New genus-specific primers for the PCR identification of novel isolates of the genus *Streptomonospora*

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Received 7 June 2006; revised 2 July 2006; accepted 10 July 2006.
First published online 15 August 2006.

DOI:10.1111/j.1574-6968.2006.00402.x

Editor: Aharon Oren

Keywords
genus-specific primers; *Streptomonospora*; PCR identification

Abstract

The halophilic actinomycete genus *Streptomonospora*, forming a distinct branch in the 16S rRNA gene phylogenetic tree adjacent to the genera *Nocardiopsis* and *Thermobifida*, was first proposed by Cui *et al*. to accommodate the species type *Streptomonospora salina*. During a biodiversity and taxonomic study on halophilic filamentous actinomycetes from a saline lake in western China, numerous new halophilic actinomycetes strains were isolated. To confirm whether they are members of the genus *Streptomonospora*, one set of genus-specific oligonucleotides was designed which allows rapid detection of members of the genus *Streptomonospora* by means of PCR amplification. The genus specificity of these primers was validated with reference strains as well as with wild-type isolates, which exhibited morphological characteristics common to this genus.

Introduction

Historically, natural product screening projects have focused on the order *Actinomycetales*, especially on the genus *Streptomyces*, which has been extensively exploited and has efficiently demonstrated its ability to produce a large diversity of new secondary metabolites (Sanglier *et al*., 1993). Extremophiles are a new important resource as potential producers of novel bioactive compounds, as well as in evolutionary and physiological studies. Halophiles, which require a minimum concentration of sodium chloride in their environment, were also isolated in the order *Actinomycetales*. The genera *Actinopolyspora* and *Streptomonospora* are two groups of strictly halophilic filamentous actinomycetes. In other words, all species of the genera *Actinopolyspora* and *Streptomonospora* are halophiles rather than halotolerant microorganisms. The genus *Streptomonospora*, forming a distinct branch in the 16S rRNA gene phylogenetic tree adjacent to the genera *Nocardiopsis* and *Thermobifida*, family *Nocardiopsaceae*, suborder *Streptosporangineae* (Stackebrandt *et al*., 1997), was proposed by Cui *et al*. (2001). At present, it has only two validly described species, *Streptomonospora salina* (Cui *et al*., 2001) and *Streptomonospora alba* (Li *et al*., 2003). During a biodiversity and taxonomic study on halophilic filamentous actinomycetes, numerous new strains were isolated from other dry hypersaline soil samples. The sampling site was close to Aiding Lake in Xinjiang Province in western China.

Members of the family *Nocardiopsaceae* are difficult to identify on the basis of their morphological characteristics only. Meanwhile, the biochemical characterization of each novel isolate is a time-consuming task, which cannot ever be undertaken when handling large numbers of new isolates, as is the case in a biodiversity study or in a natural product screening program. Advancement in nucleic acid techniques, based on data derived from the comparative analysis of 16S rRNA gene sequences, have allowed the development of novel and powerful tools that can be applied to the study of microorganisms (Ludwig & Schleifer, 1994). One important application is to develop specific probes that can be applied at different taxonomic levels. 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 al*., 1996). The development of new identification techniques based on precise genotypic information is not influenced by cultivation conditions. Consequently, DNA-based detection method has applied in differentiating some pathogenic agents

Table 1. Reference strains used in this study and results obtained from selective PCR amplification with Stmp1/Stmp2

