Intragenetic Relationships of the Actinomycete Genus *Micromonospora*

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The intragenetic structure of the genus *Micromonospora* was determined by analyzing the 16S ribosomal DNAs of the type strains of all but one of the 15 validly described species and four subspecies and 19 other *Micromonospora* strains, most of which represented invalidly described species. One of the 19 other *Micromonospora* strains grouped outside a phylogenetically tight genus *Micromonospora*, which appeared to be closely related to other genera belonging to the family *Micromonosporaceae*. The level of sequence similarity for the 16S ribosomal DNAs of most of the type strains was 98%. Values in the same range were also found when the sequences of the type strains and most of the other *Micromonospora* strains were compared. Whether the invalidly described species actually represent taxa that are worthy of species status should be investigated by performing DNA reassociation studies and thoroughly comparing physiological and chemotaxonomic properties.

The genus *Micromonospora* Ørskov 1923 (12) is the type genus of the family *Micromonosporaceae* Krasil'nikov 1938 (9), emend. Goodfellow, Stanton, Simpson, and Minnikin 1990 (6). This family now contains several genera, including the genera *Micromonospora*, *Actinoplanes*, *Dactylosporangium*, and *Pilimelia*, which are morphologically distinct but chemotaxonomically similar (6). Recently, workers have described two new genera, the genus *Catenuloplanes* (22) and the genus *Couchioplanes* (19), which can also be assigned to this family on the basis of their phylogenetic positions. The inclusion of these genera increases the phenotypic and chemotaxonomic heterogeneity of the family *Micromonosporaceae* (22). Phylogenetically, this family forms one of the major sublines within the order *Actinomycetales* (4, 16, 19); however, with the exception of *Couchioplanes* strains (19), very few representatives of each genus have been included in 16S rRNA cataloging studies (17), analyses of 16S rRNA by the reverse transcriptase method (19), or analyses of 16S ribosomal DNA (rDNA) by the PCR method (19). Of the 15 validly described *Micromonospora* species, only a single strain of *Micromonospora chalicea* (strain NRRL B-2344) was included in a phylogenetic analysis in which partial 16S rRNA sequences were used (16) (the Approved Lists of Bacterial Names [14a] includes 12 species, and three species were validly described after 1980; however, according to Kawamoto [8] no type strain of *Micromonospora gallica* is available). In order to provide a basis for determining the phylogenetic relationships among the species and to determine whether species whose taxonomic positions are not clear (8) should be considered authentic *Micromonospora* species, we analyzed the 16S rDNAs of all of the type strains of *Micromonospora* species and subspecies available, as well as several strains of invalid species.

MATERIALS AND METHODS

Strains investigated and culture conditions. The strains investigated in this study are listed in Table 1. All of these strains, which are deposited in the DSMZ-German Collection of Microorganisms and Cell Cultures GmbH, were

cultivated on medium 65 (*Streptomyces* medium) as described in the DSM-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH *Catalogue of Strains* (3).

Analysis of 16S rDNA. Genomic DNA was extracted and the 16S rDNA was amplified as described previously (13). PCR products were sequenced directly by using a Taq DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Foster City, Calif.) as recommended by the manufacturer. The sequence reaction mixtures were electrophoresed by using an Applied Biosystems model 373A DNA sequencer.

Phylogenetic analysis. The 16S rDNA sequences which we determined were compared with previously described 16S rDNA sequences for members of the order *Actinomycetales* available from the Ribosomal Database Project (11). Similarity values were transformed into phylogenetic distance values that compensated for multiple substitutions at any given site in the sequence (7). The least-squares distance method of De Soete (2) and the neighbor-joining and maximum-likelihood programs contained in the PHYLIP package (5) were used to construct phylogenetic dendrograms. To calculate bootstrap values, we analyzed 300 trees by using the NJFIND and NJBOOT programs.

Nucleotide sequence accession numbers. The 16S rDNA sequences which we determined have been deposited in the EMBL data library under accession numbers X92594 to X92631.

RESULTS AND DISCUSSION

We determined the almost complete 16S rDNA sequences of the type strains of 14 *Micromonospora* species and four subspecies, as well as *Dactylosporangium thailandense* DSM 43158. In addition, 19 strains representing invalidly published species, as well as a single undescribed *Micromonospora* strain, were included in this study (Fig. 1). The sequences which we determined were aligned with the sequences of members of the order *Actinomycetales*, and the phylogenetic positions of the strains were determined. All but one of the *Micromonospora* strains formed a phylogenetically tight cluster which occurred in the neighborhood of other members of the family *Micromonosporaceae*, such as *Actinoplanes philippinensis*, *Couchioplanes caeruleus* (19), *Catenuloplanes japonicus* (19), and *Dactylosporangium thailandense* (this study). As the number of previously published 16S rDNA sequences of strains belonging to other genera of the family *Micromonosporaceae* is minute compared with the number of validly described species, we did not produce a phylogenetic tree of relatedness at this stage of our investigation as the intrafamily relationships may change significantly when more organisms are included.

