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ORIGINAL PAPER

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Rapid detection and high-resolution discrimination of the genus *Streptomyces* based on 16S–23S rDNA spacer region and denaturing gradient gel electrophoresis

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Abstract As the leading source of antibiotics, *Streptomyces* species are the subject of widespread investigation. Many approaches have been tried to aid in the classification of *Streptomyces* isolates to the genus, species, and strain levels. Genetic methods are more rapid and convenient than classification methods based on phenotypic characteristics, but a method that is universal in detecting all *Streptomyces* yet selective in detecting only *Streptomyces* is needed. The highly conserved nature of the 16S rRNA gene (16S rDNA) combined with the need to discriminate between closely related strains results in analyses of ribosomal intergenic spacer (RIS) regions being more productive than analyses of 16S rRNA genes. PCR primers were designed to amplify the RIS region as well as a sufficient length of the 16S rRNA gene to enable phylogenetic analyses of *Streptomyces*. Improved selectivity and specificity for the amplification of RIS sequences from *Streptomyces* with environmental samples was demonstrated. The use of RIS–PCR and denaturing gradient gel electrophoresis (DGGE) was shown to be a convenient means to obtain unique genetic "fingerprints" of *Streptomyces* cultures allowing them to be accurately identified at species, and even strain classification levels. These RIS–PCR and DGGE approaches show potential for the rapid characterization of environmental *Streptomyces* populations.

Keywords *Streptomyces* · RIS · 16S rRNA · 23S rRNA · Intergenic

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Introduction

Streptomyces are gram-positive, aerobic, high-GC, filamentous soil bacteria [34] known for the production of secondary metabolites and biologically active materials. The vast majority of natural antibiotics in clinical usage are made by *Streptomyces* and fungi [17]. The identification and classification of *Streptomyces* has relied on phenotypic characteristics and, more recently, on genetic approaches. The phenotypic methods included fatty acid analysis, whole cell analysis, biochemical tests, antisera specificity test, phage typing, and protein profiling [2, 12, 20, 30, 36, 39]. The genotypic methods are based on molecular data such as DNA–DNA hybridization, restriction digestions of total chromosomal DNA, randomly amplified polymorphic DNA, PCR assays, and nucleic acid sequence comparison [10, 11, 14, 21, 27, 37].

The detection and classification of *Streptomyces* in both environmental and pure culture samples is now most commonly performed by molecular approaches based on oligonucleotide probing or selective PCR amplification [22]. While some attempts to develop *Streptomyces*-specific detection methods target streptomycin biosynthesis genes [14, 15, 19, 23, 29, 35, 38] the majority of genetic methods developed to achieve more precise knowledge of the occurrence of *Streptomyces* in different environments targeted the 16S rRNA gene [8, 28]. Group-specific probes targeting *Streptomyces*-specific regions of 16S rRNA genes have been developed [35] and the comparison of PCR-amplifed rRNA sequences is a prevailing tool in *Streptomyces* taxonomy. To examine sequence variations within the *Streptomyces* genus, three regions (α, β, and γ) in the 16S rRNA gene corresponding to nucleotides 982–998, 1102–1122, and 158–203, respectively, of *S. coelicolor* are generally investigated [2]. Although the study of these 16S rRNA variable regions, especially the γ region, have been used to resolve inter-species relationships within the *Streptomyces*, detecting strain level differences has not been possible with this approach [13]. Several studies have

tried to use sequence data from variable regions of 16S rRNA to set up taxonomic structure within the genus, but the variation was regarded as too limited to help resolve problems of species differentiation [2, 17, 29].

Ribosomal intergenic spacer (RIS) regions show faster rates of evolution than 16S or 23S rRNA genes, and the analysis of intergenic 16S–23S rRNA spacer regions has been shown to be more useful for detecting species and strain-level relationships of *Streptomyces* cultures than the analysis of 16S rRNA gene sequences [13]. The RIS comprises conserved regions as well as regions highly varied in length and sequence in *Streptomyces* [33]. Therefore, RIS regions can be expected to show clear differences between closely related organisms, especially at the species/strain level. The corresponding DNA fragments can be amplified, using conserved nucleotide regions in the flanking 16S and 23S rRNA gene sequences. Moreover, the target region may include some part of the 16S rRNA gene so that DNA sequence comparisons can enable traditional genetic taxonomic classification [22, 40]. The combined use of denaturing gradient gel electrophoresis (DGGE) and the analysis of RIS-region DNA fragments to characterize bacterial cultures is rare [3, 6, 16], and this technique has not been employed for the analysis of *Streptomyces* cultures [33].