Reference strains	Stmp1/Stmp2 58 °C
Streptomonospora salina YIM 90002^T	1
Streptomonospora alba YIM 90003^T	1
Nocardiopsis synnemataformans DSM 44143^T	0
Nocardiopsis composta DSM 44551^T	0
Nocardiopsis lucentensis DSM 44048^T	0
Nocardiopsis halotolerans DSM 44410^T	0
Nocardiopsis halophila DSM 44494^T	0
Nocardiopsis tropica DSM 44381^T	0
Nocardiopsis kunsanensis DSM 44524^T	0
Nocardiopsis aegyptia DSM 44442^T	0
Nocardiopsis umidischolae DSM 44362^T	0
Nocardiopsis listeri DSM 40297^T	0
Nocardiopsis dassonvillei subsp. *dassonvillei* KCTC 9190^T	0
Nocardiopsis xinjiangensis YIM 90004^T	0
Nocardiopsis salina YIM 90010^T	0
Nocardiopsis baichengensis YIM 90130^T	0
Nocardiopsis gilva YIM 90087^T	0
Nocardiopsis rosea YIM 90094^T	0
Nocardiopsis rhodophaea YIM 90096^T	0
Nocardiopsis chromatogenes YIM 90109^T	0
Thermobifida alba DSM 43795^T	0
Thermobifida cellulosilytica DSM 44535^T	0
Streptosporangium yunnanense DSM 44663^T	0
Streptosporangium purpuratum DSM 44688^T	0
Actinomadura atramentaria DSM 43919^T	0
Actinopolyspora mortivallis DSM 44261^T	0
Actinopolyspora halophila DSM 43834	0
Prauserella halophila YIM 90001^T	0
Prauserella alba YIM 90005^T	0
Prauserella rugosa DSM 43194^T	0
Saccharomonospora paurometabolica YIM 90007^T	0
Saccharomonospora halophila DSM 44411^T	0
Nocardioides albus DSM 43109^T	0
Kribbella yunnanensis YIM 30006^T	0
Kribbella alba YIM 31075^T	0
Actinoplanes philippinensis ATCC12427^T	0
Micromonospora chalcea DSM 43868^T	0
Catellatospora citrea subsp. *citrea* IFO14495^T	0
Streptomyces yunnanensis YIM 41004^T	0

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

from others by using their unique DNA sequence regions (Relman, 1993). These unique regions are used as DNA signatures for such pathogens. If some region of DNA sequences is present in only one pathogen, but not the others, that region can be used as a genetic marker to detect a certain microbe. Relatively, the 16S rRNA gene sequences of different genera have more dissimilarity. Thus, genus-level differentiation could be more easily accomplished. The application and usefulness of the genus-specific primers has already been reported for different members of the family *Pseudonocardiaceae* (Morón *et al.*, 1999; Salazar *et al.*, 2000) and the family *Nocardiopsaceae* (Salazar *et al.*, 2002). In this report, the 16S rRNA gene sequences of reference strains related to the genus *Streptomonospora* were downloaded from the GenBank and analyzed to find the *Streptomonospora*-specific signature region in order to design primers for PCR identification. On the basis of this PCR identification, we differentiated the *Streptomonospora* spp. from other halophilic actinomycetes effectively.

Materials and methods

Actinomycete strains

The Actinomycete reference strains used in this study are listed in Table 1. Wild-type isolates were obtained from the laboratory culture collection. The *Thermobifida* species were grown at 45 °C, on Czapek peptone agar plates (3.0% sucrose, 0.3% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4 \times 7H_2O$, 0.05% KCl, 0.001% $FeSO_4 \times 7H_2O$, 0.2% yeast extract, 0.5% peptone, and 1.5% agar). The *Actinopolyspora* species were grown on saline ISP medium 4 agar plates complemented with 15% (w/v) NaCl. All other strains were grown at 28 °C on GYM (0.4% glucose, 0.4% yeast extract, 1.0% malt extract and 0.2% $CaCO_3$) agar plates, or on alkaline GYM agar plates (adjusting pH value to 9.0 with saturated NaOH solution) in the case of some alkalipilic strains of *Nocardiopsis*, or on saline GYM agar with 10% (w/v) NaCl in case of some halophilic strains.

Design of oligonucleotide primers

The 16S rRNA gene sequences of reference strains were obtained from GenBank. Alignment of multiple sequences was performed by using the alignment program CLUSTAL_X (Thompson *et al.*, 1997) to determine the regions conserved only among *Streptomonospora* species, from which the genus-specific primers were derived. We used PRIMER PREMIER (version 5.0) to search PCR primers in these conserved regions. The genus specificity of oligonucleotides was tested against all DNA sequences available in the EMBL database with the FASTA program (Pearson *et al.*, 1990) and in the BLAST program (Altschul *et al.*, 1990), respectively. The melting temperature (T_m) was estimated according to Thomas and Dancis and the Lathe's formulae (Stahl & Amann, 1991). Relative T_m values obtained using 0.3 M as a standard salt concentration helped with the design of a pair of primers with similarly high melting temperatures. The probabilities of primer-dimer formation, autofolding and false-priming were also studied by using the OLIGO program (version 6.0), in order to keep them as low as possible. The oligonucleotides that were designed were synthesized by Sangon Biotech.

