A Taxonomic Review of the Genus *Microbispora* by Analysis of Ribosomal Protein AT-L30

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We analyzed the ribosomal AT-L30 proteins from 13 type strains of species belonging to the genera *Microbispora* and *Actinomadura*. The electrophoretic mobilities of the AT-L30 preparations from *Microbispora* strains, as determined by two-dimensional polyacrylamide gel electrophoresis, revealed that the members of the genus *Microbispora* are phylogenetically homogeneous. The results of partial amino acid sequencing of AT-L30 preparations from several representative *Microbispora* strains supported the separation of the genus *Microbispora* from other related genera. The amino acid sequences of the AT-L30 proteins from strains of species belonging to the genus *Actinomadura* sensu stricto displayed a diversity that exemplified the low levels of amino acid sequence homology within the genus. This diversity was considered to be a characteristic typical of the genus *Actinomadura*.

Analysis of rRNAs is already a well-accepted approach for studying the evolutionary relationships among microorganisms. This method has been applied successfully to the taxonomy of actinomycetes (24, 25). Ribosomal proteins could also be an excellent object of study, because ribosomal proteins have been shown to have lower evolutionary rates than other proteins (8). Since the recent discovery of heterogeneity among the ribosomal proteins of *Streptomyces* species (15), Ochi and coworkers have explored the phylogenetic relationships among groups of actinomycetes as determined by ribosomal protein AT-L30 analysis (17–21). Analysis of AT-L30 proteins by two-dimensional polyacrylamide gel electrophoresis (PAGE) has been used successfully to establish the taxonomic status of the genera *Nocardia*, *Rhodococcus*, *Gordona*, and *Tsukamurella* (17). Furthermore, the taxonomic status of representatives of the validly described genera classified in the family *Pseudonocardaceae* has also been determined by analysis of their AT-L30 proteins (21). In particular, this new approach was effective for investigating the phylogenetic relationships among the taxa belonging to the family *Streptosporangiaceae*, which was previously called *maduromycetes* by Goodfellow and Cross (5). Ochi et al. (20) analyzed the AT-L30 proteins of members of the genera *Actinomadura* and *Microtetraspora* by using two-dimensional PAGE and demonstrated that these genera can be separated by using the electrophoretic properties of the AT-L30 proteins; the results were completely consistent with the previous conclusion of Kroppenstedt et al. (10), which was based on chemical, molecular, and numerical taxonomic evidence. Subsequently, on the basis of amino acid sequence homologies of AT-L30 proteins, we stressed that each of the three morphologically distinct groups of the genus *Streptosporangium* (14) merits separate generic status (19). As recently proposed by Miyadoh et al. (11), the genus *Microbispora*, another member of the family *Streptosporangiaceae*, currently contains only two species (*Microbispora rosea* and *Microbispora bispora*) as a result of the combining of numerous species into *Microbispora rosea*. In this study, we applied our new approach to the genus *Microbispora* to clarify the taxonomic status of this genus.

MATERIALS AND METHODS

Bacterial strains. The strains used in this study are listed in Table 1. All of these strains 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. Most of the strains were grown to mid-exponential phase in soluble starch-Polypeptone-yeast extract medium (15) at 30°C for 20 to 40 h; the exceptions were *Microbispora bispora*, which was grown at 55°C, and *Microbispora aerata*, *Microbispora thermodiastatica*, and *Microbispora thermorosea*, which were grown at 45°C.

Two-dimensional PAGE. Two-dimensional PAGE by the method of Kaltschmidt and Wittmann (9) has been described in detail previously (15). After each slab gel was stained with Coomassie blue, the AT-L30 protein spot was identified. The electrophoretic mobilities (in the first dimension) of the AT-L30 proteins were expressed as relative electrophoretic mobilities (REMs) compared with the electrophoretic mobility of *Saccharomonospora viridis* AT-L30 protein, which exhibited the greatest mobility of all of the actinomycete AT-L30 proteins examined. The reference point for the REMs (a value of zero) was defined as the center point of each sample gel disk in the first electrophoretic dimension. Gels were run twice for each ribosomal protein sample to confirm the reproducibility of the results obtained.

