Discrimination of *Streptomyces albidojlavus* Strains Based on the Size and Number of 16S-23S Ribosomal DNA Intergenic Spacers

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In an attempt to develop a rapid and accurate method for discrimination of streptomycetes at the strain level, 21 strains identified by fatty acid analysis as *Streptomyces albidojlavus* and the type strains of nine subjective synonyms of *S. albidojlavus* were selected for a full or partial 16S ribosomal DNA (rDNA) sequence analysis and an investigation of the 16S-23S rDNA intergenic spacer region. 16S rDNA sequence analysis showed that 27 of the strains exhibited 100% sequence similarity; these strains included the type strain of *S. albidojlavus* and the type strains of the subjective synonyms *Streptomyces canescens*, *Streptomyces coelicolor*, *Streptomyces fulvissimus*, *Streptomyces limosus*, *Streptomyces odorifer*, and *Streptomyces spongiosus*. The type strains of the other subjective synonyms of *S. albidojlavus* (i.e., *Streptomyces gauereottii*, *Streptomyces intermedius*, and *Streptomyces rutgersensis*) were found to have levels of 16S rDNA sequence difference of 1.0 to 1.1% when they were compared to the type strain of *S. albidojlavus*. In order to discriminate between the strains which had identical 16S rDNA sequences, the 16S-23S rDNA intergenic spacer regions were amplified and cloned, and the sequences of the spacer regions were determined for four *S. albidojlavus* strains, including the type strain. The 16S-23S rDNA intergenic spacer region was found to vary in length and sequence composition among the strains and within each strain. The sizes and numbers of 16S-23S rDNA intergenic spacer regions for the 27 strains with identical 16S rDNA sequences were determined by high-resolution electrophoresis of FAM-labeled PCR products and a subsequent size analysis with GeneScan 672 software. This was shown to be a useful method for discrimination of *S. albidojlavus* strains. Strains with the same 16S-23S rDNA intergenic spacer band pattern, as determined by high-resolution electrophoresis of FAM-labeled PCR products, could be further discriminated on the basis of the sequence composition of the spacer region.

With 464 validly described species and 45 subspecies (6), the genus *Streptomyces* currently contains the largest number of species of any genus in the domain Bacteria. The ability of the streptomycetes to produce secondary metabolites, including antibiotics, makes them organisms of great commercial importance and the subject of extensive study by the pharmaceutical industry. Over the years, numerous methods have been applied with the aim of identifying, characterizing, and discriminating between streptomycete strains. The primary aim of such investigations has been to develop the most rapid and accurate methods that have been utilized include numerical analysis of phenotypic characteristics (12, 30), fatty acid profiling (25), phage susceptibility studies (14), ribosomal protein sequence analysis (22), restriction fragment length polymorphism analysis (2, 4), DNA-DNA hybridization studies (16-19), and 16S ribosomal DNA (rDNA) sequence analysis (13, 21, 26, 27). Each of these methods has been successful to varying degrees, depending on the level of discrimination required. From the numerical taxonomy analyses of phenotypic characteristics (12, 30), it is clear that the yellow streptomycetes fall into two main clusters, one containing *Streptomyces albidojlavus* and related species and the other comprising *Streptomyces griseus*, *Streptomyces albidofluvus*, and related taxa. Such clusters provide ample species and strains for comparative studies. The data made available through such studies allow the selection of representative species groups for study at the strain level.

In the majority of bacterial taxa investigated, the genes coding for components of the rRNA are arranged in the order 16S-23S-5S, as is found in *Escherichia coli* (3). The rRNA operon is often found in multiple copies in the bacterial chromosome, and the number of copies ranges from 1 to 14 (31). These genes are usually linked and separated by spacer regions which may vary in length and sequence composition. The length and sequence polymorphisms in such spacers within the rRNA operon have been shown to be useful in discriminating between different bacterial species (1, 8-11) and also in the identification of fungal species (7, 28). Jensen et al. (10) demonstrated the usefulness of this method for distinguishing certain taxa at the species level by determining size differences in PCR-amplified 16S-23S rDNA spacer fragments.

Here we report the usefulness of 16S-23S rDNA intergenic spacer analysis for discrimination of streptomycete strains when the species *S. albidojlavus* was used as an example and demonstrate the use of high-resolution polyacrylamide gel electrophoresis of dye-labeled PCR products as a rapid method for determining spacer size and number.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** The *Streptomyces* strains used in this study are shown in Table 1. All of the strains were cultivated on glucose-yeast extract-malt extract agar containing (per liter) 4 g of yeast extract, 10 g of malt extract, 4 g of r-glucose, 2 g of CaCO₃, and 15 g of agar. Plates were incubated aerobically at 28°C.