DNA extraction

Total genomic DNA from the different reference strains and all wild-type isolates were extracted and purified by using the method described previously (Cui *et al.*, 2001).

PCR amplification and 16S rRNA gene sequencing

DNA preparations were used as templates for *Taq* polymerase. 16S rRNA genes of the wild-type isolates were amplified by PCR using primers A 8-27f (5′-CCG TCG ACG AGC TCA GAG TTT GAT CCT GGC TCA G-3′) and B 1523–1504r (5′-CCC GGG TAC CAA GCT TAA GGA GGT GAT CCA GCC GCA-3′), according to the method described by Cui *et al.* (2001). The amplified 16S rRNA gene fragment was purified by using a TaKaRa DNA fragment purification kit (Ver.2.0). The partial nucleotide sequence of 16S rRNA gene was obtained automatically by using a DNA sequencer (model 377; Applied Biosystems) and software provided by the manufacturer.

PCR reactions with primers Stmp1 and Stmp2 were performed in a final volume of 25 μL containing 0.2 mM each of the four dNTPs (TaKaRa), 0.1 μM each primer, 1 μL extracted DNA (including reference strains and wild-type isolates) and 0.5 U *Taq* polymerase (TaKaRa) with its appropriate reaction buffer. Amplification was performed in a Biometera Tpersonal Thermocycler, according to the following profile: 30 cycles of 30 s at 94 °C, 30 s at 58 °C and 2 min at 72 °C, followed by 10 min at 72 °C. Negative controls without template DNA were included for each PCR experiment. Amplification products were analyzed by electrophoresis (5 V cm^{-1}) in 1.5% (w/v) agarose gels stained with ethidium bromide. The PCR experiment with Stmp1/Stmp2 was repeated twice.

Nucleotide sequence accession numbers

The accession numbers for the partial 16S rRNA gene sequence of the newly isolated wild-type strains are listed in Table 2.

Phylogenetic analysis

The phylogenetic analysis was performed by using the MEGA (version 3.1) software packages (Kumar *et al.*, 2004) after multiple alignment of data by CLUSTAL_X (Thompson *et al.*, 1997), with gaps treated as missing data. Clustering was performed by using the neighbour-joining method (Saitou & Nei, 1987). Bootstrap analysis was used to evaluate the tree topology of the neighbour-joining data by performing 1000 resamplings (Felsenstein *et al.*, 1985).

Results and discussion

Design of genus-specific primers

The design of one set of genus-specific primers for the selective amplification of the 16S rRNA gene regions of the genus *Streptomonospora* has been based on the search for conserved sequences within this genus after a comparative analysis of the variable regions of known 16S rRNA gene sequences. An alignment of the 16S rRNA gene sequences

Table 2. Wild-type isolates used in PCR experiments with genus-specific primers Stmp1/Stmp2

Strains	Accession number	Stmp1/ Stmp2
***Streptomonospora* spp.**		
YIM 90487*, YIM 90494*, YIM 90554*, YIM 90562*, YIM 90564*, YIM 90566*, YIM 90573*, YIM 90576*, YIM 90577*, YIM 90578*, YIM 90580*, YIM 90586*, YIM 90593*, YIM 90594*, YIM 90598*, YIM 90599*, YIM 90604*, YIM 90606*, YIM 90608*, YIM 90612*, YIM 90614*, YIM 90640*	DQ667022-DQ667043	1
***Nocardiopsis* spp.**		
YIM 90567*, YIM 90568*, YIM 90571*, YIM 90632*	DQ667044-DQ667047	0
***Saccharomonospora* spp.**		
YIM 90521*, YIM 90557*, YIM 90561*, YIM 90581*, YIM 90592*	DQ656336, DQ656337, DQ656340, DQ656341, DQ656342	0
***Prauserella* spp.**		
YIM 90625*, YIM 90636*	DQ656334, DQ656335	0
Unidentified filamentous actinomycetes		
YIM 90532, YIM 90588, YIM 90589, YIM 90600, YIM 90601, YIM 90610, YIM 90615, YIM 90617, YIM 90618, YIM 90630, YIM 90646, YIM 90645		0

