Polyacrylamide Gel Electrophoresis Analysis of Ribosomal Protein AT-L30 as a Novel Approach to Actinomycete Taxonomy: Application to the Genera Actinomadura and Microtetraspora

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Actinomycete ribosomal protein AT-L30 exhibits electrophoretic mobility that is specific for each genus. On the basis of this fact, we analyzed ribosomal AT-L30 proteins from 26 type strains of species belonging to the genera Actinomadura and Microtetraspora. The electrophoretic mobilities of AT-L30 preparations from these strains, as determined by two-dimensional polyacrylamide gel electrophoresis, revealed that they could be divided into two groups, one group with relative electrophoretic mobilities of 14.0 to 41.5 and another group with relative electrophoretic mobilities of -6.5 to 0. The first group corresponded to the genus Actinomadura, and the second group corresponded to the genus Microtetraspora. Partial amino acid sequencing of AT-L30 preparations from several strains proved that we were indeed dealing with the specified protein homologous to ribosomal protein L30 of Escherichia coli. Our results strongly supported the conclusions of previous work and thus proved the efficacy of ribosomal protein analysis as a novel approach for taxonomy of actinomycetes.

The ribosomal proteins of Escherichia coli have been studied in great detail (26-28). However, only a few reports are available on the ribosomal proteins of actinomycetes (16, 23). The use of two-dimensional separation of ribosomal proteins for identification and classification has been extended to the family Enterobacteriaceae, the family Bacillaceae, and several other bacteria (2, 4, 5). Despite the similarity of ribosomal protein patterns within related genera or species, Ochi (19, 21) has found that several Streptomyces species exhibit considerable electrophoretic variability in ribosomal protein patterns and has proposed that the members of the genus Streptomyces have ribosomal protein patterns that are specific to each taxon. The practical application of ribosomal protein patterns to Streptomyces taxonomy has also been demonstrated (19, 20). In the course of such studies, it has been discovered that there is striking variability in the electrophoretic mobilities of certain ribosomal proteins (the AT-L30 proteins) among genera of actinomycetes; nevertheless, these ribosomal proteins appear to be highly conserved (with respect to electrophoretic mobility) within each genus (21a). Therefore, it should be possible to utilize this fact in the taxonomy of actinomycetes. To demonstrate the efficacy of this new method, we performed an electrophoretic analysis of ribosomal AT-L30 proteins to establish the taxonomic status of the genera Actinomadura and Microtetraspora, whose taxonomy has been much confused to date. Goodfellow (8) has recommended that the genus Actinomadura should be restricted to Actinomadura madurae and related species (the A. madurae group) and that Actinomadura pusilla and related taxa (the A. pusilla group) merit separate generic status. Subsequently, Miyadoh et al. (18) suggested that the A. pusilla group and the genus Microtetraspora should be combined at the genus level on the basis of chemotaxonomic and DNA-DNA hybridization results. More recently, Kroppenstedt et al. (15) proposed a revision of the genus Actinomadura; on the basis of chemical, molecular, and numerical taxonomic evidence, they proposed that the A. pusilla group and the genus Microtetraspora should be combined at the genus level. The results of our ribosomal protein AT-L30 analysis are consistent with these previous proposals. In this study we demonstrated the efficacy of this novel method in the taxonomy of actinomycetes.

MATERIALS AND METHODS

Bacterial strains. The strains used in this study are listed in Table 1. All were type strains obtained from the Japan Collection of Microorganisms (Saitama, Japan), the Institute for Fermentation (Osaka, Japan), and the American Type Culture Collection (Rockville, Md.).