Determination of amino acid sequence. The amino acid sequence analysis of each AT-L30 protein was carried out as described previously (19) by using a model 470A protein sequencer (Applied Biosystems, Foster City, Calif.). Determination of a maximum of 20 N-terminal amino acids was possible; an amino acid residue of this size represents about one-third of the whole AT-L30 protein, assuming that the L30 protein from *Escherichia coli* (58-amino-acid residue) and the AT-L30 protein from actinomycetes are of the same size. The levels of similarity of the amino acid sequences (SAS values) were expressed as numbers of amino acid positions that were the same per number of positions compared. Positions that were not determined were not used in the calculations of SAS values.

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TABLE 1. Electrophoretic mobilities of ribosomal AT-L30 proteins from the type strains of species belonging to the genus *Microbispora* and related genera

Strain ^a	Mobility of AT-L30 protein (mm) ^b	REM ^c
<i>Microtetraspora glauca</i> JCM 3300 ^T	-3.5	-5.0 ^d
<i>Microbispora aerata</i> IFO 12581 ^T	4.3	6.5
<i>Microbispora chromogenes</i> JCM 3022 ^T	5.0	7.5
<i>Microbispora karnatakensis</i> ATCC 35927 ^T	5.7	8.5
<i>Microbispora parva</i> JCM 3024 ^T	5.7	8.5
<i>Microbispora thermorosea</i> IFO 14047 ^T	7.1	10.5
<i>Microbispora thermodiastatica</i> IFO 14046 ^T	8.6	12.5
<i>Microbispora amethystogenes</i> JCM 3021 ^T	9.3	13.5
<i>Microbispora diastatica</i> JCM 3023 ^T	9.3	13.5
<i>Microbispora bispora</i> ATCC 19993 ^T	14.3	21.0
<i>Microbispora indica</i> ATCC 35926 ^T	20.0	29.0
<i>Microbispora rosea</i> JCM 3006 ^T	20.0	29.0
<i>Actinomadura rugatobispora</i> (<i>Microbispora viridis</i>) IFO 14382 ^T	10.7	15.5
<i>Actinomadura atramentaria</i> JCM 6250 ^T	11.5	16.5 ^d
<i>Actinomadura madurae</i> ATCC 19425 ^T	13.0	19.0 ^d
<i>Actinomadura malachitica</i> JCM 3297 ^T	14.5	21.0 ^d
<i>Actinomadura echinospora</i> (<i>Microbispora echinospora</i>) ATCC 27300 ^T	15.7	23.0
<i>Actinomadura cremea</i> IFO 14182 ^T	19.5	28.5 ^d
<i>Streptosporangium roseum</i> JCM 2178 ^T	14.5	21.0 ^d
<i>Saccharomonospora viridis</i> JCM 3036 ^T	68.5	100

^a The names in parentheses are former names.

^b Mobility of the AT-L30 protein in the first dimension. A negative value indicates that the protein moved toward the reverse side (the anode side).

^c The mobility of protein AT-L30 from *Saccharomonospora viridis* JCM 3036^T was defined as 100.

^d Data from references 19 and 20.

