignment of all these sequences that allowed the localization of six variable regions in the 16S rDNA sequence corresponding to nt 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) correspond in fact 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 790–830 appeared to be one of the most promising, given the high sequence conservancy found among the members of the genus. This homology was high enough to consider the design of genus-specific primers for the sequences available for all species of the genus. The region around nt 560–640 also showed an appropriate degree of conservation within the genus. In contrast, analysis of the sequences in the other hypervariable regions showed such a low homology within the genus that these sequences can only be used at the species or even strain level. The regions containing species-specific signature nucleotides have been used to define species-specific primers (Yoon *et al.*, 1996b).

Specific primers for the genus *Saccharomonospora*

When nt 790–830 of the 16S rRNA region from different representatives of the genus *Saccharomonospora* was examined in detail, it was observed that not all positions were unique for all the members of this genus; some positions were also shared by other genera of the family *Pseudonocardiaceae*. Within this region, a short sequence of 16 nt was identified that harboured the highest number of conserved positions in the genus at nt 811–826; it presented one single base change T:G at nt 816 in the case of the type strain of *Saccharomonospora viridis* ATCC 15386^T (X54286). The sequence of 16 nt was used as the basis for the design of the reverse primer SM1 (5'-ACGGCACGGGACACGTGMACAGC-3') that included the degeneration in

this position (M = C:A) and that was extended to 23 nt by addition of five conserved nucleotides to its 3' end to stabilize hybridization. According to its sequence, the primer SM1 presented a very high T_m (between 61.18 and 62.96 °C).

To define a specific oligonucleotide to be used as a forward PCR primer, the upstream variable sequences were studied and the region included within positions 560–640 was selected. In this case, identical sequences were found for all the different strains of *Saccharomonospora* studied. Again, the most specific sequence included only 18 nt between positions 572 and 589 and presented a very low T_m which was not compatible with the high T_m of the primer SM1. The sequence was elongated to 22 nt including five bases of well-conserved positions flanking this region (5'-CGTCTGC-CGTGAAAACCTGCGGC-3'). This elongation stabilized the primer by increasing its T_m to 61.18 °C, which is in the same range as that of primer SM1. According to the sequence, the expected size of the amplification product using this pair of oligonucleotides was 266 bp.

To test the genus specificity of this pair of oligonucleotides, PCR assays were performed using template DNAs isolated from a series of reference strains of 13 different genera (Table 1). Using the pair of primers SM1/SM2 and an annealing temperature of 53 °C, the specific amplification product of 266 bp was obtained from all strains of the six different species of the genus *Saccharomonospora* tested [*Saccharomonospora azurea*, '*Saccharomonospora caesia*', *Saccharomonospora cyanea*, *Saccharomonospora glauca*, '*Saccharomonospora internatus*' and *Saccharomonospora viridis* (two strains)]. No amplification products were detected in control experiments performed with DNAs from all the other bacterial strains analysed (Table 1, Fig. 1). These results are in accordance with the sequences published most recently for many of the strains tested that complement exactly the sequence of the primers. There is only one exception with the sequence of the strain of *Saccharomonospora cyanea* K168^T in the region corresponding to the sequence of SM1. In this case, one difference was found at position 821 (T:C) that apparently does not affect the amplification result with this strain.

To confirm that this lack of amplification with the reference strains other than *Saccharomonospora* strains was not due to the 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), using the conditions recommended (Heuer *et al.*, 1997). The expected amplification product of 271 bp was observed in all strains of actinomycetes tested. The specificity of the pair of primers SM1/SM2 was tested at different annealing temperatures and it was found that an increase of this temperature up to 72 °C still allowed amplification from at least the strains of *Saccharomonospora azurea* and *Saccharomonospora viridis*.

