Heterogeneity of Ribosomal Proteins among *Streptomyces* Species and its Application to Identification

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The ribosomal proteins from 11 *Streptomyces* strains representing various numerical taxonomic clusters were compared by two-dimensional PAGE. The protein patterns were specific for each species and were unaffected by acridine dye treatment, suggesting genetic stability of ribosomal proteins. An attempt was made to identify one strain of *Streptomyces* by both traditional taxonomic methods and analysis of the ribosomal protein patterns. Both methods identified the strain as *Streptomyces lavendulae*, and protein pattern analysis also showed that *Streptomyces avidinii* was closely related to this species. The practical application of ribosomal protein patterns in *Streptomyces* taxonomy was therefore demonstrated.

INTRODUCTION

Although ribosomal proteins and RNA molecules have been studied in great detail in *Escherichia coli* (Wittmann, 1976; Kurland, 1977; Nomura *et al.*, 1984; Lindahl & Zengel, 1986) and to a lesser extent in the genus *Bacillus* (Geisser *et al.*, 1973*b*; Wienen *et al.*, 1979), little is known about the detailed structure of ribosomes from other bacterial species. The two-dimensional gel electrophoresis technique developed by Kaltschmidt & Wittmann (1970) has been especially useful for comparison of the ribosomal proteins from different species. Geisser *et al.* (1973*a*) found that the ribosomal proteins from eight species in the Enterobacteriaceae were very similar in their electrophoretic properties, and that the ribosomal proteins of several *Bacillus* species were also similar to one another (Geisser *et al.*, 1973*b*). The use of two-dimensional separation of ribosomal proteins for identification and classification purposes has been further extended to other groups of organisms such as Gram-negative bacteria (Böck, 1985), fungi (Goff & Begueret, 1984) and mammals (Madjar & Traut, 1980). The method was especially useful in the analysis of methanogenic bacteria for which classical identification criteria are not readily available (Böck, 1985). One-dimensional gel electrophoresis was also successfully applied to correlate the relatedness of different yeast species suggested by ribosomal protein patterns with that based on DNA–DNA homology (Adoutte-Panvier *et al.*, 1980). Despite similarity of ribosomal protein patterns within related genera or species, studies on *relC* mutants of several *Streptomyces* species revealed considerable electrophoretic variability in the ribosomal protein patterns (unpublished data). This paper describes the results of a more systematic study of the variability of streptomycete ribosomal proteins, and discusses the applicability of such variability to the taxonomy of *Streptomyces*.

METHODS

Strains. The strains used are listed in Table 1. *Streptomyces* strain MA406-A-1 is a prototrophic wild-type strain which produces formycin (Ochi, 1986). Curing of formycin production in strain MA406-A-1 was performed by growing the cells with acriflavin as described previously (Ochi, 1986).

Abbreviations: MBA, *N,N'*-methylene-bis-acrylamide; TEMED, *N,N,N',N'*-tetramethylethylenediamine.

Table 1. *Streptomyces* strains used in this study

Species	Cluster group	Cluster no.*	Strain no.†	Antibiotic produced	Source‡
<i>Streptomyces coelicolor</i>	A	1A	A3(2)	Methylenomycin	JII
<i>Streptomyces griseus</i>	A	1B	IFO 13189	Streptomycin	IFO
<i>Streptomyces venezuelae</i>	A	6	ISP 5230	Chloramphenicol	IFO
<i>Streptomyces violaceus</i>	A	6	ISP 5082	Mycetin	IFO
<i>Streptomyces parvulus</i>	A	12	ISP 5048	Actinomycin D	IFO
<i>Streptomyces antibioticus</i>	A	31	ATCC 14888	Actinomycins	ATCC
<i>Streptomyces hygroscopicus</i>	A	32	ISP 5578	Hygromycin	IFO
<i>Streptomyces griseoflavus</i>	A	37	FERM 1805	Bicozamycin	FERM
<i>Streptomyces avidinii</i>	F	56	ISP 5526	Streptavidin	IFO
<i>Streptomyces lavendulae</i>	F	61	ISP 5069	Streptothricin	IFO
<i>Streptomyces griseolavendus</i> (<i>Streptomyces lavendulae</i> subsp. <i>grasseri</i>)	F	61	ISP 5385	Grasseriomycin	IFO
<i>Streptomyces</i> sp.	NA	NA	MA406-A-1	Formycin	IMC

NA, Not applicable. * According to Williams *et al.* (1983).