RESULTS

Two-dimensional PAGE analysis of ribosomal proteins. The ribosomal proteins of 11 species of the genus *Microbispora* were extracted with acetic acid and separated by two-dimensional PAGE. The result obtained for *Microbispora indica* is shown in Fig. 1 as an example. When the ribosomal protein patterns of *Microbispora* species were compared, there were extensive similarities among 10 species (*Microbispora aerata*, *Microbispora amethystogenes*, *Microbispora chromogenes*, *Microbispora diastatica*, *Microbispora indica*, *Microbispora karnatakensis*, *Microbispora parva*, *Microbispora rosea*, *Microbispora thermodiastatica*, and *Microbispora thermorosea*); the only exception was *Microbispora bispora*, which produced a pattern that was very different from the patterns of the other *Microbispora* species (data not shown). It was also immediately evident that some of the spots represented homologous proteins. These proteins were designated AT-L30 proteins (Fig. 1, arrow) because of their levels of amino acid sequence homology (see below). The electrophoretic mobilities of the AT-L30 proteins in the first dimension of gel electrophoresis were different for the *Microbispora* species which we 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 of all of the AT-L30 proteins from actinomycetes examined so far (17). The experimental error for the REMs defined in this way was $\leq 5\%$, as determined from several gel runs of the same

FIG. 1. Two-dimensional PAGE of total ribosomal proteins from *Microbispora indica* ATCC 35926^T (T = type strain). O, origin in the first dimension. The arrow indicates the position of the AT-L30 protein.

sample. Table 1 shows that the members of the genus *Microbispora* that we tested displayed a relatively wide range of REMs (6.5 to 29.0). *Actinomadura rugatobispora* and *Actinomadura echinospora* were previously classified in the genus *Microbispora* (13). The REMs of these strains (15.5 and 23.0) (Table 1) fell within the REM ranges not only for the genus *Actinomadura* (REMs, 14.0 to 41.5) (20) but also for the genus *Microbispora*. Thus, to clarify the taxonomic status of the genera *Microbispora* and *Actinomadura*, it was necessary to conduct an amino acid sequence analysis of the AT-L30 proteins of these taxa.

Amino acid sequences of the AT-L30 proteins. As Fig. 2 shows, highly homologous sequences were found for the five *Microbispora* species examined. (*Microbispora rosea*, the type species of the genus, was not included in this study because of a difficulty in obtaining a sufficient amount of its AT-L30 protein for amino acid sequence analysis.) In contrast, sequences that exhibited less homology were found for the seven species of the genus *Actinomadura* examined. A comparison of the sequence of each AT-L30 protein with the known sequence of *Escherichia coli* L30 ribosomal protein (Ala-Lys-Thr-Ile-Lys-Ile-Thr-Gln-Thr-Gln-Thr-Arg-Ser-Ala-Ile-Gly. . .) (23) showed considerable homology. These results provide strong evidence that the AT-L30 proteins in the *Microbispora* species which we tested are homologous to protein L30 of *E. coli*. To express quantitatively the levels of similarity of the amino acid sequences (SAS values), we determined the frequency of appearance of the same amino acid in 10 to 18 N-terminal amino acids of each AT-L30 protein. The SAS values determined in this way for every combination of strains tested are shown in Table 2. *Actinomadura verrucososporea* was not included because of the low number of amino acids determined. It was striking that the members of the genus *Microbispora* had very high SAS values (91 to 100%) in comparisons with each other, indicating that the genus *Microbispora* is phylogenetically homogeneous. In contrast, the members of the genus *Actinomadura* had low SAS values (down to 67%); SAS values greater than 90% were detected only in comparisons of *Actinomadura madurae*, *Actinomadura echinospora*, and *Actinoma-*

