Polyacrylamide Gel Electrophoresis Analysis of Ribosomal Protein AT-L30 from an Actinomycete Genus, *Streptosporangium*

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We analyzed the ribosomal AT-L30 proteins from 11 type strains of species belonging to the genus *Streptosporangium*. The electrophoretic mobilities of the AT-L30 preparations from these strains, as determined by two-dimensional polyacrylamide gel electrophoresis, revealed that they could be divided into three groups. The first group contained *Streptosporangium viridogriseum*, *S. viridogriseum* subsp. *kofuense*, and *S. albidum*, while the second group contained *S. roseum*, *S. album*, *S. vulgare*, *S. nondiastaticum*, *S. fragile*, *S. violaceochromogenes*, and *S. amethystogenes*. *S. corrugatum* was a member of the third group. These groups were completely consistent with Nonomura's previous classification, which was based on morphological criteria. The results of partial amino acid sequencing of AT-L30 preparations from several representative strains strongly supported the hypothesis that each of the three groups of the genus *Streptosporangium* merits separate generic status.

In previous publications, Ochi and coworkers have begun to explore the phylogenetic relationships among groups of actinomycetes as determined by ribosomal protein AT-L30 analysis (14–17). This method depends on the heterogeneity of ribosomal AT-L30 proteins among genera of actinomycetes. Analysis of AT-L30 proteins by two-dimensional polyacrylamide gel electrophoresis (PAGE) has been used successfully to establish the taxonomic status of the genera *Actinomadura* and *Microtetraspora* (16). Subsequently, the taxonomic status of representatives of the validly described genera classified in the family *Pseudonocardiaceae* has been determined by analyzing of their AT-L30 proteins (17). Analysis of AT-L30 proteins by two-dimensional PAGE has also been used successfully to investigate the taxonomic status of the genera *Nocardia*, *Rhodococcus*, *Tsukamurella*, and *Gordona* (15). In this study, we used our PAGE analysis method to examine the taxonomy of the genus *Streptosporangium*.

The term *maduromycetes* was first introduced by Goodfellow and Cross (5) for a member of poorly circumscribed sporoactinomycetes that have a number of chemical and morphological features in common (4). The *maduromycetes* currently include the sporangiate genera *Streptosporangium*, *Planobispora*, and *Planomonospora*, as well as the oligosporic genus *Microbispora* and the revised genus *Microtetraspora* (3, 11). Goodfellow et al. recently proposed that this term could also accommodate organisms that are assigned to the new family *Streptosporangiaceae* (6). These organisms contain *meso*-diaminopimelic acid and madurose (3-*O*-methyl-D-galactose) in their whole-cell hydrolysates. Among the genera of *maduromycetes*, the genus *Streptosporangium* is characterized by a distinctive morphological property (i.e., formation of spore vesicles containing numerous nonmotile spores). Interestingly, as described by Nonomura (13), the members of the genus *Streptosporangium* can be divided into three groups on the basis of their morphological characteristics. Group 1 is characterized by thick, strong spore vesicle walls and contains *Streptosporangium viridogriseum*, *Streptosporangium viridogriseum* subsp.

kofuense, and *Streptosporangium albidum*. Group 2 is characterized by thin, fragile spore vesicle membranes and contains *Streptosporangium album*, *Streptosporangium amethystogenes*, *Streptosporangium longisporum*, *Streptosporangium nondiastaticum*, *Streptosporangium pseudovulgare*, *Streptosporangium roseum*, *Streptosporangium violaceochromogenes*, *Streptosporangium viridialbum*, *Streptosporangium vulgare*, and *Streptosporangium fragile*. Group 3 is characterized by small spore vesicles; *Streptosporangium corrugatum* belongs to this morphological group. The aim of this study was to clarify the taxonomic status of these three groups as determined by ribosomal AT-L30 protein analysis.