Fig. 1. Agarose gel electrophoresis of PCR products: selective amplification of the 266 bp fragment with primers SM1/SM2. Lanes: 1, control reaction without DNA; 2, *Saccharomonospora azurea* ATCC 43670^T; 3, '*Saccharomonospora caesia*' ATCC 49227; 4, *Saccharomonospora cyanea* ATCC 43724^T; 5, *Saccharomonospora glauca* DSM 43769^T; 6, '*Saccharomonospora internatus*' ATCC 33517; 7, *Saccharomonospora viridis* ATCC 15386^T; 8, *Pseudonocardia thermophila* ATCC 19285^T; 9, *Amycolatopsis orientalis* subsp. *orientalis* DSM 40040^T; 10, *Saccharopolyspora hirsuta* subsp. *hirsuta* NRRL B5792^T; 11, *Saccharothrix syringae* DSM 43886^T; 12, *Actinomadura hibisca* ATCC 53557^T; 13, *Streptomyces avermitilis* ATCC 31267; L, molecular mass marker (100 bp ladder; Pharmacia Biotech).

These results show that the pair of primers SM1/SM2 allow the identification of all the tested strains belonging to the genus, in spite of the small differences observed in their sequences. The pair SM1/SM2 is genus-specific at a low annealing temperature (53 °C). These low stringency conditions used in the PCR reaction are not selective enough to distinguish between sequences of the same genus which differ in just one nucleotide, therefore providing a very accurate tool for the detection and identification of members of this genus.

To rule out a possible PCR amplification with primers SM1/SM2 of 16S rDNA sequences other than those of the genera already tested, a FASTA analysis was also performed against all the DNA sequences available in GenBank. Results confirmed the complete homology of both primers only with sequences of *Saccharomonospora* strains.

Isolation and identification of wild-type isolates

Once the genus specificity of the oligonucleotides was verified on the reference strains from culture collections, the probes were tested as detection tools for new members of this genus. The isolation of wild-type strains of the genus *Saccharomonospora* was attempted from a series of 50 soils obtained from diverse geographical origins; 57 wild-type isolates were finally obtained from only nine of the 50 soil samples tested (Table 2). All these strains, tentatively assigned to the genus *Saccharomonospora* on the basis of their microscopic morphological characters, were positively amplified with the pair of genus-specific primers producing an amplification product of the expected size.

To determine that the strains positively identified with the pair of primers effectively corresponded to this genus, a representative subset of 15 wild-type isolates from different origins was selected (strains 12-82,

02-103, 42-17, 42-27, 42-78, 42-86, 42-146, 42-147, 42-157, 42-161, 42-162, 42-181, 42-190, 42-193, 42-197), including two additional strains from the laboratory culture collection (Table 2). The 16S rDNA region between nt 530–907, including the annealing sites of primers SM1 and SM2, was sequenced. All the sequences at positions 811–833 and 568–590 perfectly matched the sequences of SM1 and SM2, respectively (Table 3). In all cases, it was confirmed that the sequences of wild-type isolates were identical to the sequence of the type species of *Saccharomonospora glauca* and *Saccharomonospora azurea* in the SM1 region. The conservancy of the sequences found in wild-type isolates supports the specificity of the primers used for identification of the strains.

PCR detection of *Saccharomonospora* strains in soil DNAs

The primers SM1/SM2 were then tested to detect the presence of members of the genus *Saccharomonospora* directly from soils. Total DNAs were purified from the nine soils that were shown to contain representatives of this genus, as well as from soils that gave negative results in the isolation experiment. A specific amplification product of the expected size was obtained exclusively in the samples that contained *Saccharomonospora* strains, confirming the ability of this pair of primers to detect the presence of this genus directly from crude extracts of soil DNA.