† ISP, International *Streptomyces* Project.

‡ JII, John Innes Institute, Norwich, UK; IFO, Institute of Fermentation, Osaka, Japan; ATCC, American Type Culture Collection, Maryland, USA; FERM, Fermentation Research Institute, Agency of Industrial Science and Technology, Tsukuba, Japan; IMC, Institute of Microbial Chemistry, Tokyo, Japan.

Identification procedures. Species were identified according to the methods of Shirling & Gottlieb (1966). Each strain was examined for the following characters: morphology, micro-morphology, spore morphology, aerial mass colour, reverse colour, soluble colours, melanin production and carbon utilization. All characteristics were determined using ISP media 2-9 (Difco) as recommended by Shirling & Gottlieb (1966). Inoculated media were incubated at 30 °C, and all tests were done twice to confirm reproducibility. Spore chain morphology was examined by light and scanning electron microscopy of 14-d-old cultures on ISP media 3 and 4. Spore surface ornaments were determined on scanning electron microscopy preparations. Aerial spore-mass colour was determined with cultures on ISP media 2, 3, 4 and 5. Substrate mycelial pigments were observed after 14 d on ISP medium 4. The production of soluble pigments was determined on ISP medium 5 after 14 d. Melanin production was determined on ISP media 6 and 7 after 4 d. Carbon-source utilization was determined on ISP medium 9 supplemented with an ether-sterilized carbon source after 14 d and 21 d.

Preparation of ribosomes. All of the strains were grown to mid-exponential phase (usually 10-15 h) in SPY medium (Ochi, 1987) containing a reduced amount (0.1%) of anhydrous MgSO₄. Cells were collected on a filter paper (diameter 9 cm, no. 2, Toyo Roshi), and washed with deionized water; the cell-paste (about 7 g wet weight) was then suspended in 15 ml of buffer containing 20 mM-Tris/HCl (pH 8.0), 10 mM-magnesium acetate, 20 mM-ammonium chloride, 5 mM-2-mercaptoethanol and 0.1 mM-phenylmethylsulphonyl fluoride (PMSF). The cells were disrupted by sonication for 3 min in an ice-cold bath. Deoxyribonuclease (3 µg ml⁻¹, final concn) was then added and after 10 min at 0 °C, cell debris was removed by centrifugation at 16000 g for 30 min. Ribosomes present in the supernatant were then pelleted at 110000 g for 4 h. The supernatant was discarded, and the crude ribosomes were resuspended in 15 ml of buffer containing 20 mM-Tris/HCl (pH 7.6), 10 mM-magnesium acetate, 1 M-ammonium chloride, 5 mM-2-mercaptoethanol and 0.1 mM-PMSF. After standing overnight at 4 °C, the ribosomes were again pelleted by centrifugation at 110000 g for 4 h, and the supernatant was discarded. The 'washed' ribosomes thus obtained were used as the 70S ribosome preparation for the subsequent experiments.