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

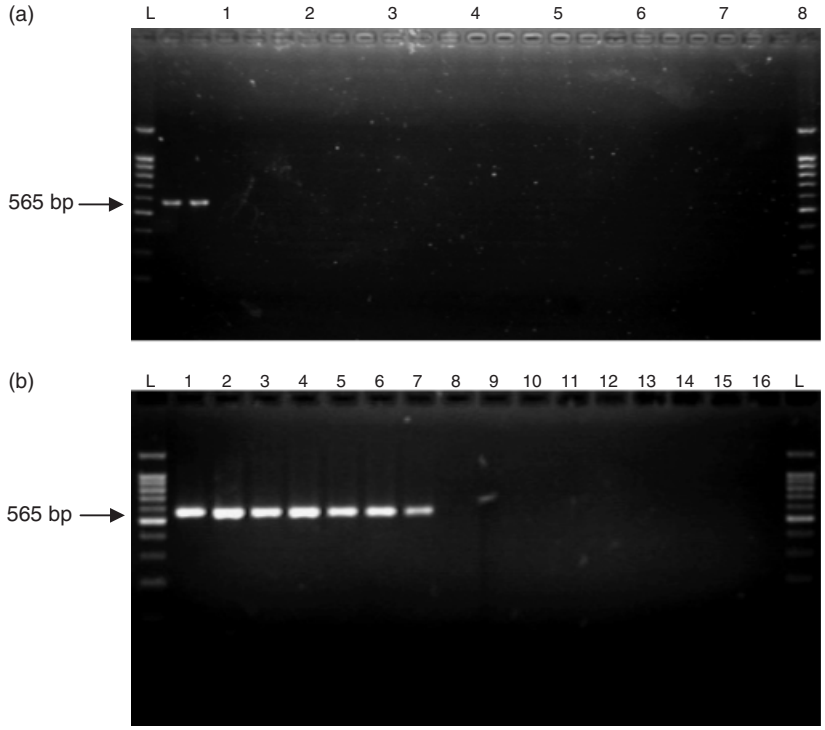

Fig. 1. Agarose gel electrophoresis of PCR products from DNA. Selective amplification at 58 °C of a 565-bp fragment using primers Stmp1/Stmp2 specific for the genus *Streptomonospora*. (a) lanes: L, DNA size ladder; 1, *Streptomonospora salina* YIM 90002^T; 2, *Streptomonospora alba* YIM 90003^T; 3, *Nocardiopsis synnemataformans* DSM 44143^T; 4, *Nocardiopsis halophila* DSM 44494^T; 5, *Nocardiopsis gilva* YIM 90087^T; 6, *Nocardiopsis salina* YIM 90010^T; 7, *Nocardiopsis composta* DSM 44551^T; 8, *Nocardiopsis tropica* DSM 44381^T; 9, *Nocardiopsis rosea* YIM 90094^T; 10, *Nocardiopsis xinjiangensis* YIM 90004^T; 11, *Nocardiopsis baichengensis* YIM 90130^T; 12, *Nocardiopsis chromatogenes* YIM 90109^T; 13, *Thermobifida alba* DSM 43795^T; 14, *Thermobifida cellulosilytica* DSM 44535^T; 15, *Streptosporangium yunnanense* DSM 44663^T; 16, *Actinopolyspora mortivallis* DSM 44261^T; 17, *Prauserella alba* YIM 90005^T; 18, *Saccharomonospora paurometabolica* YIM 90007^T; 19, *Nocardioides albus* DSM 43109^T; 20, *Kribbella yunnanensis* YIM 30006^T; 21, *Micromonospora chalcea* DSM 43868^T; 22, *Streptomyces yunnanensis* YIM 41004^T; 23, control reaction without DNA. (b) lanes: L, DNA size ladder; 1, YIM 90487; 2, YIM 90554; 3, YIM 90562; 4, YIM 90573; 5, YIM 90612; 6, YIM 90593; 7, YIM 90640; 8, YIM 90567; 9, YIM 90568; 10, YIM 90632; 11, YIM 90561; 12, YIM 90592; 13, YIM 90625; 14, YIM 90588; 15, YIM 90630; 16, control reaction without DNA.