To evaluate the level of detection of our primers in amplification reactions with crude soil DNA preparations, the lowest concentration of total DNA from *Saccharomonospora* strains that could be detected by specific PCR amplification in about 10^{-13} µg µl⁻¹ was estimated initially. The level of detection was not affected by the extraction procedure nor by the presence of exogenous DNA. The same results were observed in competition experiments performed with *Saccharomonospora* DNA that was co-extracted with

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

Strain	Nucleotide sequence region		EMBL no.
	566–588	811–833	
SM2	CGTCTGCCGTGAAAACCTGCGGC	GCTGTTCACGTGTCCCGTGCCGT	SM1*
12-82	CGTCTGCCGTGAAAACCTGCGGC	GCTGTTCACGTGTCCCGTGCCGT	AJ270373
02-103	CGTCTGCCGTGAAAACCTGCGGC	GCTGTTCACGTGTCCCGTGCCGT	AJ270374
42-146	CGTCTGCCGTGAAAACCTGCGGC	GCTGTTCACGTGTCCCGTGCCGT	AJ270375
42-147	CGTCTGCCGTGAAAACCTGCGGC	GCTGTTCACGTGTCCCGTGCCGT	AJ270376
42-157	CGTCTGCCGTGAAAACCTGCGGC	GCTGTTCACGTGTCCCGTGCCGT	AJ270377
42-161	CGTCTGCCGTGAAAACCTGCGGC	GCTGTTCACGTGTCCCGTGCCGT	AJ270378
42-162	CGTCTGCCGTGAAAACCTGCGGC	GCTGTTCACGTGTCCCGTGCCGT	AJ270389
42-17	CGTCTGCCGTGAAAACCTGCGGC	GCTGTTCACGTGTCCCGTGCCGT	AJ270380
42-182	CGTCTGCCGTGAAAACCTGCGGC	GCTGTTCACGTGTCCCGTGCCGT	AJ270381
42-187	CGTCTGCCGTGAAAACCTGCGGC	GCTGTTCACGTGTCCCGTGCCGT	AJ270382
42-190	CGTCTGCCGTGAAAACCTGCGGC	GCTGTTCACGTGTCCCGTGCCGT	AJ270383
42-193	CGTCTGCCGTGAAAACCTGCGGC	GCTGTTCACGTGTCCCGTGCCGT	AJ270384
42-27	CGTCTGCCGTGAAAACCTGCGGC	GCTGTTCACGTGTCCCGTGCCGT	AJ270385
42-78	CGTCTGCCGTGAAAACCTGCGGC	GCTGTTCACGTGTCCCGTGCCGT	AJ270386
42-82	CGTCTGCCGTGAAAACCTGCGGC	GCTGTTCACGTGTCCCGTGCCGT	AJ270387
42-86	CGTCTGCCGTGAAAACCTGCGGC	GCTGTTCACGTGTCCCGTGCCGT	AJ270388

* For the best comparison with the sequenced region, complementary sequences are shown for reverse primer SM1.

Fig. 2. Agarose gel electrophoresis of PCR products from soil DNA. (a) Selective amplification of the 266 bp fragment using primers SM2/SM1 specific for the genus *Saccharomonospora*. (b) Selective amplification of the 271 bp fragment using primers F243/R513 specific for actinomycetes and Gram-positive bacteria, respectively. In all cases, serial dilutions of 10^{-4} , 10^{-5} , 10^{-6} , 10^{-7} and 10^{-8} $\mu\text{g } \mu\text{l}^{-1}$ soil DNA were used in amplification reactions. Lanes: 1–5, PCR products from soil no.1 (Sri Lanka); 6–10, PCR products obtained from soil no. 8 (Mexico); 11–15, PCR products obtained from control soil containing actinomycetes other than *Saccharomonospora* strains; 16, control reaction without DNA; L, molecular mass marker (100 bp ladder; Pharmacia Biotech).

total DNA from different soils that did not produce an amplification product with the probes.

To evaluate the detection range in crude total soil DNA extracts, the minimum soil DNA concentration necessary to detect the presence of this genus was determined with the probes. Serial dilutions of the nine soil DNAs were used in PCR amplification experiments and, in all cases, the minimum concentration

required to detect an amplification product was around 10^{-7} $\mu\text{g } \mu\text{l}^{-1}$ (Fig. 2).