Preparation of total ribosomal proteins. Ribosomal proteins were prepared by the method of Hardy *et al.* (1969) with modifications as follows. The 70S ribosomal preparation described above was dissolved in 2.5 ml of a solution containing 10 mM-Tris/HCl (pH 8.0), 3 mM-succinic acid, 10 mM-magnesium chloride and 0.1 mM-PMSF. Then 0.25 ml of 1 M-magnesium chloride and 5 ml acetic acid (99%) were added consecutively, followed by continuous stirring in an ice bath. After 45 min the precipitated RNA was separated from the supernatant (containing the ribosomal protein) by centrifugation at 12000 g for 10 min. For further extraction of the ribosomal proteins, the precipitated RNA was dissolved in 3 ml 67% (v/v) acetic acid containing 33 mM-magnesium chloride, and stirred for 20 min in an ice bath. After centrifugation at 12000 g for 10 min the supernatant was collected. The supernatants were combined, dialysed overnight against 5 litre 2% (v/v) acetic acid, lyophilized for 20 h and then redissolved in 1-1.5 ml of deionized water. A small amount of insoluble material was removed by centrifugation at 5000 g for 10 min. The supernatant fluid was stored at -20 °C and used as the total ribosomal protein sample for gel electrophoresis. It contained 20 to 30 mg protein ml⁻¹. All the procedures used in the preparation of the ribosomes and ribosomal proteins were at 0-4 °C.

Two-dimensional PAGE. The method described by Kaltschmidt & Wittmann (1970) was used. All the reagents were of a special grade prepared for electrophoresis. The separation gel used for the first dimension contained, per 100 ml: 36 g urea, 4 g acrylamide, 0.1 g *N*-*N'*-methylene-bis-acrylamide (MBA), 0.8 g disodium EDTA, 3.2 g boric acid, 4.8 g Tris base and 0.3 ml *N,N,N',N'*-tetramethylethylenediamine (TEMED). This was polymerized by adding 0.1 ml 7% (w/v) ammonium persulphate, per 10 ml. The sample gel contained, per 100 ml: 48 g urea, 4 g acrylamide, 0.2 g MBA, 0.085 g disodium EDTA, 0.32 g boric acid and 60 µl TEMED. This was polymerized by adding 0.1 ml each of 0.5% ammonium persulphate and 0.05% riboflavin, per 10 ml. The electrode buffer used in the first dimension contained, per litre: 360 g urea, 2.4 g disodium EDTA, 9.6 g boric acid and 14.6 g Tris base. For the first dimension electrophoresis a glass tube 152 mm long and 5 mm inner diameter was used. The sample gel volumes amounted to 0.1–0.12 ml, and contained 400–500 µg of total ribosomal protein. Electrophoresis was carried out for 30 h at a constant current of 2 mA, and then the gel was dialysed immediately against 100 ml of dialysing buffer [containing, per litre: 480 g urea, 0.74 ml acetic acid (99%) and 2.4 ml 5 M-KOH] for 1 h. The dialysis procedure was repeated once. The dialysed gel was immediately used for the second dimension. The gel used for second dimension contained, per 100 ml: 36 g urea, 18 g acrylamide, 0.5 g MBA, 5.2 ml acetic acid (99%), 0.96 ml 5 M-KOH and 0.58 ml TEMED. This was polymerized by adding 3.3 ml 10% (w/v) ammonium persulphate, per 100 ml. The electrode buffer for the second dimension contained, per litre: 14 g glycine and 1.5 ml acetic acid (99%). The size of the gel slab prepared for the second dimension electrophoresis was 125 × 138 × 3 mm. Electrophoresis was carried out for 20 h at a constant voltage of 90 V (initial current 22 mA).

After the run, the slab was placed for 2 h in 150 ml methanol/acetic acid/water (5:1:5, by vol.) containing 0.3 g Coomassie brilliant blue R-250, and was then destained in 500 ml of a solution containing 7.5% (v/v) acetic acid and 10% (v/v) methanol with gentle stirring for 3 d. The solution was replaced at intervals with a fresh one.

The gels were run three or four times for each ribosomal protein sample to confirm the reproducibility of the results obtained.