The objective of this study was to develop a convenient, rapid, and accurate method allowing the detection and identification of isolates of the genus *Streptomyces*. For this, a set of PCR primers specific for *Streptomyces* was designed, allowing the amplification of RIS together with a phylogenetically significant part of 16S rDNA. The validity of this primer set was assessed using known *Streptomyces* and non-*Streptomyces* cultures as well as environmental soil isolates. Sequence polymorphisms of RIS fragments among species were detected by DGGE.

Materials and methods

Microbial strains and environmental isolates

The *Streptomyces* strains were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany) or American Type Culture Collection (ATCC, Manassas, VA, USA). The *Streptomyces* strains and other bacterial cultures used in this study are shown in Table 1. All of the *Streptomyces* strains were cultivated on glucose–yeast–malt extract agar containing (per liter) 4 g of yeast extract, 10 g of malt extract, 4 g of D-glucose, 2 g of $CaCO_3$, and 15 g of agar. Plates were incubated aerobically at 28°C. Garden soil from the Gas Technology Institute in Des Plaines, IL, USA was the source for environmental samples.

Extraction of genomic DNA

Genomic DNA was purchased from the American Type Culture Collection (ATCC) or extracted from lyophilized pure culture samples obtained from ATCC, from reference strains in our lab (Table 1), and from soil samples using a BIO101 kit (Qbiogene, Carlsbad, CA, USA). The DNA pellet was resuspended in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0), stored at −20°C, and used as template DNA in the PCR to amplify 16S RNA or RIS regions.

Design of Streptomyces-specific primers

16S rRNA and 23S rRNA gene sequences for *Streptomyces* spp were obtained from GenBank, and were aligned with Vector NTI software version 5.6 (Informax Inc., Carlsbad, CA, USA). This alignment was used to determine conserved regions for PCR primer design. The matching of sequences was assessed in the Ribosome Database Project (RDP) II [5]. The *Streptomyces*-specific RIS region PCR primers developed and used in this study were Strep 5′RIS 5′-TGTCTTGGGCTGCA-CACGT-3′ and Strep 3′RIS 5′-GGTCCTTGGCTAC TTC-3′ that annealed to conserved regions of the 16S (nucleotides 1197–1213) and 23S (nucleotides 39–54) rRNA genes, respectively. The primers used in this study were synthesized by MWG Biotech (High Point, NC, USA).

PCR amplification

The PCR amplifications of RIS DNA fragments from template DNA obtained from pure cultures of *Streptomyces* species and from environmental samples were performed as follows. Fifty microliters of PCR mixture contained 1 μl template DNA (10–40 ng), 400 nM of each primer, 10× PCR reaction buffer with Mg^{2+}, 200 μM of each deoxynucleoside triphosphate, and 1 U of Eppendorf MasterTaq (Eppendorf, Westbury, NY, USA). The amplifications were performed with a Mastercycler Gradient thermocycler (Eppendorf AG, Hamburg, Germany). The PCR condition for the RIS amplification was subjected to an initial denaturation at 94°C for 2 min and the subsequent cycles consisted of 30 s denaturation at 94°C, 30 s annealing at 50°C, and a 2-min extension step at 72°C. After 35 cycles, there was a final 7-min extension at 72°C. The 16S rRNA gene of some *Streptomyces* sp. were amplified by using forward SSU139F 5′-ACAAGCCCTGGAAACGGGGT-3′ and reverse SSU657R 5′-CACCAGGAATTCCGATCT-3′ which are located at positions 139–158 and 640–657 (*S. ambofaciens* numbering) [23], respectively. The PCR amplification was performed as described above.

DGGE analysis, cloning and sequencing

For DGGE analysis, RIS and 16S rRNA regions of *Streptomyces* sp. were directly amplified with primer pair Strep 5′RIS–Strep 3′RIS and SSU139F–SSU657R,

