Nucleotide sequences of streptomycete 16S ribosomal DNA: towards a specific identification system for streptomycetes using PCR

Annette Mehling, Udo F. Wehmeier and Wolfgang Piepersberg

Author for correspondence: Udo F. Wehmeier. Tel: +49 202 439 2802. Fax: +49 202 439 2698.

Bergische Universität GH
Wuppertal, AG Chemische
Mikrobiologie, D-42097
Wuppertal, Federal Republic
of Germany

To facilitate the differential identification of the genus *Streptomyces*, the 16S rRNA genes of 17 actinomycetes were sequenced and screened for the existence of streptomycete-specific signatures. The 16S rDNA of the *Streptomyces* strains and *Amycolatopsis orientalis* subsp. *lurida* exhibited 95–100% similarity, while that of the 16S rDNA of *Actinoplanes utahensis* showed only 88% similarity to the streptomycete 16S rDNAs. Potential genus-specific sequences were found in regions located around nucleotide positions 120, 800 and 1100. Several sets of primers derived from these characteristic regions were investigated as to their specificity in PCR-mediated amplifications. Most sets allowed selective amplification of the streptomycete rDNA sequences studied. RFLPs in the 16S rDNA permitted all strains to be distinguished.

Keywords: *Streptomyces*, identification, 16S rRNA genes, diagnostic probes, RFLP

INTRODUCTION

Members of the genus *Streptomyces* are filamentous Gram-positive soil bacteria with a typical base composition of 72–75 mol% G+C (Enquist & Bradley, 1971; Piepersberg, 1993). Streptomycetes undergo complex morphological differentiation including growth of substrate mycelia in the initial phase, followed by development of aerial mycelium and its subsequent conversion to spores (Piepersberg, 1993). Streptomycetes possess extensive secondary metabolic pathways leading to the production of a wide array of bioactive compounds including many commercially important substances such as antibiotics. The metabolic diversity of these indigenous soil micro-organisms has been widely exploited in industry and agriculture. Selective methods for detecting and identifying these micro-organisms are needed in order to gain insights into the population and genome dynamics of *Streptomyces* in natural environments, in particular the distribution of unstable genetic material such as the biosynthesis genes for antibiotic production (Piepersberg, 1993; Phillips & Wellington, 1992); for detecting novel strains of biotechnological importance;

and for assessing the fate of genetically engineered micro-organisms.

A number of methods have been developed to monitor micro-organisms in their habitats. These include culturing methods using selective plating techniques (Kuster & Williams, 1964), hybridization probes (Holben *et al.*, 1988), construction of genetic marker systems allowing phenotypic and genotypic detection of heterologous genes and their gene products (Wipat *et al.*, 1991), and DNA amplification of target sequences employing PCR (Steffan & Atlas, 1988). Considerable evidence has also been provided by Liesack & Stackebrandt (1992) indicating that only a fraction of bacteria in soil have been defined by conventional methods. A promising method for simultaneous and selective detection of both culturable and nonculturable bacteria of defined taxonomic groups is the amplification of 16S ribosomal DNA (rDNA) or ribosomal RNA (rRNA) sequences using PCR.

Sequence comparisons of small subunit rRNA have been used as a source for determining phylogenetic and evolutionary relationships among organisms of the three kingdoms Archaea, Eucarya and Bacteria. The present compilation of complete genes for the small subunit rRNA contains over 2200 16S and 16S-like sequences (Gutell *et al.*, 1994). The 16S rRNAs are highly conserved, sharing common three-dimensional structural elements of similar function. The primary structures are well investi-

Abbreviations: Act., *Actinoplanes*; Amy., *Amycolatopsis*; B., *Bacillus*; Br., *Brevibacterium*; E., *Escherichia*; M., *Mycobacterium*; S., *Streptomyces*; Stv., *Streptoverticillium*.

gated and conserved, and variable regions have been determined (Woese, 1987). Primers located in highly conserved regions have been published, allowing the amplification of 16S rDNA and subsequent sequence analysis (Edwards *et al.*, 1989). Certain signatures in the nucleotide sequence can be unique for particular phylogenetic groups, offering the opportunity to design genus-specific probes (Salama *et al.*, 1991), whereas the variable regions can be used to assign organisms to lower taxonomic groups (Klijn *et al.*, 1991). Three variable regions in the 16S rRNAs of *Streptomyces* were defined and termed by Stackebrandt *et al.* (1991), as α - (positions 982–998, *S. ambofaciens* nomenclature), β - (1102–1122) and γ - (150–200) regions. The comparison of the partial rRNA gene sequences revealed substantial variations between these rRNA regions of this genus. Although no genus-specific signature was found, three variable regions, V2, V6 and V7, were identified (Stackebrandt *et al.*, 1991), which could further assist in designing genus-, species- and strain-specific probes.