that were used for the design of the primers is available as Fig. S1. In this alignment, two regions, covering nucleotides 455–495 and 1010–1035, corresponding to nucleotide positions of 16S rRNA gene of the *Escherichia coli* (GenBank accession number J01695), were found to be highly conserved among the different species of the genus. These sequence homologies were high enough to consider the design of genus-specific primers. The two primers, Stmp1 (5′-TCT GTG CGG GTT GAC GG TAG-3′) and Stmp2 (5′-CGA AGG CAC CCT CCG TCT C-3′), that were designed for the selective amplification respectively nested at positions 463–488 and 1014–1032 of the 16S rRNA gene.

Specificity of the primers for the genus *Streptomonospora*

The sequence alignment with primer Stmp1 was completely identical to those sequences of *S. Salina* and *S. alba* (see Fig. S1). The alignment with the sequences of the genus *Nocardiopsis* showed two to five no-match positions with the primer sequence. The alignment with the sequences of the genus *Thermobifida* showed four to five no-match positions with the primer sequence. The primer Stmp2 showed only one mismatching position in the *Streptomonospora* species, which was located at the site of the eighth nucleotide in the sequence of Stmp2. The only difference between Stmp2 and template-matching with Stmp2 came from purposive manual modification. The purpose of reducing the G+C content of Stmp2 was to make Stmp1 and Stmp2 have close T_m values, so as to make the forward primer and reverse primer anneal simultaneously. The alignment with the sequences of the genus *Nocardiopsis* showed from five to 12 no-match positions with the sequence of Stmp2. The alignment with those sequences of the genus *Thermobifida* showed three no-match positions with the primer sequence.

When the primer pair Stmp1/Stmp2 was tested in PCR using an annealing temperature of 58 °C, defined as optimal for them, we obtained the expected amplification product of 565 bp from all type strains of the genus *Streptomonospora*. Meanwhile, no amplification products were obtained with