Preparation of total ribosomal proteins. Since the members of the genera Actinomadura and Microtetraspora generally grew slowly in a soluble starch-peptone-yeast extract medium (19), a medium suitable for the growth of other actinomycetes, all of the strains used in this study were grown in a glucose-yeast extract medium (18) on a rotary shaker (220 rpm) at 30°C. After cultivation for 20 to 30 h (mid-exponential phase), the cells were collected, washed, and then disrupted by sonication for 3 to 5 min, as described previously (19). The cells of several Actinomadura species were hardly disrupted by sonic treatment, but the cells were not treated for more than 5 min to avoid destruction of ribosomes. The 70S ribosomes were pelleted by centrifugation at 110,000 × g for 4 h. Ribosomal proteins were prepared from the 70S ribosomes by extraction with acetic acid, as previously described (19), using the method of Hardy et al. (10). The total ribosomal protein samples obtained in this way contained 5 to 12 mg of protein per ml. The relatively low protein concentrations in the samples compared with samples from other actinomycete genera (20 to 30 mg of protein per ml) (19) were apparently due to the difficulty encountered in disrupting cells of Actinomadura species.

Two-dimensional PAGE. For polyacrylamide gel electrophoresis (PAGE), the method of Kaltschmidt and Wittmann...
(14) was used, as described in detail previously (19). The gels were run twice for each ribosomal protein sample to confirm the reproducibility of the results obtained.

**Determination of amino acid sequences.** After two-dimensional PAGE, the spots (usually containing 20 to 50 μg of protein) assigned to AT-L30 were cut from the gels and washed with 100 ml of a solution containing 10% (vol/vol) acetic acid and 50% (vol/vol) methanol with gentle shaking for 5 days. This procedure was effective for removing the pigment (Coomassie brilliant blue) which bound to the protein. The proteins were extracted from the gels by electrophoresis. Sequence analyses of the extracted proteins were performed with model 470A protein sequencer (Applied Biosystems). Complete details of the sequencing procedures will be given elsewhere.

**RESULTS**

Two-dimensional PAGE analysis of ribosomal proteins. All of the organisms belonging to the genera *Actinomadura* and *Microtetraspora* examined were type strains (Table 1); the nomenclature for these bacteria was based on the proposal by Kroppenstedt et al. (15). The ribosomal proteins of these strains were extracted with acetic acid and separated by two-dimensional PAGE. A few examples of the results of this analysis are shown in Fig. 1A through I (the spots assigned to ribosomal protein AT-L30 are indicated by arrows). The results for *Saccharomonospora viridis*, whose protein AT-L30 was taken as unity for determining relative electrophoretic mobilities (REMs), are shown in Fig. 1J. The relative electrophoretic mobility (REM) defined in this way could make it possible to compare the AT-L30 mobilities in different organisms belonging to the genera *Actinomadura* and *Microtetraspora*, whose nomenclature for these bacteria was based on the proposal by Kroppenstedt et al. (15); the old names are given in parentheses.