Diversity of *Saccharomonospora* isolates

To determine the diversity of the isolates that, within this genus, could be detected with the use of these oligonucleotides, the existing relationship between our isolates and the reference strains was studied by

Fig. 3. Cluster analysis of PCR fingerprints showing the diversity of the wild-type isolates of *Saccharomonospora* using the UPGMA method. The schematic representation of the intergenic 16S–23S amplification patterns obtained with the primers L1/G1 was performed with the GELCOMP software package.

analysis of their PCR fingerprinting pattern generated by specific amplification of the 16S–23S rDNA ITS. It has been shown that these spacers have a high degree of length and sequence variation at the genus and species level, due in part to variations in the number and type of tRNA sequences (Loughney *et al.*, 1982; Brosius *et al.*, 1981). The high-resolution fingerprinting primers described by Jensen *et al.* (1993) under the conditions recommended by Hirsch & Sigmund (1995) were used. The PCR amplification patterns of a group

of 66 strains, including six reference strains of *Saccharomonospora* (*Saccharomonospora azurea*, *Saccharomonospora cyanea*, *Saccharomonospora viridis*, ‘*Saccharomonospora internatus*’, *Saccharomonospora glauca* and ‘*Saccharomonospora caesia*’) and a subset of 60 wild-type *Saccharomonospora* isolates were analysed. Their relationship was established in a dendrogram generated by treatment of the Euclidian distances of the amplification patterns by UPGMA (Fig. 3).

Fig. 4. Phylogenetic analysis of the aligned 16S–23S ITS sequences of the wild-type isolates and reference strains of *Saccharomonospora* using the maximum-parsimony method in PAUP 3.1.1. The numbers above branches indicate the bootstrap percentage from 1000 bootstrap replicates that was used as a measure of statistical confidence. A grouping found on 95% bootstrap replicates was considered to be statistically significant. GenBank accession numbers of sequences from the strains of *Saccharomonospora* included in the analysis are as follows: U73402 (*S. cyanea* K168^T); U73397 (*S. azurea* K161^T); U73428 (*S. caesia* K76^T); U73400 (*S. caesia* K163); U73410 (*S. caesia* K182); U73422 (*S. caesia* K200); U73430 (*S. caesia* SB-01); U73432 (*S. caesia* SB-22); U73440 (*S. caesia* SB-58); U73408 (*Saccharomonospora* sp. K180); U73426 (*S. viridis* K73^T); U73412 (*S. viridis* K185); U73414 (*S. viridis* K191); U73420 (*S. viridis* K197); U73434 (*S. viridis* SB-31); U73436 (*S. viridis* SB-33); U73404 (*S. glauca* K169^T); U73406 (*S. glauca* K179); U73416 (*S. glauca* K194); U73418 (*S. glauca* K195); U73424 (*S. glauca* K202); and U73438 (*S. glauca* SB-37). The tree was rooted with the ITS sequence of *Actinopolyspora halophila* JCM 3278^T (Z46603).

Analysis has shown the existence of four major clusters of strains among our isolates. It was observed that although most of the wild-type isolates of the genus *Saccharomonospora* are associated with the same main cluster, many strains also appear on separate branches

of the dendrogram and do not match any of the clusters defined by the amplification patterns of known species. Clustering analysis based on PCR fingerprints was only used to show the large diversity of new isolates that could be identified to the genus level by

means of a selective PCR amplification with the genus-specific primers. This fingerprinting pattern was also used to identify the presence of redundant isolates among strains obtained from the same source. With the exception of four clusters of isolates obtained from three different sources that seemed to correspond to identical strains, the remaining cultures appeared as unique strains as they were obtained from different environments.