The present study describes the cloning and sequence analysis of the complete 16S rRNA genes from 17 actinomycetes. Strains were largely selected by their ability to produce streptomycin-like or lincomycin-like antibiotics. The comparative analysis of these sequences provided the basis for the design of diagnostic probes with potential for genus-specific detection of streptomycetes in PCR assays. Probes allowing strain-specific detection were also developed. Furthermore, the strains investigated in this study were characterized by ribotyping experiments.

METHODS

Bacterial strains and media. The streptomycetes and other bacteria investigated in this study are listed in Table 1. They were obtained from the American Type Culture Collection (ATCC), Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Institut für Mikrobiologie und experimentelle Therapie (IMET), International Streptomyces Project (ISP) and Northern Regional Research Laboratories (NRRL). Actinomycete strains were cultured in tryptic soy broth (TSB, Hopwood *et al.*, 1985) liquid medium or on soy mannitol agar (SMA, Distler *et al.*, 1985). All other strains were grown in Luria Bertani broth (LB) liquid medium or on LB plates containing 1.6% (w/v) agar. *Escherichia coli* strains harbouring plasmids were grown on LB medium supplemented with 100 mg ampicillin ml⁻¹.

Extraction of genomic DNA. Streptomycete strains were grown to the late exponential phase in TSB at 28 °C, and washed twice with Tris/HCl-EDTA buffer or 10.3% (w/v) sucrose prior to DNA preparation. Chromosomal DNA was isolated (A) by the CTAB (hexadecyltrimethylammonium bromide) isolation method, according to the protocol of Wilson (1990), and (B) by a method adapted from a protocol developed for maize (Dellaporta *et al.*, 1985). The following modifications were made to method A: (1) mycelia (0.2–0.4 g) were incubated in lysis buffer with increased concentrations of lysozyme (10 mg per ml lysis buffer) and proteinase K (200 µg per ml lysis buffer) at 37 °C for 60–120 min until the suspension cleared; (2) DNA was extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1, by vol.) to obtain DNA of higher purity. All other steps were as described in the original protocol. The following

Table 1. Bacteria examined in this study

Strain†	Reference/origin‡
<i>Actinoplanes utabensis</i> *	ATCC 31044
<i>Amycolatopsis orientalis</i> subsp. <i>lurida</i> *	DSM 43187 (SM)
<i>Amycolatopsis orientalis</i> subsp. <i>orientalis</i>	DSM 40040 ^T
<i>Bacillus subtilis</i>	DSM 347
' <i>Brevibacterium flavum</i> '	ATCC 14067
' <i>Brevibacterium lactofermentum</i> '	ATCC 13869
<i>Corynebacterium glutamicum</i>	ATCC 13032 ^T
<i>Enterobacter aerogenes</i>	DSM 30053 ^T
<i>Escherichia coli</i> DH5 α	Hanahan (1983)
<i>Escherichia coli</i> GM1674	Woodcock (1989)
<i>Escherichia coli</i> JM83	Yanisch-Perron <i>et al.</i> (1985)
<i>Escherichia coli</i> JM109	Yanisch-Perron <i>et al.</i> (1985)
<i>Hyphomicrobium</i> EG	Laboratory strain
<i>Pseudomonas</i> BL3	Laboratory strain
<i>Salmonella typhimurium</i> LT2	Laboratory strain
<i>Streptomyces albus</i> GR2	Laboratory strain
<i>Streptomyces bikiniensis</i> *	NRRL B-1049 ^T (SM)
<i>Streptomyces bluensis</i> *	ISP 5564 ^T (SM)
<i>Streptomyces caelestis</i> *	NRRL 2418 ^T (LM)
' <i>Streptomyces espinosus</i> '*	NRRL 3890 (LM)
' <i>Streptomyces espinosus</i> '*	NRRL 5729 (LM)
<i>Streptomyces fradiae</i>	DSM 40063 ^T
<i>Streptomyces galbus</i> *	DSM 40480 (SM)
<i>Streptomyces galbus</i> *	DSM 40089 ^T
<i>Streptomyces glaucescens</i> GLA.0*	DSM 40716 (SM)
<i>Streptomyces griseofuscus</i>	DSM 40191 ^T
<i>Streptomyces griseus</i> N2-3-11*	Kaken Chem. Co., Tokyo (SM)
<i>Streptomyces griseus</i> *	DSM 40236 ^T (SM)
<i>Streptomyces hygroscopicus</i> subsp. <i>glebosus</i> *	DSM 40823 ^T (SM)
<i>Streptomyces hygroscopicus</i> subsp. <i>limoneus</i>	ATCC 21431
<i>Streptomyces lavendulae</i>	IMET 40330 ^T
<i>Streptomyces lincolnensis</i> *	NRRL 2936 ^T (LM)
<i>Streptomyces lincolnensis</i> *	NRRL 5321 (LM)
<i>Streptomyces lincolnensis</i> 78-11*	Zhang <i>et al.</i> (1992) (LM)
<i>Streptomyces lividans</i> TK23*	Hopwood <i>et al.</i> (1985)
' <i>Streptomyces longissimus</i> '	ATCC 14562
<i>Streptomyces netropsis</i>	DSM 40259 ^T
' <i>Streptomyces ornatus</i> '*	DSM 40307 (SM)
<i>Streptomyces pactum</i>	NRRL 2939 ^T
<i>Streptomyces parvulus</i>	NRRL 2283
<i>Streptomyces pseudogriseolus</i> *	NRRL 3985 (LM)
<i>Streptomyces rimosus</i> subsp. <i>paromycinus</i>	ATCC 21484
<i>Streptomyces spectabilis</i>	DSM 40512
<i>Streptomyces subrutilis</i> *	DSM 40445 ^T (SM)
' <i>Streptomyces vellosus</i> '	NRRL 8037 (LM)
<i>Streptomyces violaceus</i>	IMET 43085 ^T
' <i>Streptoverticillium griseocarneum</i> '*	DSM 40004 ^T (SM)
' <i>Streptoverticillium mashuense</i> '*	DSM 40221 ^T (SM)