Table 1 Microbes used in this study

Species	Sources	Major *Streptomyces* species or group clusters[a]
Streptomyces rutgersensis Subsp. *rutgersensis*	ATCC 3350	*S. albidoflavus*/*S. griseus*/*S. antibioticus*
Streptomyces bikiniensis	ATCC 11062	*S. lavendulae*/*S. fradiae*/*Streptoverticillum*
Streptomyces lavendulae Subsp. *Lavendulae*	ATCC 25233	*S. albidoflavus*/*S. griseus*/*S. antibioticus*
Streptomyces roseoviolaceus	ATCC 25493	*S. violaceus*
Streptomyces venezuelae	ATCC 10712	*S. exfollatus*
Streptomyces viridochromogenes	ATCC 14920	*S. albidoflavus*/*S. griseus*/*S. antibioticus*
Streptomyces viridochromogenes	ATCC 3356	*S. albidoflavus*/*S. griseus*/*S. antibioticus*
Streptomyces viridifaciens	ATCC 11989	*S. lavendulae*/*S. fradiae*/*Streptoverticillum*
Streptomyces parvulus	ATCC 12434	*S. parvulus*
Streptomyces olivochromogenus	ATCC 3336	*S. violaceus*
Streptomyces varsoviensis	ATCC 14631	*S. varsoviensis*
Streptomyces setae	ATCC 33774	*S. albidoflavus*/*S. griseus*/*S. antibioticus*
Streptomyces rochei	ATCC 10739	*S. rochei*
Streptomyces coelicolor	DSM 40675	*S. albidoflavus*/*S. griseus*/*S. antibioticus*
Streptomyces coelicolor	DSM 40682	*S. albidoflavus*/*S. griseus*/*S. antibioticus*
Streptomyces antibioticus	ATCC 8663	*S. albidoflavus*/*S. griseus*/*S. antibioticus*
Streptomyces minutiscleroticus	ATCC 17757	*S. rochei*
Streptomyces recifensis	ATCC 19803	*S. albidoflavus*/*S. griseus*/*S. antibioticus*
Streptomyces sp.	ATCC 14077	NA
Streptomyces kanamyceticus	ATCC 12853	*S. albidoflavus*/*S. griseus*/*S. antibioticus*
Streptomyces ambofaciens	ATCC 13877	*S. rochei*
Streptomyces griseus Subsp. *griseus*	ATCC 10137	*S. albidoflavus*/*S. griseus*/*S. antibioticus*
Streptomyces althioticus	ATCC 19724	*S. rochei*
Other bacteria		
Finegoldia magna	ATCC 15794	
Clostridium beijerinckii	ATCC 8260	
Desulfomonas pigra	ATCC 29098	
Rahnella aquatilis	ATCC 33071	
Lactobacillus gasseri	ATCC 33323	
Duganella zoogloeoides	ATCC 25935	
Escherichia coli	ATCC 11775	
Serratia marcescens	ATCC 13880	
Succinivibrio dextrinosolvens	ATCC 19716	
Klebsiella oxytoca	ATCC 13182	
Actinomyces meyeri	ATCC 35568	
Butyrivibrio fibrisolvens	ATCC 19171	
Actinomyces viscosus	ATCC 27044	
Desulfovibrio sp.	ATCC 700045	
Bacterodies fragilis	ATCC 25285	
Pseudomonas aeruginosa PAO-1	ATCC 39018	
Rhodobacter sphaeroides	ATCC 17023	
Pseudomonas resinovorans CA10	[25]	
Sphingomonas sp. GTIN11	[18]	

[a]Cluster data were derived from Kampfer et al. [13]

respectively. The Strep 5′ RIS and SSU139F primers included a GC clamp at the 5′ end (5′-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCC-G-3′) [29]. Prior to DGGE analysis, PCR products were purified and concentrated using the QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA, USA) as instructed by the manufacturer. The DNA concentration of samples was determined by comparison with a DNA quantification standard (GenSura Laboratories, Inc., San Diego, CA, USA). Four hundred nanograms of DNA were loaded onto an 8% (wt/vol) polyacrylamide gel in TAE buffer (40 mM Tris, 20 mM acetate, 1 mM EDTA, pH 8.0). The 8% polyacrylamide gel (40% acrylamide-*N*,*N*′-methylenebisacrylamide stock, 37:1) was made with denaturing gradients ranging 45–75% (100% denaturant contains 7 M urea and 40% formamide). DGGE was performed on a Dcode Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA, USA) at 60°C overnight at 100 V. DNA bands were visualized by silver staining, and DNA was obtained from bands in the gel by removing gel plugs with a sterile pipette tip and elution into 30 μl of buffer (1 mM Tris, 0.1 mM EDTA, pH 8) at 4°C overnight. Three microliters of eluate from individual bands was used for reamplification by PCR with primers and conditions described above, but 0.1% bovine serum albumin was added to the PCR reaction. PCR products were purified and concentrated using the QIAquick PCR Purification Kit prior to ligation into the pGEM-T easy cloning vector (Promega Corp., Madison, WI, USA). Plasmid DNA was isolated from the transformants and used as template for reamplification of the RIS and 16S

rRNA inserts with M13 forward and M13 reverse primers. The amplified insert DNA was digested with restriction enzyme *Msp*I and *Mbo*I, and the resulting restriction enzyme fragment patterns were compared and sorted visually on 3% NuSieve agarose gel (BioWhittaker Molecular Applications, Rockland, ME, USA). Clones with similar patterns were compared in adjacent lanes of a second 3% NuSieve gel to detect small differences in the patterns. DNA sequence data was generated from unique clones using M13 forward and M13 reverse primers at SeqWright, Inc. (Houston, Texas, USA). The sequence data were inspected for the presence of ambiguous base assignments, and unreliable sequences were removed. The sequences were also subjected to the Check Chimera program from the Ribosomal Database Project before the sequences were submitted for similarity and homology searches, which were performed with the Blast and Vector NTI AlignX programs.