- 1) Ala-Gln-Leu-Lys-Ile-Thr-Gln-Val-Lys-Ser- ? -Ile-Gly-Gly- ? -Gln-Asn- ? - ? -Asp
- 2) Ala-Lys-Leu-Lys-Ile-Thr-Gln-Thr-Lys-Ser-Val-Ile-Gly-Gly-Gln-Gln-Asn-Gln-Gln-Lys
- 3) Ser-Gln-Leu-Lys-Ile-Thr-Gln-Lys-Lys-Ala-Val-Ile- ? - ? -Gln-Gln-Asn-Gln-Gln-Asp
- 4) Ala-Gln-Leu-Lys-Ile-Thr-Gln-Ile-Lys-Ser- ? -Ile-Gly-Gly-Gln-Gln-Asn
- 5) Ala-Gln-Leu-Lys-Ile-Thr-Gln-Thr-Lys-Ala-Val-Ile-Asn
- 6) Ala-Gln-Leu-Lys-Ile-Thr-Gln-Val- ? -Ser-Lys-Ile-Gly-Gly-Gln-Gln-Asn-Gln
- 7) Ala- ? -Leu-Lys-Ile-Thr
- 8) Ala-Lys-Leu-Lys-Ile-Thr-Gln-Thr-Lys-Ser-Ser-Ile-Gly- ? -Gln-Gln- ? - ? - ? -Asp
- 9) Ala- ? -Leu-Lys-Ile-Thr-Gln-Thr- ? -Ser-Lys-Ile-Gly-Gly-Gln-Gln-Asn-Gln
- 10) Ala-Gln-Leu-Lys-Ile-Thr-Gln-Thr- ? -Ser- ? -Ile-Gly-Gly-Gln-Gln-Asn-Gln
- 11) Ala- ? -Leu-Lys-Ile-Thr-Gln-Val- ? -Ser- ? -Ile-Gly-Gly-Gln-Gln-Asn-Gln
- 12) Ala- ? -Leu-Lys-Ile-Thr-Gln-Val- ? -Ser- ? -Ile-Gly-Gly-Gln
- 13) Ala- ? -Leu-Lys-Ile-Thr-Gln-Val-Arg-Ser-Lys-Ile-Gly-Gly-Lys-Gln-Asn-Gln- ? -Asp
- 14) Gly-Ala-Glu-Ile-Val-Asp-Ala-Leu-Gln-Phe-Val- ? -Gly-Glu-Arg-Gly-Phe-Tyr-Phe-Asn

FIG. 2. Primary structures of N termini of AT-L30 proteins from several species of the genera *Actinomadura* and *Microbispora*. The data for *Microtetraspora glauca* and *A. malachitica* are from reference 20, and the data for *Streptosporangium roseum* are from reference 19. Question marks indicate amino acids that were not determined. Line 1, *A. madurae* ATCC 19425^T (T = type strain); line 2, *A. atramentaria* JCM 6250^T; line 3, *A. cremea* IFO 14182^T; line 4, *A. echinospora* ATCC 27300^T; line 5, *A. malachitica* JCM 3297^T; line 6, *A. rugatobispora* IFO 14382^T; line 7, *A. verrucospora* JCM 3147^T; line 8, *Microbispora amethystogenes* JCM 3021^T; line 9, *Microbispora indica* ATCC 35926^T; line 10, *Microbispora aerata* IFO 12581^T; line 11, *Microbispora thermodiastatica* IFO 14046^T; line 12, *Microbispora thermorosea* IFO 14047^T; line 13, *Streptosporangium roseum* JCM 2178^T; line 14, *Microtetraspora glauca* JCM 3300^T.

dura rugatobispora. Three other *Actinomadura* species (*Actinomadura atramentaria*, *Actinomadura cremea*, and *Actinomadura malachitica*) displayed low SAS values (72 to 83%) in comparisons with each other (Table 2). It should also be pointed out that *Actinomadura cremea* had serine in the N terminus of its AT-L30 protein at a position that is occupied by alanine in the other AT-L30 proteins that have been examined so far (Fig. 2). Perhaps, this is a reflection of the observed diversity of the *Actinomadura* AT-L30 proteins. A dendrogram of the data in Fig. 2 is shown in Fig. 3.