† Invalid names are indicated by quotation marks; strains used in RFLP analyses are indicated by *.

‡ Type strains are indicated by a superscript T; producers of streptomycin-like or lincomycin-like antibiotics are indicated by (SM) or (LM), respectively.

Table 2. Primers tested in this study

Primer	Nucleotide sequence†	Corresponding region‡
pA	AGAGTTTGATCCTGGCTCAG	nt 8 (Edwards <i>et al.</i> , 1989)
pH*	AAGGAGGTGATCCAGCCGCA	nt 1530 (Edwards <i>et al.</i> , 1989)
AM33	AGATCGGAATTCCTGG	nt 640
AM42	CAAGGGCATCCACCGT	Identical to L1 (Jensen <i>et al.</i> , 1993); nt 20 of the 23S rDNA
AM44	CTTCGGGGTG(ATC)TGGGGACTCAC	nt 1100
AM45	GTGAGTCCCCA(GAT)CACCCCGAAG	nt 1100 (comp.)
AM46	TGACCATCTTGGGCATCCTTGAT	<i>Streptomyces galbus</i> DSM 40480 specific probe γ -region
AM47	GTGGGCAATCTGCCCT(TG)CACTCT	nt 120
AM48	CCCT(TG)CACTCTGGGACAAGCCCT	nt 130
AM51	ACATTCCACGT(CT)GTC(GC)GTGCC	nt 800
AM52	GGCAC(GC)GAC(GA)ACGTGGAATGT	nt 800 (comp.)

† Ambiguous nucleotides are represented by parentheses.

‡ The position of the primers within the 16S rRNA genes is indicated by the nt position. Sequences found in the complementary DNA strand are marked '(comp.)'. Primers were developed in this work except where noted.

is a brief description of method B. Approximately 0.5–1.0 g mycelia were resuspended in 5 ml lysis buffer (25 mM Tris/HCl, 25 mM EDTA, 10–15 mg lysozyme; 50 μ g RNaseA ml⁻¹; pH 8.0) and incubated for 30–80 min at 37 °C. After 500 μ l of 5 M NaCl solution had been added, the suspension was agitated on a vortex-mixer until it became translucent. Cells were lysed by the addition of 1.2 ml of 10% (w/v) SDS. The lysates were incubated for 15–30 min at 65 °C. After the addition of 2.4 ml 5 M potassium acetate, the solution was mixed by vortexing, and left on ice for a minimum of 20 min. The precipitate was removed by centrifugation for 30 min at 6000 r.p.m. (or 10 min at 12000 r.p.m., Sorvall SS34), and the volume of the supernatant adjusted to 8 ml. The DNA was recovered by precipitation with 2-propanol and dissolved in 700 μ l 50 mM Tris/HCl-10 mM EDTA, pH 8.0. The aqueous phase was transferred to a 1.5 ml microfuge tube. After 75 μ l 3 M sodium acetate and 500 μ l 2-propanol had been added, the solution was centrifuged for 0.5–2 min. The precipitate was washed with cold 70% (v/v) ethanol, dried and dissolved in 100 μ l TE (10 mM Tris/HCl-1 mM EDTA, pH 8.0) or distilled water.