Nucleotide sequence accession number

Streptomyces RIS region DNA sequences were deposited with GenBank and are available under accession numbers AY956502 to AY956540.

Results and discussion

Primer specificity test on *Streptomyces* and other bacteria species

The lengths of 16S rRNA and 23S rRNA sequences included in RIS amplicons are an important consideration in selecting PCR primers because RIS fragments can be recovered from bands on electrophoresis gels and sequenced, or sequenced directly from PCR reactions, to enable phylogenetic analyses. If only the intergenic spacer region is amplified, then identification of the bacterial species from which the DNA fragment was derived is generally not possible. However, if 16S rRNA and/or 23S rRNA gene sequences are included, then species identification can be made, but the accuracy of such identifications depends on the length of DNA sequence available and the size of the database used for analysis. Accordingly, PCR primers used in RIS analyses should include a minimum of 200 bp of 16S rRNA and/or 23S rRNA gene sequences in their amplicons, as this is the minimum length of DNA that can be used in meaningful analyses of phylogeny [32]. The accuracy of phylogenetic analyses increases with the length of DNA sequence available [5], but increased fragment length can decrease the efficiency of the PCR [7] and the effectiveness of DGEE [24].

Previous primers used for RIS analysis were limited by amplifying the RIS region [13, 26, 33, 35], while the RIS primers in this study can also amplify about 350 bp of 16S rRNA sequence. The design of *Streptomyces*-specific RIS primers was based on sequences available in the GenBank database and targeted 1197–1213 for 16S rDNA sequences (Strep 5′ RIS), and 39–54 for 23S rDNA sequences (Strep 3′ RIS). To facilitate the analysis of amplicons in DGGE, primers were selected to amplify fragments that were 700 bp or less and contained about 350 bp of 16S rDNA, which includes the V8 variable region, to assist with phylogenetic analysis of subsequent DNA sequence data. The Strep 5′ RIS sequence displayed a perfect match with 809 out of 823 16S rDNA *Streptomyces* sequences (of sequences over 1,200 bp) in the RDP II database. Limited data were available for the definition of a consensus oligonucleotide for the 5′end of the *Streptomyces* 23S rDNA. Only a few full and partial 23S rDNA sequences of *Streptomyces* cultures were available in the GenBank database (*S. coelicolor, S. griseus, S. avermitilis, S. nodosus, S. rimosus, S. lividans*, and *S. ambifaciens*). The literature suggests that the 23S rDNA sequences from all available microbial species contain a highly conserved region near the 5′ termini [1, 4, 9], and this region was targeted in the design of primer Strep 3′ RIS. The Strep 3′ RIS primer showed a perfect match with all known *Streptomyces* 23S rDNA.

The specificity of the primer pairs Strep 5′RIS and Strep 3′RIS were experimentally confirmed by PCR amplification from 23 *Streptomyces* strains and 19 non-*Streptomyces* strains listed in Table 1. The RIS region was successfully amplified from all *Streptomyces* strains tested and usually generated PCR products that were about 700 bp. None of the 19 non-*Streptomyces* collection strains in Table 1 generated a PCR amplicon with Strep 5′RIS and Strep 3′RIS primers. Both *Actinomycetes meyeri*, and *Actinomyces viscosus*, which are phylogenetically closely related to *Streptomyces*, were not amplified demonstrating the specificity of these primers. To validate that DNA suitable for PCR was used in RIS amplification, the V3 region of the 16S rDNA was successfully amplified from each of the genomic DNA samples used in RIS amplifications (data not shown).

Determination of primer and reaction sensitivity

The sensitivity of the PCR amplification was determined by using DNA isolated from *S. griseus* in serial tenfold dilutions as the template. Template concentrations ranging from 1 pg to 10 ng were used. Good amplification was observed with 100 pg of template DNA and an amplicon band was faintly visible with 10 pg, but no amplification was observed with 1 pg of template DNA (data not shown). The amplification of *Streptomyces* RIS fragments from mixtures of DNA from various species and at various concentration ratios was also performed. The concentration of DNA from three bacterial species (*Rahnella aquatilis, Serratia marcencens* and *Desulfvibrio* sp) was held constant at 10 ng each while the concentration of *S. griseus* was varied from 1 to 0.001 ng the results obtained were essentially the same

as PCR reactions employing pure *S. griseus* template DNA: a single amplicon of about 700 bp was observed and the limit of detection was 10 pg of *S. griseus* DNA template (data not shown).