**DNA extraction.** Genomic DNA was isolated and purified as described by Rainey et al. (24).

**PCR amplification of the 16S rDNA.** Amplification of the 16S rDNA was performed as described previously (24) by using Taq polymerase (Boehringer, Mannheim, Germany) and the following primers: 5′ GAGTTTGATCCTGCT CAG 3′ (positions 9 to 27, *Escherichia coli* numbering [3]) and 5′ AGAAAGG AGGTTACGGCC 3′ (positions 1525 to 1545). A total of 28 cycles of amplification were performed with a model 480 DNA thermal cycler (Perkin-Elmer Cetus, Foster City, Calif.) by using the following thermal profile: 52°C for 1 min, 72°C for 2 min, and 95°C for 1 min. An additional final extension step consisting of 72°C for 5 min was performed. The PCR products were purified by extraction with chloroform; this was followed by final purification with a Prep-A-Gene DNA purification kit (Bio-Rad, Hercules, Calif.), used according to the manufacturer’s instructions. Elution of DNA was done with 50 μl of water.

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The PCR products were diluted 1:80 with sterile water, and 1.2 μl of each dilution was added to 1.8 μl of a ROX-labeled internal lane standard (GENESCAN-200 ROX, Applied Biosystems) premixture, which was prepared as recommended by the manufacturer. The 3×1-lane samples were deionized for 2 min at 98°C and placed on ice. The samples were electrophoresed on a 6% (wt/vol) polyacrylamide gel (length, 12 cm) containing 8 M urea and 1× Tris-borate-EDTA by using an Applied Biosystems 377A DNA sequencer under the following conditions: 2.500 V, 45 mA, and 30 W for 4 h. Fragment sizes were determined by using GENESCAN 672 software (Applied Biosystems) by comparison with the ROX-labeled internal lane standard (GENESCAN-200 ROX, Applied Biosystems).

Nucleotide sequence accession numbers. The complete 16S rDNA sequences determined in this study have been deposited in EMBL database under accession numbers Z67687 to Z67688 (Table 1). The partial 16S rDNA sequences determined in this study have been deposited in EMBL database under accession numbers Z67689 to Z67705. The 16S-23S rDNA intergenic spacer region sequences of S. albidoflavus DSM 40455, (T = type strain) and DSM 40792 have been deposited in EMBL database under accession numbers Z77332 to Z77351.

RESULTS AND DISCUSSION

16S rDNA sequence comparison. Strains of S. albidoflavus, a polyene antifungal antibiotic producer (23), were chosen for comparison in this study. Thirty Streptomyces strains were selected for analysis. These included the four S. albidoflavus strains listed in the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH Catalogue of Strains (5), members of nine species considered to be subjective synonyms of S. albidoflavus (29), and 18 strains identified as S. albidoflavus strains on the basis of fatty acid profile analysis (15) although they were identified as members of other species by the original isolaters or depositors (5).

Partial 16S rDNA sequence data covering the most variable regions in streptomycetes, the γ, α, and β regions (26), were obtained for all 32 strains. In the two subsequences (positions 22 to 499 and 970 to 1249, E. coli numbering [1]), which comprised 870 nucleotide positions, there was 100% 16S rDNA sequence similarity among 27 of the 32 strains, including the type strain of S. albidoflavus, strain DSM 40455. Full 16S rDNA sequence data for 1,474 to 1,476 nucleotide positions were determined for the four S. albidoflavus strains (DSM 40455, DSMS 40792, DSM 40880, and DSM 46452) and the nine subjective synonyms. A comparison of the sequences showed that 10 of these 13 strains, including six of the nine subjective synonyms, were identical both in the most variable regions (γ, α, and β regions) and in the remaining conserved regions for the 1,474 nucleotide positions determined. Of the three of the subjective synonyms, Streptomyces intermedius DSM 40372, Streptomyces gourieoti DSM 40324, and Streptomyces rutgersensis DSM 40077, exhibited 1.0 to 1.1% sequence differences over the 1,474 nucleotide positions compared to the type strain of S. albidoflavus, strain DSM 40455. These data support the findings of Williams et al. (29), who reported that Streptomyces canescens, Streptomyces coelicolor, Streptomyces felleus, Streptomyces limosus, Streptomyces odorus, and Streptomyces sampsonii are subjective synonyms of S. albidoflavus. However, the levels of 16S rDNA sequence difference between three of the strains, S. intermedius DSM 40372, S. gourieoti DSM 40324, and S. rutgersensis DSM 40077, and type strain DSM 40455 of S. albidoflavus (1.0 to 1.1%) cast doubt on the status of these species as subjective synonyms of S. albidoflavus; recent DNA-DNA hybridization data for these species and S. albidoflavus DSM 40455 confirm this finding (17). S. intermedius DSM 40372, S. gourieoti DSM 40324, and S. rutgersensis DSM 40077 show between 99.8 and 100% 16S rDNA sequence similarity to each other. As more 16S rDNA sequence data become available for additional species of the genus Streptomyces, the true species identity of these strains should become clear. Three of these species were not investigated further in this study.
16S-23S rDNA intergenic spacer region sequence comparison. Direct sequencing of the PCR products of the 16S-23S rDNA intergenic spacer regions of four *S. albidojavanus* strains (DSM 40455\textsuperscript{T}, DSM 40792, DSM 40880, and DSM 46452) yielded unreadable mixed sequence data, indicating that heterogeneous sequences were present in a single PCR product. In order to determine the extent of sequence heterogeneity, the PCR products were cloned prior to sequencing. A comparison of the 16S-23S rDNA intergenic spacer region clone sequences showed that they vary in number, size, and composition within and between the four strains.