MATERIALS AND METHODS

Bacterial strains. The strains which we used are listed in Table 1. All of the organisms were type strains that were obtained from the Japan Collection of Microorganisms, Saitama, Japan, the Institute of Fermentation, Osaka, Japan, and the American Type Culture Collection, Rockville, Md. The strains were grown to mid-exponential phase in soluble starch-Polypeptone-yeast extract medium (14) at 30°C for 20 to 40 h.

Two-dimensional PAGE. Ribosomal proteins were prepared from 70S ribosomes by extraction with acetic acid, as previously described (14), using the method of Hardy et al. (7). For PAGE, the method of Kaltschmidt and Wittmann (10) was used, as described previously (14). The gels were run two to three times for each ribosomal protein sample to confirm the reproducibility of the results obtained.

Determination of amino acid sequence. When we were preparing the AT-L30 protein samples for amino acid sequence analysis, we applied large amounts (2 to 3 mg) of the total ribosomal proteins to the PAGE gels. After two-dimensional PAGE, the spots (containing about 50 µg of protein) assigned to AT-L30 protein were cut from the gels. The proteins were extracted from the gels by electrophoresis with a model TB-8 blotting cell (TEFCO) as described below. (Although we originally described a washing procedure for removing the pigment from the cut gel [16], this procedure was later omitted since we found that the pigment

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TABLE 1. Electrophoretic mobilities of ribosomal AT-L30 proteins from the type strains of the members of the genus *Streptosporangium*

Strain	Mobility of AT-L30 (mm) ^a	REM ^b
<i>Streptosporangium corrugatum</i> JCM 3181 ^{Tc}	2.0	3.0
<i>Streptosporangium album</i> JCM 2177 ^T	14.5	21.0
<i>Streptosporangium roseum</i> JCM 2178 ^T	14.5	21.0
<i>Streptosporangium vulgare</i> JCM 3082 ^T	15.7	23.0
<i>Streptosporangium nondiastaticum</i> JCM 3114 ^T	17.1	25.0
<i>Streptosporangium fragile</i> IFO 14311 ^T	17.8	26.0
<i>Streptosporangium violaceochromogenes</i> JCM 3281 ^T	20.0	29.0
<i>Streptosporangium amethystogenes</i> JCM 3026 ^T	21.0	30.5
<i>Streptosporangium viridogriseum</i> JCM 3282 ^T	34.6	50.5
<i>Streptosporangium albidum</i> IFO 13901 ^T	35.0	51.0
<i>Streptosporangium viridogriseum</i> subsp. <i>kofuense</i> JCM 3157 ^T	45.7	66.5
<i>Kibdelosporangium aridum</i> ATCC 39323 ^T	28.6	41.5
<i>Saccharomonospora viridis</i> JCM 3036 ^T	68.5	100

^a Mobility of the AT-L30 protein in the first dimension.^b The mobility of protein AT-L30 from *Saccharomonospora viridis* JCM 3036^T was defined as unity (100).^c T = type strain.

had no effect on the amino acid sequence.) Electrophoresis was carried out for 24 h at a constant current of 300 mA (initial voltage, 8 V); the protein was extracted from each gel on a membrane (ProBlott; Applied Biosystems, Foster City, Calif.), which was placed on the cathode side. The buffer solution which we used contained (per liter) 300 g of urea, 14 g of glycine, and 52 ml of acetic acid (adjusted to pH 4 by 5 N KOH). Sequence analyses of the extracted proteins were performed with a model 470A protein sequencer (Applied Biosystems).