Use of DGGE of RIS region DNA for species discrimination

PCR was used to amplify 16S rRNA and RIS fragments from 13 *Streptomyces* cultures. Then, DGGE was applied to evaluate the ability of both approaches to differentiate *Streptomyces* sp. The pattern obtained in Fig. 1 confirmed the high-resolution power of DGGE gels in separating DNA fragments of similar sizes but different sequence composition. Furthermore, it is immediately apparent that the number of RIS fragments (Fig. 1, panel B) is far greater than the number of 16S rRNA gene fragments derived from the same samples (Fig. 1, panel A). Consequently, DGGE analysis of RIS fragments allows far more information than DGGE analysis of 16S rRNA gene fragments. Several pairs of *Streptomyces* cultures that yielded highly similar results with DGGE analysis of 16S rRNA fragments such as *S. coelicolor* DSM 40682 and *S. coelicolor* DSM 40675 (lanes 8 and 10 of Fig. 1) or *S. antibioticus* ATCC 8663 and *S. ambofaciens* ATCC 13877 (lanes 11 and 13 of Fig. 1) were clearly differentiated by DGGE analysis of RIS fragments: compare panel A versus panel B of Fig. 1.

The data shown in Fig. 1 demonstrate that the resolution power of RIS sequences and DGGE in distinguishing phylogenetically closely related organisms, such as in the case of *S. coelicolor* DSM 40675 and *S. coelicolor* DSM 40682 is superior to the analysis of 16S rRNA gene fragments for the characterization of *Streptomyces* cultures. To verify that the multiple bands observed in DGGE analysis of RIS amplification products are derived from RIS regions, a total of 39 bands were eluted from DGGE gels and the DNA sequences of each of these fragments was determined. All 39 DNA sequences derived from both known *Strepto-*