The extent of sequence length heterogeneity between and within the four *S. albidojavanus* strains investigated suggested that these strains could be discriminated on the basis of spacer length and/or sequence composition. The application of this observation to discrimination of streptomycete strains required the development of a rapid and accurate method for determining the number and size of the 16S-23S rDNA intergenic spacer fragments. PCR fragments which differ in length by <10 bp cannot be readily resolved by normal agarose gel electrophoresis. Cloning and sequencing of 16S-23S rDNA spacer regions on a routine basis would be both costly and time-consuming. High-resolution polyacrylamide gel electrophoresis as used by Jensen et al. (10) provides a method to separate such fragments. In this study we used high-resolution polyacrylamide gel electrophoresis of dye-labeled spacer fragments and GENESCAN 672 software for rapid and accurate determination of fragment size and number.

16S-23S rDNA intergenic spacer region fragment size analysis. Dye-labeled amplification products of the 16S-23S rDNA intergenic spacer region for the 27 strains shown to have identical 16S rDNA sequences. Electrophoresis and fragment size analysis of these products revealed extensive variability in the number and size of spacer regions and resulted in 19 distinct band patterns (Fig. 1). The number of bands ranged from two to five, and the band sizes were between 347 and 363 bp (including the length of amplification primers). The GENESCAN 672 software calculates fragment size to two decimal places. However, in this study we approximated the values to the nearest whole number. The process of amplification, electrophoresis, and size calculation was repeated three times for each strain. The greatest variation found between replicates was 0.5 bp. The method can therefore be considered highly reproducible, and the patterns obtained in different experiments are comparable. When this approach was used, strains which have been found to have identical partial or complete 16S rDNA sequences could in many cases be discriminated at a lower level on the basis of the band pattern.

Of the 19 different band patterns, 4 were exhibited by more than one strain (Fig. 1). The presence of identical band patterns in these strain groups may indicate that the strains are identical. However, a sequence analysis of the cloned spacer regions indicated that a band of a particular size may represent more than one sequence with the same number of nucleotides but different sequence compositions. This was demonstrated for strains DSM 40455\textsuperscript{T} and DSM 40792, which had identical band patterns but differed in sequence type within each strain. Ten clones of each strain were randomly chosen, and the inserts were sequenced. The sequences of the 16S-23S rDNA intergenic spacers were between 311 and 322 nucleotides long and contained six highly conserved regions and five variable regions (Fig. 2 and Table 2). Highly conserved regions c1 to c6 were 15, 68, 20, 31, 19, and 49 nucleotides long, respectively. For each of the five variable regions, regions v1 to v5, more than one sequence composition was found. In the case of variable regions v1 (19 to 28 nucleotides long) and v5 (29 to 32 nucleotides long) three different sequence compositions were determined. Variable regions v2 and v3 were 21 and 27 nucleotides long, respectively, while v4 was either 11 or 12 nucleotides long. In addition, more than one sequence type consisting of combinations of different variable regions was found within each strain. From the sequence data for 10 clones of *S. albidojavanus* DSM 40455\textsuperscript{T}, four different spacer sequences were identified, while eight different sequences were found among ten clones of *S. albidojavanus* DSM 40792. In all, 10 different types were found within the two strains. Two sequence types were found in both strains, while two sequence types were found to be unique to strain DSM 40455\textsuperscript{T} and six were unique to strain DSM 40792.

Conclusions. 16S rDNA sequence analysis, which was previously shown to be useful for differentiating *Streptomyces* species, was found to be of no value for discriminating *S. albidojavanus* strains. This study demonstrates the usefulness of the 16S-23S rDNA intergenic spacer region for discrimination at the strain level. By using the combination of dye-labeled PCR-generated 16S-23S rDNA intergenic spacer fragments and the accurate size determination facility of the GENESCAN 672 software, it is possible to produce strain-specific band patterns even when fragments differ in size by only a few base pairs. From the 16S-23S rDNA intergenic spacer sequence data obtained for two *S. albidojavanus* strains as described above, and given that strain-specific sequences were observed, it should be possible to design strain-specific oligonucleotide probes or PCR primers which could be used both for identification and for environmentally oriented studies. The high reproducibility...
and high throughput potential of the 16S-23S rDNA intergenic spacer fragment size analysis method has far-reaching implications for the establishment of a database for strains of various *Streptomyces* species. The method may also have applications within other actinomycete groups or across the broad bacterial spectrum.

**REFERENCES**