**Table 1. Electrophoretic mobilities of ribosomal AT-L30 proteins from the type strains of members of the genera Actinomadura and Microtetraspora**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mobility of AT-L30 (mm)</th>
<th>REM</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Microtetraspora niveoalba</em> JCM 3149T</td>
<td>-4.5</td>
<td>-6.5</td>
</tr>
<tr>
<td><em>Microtetraspora pusillia</em> (Actinomadura pusillia) ATCC 27295T</td>
<td>-4.0</td>
<td>-6.0</td>
</tr>
<tr>
<td><em>Microtetraspora glauca</em> JCM 3300T</td>
<td>-3.5</td>
<td>-5.0</td>
</tr>
<tr>
<td><em>Microtetraspora fusca</em> ATCC 23058T</td>
<td>-3.0</td>
<td>-4.5</td>
</tr>
<tr>
<td><em>Microtetraspora roseoviolacea</em> (Actinomadura roseoviolacea) JCM 3145T</td>
<td>-3.0</td>
<td>-4.5</td>
</tr>
<tr>
<td><em>Microtetraspora helvata</em> (Actinomadura helvata) JCM 3143T</td>
<td>-2.0</td>
<td>-3.0</td>
</tr>
<tr>
<td><em>Microtetraspora fastidiosa</em> (Actinomadura fastidiosa) JCM 3323T</td>
<td>-1.0</td>
<td>-1.5</td>
</tr>
<tr>
<td><em>Microtetraspora spiralis</em> (Actinomadura spiralis) IFO 14097T</td>
<td>-1.0</td>
<td>-1.5</td>
</tr>
<tr>
<td><em>Microtetraspora roseola</em> (Actinomadura roseola) JCM 3323T</td>
<td>-1.0</td>
<td>-1.5</td>
</tr>
<tr>
<td><em>Microtetraspora africana</em> (Nocardiosis africana) JCM 6240T</td>
<td>-1.0</td>
<td>-1.5</td>
</tr>
<tr>
<td><em>Microtetraspora ferruginea</em> (Actinomadura ferruginea) JCM 3283T</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td><em>Microtetraspora salmonea</em> (Actinomadura salmonea) JCM 3324T</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td><em>Microtetraspora angiospora</em> (Micropoly- spora angiospora) JCM 3109T</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td><em>Actinomadura spadix</em> IFO 14099T</td>
<td>9.5</td>
<td>14.0</td>
</tr>
<tr>
<td><em>Actinomadura kijianata</em> JCM 3306T</td>
<td>11.0</td>
<td>16.0</td>
</tr>
<tr>
<td><em>Actinomadura verrucosalza</em> JCM 6053T</td>
<td>11.0</td>
<td>16.0</td>
</tr>
<tr>
<td><em>Actinomadura atroventraria</em> JCM 6250T</td>
<td>11.5</td>
<td>16.5</td>
</tr>
<tr>
<td><em>Actinomadura madurae</em> ATCC 19425T</td>
<td>13.0</td>
<td>19.0</td>
</tr>
<tr>
<td><em>Actinomadura malachitica</em> JCM 3297T</td>
<td>14.5</td>
<td>21.0</td>
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<tr>
<td><em>Actinomadura creneia</em> IFO 14182T</td>
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<td>28.5</td>
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<tr>
<td><em>Actinomadura libanotica</em> IFO 14095T</td>
<td>25.0</td>
<td>36.5</td>
</tr>
<tr>
<td><em>Actinomadura vinacea</em> JCM 3325T</td>
<td>25.5</td>
<td>37.0</td>
</tr>
<tr>
<td><em>Actinomadura coerulea</em> JCM 3320T</td>
<td>28.5</td>
<td>41.5</td>
</tr>
<tr>
<td><em>Actinomadura luteoflorescens</em> JCM 4203T</td>
<td>28.5</td>
<td>41.5</td>
</tr>
<tr>
<td><em>Actinomadura pelletier</em> JCM 3388T</td>
<td>28.5</td>
<td>41.5</td>
</tr>
<tr>
<td><em>Actinomadura verrucosalza</em> JCM 3147T</td>
<td>28.5</td>
<td>41.5</td>
</tr>
<tr>
<td><em>Nocardia asteroides</em> JCM 3384T</td>
<td>40.5</td>
<td>59.0</td>
</tr>
<tr>
<td><em>Escherichia coli</em> ATCC 11775T</td>
<td>50.0</td>
<td>73.0</td>
</tr>
<tr>
<td><em>Saccharomonospora viridis</em> JCM 3036T</td>
<td>68.5</td>
<td>100.0</td>
</tr>
</tbody>
</table>

ª The nomenclature of bacteria is based on the proposal of Kroppenstedt et al. (15); the old names are given in parentheses.

ª Mobility of AT-L30 in the first dimension. Negative values indicate that the protein moved toward the reverse side (the anode side).

ª The mobility of protein AT-L30 from *S. viridis* JCM 3036T was defined as unity (100).

ª T = type strain.