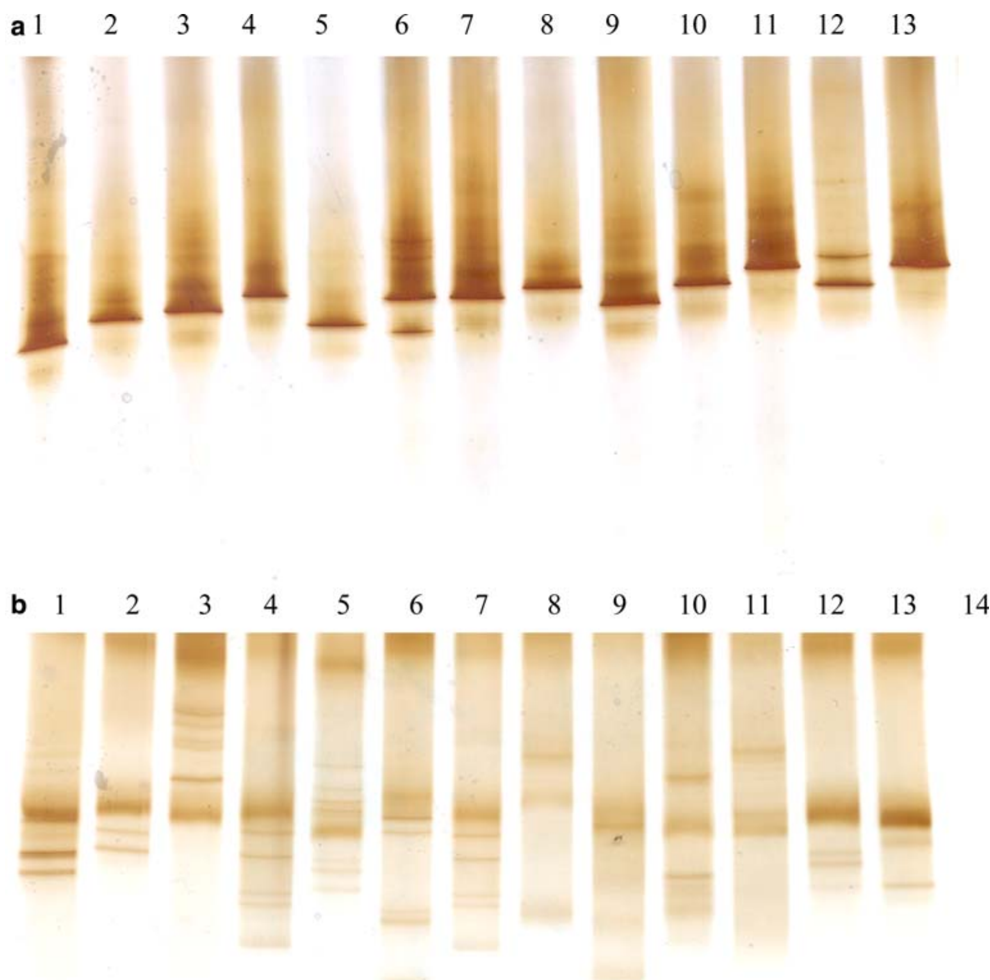

Fig. 1 DGGE gel of 16S rRNA (**a**) and RIS (**b**) of selected *Streptomyces* sp amplifications. *Lane 1 S. bikiniensis* ATCC11062, *Lane 2 S. roseoviolaceus* ATCC25493, *Lane 3 S. venezuelae* ATCC 10712, *Lane 4 S. viridochromogenes* ATCC 14920, *Lane 5 S. viridifaciens* ATCC11989, *Lane 6 S. parvulus* ATCC12434, *Lane 7 S. viridochromogenes* ATCC 3356, *Lane 8 S. coelicolor* DSM 40682, *Lane 9 S. rochei* ATCC 10739, *Lane 10 S. coelicolor* DSM 40675, *Lane 11 S. antibioticus* ATCC 8663, *Lane 12 S. recifensis* ATCC 19803, *Lane 13 S. ambofaciens* ATCC 13877