PCR amplification of the 16S rDNAs. Primers pA and pH* (Table 2), as described by Edwards *et al.* (1989), were used for the amplification of 16S rDNA sequences. Primers were supplied by BIOMETRA in PCR-grade or were synthesized on a solid support by using a model 381A DNA synthesizer (Applied Biosystems). Approximately 500 ng genomic template DNA was used with 100 pmol of each primer per 100 μ l reaction volume. To improve the denaturation of the DNA, 10% (v/v) DMSO was added to the reaction mixture. A few drops of mineral oil were layered over the reaction mixture to prevent evaporation. Amplifications were performed in automated thermocyclers according to the following amplification profile: 98 °C (5 min) followed by 30 cycles of 95 °C (1 min), 52 °C (40 s), and 72 °C (2 min), adding Vent DNA polymerase (New England Biolabs) after a 'hot start'. To ensure that no contaminating DNAs would give false positive results, one sample lacking a template was included in each series of reactions. Aliquots of the amplification products were analysed by agarose gel electrophoresis.

Cloning and sequencing of the PCR products. Amplification products were subjected to agarose gel electrophoresis. The fragments corresponding to the size of the 16S rDNA were excised and subsequently purified using a JetSorb Kit (GENOMED). The fragments were cloned into the vector pUC18 (Yanisch-Perron *et al.*, 1985) using standard methods (Sambrook *et al.*, 1989). DNA was sequenced according to the manufacturer's specifications on an automatic DNA sequencer (ALF, Pharmacia), using a T7 polymerase sequencing kit and primers labelled with fluorescent dye (Pharmacia). To minimize sequence errors made by the Vent-DNA polymerase, a minimum of three independent clones containing 16S rDNA inserts were analysed for each organism.

Genomic Southern blot hybridization. Genomic DNA was cleaved with restriction enzymes (Gibco-BRL) according to the manufacturer's recommendations. Southern blots were prepared by transferring the DNA fragments onto Hybond N⁺ membranes (Amersham) by the capillary transfer method using 0.4 M NaOH (Southern, 1975). Hybridization was carried out with ³²P-end-labelled oligonucleotide probes or nick-translated DNA fragments labelled with [³²P]dCTP at 65 °C or 72 °C, respectively. Filters were washed twice in 6 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.2) and 0.5% SDS, once in 0.5 × SSC and 0.5% SDS at hybridization temperature and subsequently autoradiographed for 6–60 h.

Nucleotide sequence accession numbers. The EMBL accession numbers of the 16S rDNAs determined in this study are as follows: *Actinoplanes utahensis* ATCC 31044, X80823; *Amycolatopsis orientalis* subsp. *lurida* DSM 43187, X81573; *Streptomyces bikiniensis* DSM 40581, X79851; *Streptomyces bluensis* ISP 5564, X79324; *Streptomyces caelestis* NRRL 2418, X80824; '*Streptomyces espinosus*', NRRL 3890, X81574; '*Streptomyces espinosus*' NRRL 5729, X80826; *Streptomyces galbus* DSM 40089, X79852; *Streptomyces galbus* DSM 40480, X79325; *Streptomyces glaucescens* DSM 40716, X79322; *Streptomyces hygroscopicus* subsp. *glebosus* DSM 40823, X86067; *Streptomyces hygroscopicus* subsp. *limoneus* ATCC 21431, X79853; *Streptomyces lincolnensis* NRRL 2936, X79854; '*Streptomyces ornatus*' DSM 40307, X79326; *Streptomyces pseudogriseolus* NRRL 3985, X80827; *Streptomyces*

Fig. 1. For legend see facing page.

subtilis DSM 40445, X80825, '*Streptovercillium mashuensis*' DSM 40221, X79323.

RESULTS AND DISCUSSION

Cloning of the 16S rRNA genes

The 16S rDNA was amplified as described in Methods. In general, only one amplification product was obtained in PCR assays using the primers pA and pH* (Table 2). When additional amplification products were obtained, 16S rRNA genes were initially identified by their fragment lengths, as deduced from other published 16S rDNAs and estimated to be approximately 1530 bp. Furthermore, the patterns of restriction fragment lengths obtained following digestion with *Eco*RI, *Kpn*I and *Hinc*II were conserved in all strains, thus permitting further identification. The 1.5 kb PCR fragments were cloned in pUC18

for further analysis. Subclones were generated by isolation of the *Kpn*I/*Hinc*II fragments.