Actinomadura until Kroppenstedt et al. (15) revised the taxonomic status of these species. It is striking that these species all had REMs similar to those obtained for authentic *Microtetraspora* species (e.g., *Microtetraspora glauca* but not for members of the genus *Actinomadura* sensu stricto. These results strongly support the previous proposal of Kroppenstedt et al. (15). *Microtetraspora angiospora* and *Microtetraspora africana* have been previously called *Micropoly- spora angiospora* and *Nocardiosis africana*, respectively. Chemo- taxonomic studies of these species have shown that they are closely related to *Microtetraspora pusillia* and related species (9, 22), and recently Kroppenstedt et al. (15) validly classified these two species in the genus *Microtetraspora*. Our results supported this proposal, since the REMs for these organisms were similar to the
REMs for Microtetraspora species (Table 1). "Parvopoly-
spora pallida," which was presumed to belong to the genus
Actinomadura by Itoh et al. (13) and Miyadoh et al. (17), was
found to have an REM (16.0) in the range observed for the
genus Actinomadura (Table 1), supporting the presumption
of Itoh et al. and Miyadoh et al. Thus, despite the entirely
different taxonomic approaches used in the different labora-
tories, we reached the same conclusion.

Amino acid sequence of the protein assigned to AT-L30. The
complete amino acid sequence of E. coli protein L30 is
known, as reported by Ritter and Wittmann-Liebold (24); its
N-terminal amino acid sequence is shown in Fig. 2. This
protein contains 58 amino acid residues, and the molecular
weight calculated from the sequence is 6,411. We deter-
dined the N-terminal amino acid sequences of AT-L30
proteins from several species of the genera Actinomadura
and Microtetraspora. As shown in Fig. 2, the same amino
acid sequence (Lys-Ile-Thr-Gln-Thr) was found in both E.
coli and Actinomadura malachitica. These results provide
strong evidence that the protein assigned to AT-L30 in A.
malachitica is indeed homologous to protein L30 of E. coli.
Almost no similarity was found between L30 from E. coli
and AT-L30 from Microtetraspora helvata or Microtetra-
spora glauca. Nevertheless, these AT-L30 proteins can be
considered homologous since they share a sequence signa-
ture in their N termini (Fig. 2, box). In addition, a compar-
ison of the sequence of each AT-L30 protein with the known
sequences of E. coli ribosomal proteins other than L30
showed no homology at all. Thus, it is evident that we were
indeed dealing with the proteins (named AT-L30 proteins)
homologous to protein L30 of E. coli.

It should also be emphasized that Microtetraspora helvata
and Microtetraspora glauca, a single generic taxon, exhibited an extremely high level of homology in the amino acid sequences of their AT-L30 proteins; in contrast, there was little homology between A. malachitica and these two organisms (Fig. 2). This implies that there is an evolutionally estranged relationship between the genus Microtetraspora and the genus Actinomadura. Also, although the sequencing experiment was conducted for only one-third of the whole AT-L30 protein, the high frequency of acidic amino acids and the low frequency of basic amino acids in Microtetraspora glauca compared with E. coli account for the observed low electrophoretic mobility of the AT-L30 protein of Microtetraspora glauca (Fig. 1C).

**DISCUSSION**

Bacterial ribosomes consist of three species (5S, 16S, and 23S) of RNA and more than 50 species of ribosomal proteins (for reviews, see references 26 through 28). Because of many structural and functional constraints, the variability of ribosomal proteins (and also rRNA) is more limited than the variability of other proteins; thus, ribosomal proteins have much lower evolutionary rates than other proteins (11, 12). Therefore, analysis of ribosomal proteins and rRNAs could be an excellent approach for studying the evolutionary relationships among organisms. Indeed, classification of

![Image of gel analysis](https://example.com/gel_analysis.png)

**FIG. 1.** Two-dimensional PAGE of total ribosomal proteins from actinomycetes. The gel system was based on the system of Kaltschmidt and Wittmann (14). O in panel A indicates the origin in the first dimension. The arrows indicate the positions of AT-L30 proteins. Strains were arranged in order of protein AT-L30 mobility, with less mobility to the cathode side. (A) Microtetraspora pusilla. (B) Microtetraspora nivealba. (C) Microtetraspora glauca. (D) Microtetraspora fusca. (E) Microtetraspora helvata. (F) Microtetraspora ferruginea. (G) Actinomadura madurae. (H) Actinomadura malachitica. (I) Actinomadura pelletieri. (J) Saccharomonospora viridis.