RESULTS

Two-dimensional PAGE of ribosomal proteins

The ribosomal proteins of the type strains of *S. venezuelae*, *S. violaceus*, *S. parvulus* and *S. hygroscopicus* and of strains of four other species (*S. griseus*, *S. coelicolor*, *S. antibioticus* and *S. griseoflavus*) from various numerical taxonomic clusters (Williams *et al.*, 1983) were extracted with acetic acid and separated by two-dimensional PAGE (Fig. 1*a–h*). The protein patterns varied extensively among these strains. For example, it is immediately evident that at least five proteins showed considerable variation in their migration between strains, and more than ten proteins showed little variation. Few proteins appeared to be highly conserved in these eight species. This contrasts with the situation in *E. coli* and *Proteus mirabilis* where only three proteins showed little variation and other proteins had the same electrophoretic mobility (Böck, 1985).

The protein patterns of all *Streptomyces* strains were unaffected [Fig. 1(*i*), (*j*)] when the ribosomal proteins were prepared from cells grown to mid-exponential phase in a different medium (maltose/polypeptone/yeast extract medium; Ochi, 1986). These results also demonstrate that protein patterns are highly reproducible in different runs. Thus, the genus *Streptomyces* appears to have ribosomal protein patterns specific for each taxon.

Identification of Streptomyces strain MA406-A-1

Species identification of strain MA406-A-1 was attempted using the methods of Shirling & Gottlieb (1966). Colonial growth of the strain was reddish brown (red colour-series) on most media. Substrate mycelia were brown and a diffusible pigment was not produced. Spores were produced in short open-looped or terminal spiral chains, but some spore chains were flexuous or straight. The spore surface was smooth. The strain utilized D-glucose, D-fructose, L-arabinose and D-xylose, but not raffinose, rhamnose, sucrose, D-mannitol or L-inositol. Melanin formation was positive. The organism most closely resembled *S. griseolavendus* and to a lesser extent *S. lavendulae* and *S. avidinii*. Since *S. griseolavendus* is currently regarded as a subspecies of *S. lavendulae* (Skerman *et al.*, 1980), *Streptomyces* spp. MA406-A-1 was considered to be a variant of *S. lavendulae* (Waksman & Curtis) Waksman & Henrici. It should be noted that the strain differed from *S. lavendulae* or *S. griseolavendus* type cultures in its utilization of arabinose and xylose, which were not utilized by the *S. lavendulae* or *S. griseolavendus* type cultures, as determined in parallel experiments.

Fig. 1. Two-dimensional PAGE of total ribosomal proteins. The gel system was based upon that of Kaltschmidt & Wittmann (1970). (a) *S. venezuelae*; (b) *S. violaceus*; (c) *S. parvulus*; (d) *S. hygrosopicus*; (e) *S. griseus*; (f) *S. coelicolor*; (g) *S. antibioticus*; (h) *S. griseoflavus*; (i) *S. coelicolor* (grown in MPY-medium); (j) *S. griseoflavus* (grown in MPY-medium).

The ribosomal protein patterns of *Streptomyces* strain MA406-A-1, *S. lavendulae*, *S. griseolavendus* and *S. avidinii* were compared (Fig. 2*a-d*). There were extensive similarities: *S. lavendulae* and *S. griseolavendus* in particular could not be distinguished from each other. It was difficult to conclude, from these data alone, that the protein pattern of strain MA406-A-1 is closer to that of either *S. lavendulae* or *S. avidinii*. Since co-electrophoresis of mixed samples is the best comparison, ribosomal proteins from strain MA406-A-1, *S. lavendulae* and *S. avidinii* were analysed. Four proteins from strain MA406-A-1 migrated differently from those from *S. lavendulae*, and five differently from those from *S. avidinii* (Fig. 2*e, f*). It appears, therefore, to be difficult to identify unambiguously strain MA406-A-1 as either *S. lavendulae* or *S. avidinii*, if *S. avidinii* really represents a single taxon.

Genetic stability of ribosomal proteins

Streptomyces strains are often genetically unstable (Schrempf *et al.*, 1988). Culture of strain MA406-A-1 with acridine dye also resulted in a high frequency (about 50%) of colonies with altered phenotype (Ochi, 1986). Two representative mutants (derived from strain MA406-A-1 with acridine dye treatment) that had lost the ability to produce formycin, melanin pigment and aerial mycelium were analysed; there were no differences in either the number of ribosomal proteins or their mobility from those of the original strain (figures not shown).