Sequence analysis

The sequences of the 16S rRNA genes analysed in this study contained approximately 56–59 mol% G+C, whereas the G+C content of total genomic DNA of actinomycetes was estimated to be 72–75 mol% (Enquist & Bradley, 1971). The lengths of the PCR-amplified rRNA genes varied between 1518 and 1523 bp; no differences were detected between the sequences of the three independent clones of each strain. The sequences of the streptomycete strains investigated in this study are highly related (over 95% identical bases) when compared to each other and to previously published 16S rRNA sequences of other streptomycetes (EMBL Data Library;

Fig. 1. Sequence comparison of the 16S rDNA from representative strains: a, *S. ambofaciens* (Pernodet *et al.*, 1989); b, *S. griseus* (Kim *et al.*, 1993); c, *S. bluensis*; d, *S. bikiniensis*; e, *S. caelestis*; f, *S. galbus* DSM 40480; g, *S. glaucescens*; h, *S. lincolnensis*; i, '*Stv. mashuensis*'; j, *Amy. orientalis* subsp. *lurida*; k, *Act. utahensis*; l, *Frankia* sp. (EMBL M88466); m, *Mycobacterium bovis* (EMBL X55589); n, *Clostridium butyricum* (Hutson *et al.*, 1993); o, *Bacillus subtilis* (Ash *et al.*, 1991), p, *E. coli* (Carbon *et al.*, 1979). All sequences are compared to *S. ambofaciens*. Identical bases are indicated by dots; basepair exchanges are represented by the letters (N denotes an ambiguous nucleotide); dashes denote sequence gaps. Nucleotide positions are noted by the numbers in parentheses. Numbers above the sequences represent covariance patterns (see text). Regions from which specific primers were derived are overlined and labelled according to Table 2. The position of the genus-specific nucleotide according to Stackebrandt *et al.* (1991) and the corresponding basepair (see text) are indicated by * and +, respectively.

Fig. 1). The sequences of the 16S rDNA of *S. griseus* subsp. *griseus* (Kim *et al.*, 1993) and of '*S. ornatus*' were identical in the α -region and γ -region. These sequences otherwise differed only in two nucleotide positions in which bases were missing in the corresponding location in *S. griseus*. Both strains were classified as members of group A 1B in the cluster analysis of Williams *et al.* (1983), and as members of the subcluster 1-3, *S. griseus*, as determined by numerical classification by Kämpfer *et al.* (1991). These data indicate that these strains are very closely related and should possibly be classified as the same species. However, as shown below, the two strains have somewhat different restriction fragment length patterns of the rRNA operons, implying that they are distinct strains. Sequence comparison also revealed a high degree of similarity between the 16S sequences of *Amy. orientalis* subsp. *lurida* and those of the streptomycetes studied, giving evidence that this strain may actually be a non-sporeforming strain belonging to the genus *Streptomyces*. The sequence of the 16S rDNA of *S. lincolnensis* NRRL 2936 perfectly matched that of its derivative, the industrial strain 78-11, proving that they were still the same clonal cell line.

The majority of basepair exchanges among the actinomycetes are located in the variable γ -region between nt positions 150 and 200 (according to the nomenclature of the *S. ambofaciens*, Pernodet *et al.*, 1989) and the variable α -

regions between nt positions 982 and 998 (Fig. 1; not all data shown). The β -region (position 1102–1120) showed much less variability among the various actinomycetes analysed. However, the comparison of the β -region with the 16S rDNA sequences of other bacteria [e.g. *Frankia* sp., *E. coli*, *B. subtilis*, *M. bovis*, cf. Fig. 1 (not all data shown)] revealed an insertion of 5 to 6 nt common to all the streptomycete sequences and the sequence of *Amy. orientalis* subsp. *lurida*. No comparable sequence was found in other 16S rDNA sequences available in the EMBL Data Library. The alignment of the 16S rDNA sequences from the actinomycetes investigated also revealed a deletion of 20 nt around nt position 450 when compared to *E. coli* and *B. subtilis*. This deletion in *S. ambofaciens* was noted by Pernodet *et al.* (1989) and can also be found in members of other actinomycete and related genera, such as *Frankia* sp., *M. bovis*, *Arthrobacter simplex*, *Dermatophilus congolensis* and *Kibdellosporangium aridum* (Fig. 1; not all data shown). A further deletion of varying length is found within the region of nt 70–90 in most Gram-positive bacteria. Another region with prominent features is located around nt position 800 containing the sequence 5'-ACATTCACGTCGTCG-3', which is conserved only in the *Streptomyces* strains but not in the representatives of closely related taxa of actinomycetes or other bacteria (Fig. 1, not all data shown). When compared to the

Streptomyces strains, the sequences of the corresponding regions of *Act. utabensis* and *Frankia* are identical and contain six basepair exchanges. These data are in good accordance with the highest degree of sequence similarity having been found between the complete *Act. utabensis* 16S rDNA and the *Frankia* sequences deposited in the EMBL DataBank. All streptomycete strains examined and *Amy. orientalis* subsp. *lurida* exhibited the genus-specific C at nt position 929 found in the four previously published strains as inferred by Stackebrandt *et al.* (1991). The comparison of complete 16S rDNA sequences revealed an additional potential genus-specific nucleotide signature located around the G at nt position 1195, which corresponds to the genus-specific C and part of its flanking regions within the secondary structure of the 16S rRNA (Fig. 1). These nucleotides are located at the base of a stem structure in the mature 16S rRNA, according to the 16S secondary structure model of *S. ambofaciens* as proposed by Pernodet *et al.* (1989).