RESULTS AND DISCUSSION

Two-dimensional PAGE analysis of ribosomal proteins. The ribosomal proteins of 11 species of the genus *Streptosporangium* were extracted with acetic acid and separated by two-dimensional PAGE. The results obtained for several species are shown in Fig. 1. When we compared the ribosomal protein patterns, it was immediately evident that some of the spots (Fig. 1, arrows) were homologous proteins. These proteins were designated AT-L30 proteins because of their amino acid sequence homology (see below). The results for *Saccharomonospora viridis*, whose AT-L30 protein was defined as unity for determining relative electrophoretic mobilities (REMs), are shown in Fig. 1F. Figure 1 shows that the electrophoretic mobilities of the AT-L30 proteins in the first dimension of gel electrophoresis differed among the species examined. The distances that the AT-L30 proteins moved in the first dimension in the original slab gels are shown in Table 1. Table 1 also shows the electrophoretic mobility of the AT-L30 protein from each species relative to the electrophoretic mobility of *Saccharomonospora viridis* AT-L30 protein, which exhibited the greatest mobility among all of the AT-L30 proteins from actinomycetes examined so far (15). The experimental error for the REM defined in this way was at most 5%, as determined by several gel runs of the same sample. Table 1 shows that the species which we tested could be divided into three clusters; cluster 1 species (REM, 3.0) exhibited slight AT-L30 protein mobility in the first dimension of gel electrophoresis, and in cluster

2 species (REMs, 21.0 to 30.5) and cluster 3 species (REMs, 50.5 to 66.5) the AT-L30 proteins migrated to the cathode side (the right side in Fig. 1). The clustering is also shown in Fig. 2. *Streptosporangium corrugatum* was a member of cluster 1. Cluster 2 contained *Streptosporangium album*, *Streptosporangium roseum*, *Streptosporangium vulgare*, *Streptosporangium nondiastaticum*, *Streptosporangium fragile*, *Streptosporangium violaceochromogenes*, and *Streptosporangium amethystogenes*, while cluster 3 contained *Streptosporangium viridogriseum*, *Streptosporangium viridogriseum* subsp. *kofuense*, and *Streptosporangium albidum*. It was striking that this clustering was completely consistent with the morphological classification of Nonomura (13). Since the genera of actinomycetes have been shown to have REMs that are specific for each genus (15–17), it seems reasonable to propose that the genus *Streptosporangium* is an assembly of phylogenetically distinct taxa. To examine this idea, we determined the amino acid sequences of the AT-L30 proteins from several species.

Amino acid sequences of the AT-L30 proteins. As Fig. 3 shows, a highly homologous sequence (indicated by underlining) was found in *Escherichia coli* and the three species of *Streptosporangium* examined. These results provide strong evidence that the AT-L30 proteins in the *Streptosporangium* species which we tested are homologous to protein L30 of *E. coli*. Furthermore, a comparison of the sequence of each AT-L30 protein with the known sequences of *E. coli* ribosomal proteins other than the L30 protein showed no homology at all. Thus, it was evident that we were indeed dealing with the proteins (designated AT-L30 proteins) that are homologous to protein L30 of *E. coli*.

It should be noted that the amino acid sequences of the AT-L30 proteins of *Streptosporangium roseum* and *Streptosporangium amethystogenes*, members of cluster 2, exhibited an extremely high level of homology; in contrast, there was less homology between *Streptosporangium viridogriseum* subsp. *kofuense* and these two organisms (Fig. 3). To express quantitatively the level of similarity of the amino acid sequences (SAS values), we determined the frequency of appearance of the same amino acid in 20 N-terminal amino acids of each AT-L30 protein. The SAS values determined in this way are shown in Table 2. We found that the species belonging to the same genus had very high SAS values (90 to 100%), as exemplified by three species of the genus *Micromonospora*. On the other hand, species belonging to different genera exhibited low SAS values (20 to 62%). We also evaluated the SAS values determined for species of the genus *Streptosporangium*. As expected from the REM results, the SAS value for *Streptosporangium roseum* and *Streptosporangium amethystogenes* was high (94%), indicating that these two species should be viewed as a single taxon. In contrast, the SAS values for *Streptosporangium viridogriseum* subsp. *kofuense* and *Streptosporangium roseum* or *Streptosporangium amethystogenes* were significantly lower (76 and 71%, respectively). These SAS values are closer to the values observed for different genera than the values observed for members of the same genus. These results, together with the marked differences in REMs (Fig. 2), provide phylogenetic evidence that the species in group 1 of Nonomura (e.g., *Streptosporangium viridogriseum* subsp. *kofuense*) and those in group 2 (e.g., *Streptosporangium roseum*) are separable at the generic level. Also, although amino acid sequencing was not conducted, group 3 (e.g., *Streptosporangium corrugatum*) should be considered an independent taxon that is separable from groups 1 and 2 on the basis of the remarkable differences in their REMs