![Image of primary structures](https://example.com/primary_structures.png)

**FIG. 2.** Primary structures of N termini of AT-L30 proteins from several species of the genera Actinomadura and Microtetraspora. The data for E. coli are from reference 24. The common sequence found in E. coli and other strains is indicated by underlining. ?, Not determined; 1, Microtetraspora glauca; 2, Microtetraspora helvata; 3, Actinomadura malachitica; 4, Escherichia coli K-12.
actinomycetes has been attempted by partial sequencing of 16S rRNAs (3, 7, 25).

In our analysis of ribosomal protein AT-L30 by two-dimensional PAGE, we demonstrated that the members of the genus *Actinomadura* and the members of the genus *Microtetraspora* can be separated clearly by their AT-L30 REMs. Since the genera of actinomycetes have been shown to have REMs that are specific to each genus (21a), it seems reasonable to propose that the genera *Actinomadura* and *Microtetraspora* should be viewed as independent taxa. To our knowledge, this is the first study to deal with ribosomal protein analysis for classification of actinomycetes; the efficacy of this method was predicted by Ochi previously (19). It is especially noteworthy that several species of *Actinomadura* (for example, *Actinomadura kijanitai*, *Actinomadura libanotica*, and *Actinomadura spadix*) whose classification in the genus *Actinomadura* or the genus *Microtetraspora* has been ambiguous to date were all clearly assigned to the former genus on the basis of the REMs of their AT-L30 proteins (Table 1). It should also be pointed out that there were great similarities in the ribosomal protein patterns for the three *Microtetraspora* species (*Microtetraspora glauca*, *Microtetraspora fusca*, and *Microtetraspora niveola*) (Fig. 1B through D). These results, together with the results of a DNA-DNA pairing test (18), suggest that these species might be combined into a single species. The extensive similarities were especially impressive for *Microtetraspora roseoviolacea*, *Microtetraspora salmonea*, and *Microtetraspora verrucosospora* and for *Microtetraspora* species. The results prove that ribosomal protein analysis (especially analysis of AT-L30 proteins) is a very useful approach for both classification and identification of actinomycetes. Several attempts have been made to utilize PAGE analysis of ribosomal proteins for classification of bacteria, yeast, and fungi (1, 2, 4–6), but it has generally been difficult to obtain unambiguous conclusions, perhaps because of the extensive similarities in the ribosomal protein patterns of bacteria. Apparently, our success in classification of actinomycetes depended solely on the striking variability of the ribosomal proteins of actinomycetes. In bacteria such as *E. coli* and *Bacillus subtilis*, no such variability occurs, at least with respect to electrophoretic mobility, even in the L30 protein corresponding to AT-L30 protein (21a). It is clear that the variability of amino acid sequences (Fig. 2) is involved in part (if not solely) in the diversity of AT-L30 electrophoretic mobilities. However, we cannot explain why actinomycetes evolved AT-L30 proteins that are so divergent with respect to electrophoretic mobility. A sequence analysis of several whole AT-L30 proteins will be required for further understanding.

In contrast to the narrow ranges of REMs in the genera *Streptomyces* (14.5 to 25.5), *Streptosporangium* (21.0 to 30.5), *Saccharomonospora* (97.0 to 100), and *Microtetraspora* (−6.5 to 0), the range of REMs for members of the genus *Actinomadura* (14.0 to 41.5) is wide (21a; this study). Although the values are continuous within this range (Table 1), the data may imply that this taxon is heterologous. Perhaps the greater variability in the ribosomal protein patterns in the genus *Actinomadura* than in the genus *Microtetraspora* (see above) reflects the wide range of REMs.

As will be reported elsewhere, analysis of AT-L30 proteins by two-dimensional PAGE has been used successfully to establish the taxonomic status of the genera *Microtetraspora* and *Streptosporangium*. Therefore, it is evident that ribosomal protein analysis will be very helpful in generating polyphasic taxonomies of actinomycete taxa.

REFERENCES

19. Ochi, K. 1989. Heterogeneity of ribosomal proteins among...