Comparative analysis also revealed 16 sites exhibiting compositional covariance. The covarying basepairs are found within stem structures, probably constituting relatively neutral changes in the secondary structure, with the exception of nt position 791, which is found within a loop structure. Certain patterns propagated by covariance are conserved within particular strains of streptomycetes. The patterns consist of exchanges in the following corresponding nt positions (see Fig. 1): pattern (1), 129 and 216; pattern (2), 630 and 719; 631 and 718; 796 and 850; 806 and 826; 915 and 1322; 1093 and 1132; pattern (3), 250 and 260; 973 and 1014; single nt exchange at position 791; pattern (4) 810 and 822. These patterns were strictly conserved in the streptomycetes studied but not in '*Stv. mashuense*' or *Amy. orientalis*. Therefore, it is conceivable that these patterns may be used as a phylogenetic tool, permitting the cataloguing of strains to lower taxonomic levels. Since 10–30% of 16S rDNA sequences generated by PCR of mixed populations may be recombinant products (Liesack *et al.*, 1991), these patterns may also be helpful in detecting the chimeric nature of amplification products in environmental studies. It is interesting to note that no nucleotide transitions or transversions are present in the highly conserved regions around nt positions 516 and 905 of the streptomycin-producing strains, in which base exchanges in the 16S rRNA conveying streptomycin resistance have been observed in other bacteria (Cundliffe, 1990), suggesting that the streptomycin resistance is conferred by other mechanisms, e.g. aminoglycoside kinases (Retzlaff *et al.*, 1993), rather than changes in the 16S rRNA.

Streptomyces-specific primers

The 16S rDNA sequences of the actinomycetes investigated were compared to various other bacterial 16S rDNAs. The existence of regions exhibiting possible sequence signatures at genus, species and strain levels of the streptomycetes allowed the design of primers for use in identification of these taxonomic ranks in PCR assays (Table 2; Fig. 1). PCR primers derived from the sequences from four different regions of the 16S rDNA were chosen

for the preliminary study of the following regions. (1) The region located around nt position 1100 containing the insertion of 5 or 6 nt typical of the streptomycete 16S rDNA. This region was used for the construction of the primers AM44 and AM45. Both primers cover the same region but in opposite orientation for use in various PCR assays. (2) The region around position 120 preceding the variable γ -region. This region is also conserved in all the streptomycetes investigated but not in other published 16S rDNAs. The primers AM47 and AM48 were derived from these sequences. (3) The region around nt position 800. The primers AM51 and AM52 were constructed using the sequences containing the streptomycete-specific signature found in this region. AM52 consists of the sequence complementary to AM51. (4) An area of moderate variability located in the region of nt position 640 used to create primer AM33. Unambiguous results were obtained when using the following annealing temperatures: AM42/AM44, 45 °C; AM45/pA, 51 °C; AM45/AM33, 43 °C; AM45/47, 62 °C; AM45/AM48, 62 °C; AM47/pH*, 61 °C; AM48/pH*, 61 °C. Annealing temperatures were chosen according to the highest stringency possible for each combination.

In order to test the general applicability of the primers, PCR experiments were performed on a diverse array of type and non-type strains permitting the specific detection and amplification of streptomycete DNA in each of the PCR assays. A band in addition to the expected amplicon was obtained only when using a combination of primers AM45 and pA or AM33, along with DNA from *S. glaucescens* (Fig. 2). No amplification products, or only products of differing size, were obtained in control experiments with DNAs from all other bacterial strains listed in Table 1 (Fig. 2).

The sequences of the primers were compared to all DNA sequences available in the EMBL data library using the FASTA program. The highest scores were found in comparisons between 16S rDNAs of the streptomycetes, corresponding to 100% sequence similarity. Somewhat lower homologies, with one to three mismatches, were found when comparing sequences of related actinomycetes (e.g. *Mycobacterium* spp., *Frankia* spp., *Nocardia* spp.), while three or more mismatches were found when comparing the primers with DNAs from other bacteria. As each mismatch results in a reduction of 5–7 °C in the annealing temperature of the primer and the mismatches were not found in the 5'-region of the primer, no false positive amplification products should be generated under stringent amplification conditions using template DNA from non-streptomycete organisms.