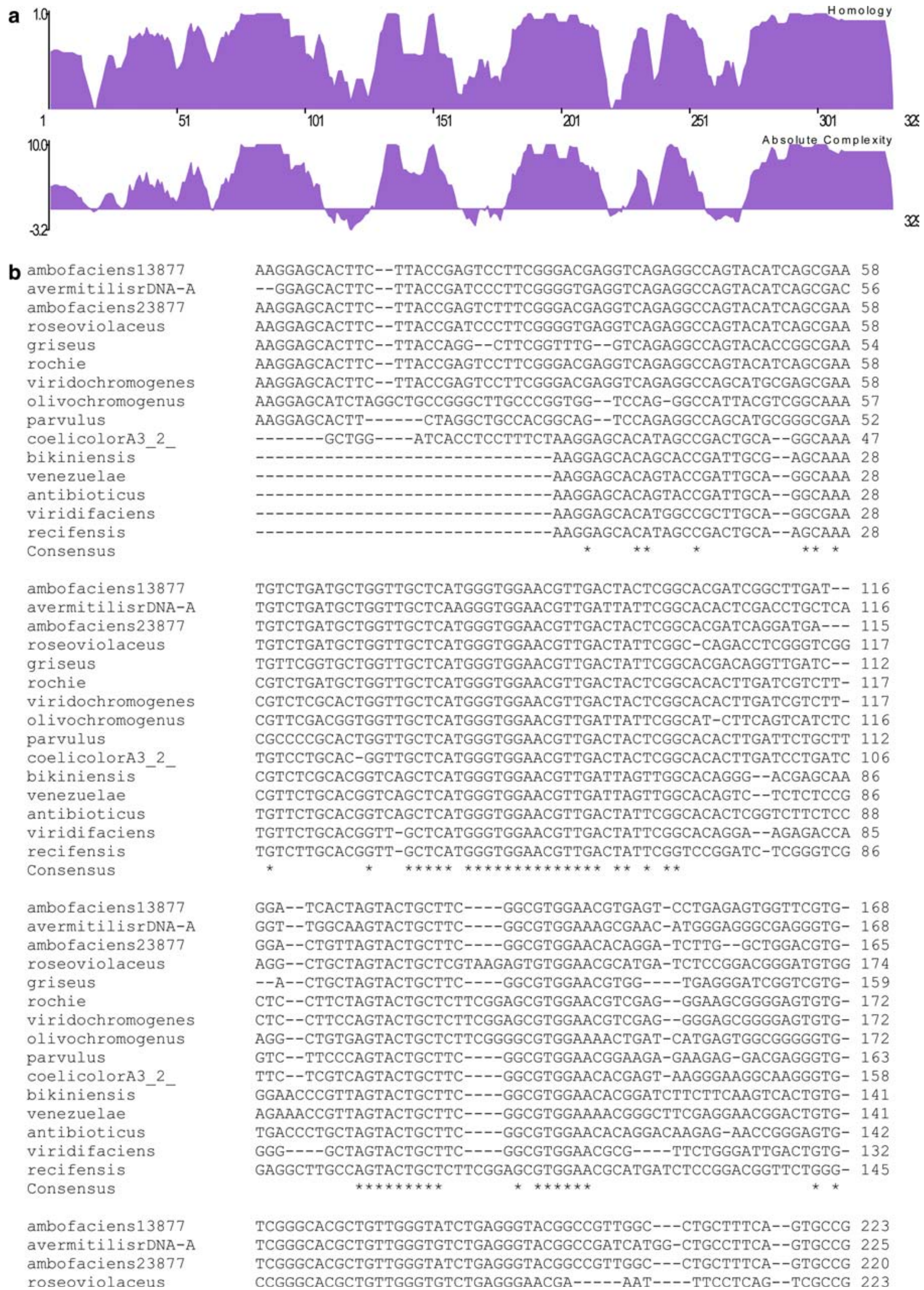

Fig. 2 Nucleotide sequence comparison of the RIS region of *Streptomyces* sp. Similarity and complexity (**a**), and sequence comparison (**b**) of 16 *Streptomyces* RIS region DNA fragments are shown. Nucleotides in (**b**) that are identical in all 15 DNA sequences are indicated by asterisks

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griseus               TCGGGCACGTTGTTGGGTGTCTGAGGGTACGGCCGATGAGG--CTGCCTTCT--GATCCG 215
rochie                TCGGGCACGCTGTTGGGTGTCTGAAGGTACGGCCGCGAGGT---CGCCTTCA--GTGCCG 227
viridochromogenes     TCGGGCACGCTGTTGGGTGTCTGAGGGAATGAAC----------TTCCTTCA--GTGCCG 220
olivochromogenus      CCGGGCACGCTGTTGGGTGTCTGAGGGTACGGCCGATTATCGGCTGCCTTCA--GTGCCG 230
parvulus              TCGGGCACGCTGTTGGGTATCTGAGGGCACGGCCGTATGGCTGGGTCTTCGG--TTGCCG 221
coelicolorA3_2_       TCGGGCACGCTGTTGGGTATCTGAGGGTACGGCCGTGAGGT--CGCCTTCAG--TTGCCG 214
bikiniensis           CCTGGCACGTTGTTGGGTGTCTGAGGGTACGGCCGTATGGTCGTCCTTCACG-GATGCCG 200
venezuelae            CTTGGCACGTTGTTGGGTGTCTGAGGGTACGGCCGTAAGGTTTGTATCTTCGCGATGCCG 201
antibioticus          TCGGGCACGCTGTTGGGTGTCTGAGGGCACGGCCGCAAGGCCTG--TCTTCGGGATGCCG 200
viridifaciens         TCGGGCACGTTGTTGGGT-CCTGAGGGAACGG-----AAGTTG---CCTCAAGGATGCCG 183
recifensis            CCGGGCACGCTGTTGGGTGTCTGAGGGAATGA-------ATCCCCTCTTTCG-----CCG 193
Consensus                ****** ********  **** ** * *                       ***

ambofaciens13877      GCCCCGGTAAAAT-CCG-CGTGAGTGGGTTG-TGACGGGTGGTTGGTCGTTGTTTGAGA 280
avermitilisrDNA-A     GCCCCGGTAAAGAT-CTG-CTTCGGCGGGTTG-TGACGGGTGGTTGGTCGTTGTTTGAGA 282
ambofaciens23877      GCCCCAGTGCACTC-GGGATGTTGTCCCGGGG-TGATGGGTGGTTGGTCGTTGTTTGAGA 278
roseoviolaceus        GCCCCAGTAAACTCGCTGGTTTTCCAGTGGGG-TGATGGGTGGCTGGTCGTTGTTTGAGA 282
griseus               GCCCCAGTGAACTCATCCGGTTTCGGGTGGGG-TGGTGGGTGGCTGGTCGTTGTTTGAGA 274
rochie                GCCCCAGTGCACTC-GAGCCGCTGGTTCGGGG-TGATGGGTGGTTGGTCGTTGTTTGAGA 285
viridochromogenes     GCCCCAGTGAACTC-CGGGT-TTTTCCGGGGG-TGATGGGTGGTTGGTCGTTGTTTGAGA 277
olivochromogenus      GCCCCAGTGCACTCGAACCGGAAGGTTCGGGG-TGATGGGTGGTTGGTCGTTGTTTGAGA 289
parvulus              GCCCCAGTGAACTTGCCCGTGAGGG--CAGGG-TGATGGGTGGTTGGTCGTTGTTTGAGA 278
coelicolorA3_2_       GCCCCGGTAAAAT--CCGCGTGAGTGGGTTG-TGACGGGTGGTTGGTCGTTGTTTGAGA 271
bikiniensis           GCCCCAGTGAACCTGGCCTGTATGGGTCAGGG-TGGTGGGTGGCTGGTCGTTGTTTGAGA 259
venezuelae            GCCCCAGTGAACTTGATTCGTTTGGATCAGGG-TGATGGGTGGCTGGTCGTTGCTTGAGA 260
antibioticus          GCCCCAGTGAACTCCGA--GTGTGACTCGGGGGTGATGGGTGGCTGGTCGTTGTTTGAGA 258
viridifaciens         GCCTCACTTGAGATGGTCCTTCGGGATTGTTGAGGGTGTGTGACTGGTCGTTGTTTGAGA 243
recifensis            GCCCCAGTGCACTCGAACCGGTTGGTTCGGGG-TGATGGGTGGCTGGTCGTTGTTTGAGA 252
Consensus             *** *  *  *                  *  *  * ***  ********* ******