FIG. 1. Two-dimensional PAGE of total ribosomal proteins from members of the genus *Streptosporangium*. The gel system was based on the system of Kaltschmidt and Wittmann (10). The O in panel A indicates the origin in the first dimension. The arrows indicate the positions of AT-L30 proteins. (A) *Streptosporangium corrugatum*. (B) *Streptosporangium roseum*. (C) *Streptosporangium amethystogenes*. (D) *Streptosporangium viridogriseum*. (E) *Streptosporangium viridogriseum* subsp. *kofuense*. (F) *Saccharomonospora viridis*.

FIG. 2. REM values for the members of the genus *Streptosporangium*. The data are from Table 1. The mobility of protein AT-L30 from *Saccharomonospora viridis* JCM 3036^T was defined as unity (100).

(Fig. 2). Therefore, we propose that each of the three groups of the genus *Streptosporangium* that were originally classified by Nonomura on the basis of morphological characteristics merits separate generic status. Since the type species of the genus *Streptosporangium* is *Streptosporangium roseum*, the members of group 2 are considered members of the genus *Streptosporangium* sensu stricto. However, it is not clear whether the members of groups 1 and 3 should be combined with species of previously described genera or whether they merit new generic status. Our proposal needs

FIG. 3. Primary structures of N termini of AT-L30 proteins from several species of the genera *Streptosporangium*, *Microtetraspora*, and *Actinomadura*. The data for *E. coli* are from reference 18, and the data for *Microtetraspora glauca*, *Microtetraspora helvata*, and *Actinomadura malachitica* are from reference 16. The common sequence found in *E. coli* and other strains is indicated by underlining. A sequence signature in the N termini, which was common to all of the actinomycetes examined, is enclosed in a box. Question marks indicate amino acids that were not determined. Line 1, *Microtetraspora glauca*; line 2, *Microtetraspora fusca*; line 3, *Microtetraspora helvata*; line 4, *Streptosporangium roseum*; line 5, *Streptosporangium amethystogenes*; line 6, *Streptosporangium viridogriseum* subsp. *kofuense*; line 7, *Actinomadura malachitica*; line 8, *E. coli* K-12.

to be studied by using other approaches, such as rRNA sequencing, DNA relatedness, or chemotaxonomy.

Streptosporangium viridogriseum and *Streptosporangium viridogriseum* subsp. *kofuense* exhibited considerably different REMs (50.5 versus 66.5) (Table 1). However, these two strains can be assigned to a single species or to closely related species since there was a great deal of similarity in their ribosomal protein patterns on two-dimensional PAGE gels (Fig. 1D and E).

The electrophoretic mobilities of protein molecules are greatly affected by the level of electric charge. Although the sequencing experiments were conducted for only one-third of the whole AT-L30 protein, the frequencies of basic amino acids and acidic amino acids in *E. coli* (REM, 73.0), *Streptosporangium roseum* (REM, 21.0), and *Microtetraspora glauca* (REM, -5.0) explain the observed electrophoretic mobilities of the AT-L30 proteins of these organisms. Thus, REM appears to be an accurate reflection of the physicochemical properties of each AT-L30 protein.