DISCUSSION

The term maduromycetes was first introduced by Goodfellow and Cross (5) for a group of poorly circumscribed sporoactinomycetes that have a number of chemical and morphological features in common. The maduromycetes, which currently accommodates organisms that are assigned to the new family *Streptosporangiaceae*, include the sporangiate genera *Streptosporangium*, *Planobispora*, and *Planomonospora*, as well as the oligosporic genus *Microbispora*

TABLE 2. SAS values for the amino acid sequences of AT-L30 proteins^a

Species	SAS values (%)											
	<i>A. madurae</i>	<i>A. atramentaria</i>	<i>A. cremea</i>	<i>A. echinospora</i>	<i>A. malachitica</i>	<i>A. rugatobispora</i>	<i>Microbispora amethystogenes</i>	<i>Microbispora indica</i>	<i>Microbispora aerata</i>	<i>Microbispora thermodiastatica</i>	<i>Microbispora thermorosea</i>	<i>Streptosporangium roseum</i>
<i>A. madurae</i>												
<i>A. atramentaria</i>	81											
<i>A. cremea</i>	79	72										
<i>A. echinospora</i>	93	88	79									
<i>A. malachitica</i>	75	77	83	75								
<i>A. rugatobispora</i>	100	82	73	93	67							
<i>Microbispora amethystogenes</i>	85	93	67	86	69	79						
<i>Microbispora indica</i>	92	94	71	93	73	94	92					
<i>Microbispora aerata</i>	93	94	79	93	82	94	92	100				
<i>Microbispora thermodiastatica</i>	100	93	77	93	70	100	92	93	93			
<i>Microbispora thermorosea</i>	100	92	70	92	70	100	91	92	92	100		
<i>Streptosporangium roseum</i>	93	72	63	80	58	94	73	82	87	93	92	
<i>Microtetraspora glauca</i>	7	6	0	6	8	6	7	7	6	7	8	6

^a The SAS value for each pair of organisms for 10 to 18 N-terminal amino acids of the AT-L30 proteins was determined on the basis of the data shown in Fig. 2.

FIG. 3. Clustering of AT-L30 protein SAS values. The dendrogram was drawn by using the data in Fig. 2. Abbreviations: Acre, *A. cremea*; Amal, *A. malachitica*; Aech, *A. echinospora*; Amad, *A. madurae*; Arug, *A. rugatobispora*; Mthd, *Microbispora thermodiastatica*; Mthr, *Microbispora thermorosea*; Sros, *Streptosporangium roseum*; Aatr, *A. atramentaria*; Maer, *Microbispora aerata*; Mind, *Microbispora indica*; Mame, *Microbispora amethystogenes*; Mtgl, *Microtetrastora glauca*.

and the revised genus *Microtetrastora* (3, 7, 10). These organisms contain *meso*-diaminopimelic acid and madurose (3-*O*-methyl-D-galactose) in their whole-cell hydrolysates. Among the genera of maduromycetes, all members of the genus *Microbispora* exhibit an unequivocal morphology characterized by the formation of longitudinally paired spores on aerial mycelia. Moreover, as determined by Miyadoh et al. (11), these organisms have common chemotaxonomic phospholipid and menaquinone compositions. Although the members of the genus *Microbispora* can be divided into two groups on the basis of growth temperature requirements (14), they exhibit high SAS values in comparisons with each other (Table 2). As demonstrated in the genera *Microtetrastora*, *Streptosporangium*, and *Streptomyces*, we have found that the members of a well-demarcated genus exhibit SAS values higher than 90% (18a, 19, 20). Therefore, our ribosomal AT-L30 protein analysis data, together with data from a previous study by Miyadoh et al. (11), confirm the phylogenetic status of the genus *Microbispora* as a single genus. Miyadoh et al. (11) also proposed that 10 *Microbispora* species other than *Microbispora bispora* should be combined into a single species with two subspecies (*Microbispora rosea* subsp. *rosea* and *Microbispora rosea* subsp. *aerata*). This view point was based mainly on the high levels of DNA relatedness among the 10 *Microbispora* species. On the other hand, Ochi (16) has shown that strains of a single species have virtually identical REM values for AT-L30 proteins, as demonstrated in various strains of *Streptomyces lavendulae*. Nevertheless, the 10 *Microbispora* strains displayed substantially different REMs (Table 1). Therefore, our data cast doubt on the previous

view of Miyadoh et al. (11), although the 10 strains which we studied are closely related to each other, as demonstrated by the extensive similarity of their ribosomal protein patterns. On the other hand, *Microbispora bispora* clearly differs from these 10 strains at the species level.