The primer combination AM42/AM44 permitted amplification of the spacer regions between the 16S and 23S genes. The amplification products obtained were of similar size to that of *S. lividans*, indicating that no tRNA genes are interspersed in the amplifiable 16S–23S spacer regions. Furthermore, the sequence analysis of the 3'-end of the 23S rRNA gene and the 5'-end of the 5S rRNA gene of various actinomycetes revealed that no tRNA genes are located in the intergenic region (Mehling *et al.*, 1995).

Fig. 2. Agarose gel electrophoresis of representative PCR products using different combinations of primers. Primers used for amplification are noted in parentheses and correspond to those listed in Table 2. The PCR conditions are described in the text. Lanes 1 and 21, molecular size markers, λ DNA cleaved with *EcoRI* and *HindIII*. Lanes 2–7 (AM45/48): 2, '*Br. flavum*'; 3, *S. bluensis*; 4, *S. lincolnensis* 78-11; 5, *S. glaucescens*; 6, *S. galbus* DSM 40480; 7, *S. griseus* N2-3-11. Lanes 8–13 (AM45/47): 8, '*Br. flavum*'; 9, *S. bluensis*; 10, *S. lincolnensis* 78-11; 11, *S. glaucescens*; 12, *S. galbus* DSM 40480; 13, *S. griseus* N2-3-11. Lanes 14–20 (AM45/pA): 14, '*Br. flavum*'; 15, *S. bluensis*; 16, *S. lincolnensis* 78-11; 17, *S. glaucescens*; 18, *S. galbus* DSM 40480; 19, *S. griseus* N2-3-11; 20, *B. subtilis*. Lanes 22–28 (AM45/AM33): 22, '*Br. flavum*'; 23, *S. bluensis*; 24, *S. lincolnensis* 78-11; 25, *S. glaucescens*; 26, *S. galbus* DSM 40480; 27, *S. griseus* N2-3-11; 28, *B. subtilis*. Lanes 29–34 (AM44/AM42): 29, '*Br. flavum*'; 30, *S. bluensis*; 31, *S. lincolnensis* 78-11; 32, *S. glaucescens*; 33, *S. galbus* DSM 40480; 34, *S. griseus* N2-3-11. Lanes 35–40 (pA/pH*): 35, *S. bluensis*; 36, *S. lincolnensis* 78-11; 37, *S. glaucescens*; 38, *S. galbus* DSM 40480; 39, *S. griseus* N2-3-11; 40, *Act. utahensis*.

These results indicate that the amplified streptomycete ribosomal rRNA gene cluster of all actinomycetes investigated is arranged in the order 16S, 23S and 5S rRNA. These conclusions are in good accordance with the pertinent experimental data of the three streptomycete rDNA operons published by other authors (Baylis & Bibb, 1987; Kim *et al.*, 1993; Pernodet *et al.*, 1989).

Strain-specific probes

The possibility of detecting a defined strain was investigated by using a strain-specific probe derived from the nucleotide sequence of the highly variable γ -region of *S. galbus* DSM 40480 (Fig. 1). The strain-specific primer AM46 was tested in various PCR assays. Multiplex PCR assays were performed on mixtures of DNA, including those from streptomycetes and other bacteria, using the strain-specific (AM46) and the genus-specific (AM45/AM47) primers, in addition to the PCR profiles for genus-specific amplification. Selective amplification of both sequences was observed only when DNA of *S. galbus* was added, although the agarose gel only weakly resolved the

difference of approximately 60 nt in fragment length (data not shown). Unambiguous results were obtained when the mixture of the genus-specific PCR products was used as a template for the strain-specific amplification. In this case, only one distinct band of the proper size was generated (data not shown).

Identification of the numbers of the 16S rRNA operons and comparison of the restriction fragment patterns

Several dispersed copies of the rRNA operon exist in many bacterial genomes, leading to diverse restriction fragment length polymorphisms (RFLPs). The four streptomycetes so far studied possess varying numbers of rRNA gene clusters: six sets are found in *S. lividans* TK21 (Suzuki *et al.*, 1988), *S. griseus* subsp. *griseus* (Kim *et al.*, 1993), and *S. coelicolor* (Baylis & Bibb, 1987), while only four are found in *S. ambofaciens* (Pernodet *et al.*, 1989). In this study, rDNA restriction patterns from 23 actinomycetes were compared (Fig. 3). Genomic DNAs hydrolysed with *Bam*HI and *Nco*I, which do not have cleavage sites within the sequenced 16S rDNAs, were immobilized on a nylon membrane and subjected to Southern hybridization. Two different probes were used for these experiments: the primer pA (Table 2) which had previously been used for the amplification of the 16S rRNA genes; and a probe consisting of the internal *Kpn*I/*Hinc*II fragment of the 16S rRNA gene of *S. galbus* DSM 40480. Both probes generated identical restriction fragment patterns, each belonging to a specific rRNA operon. In general, these probes yielded six distinct signals in the tested actinomycetes, with the exception of *S. subbrutillus* (seven signals). These results suggest that most strains contain six ribosomal operons, which is in good accordance with the data reported in the references mentioned above.