Taxonomic relationship between the genus *Streptosporangium* and allied taxa. Previous investigations have dem-

TABLE 2. Comparisons among the AT-L30 amino acid sequences

Strains compared	Level of similarity of amino acid sequences (SAS value) (%) ^a
<i>Microtetraspora glauca</i> vs <i>Microtetraspora fusca</i>	94
<i>Microtetraspora glauca</i> vs <i>Microtetraspora helvata</i>	100
<i>Microtetraspora fusca</i> vs <i>Microtetraspora helvata</i>	90
<i>Streptosporangium roseum</i> vs <i>Streptosporangium amethystogenes</i>	94
<i>Streptosporangium roseum</i> vs <i>Streptosporangium viridogriseum</i> subsp. <i>kofuense</i>	76
<i>Streptosporangium amethystogenes</i> vs <i>Streptosporangium viridogriseum</i> subsp. <i>kofuense</i>	71
<i>Streptosporangium viridogriseum</i> subsp. <i>kofuense</i> vs <i>Actinomadura malachitica</i>	62
<i>Streptosporangium roseum</i> vs <i>Actinomadura malachitica</i>	58
<i>Streptosporangium amethystogenes</i> vs <i>Actinomadura malachitica</i>	58
<i>Streptosporangium roseum</i> vs <i>Microtetraspora glauca</i>	20
<i>Streptosporangium roseum</i> vs <i>Escherichia coli</i>	47
<i>Actinomadura malachitica</i> vs <i>Escherichia coli</i>	46
<i>Streptosporangium viridogriseum</i> subsp. <i>kofuense</i> vs <i>Escherichia coli</i>	35

^a The SAS value for each pair of organisms for 20 N-terminal amino acids of the AT-L30 proteins was determined on the basis of the data shown in Fig. 3.

onstrated that the genus *Streptosporangium* is more closely related to the genus *Microtetraspora* than to the genus *Actinomadura*, as shown by the results of 16S rRNA analyses and by chemotaxonomic characteristics (1, 2, 12). In contrast, our ribosomal AT-L30 protein analyses provided the opposite result; namely, the SAS value observed for *Streptosporangium roseum* and *Actinomadura malachitica* was much higher than that observed for *Streptosporangium roseum* and *Microtetraspora glauca* (58 versus 20%) (Table 2). Furthermore, the REMS for the genus *Streptosporangium* sensu stricto (21.0 to 30.5) were closer to the REMs for the genus *Actinomadura* (14.0 to 41.5) than the REMs for the genus *Microtetraspora* (−6.5 to 0) (see reference 16). Ribosomal proteins and rRNA are both components of ribosomes, whose variability is considered to be more limited than that of the other cellular components because of many structural and functional constraints (8, 9). Therefore, it is surprising that analyses of ribosomal proteins and rRNAs led to different conclusions, but this may imply that molecular evolution of ribosomal proteins and molecular evolution of rRNAs proceed independently. The apparent incongruity between the protein and rRNA data is fascinating and does underline the importance of basing phylogenetic relationships on more than one type of molecule. However, it does seem likely that in this case the true relationships are those that are based on nucleotide sequences given the excellent agreement with the chemotaxonomic data. It is well known that ribosomal proteins have evolved at very different rates, depending on the individual proteins and their functions (21). The genus *Microtetraspora* appears to occupy an isolated position in the evolution of maduromycetes, since the amino acid sequence of the AT-L30 proteins from this genus was especially different from the other amino acid sequences (Fig. 3). It is noteworthy that *Streptosporangium corrugatum*, a member of group 3 of the genus *Streptosporangium*, had an REM (3.0) that was similar to the REMs for the genus *Microtetraspora* (−6.5 to 0). This fact implies a close phylogenetic relationship between *Streptosporangium corrugatum* and the genus *Microtetraspora*, but a comparison on the level of amino acid sequence is required to establish the taxonomic relationship of these organisms.

The genus *Kibdelosporangium* was recently described by Shearer et al. (20). Morphologically, this genus closely resembles group 1 *Streptosporangium* species, but it has cell wall chemotype IV (19). Our results supported the previous proposal of Shearer et al., since the REM for *Kibdelosporangium aridum* ATCC 39323^T did not fall within the REM ranges observed for the *Streptosporangium* groups (Fig. 2).

The novel method discussed above will contribute to polyphasic taxonomy of actinomycete taxa, especially when it is used together with 16S rRNA analysis.

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