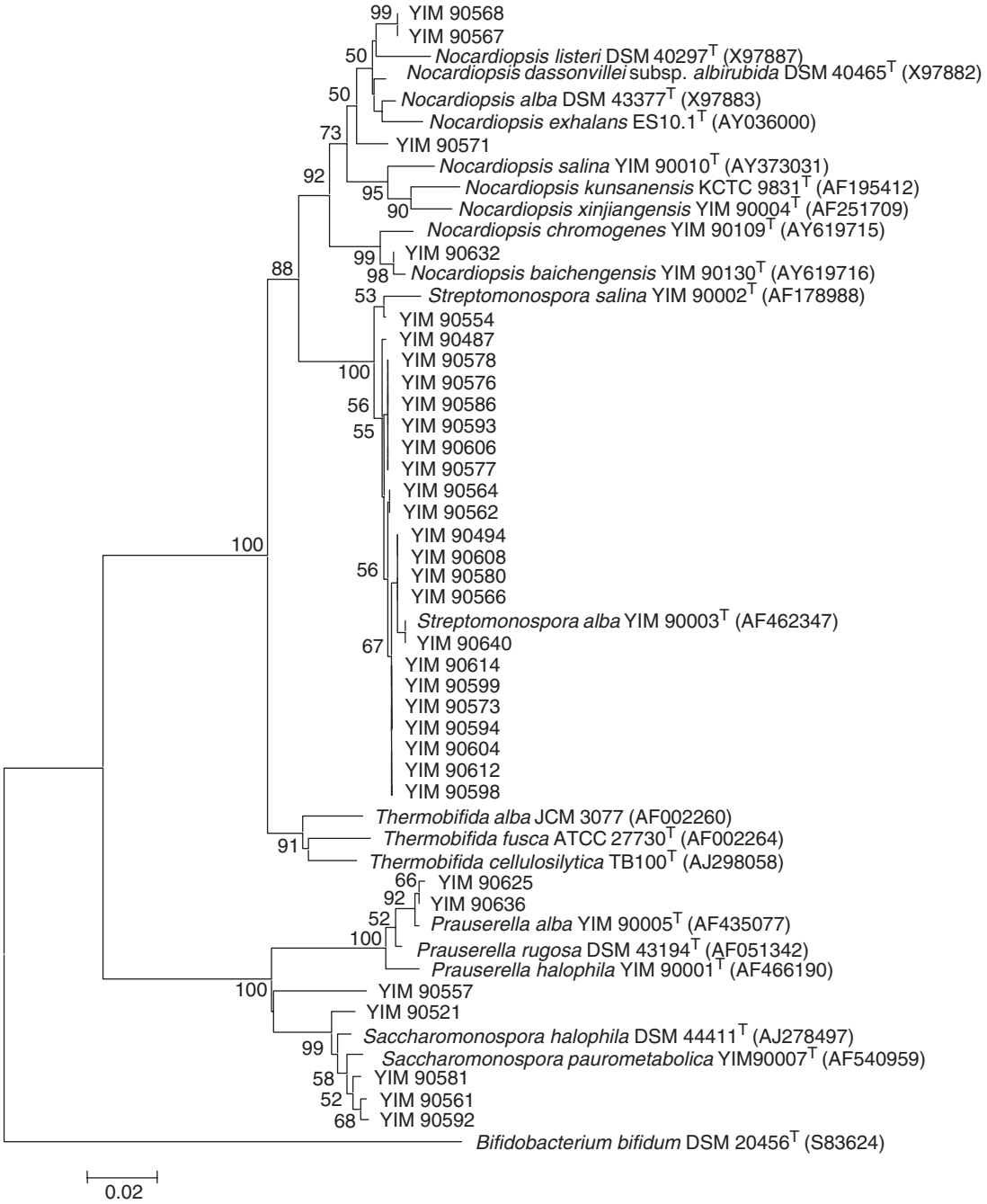

Fig. 2. Diversity of wild-type isolates identified with Stmp1/Stmp2 and their relationships to reference strains. The phylogenetic tree was constructed by the neighbor-joining method. Bootstrap values (> 50%) from 1000 analyses are shown at the nodes of the tree. The scale bar represents 2 inferred nucleotides changing per 100 nucleotides.

DNA from reference strains except the *Streptomonospora* species (Fig. 1). The same results were obtained when amplification was performed at different annealing temperatures (48 °C to 68 °C). This result indicated that the genus specificity of the primers was not affected over a wide range of temperatures.

To rule out a possible PCR amplification of 16S rRNA gene sequences with primers Stmp1/Stmp2 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 *Streptomonospora* strains.

Identification of wild-type isolates

In this work, we selected a group of 45 wild-type isolates from our culture collection, which have never been definitively assigned to any genera because of the absence of required chemotaxonomic and morphological study. DNA was extracted from each of the 45 wild-type isolates and amplified with Stmp1/Stmp2. A positive amplification was only obtained in 22 isolates (Fig. 1, partial results). A phylogenetic tree based on these 16S rRNA gene sequences was constructed using the neighbor-joining method (Fig. 2), showing the inter-and intra-specific relationships of the wild-type strains to reference strains. The topology of the tree further confirmed that our previous studies had determined the taxonomic relationships of the wild-type strains by PCR amplification.

The 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 this new primer pair for 16S rRNA gene sequences of the genus *Streptomonospora*. These data support the usefulness of this primer pair for the tentative assignment of new isolates to this genus from the large numbers of strains that are normally obtained from the environment.

Acknowledgements

The authors are grateful to Prof. R. M. Kroppenstedt of DSMZ and Dr. Jung-Sook Lee of KCTC for their kind providing some reference type strains. This research was supported by National Basic Research Program of China (Project no. 2004CB719601) and Yunnan Provincial Natural Science Foundation (Project no.2004 C0002Q). W. J. Li. was also supported by the Program for New Century Excellent Talent (NCET) in University.

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Supplementary material

The following supplementary material is available for this article online:

Figure S1. Alignment of Gen Bank 165 rRNA gene sequences of members of actinomycete taxa used to evaluate the specificity of the *Streptomonospora*-specific primers Stmp1/Stmp2.

This material is available as part of the online article from http://www.blackwell-synergy.com