Differentiation of the strains examined was possible due to the uniqueness of the RFLP patterns created (Fig. 3), with the exceptions of the related *S. lincolnensis* strains NRRL 5321, NRRL 2936 and its derivative, the lincomycin-overproducing strain 78-11, and the two *S. griseus* strains DSM 40236 and N2-3-11, which are derived from different natural isolates of streptomycin-producing *S. griseus* strains. The strains within these two groups displayed identical patterns. Different patterns were observed when the DNAs of *S. griseus* N2-3-11 and *S. ornatus* were hybridized with the ribosomal probes although the nucleotide sequences of the 16S rRNA genes were almost identical (see **Sequence Analysis** above). *S. griseus* shared five common ribosomal bands with '*S. ornatus*' while the sixth band differed between the two strains. This may be due to an additional restriction site in the vicinity of one of the 16S rRNA genes, as the recognition sites of the enzymes used are not found within the sequenced 16S rDNA. Variations in rRNA gene RFLPs of *Streptomyces* species have already been shown to be the basis for a rapid method for identifying *Streptomyces* strains (Clarke *et al.*, 1993). Although our results allow no conclusions as to the taxonomic status of the strains used, they do show the potential of RFLP analysis as an additional method of verifying the strains used in a laboratory.

Fig. 3. Schematic representation of RFLPs. Total genomic DNA was cleaved with *Nco*I and *Bam*HI and subjected to Southern hybridization with the *Kpn*I/*Hinc*II fragment of the 16S rDNA of *S. galbus* DSM 40480. Lanes: λ , λ DNA cleaved with *Eco*RI/*Hind*III (the numbers indicate the fragment size in bp); 1, *S. lincolnensis* 78-11; 2, *S. lincolnensis* NRRL 2936; 3, *S. lincolnensis* NRRL 5321; 4, *S. pseudogriseolus* (DNA prepared by the CTAB method, see Methods); 5, *S. pseudogriseolus* (DNA prepared by the modified maize method, see Methods); 6, '*S. espinosus*' NRRL 5729; 7, '*S. espinosus*' NRRL 3890; 8, *S. caelestis*; 9, *S. lividans* TK23; 10, *Act. utahensis*; 11, *S. galbus* DSM 40089; 12, *S. galbus* DSM 40480; 13, *S. griseus* DSM 40236; 14, *S. griseus* N2-3-11; 15, *S. bikiniensis*; 16, '*S. ornatus*'; 17, '*Stv. mashuense*'; 18, '*Stv. griseocarneum*'; 19, *Amy. orientalis* subsp. *lurida*; 20, *S. subrutillus*; 21, *S. glaucescens*; 22, *S. hygroscopicus* subsp. *glebosus*; 23, *S. bluensis*.

Conclusions

(1) The determination of full-length 16S rDNA sequences, as opposed to partial gene sequences, of streptomycete and some other actinomycete strains has provided data which may be useful in elucidating taxonomic levels or detecting chimeric PCR-products.

(2) The design of PCR primers with potential for the differentiation of strains at the genus, species and strain levels was made possible by sequence analysis of the complete 16S rDNA sequences. The possible combinations of genus- and strain-specific primers permit diverse assays, such as multiplex PCR or PCR with nested primers, lessening the likelihood of false-positive identification of streptomycetes and thus increasing the fidelity of the assay. Furthermore, genus-specific amplification, e.g. with the primers AM45 and AM47, and subsequent sequencing of the variable α - or γ -region would allow the identification of certain strains.

(3) A by-product of this investigation was the discovery that the distribution of antibiotic production patterns among the known streptomycin- and lincomycin-producing strains was not correlated with the taxonomic status of the producer (data not shown). This indicates that phylogenetic clustering of certain strains does not reflect antibiotic production and can therefore not be relied upon to assist in selecting potential antibiotic producers. However, taxonomically distant streptomycin- and lincomycin-producing strains can exhibit a high degree of similarity in the organization and sequences of functionally similar units within their respective gene clusters (Retzlaff *et al.*, 1993; Zhang *et al.*, 1992; G. Mayer, A. Mehling & W. Piepersberg, unpublished).

(4) The set of strains investigated represents strains with particular traits, such as antibiotic production or useful

properties as hosts for recombinant DNA. Their detection in environmental samples may now be possible by employing PCR technology and diagnostic PCR primers.

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