DISCUSSION

There is a striking variability of ribosomal protein patterns among some species of *Streptomyces*. This was rather surprising since there are extensive similarities of ribosomal protein patterns within the Enterobacteriaceae and the Bacillaceae (Geisser *et al.*, 1973*a, b*). The divergence of *Streptomyces* ribosomal proteins may reflect the genetic instability typical of the genus. The ribosome is essential for protein synthesis in all organisms and is presumed to have been conserved during evolution (Geisser *et al.*, 1973*a*). It is therefore an excellent approach for unravelling the evolutionary relationships among organisms (briefly reviewed by Böck, 1985). In this connection, classification of actinomycetes has been attempted by partial oligonucleotide sequencing of rRNA (reviewed by Stackebrandt & Woese, 1981; Goodfellow & Cross, 1984). The results reported here suggest that the ribosomal protein pattern in two-dimensional PAGE, together with the sequencing of rRNA, may offer very useful information both for classification and for identification of actinomycetes.

Fig. 2. Two-dimensional PAGE of total ribosomal proteins. (a) *Streptomyces* strain MA406-A-1; (b) *S. lavendulae*; (c) *S. avidinii*; (d) *S. griseolavendus*; (e) *Streptomyces* strain MA406-A-1 + *S. lavendulae*; (f) *Streptomyces* strain MA406-A-1 + *S. avidinii*. Arrows indicate protein spots from *Streptomyces* strain MA406-A-1, and arrowheads those from *S. lavendulae* or *S. avidinii*.

The genus *Streptomyces* is of special interest because of its industrial importance. *Streptomyces* taxonomy has been investigated by three major approaches – morphology and pigment production, numerical taxonomy and nucleic acid hybridization – but identification has generally depended on morphology and pigment production. Recent investigations suggested an ELISA (Kirby & Rybicki, 1986), rapid biochemical assay (Goodfellow *et al.*, 1987) or electrophoretic movement of whole cell extracts (Dietz, 1988) as new approaches. However, sound classification and reliable identification within the genus *Streptomyces* are difficult because of genetic and phenetic instability. From this point of view, it can be emphasized that ribosomal protein patterns were unaffected by acridine dye treatment, which caused extensive changes in the phenotype of the tested isolates (see text). Moreover, five strains, isolated and

identified as *S. lavendulae* in independent laboratories, all showed quite similar ribosomal protein patterns to that of the *S. lavendulae* type strain (unpublished), despite considerable differences in phenotype. In addition, the frequency of spontaneously arising *relC* mutants of various *Streptomyces* species with an altered ribosomal protein ST-L11 were quite low, i.e. 10^{-7} – 10^{-8} (unpublished). This contrasts with a high frequency (usually 10^{-1} – 10^{-2}) of colonies arising spontaneously with a deficiency in pigment or aerial mycelium formation. More work is, however, necessary to conclude that ribosomal proteins (in comparison with other phenetic characters) are not easily affected by genetic stress. Also, although the electrophoretic comparison indicated the extent of the divergence of the ribosomal proteins within different species, it should be noted that the extent of the differences in migration does not necessarily reflect the extent of the differences in the sequences of proteins.

The great similarity of the protein patterns of *S. lavendulae* and *S. avidinii* is of interest. The degree of variation between these two species was far less than that observed in the protein patterns of *S. venezuelae*, *S. violaceus*, *S. parvulus* and *S. hygroscopicus* (Fig. 1). This suggests that *S. lavendulae* and *S. avidinii* are closely related species (if they do not represent a single taxon). The virtual lack of difference in the protein patterns of *S. lavendulae* and *S. griseolavendus* also supports the idea that the latter is a variant of the former. Further study of the ribosomal protein patterns within the same or different species would allow us to evaluate the efficacy of this new method as a new approach for *Streptomyces* (and other actinomycetes) taxonomy.

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