The use of the PCR fingerprinting has also revealed among many of our isolates the existence of a polymorphism in the size and number of the 16S–23S rDNA ITS sequences (Fig. 2). Multiple additional bands of higher molecular mass (above 700 bp) were obtained as PCR products even under the most stringent amplification conditions tested in the study. The same result was also obtained with the use of alternative PCR primers such as 1497f and 115r directed to conserved sequences at the end and beginning of the 16S and 23S rDNAs, respectively (Lane, 1991); this confirms previous observations (Jensen *et al.*, 1993; Yoon *et al.*, 1997a).

The sequence polymorphism of these spacers has recently been shown to discriminate between different species of the genus *Saccharomonospora* (Yoon *et al.*, 1997a). The fragment that corresponded to the 16S–23S spacer of 17 isolates was sequenced producing single amplification products but with different lengths, and sequence data also show a high level of polymorphism in our isolates. The 16S–23S intergenic spacer regions of the *Saccharomonospora* wild-type isolates range from a minimum size of 181 bp (isolate 42-157) to a maximum of 192 bp (42-190). The ITS region 5' and 3' ends were deduced from the 3' ends of the 16S rDNAs and the 5' ends of the 23S rDNAs of *Saccharomonospora* spp. previously reported (Kim *et al.*, 1995; Yoon *et al.*, 1997a). Due to their small size, tRNA-like sequences were not found in the ITS region of any of the *Saccharomonospora* strains. This is consistent with previous results showing the absence of tRNA genes in the ITS sequences of *Saccharomonospora* strains, as well as in other actinomycetes (Yoon *et al.*, 1997a). The level of sequence similarity for the 16S–23S sequence among wild-type isolates was 87–99%. The most distant relationship of these strains with reference species was found with *Saccharomonospora glauca*, where similarities were between 77 and 81%. As previously reported, the strains of '*Saccharomonospora caesia*' and *Saccharomonospora azurea* show identical 16S–23S ITS sequences, a result previously used to support the proposal of '*Saccharomonospora caesia*' and *Saccharomonospora azurea* as synonyms (Yoon *et al.*, 1999). A phylogenetic tree based on these 16S–23S ITS sequences was built using the maximum-parsimony method (Fig. 4), showing the inter and intraspecific relationships of the genus *Saccharomonospora*. As previously described, the four valid species and the strain *Saccharomonospora* sp. form distinct branches. The strains of *Saccharomonospora viridis* and *Saccharomonospora glauca* are more

closely related than the synonyms *Saccharomonospora azurea*/*'Saccharomonospora caesia'* and *Saccharomonospora cyanea*, which is the most distant species. The topology of the tree confirms previous works (Yoon *et al.*, 1997a). Wild-type isolates are included in a new cluster clearly separated from reference isolates, with the exception of isolates 42-147 and 42-157, which cluster even more externally, out of the principal branches. The presence of a new cluster of wild-type isolates could suggest the existence of a possible new lineage within the genus. The topology of the tree suggests the presence of a common ancestor for the isolates of '*Saccharomonospora caesia*', *Saccharomonospora azurea*, *Saccharomonospora viridis* and *Saccharomonospora glauca*, whereas *Saccharomonospora cyanea* appears as another evolutionary lineage distant from the rest of the species. The phylogenetic tree was supported by bootstrapping analysis, which shows very high indexes of statistical confidence in branches which include isolates belonging to the same species (Fig. 4).

The sequence polymorphism of the 16S–23S ITS region has been very useful to distinguish between our *Saccharomonospora* strains. Within this genus, the intraspecific homogeneity of the 16S rDNA gene that has been reported for all species contrasts with the intraspecific differences found in the 16S–23S ITS sequences. These sequences have provided very useful information to evaluate the diversity of wild-type isolates of the genus *Saccharomonospora* that can be identified with the genus-specific probes.

Our results with reference strains as well as with wild-type isolates validate the specificity of this new pair of primers for 16S rDNA sequences of *Saccharomonospora* strains. The high diversity of the wild-type strains obtained from different habitats supports the usefulness of the application of these tools for the tentative genus assignment of new isolates of this taxon. Finally, their application in the direct detection of members of this genus from environmental DNA has also been shown.

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