A. echinospora and *A. rugatobispora*, which were previously classified in the genus *Microbispora*, both displayed high SAS values (93 and 100%, respectively) in comparisons with *A. madurae*, the type species of the genus *Actinomadura* (Table 2). In contrast, these species displayed relatively low SAS values (86 and 79%) in comparisons with *Microbispora amethystogenes*, an example of a *Microbispora* species. These observations support the view of Poschner et al. (22) and Miyadoh et al. (11) that *Microbispora echinospora* (= *A. echinospora*) and *Microbispora viridis* (= *A. rugatobispora*) should be classified as members of the genus *Actinomadura* instead of the genus *Microbispora*.

The genus *Actinomadura* has had a long and confusing taxonomic history. This genus was originally considered a member of the maduromycetes group, but is currently classified in the family *Thermonosporaceae* (6). It was surprising that, unlike the members of the genus *Microbispora*, the members of the genus *Actinomadura* displayed low SAS values (down to 67%) in comparisons within the genus. Recently, a revision of the genus *Actinomadura* has been suggested by Goodfellow (4) and Miyadoh et al. (12); subsequently, on the basis of chemical, molecular, and numerical taxonomic evidence, Kroppenstedt et al. (10) proposed that *Actinomadura pusilla* and allied species should be viewed as a taxon that is independent from members of the genus *Actinomadura sensu stricto* and should be transferred to the

genus *Microtetraspora*. The genus *Actinomadura* sensu stricto revised in this way is well defined and is characterized by many common properties among its species (10). The *Actinomadura* species used in this study were all members of *Actinomadura* sensu stricto. Therefore, the observed diversity in amino acid sequences (i.e., low SAS values) in *Actinomadura* AT-L30 proteins can be considered a characteristic of this genus. The diversity in AT-L30 proteins in the genus *Actinomadura* was expected from previous results that demonstrated that the range of REMs (14.0 to 41.5) for members of the genus *Actinomadura* sensu stricto is remarkably wide (20). These facts might imply that there is genetic instability within the genus *Actinomadura*, and such genetic instability, if any, might have led to the confusing taxonomic history of the genus *Actinomadura* and allied genera. A cluster analysis (Fig. 3) really demonstrated how confusing this situation is.

It is noteworthy that the members of the genus *Microbispora* exhibited high SAS values in comparisons with *Streptosporangium roseum*; this was especially pronounced with *Microbispora thermodiastatica* and *Microbispora thermosea* (Table 2 and Fig. 3). Apparently, this is an indication of a close relationship between the genera *Microbispora* and *Streptosporangium*. The member of the genus *Microtetraspora*, another member of the maduromycetes, displayed extremely low SAS values (less than 8%) not only in comparisons with members of the genus *Streptosporangium* but also in comparisons with members of the genus *Microbispora* (Table 2 and Fig. 3). However, previous investigations of the 16S rRNA sequences demonstrated that the genus *Microtetraspora* is closely related to the genus *Streptosporangium* (1, 2). As discussed previously regarding this apparent incongruity (19), the true relationships are the relationships based on rRNA nucleotide sequences, given their agreement with the chemotaxonomic data. In the genus *Microbispora*, no data for rRNA nucleotide sequences have been reported.

Thus, to date, Ochi and coworkers have explored the efficacy of ribosomal protein patterns (for identification at the species level) and AT-L30 protein analysis (for classification at the genus level) as new approaches for the taxonomy of actinomycetes. It would be interesting to study whether the members of the genus *Actinomadura* exhibit diversity in rRNA sequences like that observed in the AT-L30 amino acid sequences.

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