ambofaciens13877      ACTGCACAGTGGACGCGAGCATCTGT-- 306
avermitilisrDNA-A     ACTGCACAGTGGACGCGAGCATCTGTGG 310
ambofaciens23877      ACTGCACAGTGGACGCGAGCATCTGT-- 304
roseoviolaceus        ACTGCACAGTGGACGCGAGCATCTGT-- 308
griseus               ACTGCACAGTGGACGCGAGCATCTGT-- 300
rochie                ACTGCACAGTGGACGCGAGCATCTGT-- 311
viridochromogenes     ACTGCACAGTGGACGCGAGCATCTGT-- 303
olivochromogenus      ACTGCACAGTGGACGCGAGCATCTGT-- 315
parvulus              ACTGCACAGTGGACGCGAGCATCTGT-- 304
coelicolorA3_2_       ACTGC----------------------- 276
bikiniensis           ACTACACAGTGGACGCGAGCATCTGT-- 285
venezuelae            ACTACACAGTGGACGCGAGCATCTGT-- 286
antibioticus          ACTGCACAGTGGACGCGAGCATCTGT-- 284
viridifaciens         ACTGCACAGTGGACGCGAGCATCTGT-- 269
recifensis            ACTGCACAGTGGACGCGAGCATCTGT-- 278
Consensus             *** *
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Fig. 2 (Contd.)

myces cultures and from soil samples were found to be RIS region DNA fragments (data not shown).

Detection of *Streptomycetes* from environmental samples

The specificity of the Strep 5′ RIS and Strep 3′ RIS primers was further examined by PCR amplification using DNA isolated from soil samples as the template. Multiple RIS bands of the expected size (around 700 bp) were observed when DNA from soil samples was used as the template in PCR reactions and analyzed by DGGE. In order to confirm the specificity of the PCR reaction, the PCR amplification products were run on DGGE gels, then gel bands were eluted, cloned and sequenced. A total of nine DNA sequences were obtained from RIS fragments derived from soil samples in two independent experiments. All nine sequenced amplification products obtained from soil samples showed greater than 94% similarity with *Streptomyces* 16S rRNA gene sequences. The DNA sequences of these *Streptomyces* RIS fragments derived from soil samples were deposited with GenBank as accession numbers AY956502 to AY956540. No false results were detected. Moreover, analysis of RIS sequences from 30 reference strains and nine environmental isolates showed that tRNA genes were not found in this region.

These results indicate that the use of PCR amplification using the *Streptomyces*-specific PCR primers described here, combined with DGGE, may provide a rapid, accurate and sensitive approach to characterize the diversity of *Streptomyces* strains in the environment. The sequence alignment of *Streptomyces* sp. RIS frag-

ments revealed a mosaic-like structure of conserved and variable regions as shown in Fig. 2. The first graph in Fig. 2a displays the alignment quality profile (similarity). The VectorNTI software assigns specific values to each residue at a given alignment position in each aligned sequenced depending on whether the residue is identical, similar or weakly similar to the corresponding residue of the consensus sequence. The second graph in Fig. 2a shows the statistical significance profile (absolute complexity) of an alignment. It is calculated as a sum of all pairwise residue substitution scores at a given alignment position normalized by the number of pairs in the alignment. Figure 2b shows the DNA sequences of the 16 *Streptomyces* RIS region DNA fragments analyzed.

Three of the conserved DNA sequence regions within the *Streptomyces* RIS fragments are sufficiently long (28–32 bp) to serve as targets for the design of *Streptomyces*-specific PCR primers. Similarly the variable regions within the RIS fragments could also serve as targets for PCR primers that could allow species and strain specificity. Therefore, future studies of selective amplification of *Streptomyces*-specific DNA from environmental samples could employ a nested PCR amplification strategy using the 16S/23S rRNA primers described here, followed by primers targeting conserved and/or variable regions within the RIS and/or 16S rRNA gene sequences to facilitate the sensitive and accurate detection of *Streptomyces* at genus, species, and strain levels within environmental samples. The use of such primers in conjunction with DGGE could enable the detection and characterization of the diversity of *Streptomyces* cultures in environmental samples and the use of such primers in quantitative PCR could enable the quantification of *Streptomyces* cultures in environmental samples.

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