The only strain that grouped outside the radiation of *Micromonospora* strains was strain DSM 43892 (= JCM 3105), which was invalidly described as "*Micromonospora megalomi-*

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TABLE 1. Organisms investigated, their source, and their 16S rDNA accession numbers

Taxon	Strain	Accession no.
<i>Micromonospora aurantiaca</i>	DSM 43813 ^T	X92604
<i>Micromonospora brunnea</i>	DSM 43814 ^T	X92605
<i>Micromonospora carbonacea</i> subsp. <i>carbonacea</i>	DSM 43168 ^T	X92599
<i>Micromonospora carbonacea</i> subsp. <i>aurantiaca</i>	DSM 43815 ^T	X92606
<i>Micromonospora chalcone</i>	DSM 43026 ^T	X92549
<i>Micromonospora chersina</i>	DSM 44151 ^T	X92628
<i>Micromonospora coerulea</i>	DSM 43143 ^T	X92598
<i>Micromonospora echinospora</i> subsp. <i>echinospora</i>	DSM 43816 ^T	X92607
<i>Micromonospora echinospora</i> subsp. <i>ferruginea</i>	DSM 43141 ^T	X92597
<i>Micromonospora echinospora</i> subsp. <i>pallida</i>	DSM 43817 ^T	X92608
<i>Micromonospora halophytica</i> subsp. <i>halophytica</i>	DSM 43171 ^T	X92601
<i>Micromonospora halophytica</i> subsp. <i>nigra</i>	DSM 43818	X92609
<i>Micromonospora inositol</i>	DSM 43819 ^T	X92610
<i>Micromonospora olivasterospora</i>	DSM 43868 ^T	X92613
<i>Micromonospora purpurea</i>	DSM 43036 ^T	X92595
<i>Micromonospora purpureochromogenes</i>	DSM 43821 ^T	X92611
<i>Micromonospora rhodorangea</i>	DSM 1039 ^T	X92612
<i>Micromonospora rosaria</i>	DSM 803 ^T	X92631
" <i>Micromonospora brunnescens</i> "	DSM 43902	X92616
" <i>Micromonospora citrea</i> "	DSM 43903	X92617
" <i>Micromonospora echinoaurantiaca</i> "	DSM 43904	X92618
" <i>Micromonospora echinobrunnea</i> "	DSM 43913	X92625
" <i>Micromonospora floridensis</i> "	DSM 43907	X92621
" <i>Micromonospora fulvoviridis</i> "	DSM 43906	X92620
" <i>Micromonospora fulvoviolacea</i> "	DSM 43905	X92619
" <i>Micromonospora globosa</i> "	DSM 43170	X92600
" <i>Micromonospora inyoensis</i> "	DSM 46123	X92629
" <i>Micromonospora lacustris</i> "	DSM 43908	X92622
" <i>Micromonospora megalomicea</i> subsp. <i>megalomicea</i> "	DSM 43892	X92614
" <i>Micromonospora melanosporea</i> "	DSM 43126	X92596
" <i>Micromonospora narashino</i> "	DSM 43172	X92602
" <i>Micromonospora peucetica</i> "	DSM 43363	X92603
" <i>Micromonospora sagamiensis</i> subsp. <i>sagamiensis</i> "	DSM 43912	X92624
" <i>Micromonospora viridifaciens</i> "	DSM 43909	X92623
" <i>Micromonospora yulongensis</i> "	DSM 43915	X92626
" <i>Micromonospora fulvopurpureus</i> "	DSM 43918	X92627
<i>Micromonospora</i> sp.	DSM 43893	X92615
<i>Dactylosporangium thailandense</i>	DSM 43158 ^T	X92630

cea subsp. *megalomicea*." While the levels of similarity between strain DSM 43892 and authentic *Micromonospora* strains ranged from 89 to 90%, the levels of similarity between strain DSM 43892 and representatives of the genus *Streptomyces* (21) ranged from 97 to 98.6%. The relatedness of strain DSM 43892 to streptomycetes was confirmed by the presence of the diagnostic compound LL-diaminopimelic acid in whole-cell extracts and by the results of a fatty acid analysis which revealed that when the pattern for this strain was compared with the patterns available for other actinomycetes, the highest matching coefficient was with *Streptomyces viridochromogenes* (data not shown).

Phylogenetic relationships among the *Micromonospora* type strains. The levels of relatedness obtained for the type strains of the *Micromonospora* spp. were high, ranging from 97.2 to 99% (Table 2). The levels of similarity for many type strains of *Micromonospora* species were actually so high (>98.0%) that their species status could not be confirmed on the basis of

these data alone. DNA-DNA hybridization studies that could confirm the presence of genospecies that correspond to the phenotypically defined species have not been performed. However, as long as DNA-DNA reassociation values are not available, we believe that the isolated positions of the species on the phylogenetic dendrogram indicate that the species are valid.

The 16S rDNAs of the subspecies of *Micromonospora carbonacea* (i.e., *Micromonospora carbonacea* subsp. *carbonacea* and *Micromonospora carbonacea* subsp. *aurantiaca*) are virtually identical (level of similarity, 99.9%). Likewise, two of the three subspecies of *Micromonospora echinospora* (*Micromonospora echinospora* subsp. *echinospora* and *Micromonospora echinospora* subsp. *ferruginea*) have almost identical 16S rDNA sequences (level of similarity, 99.7%); the third subspecies, *Micromonospora echinospora* subsp. *pallida*, is more distantly related to the other two subspecies. Interestingly, the latter

FIG. 1. Neighbor-joining dendrogram (5, 14) showing the phylogenetic positions of validly and invalidly described *Micromonospora* species and a *Micromonospora* strain, based on the results of an analysis of almost complete 16S rDNA sequences (a similarity matrix is not shown). The arrow indicates an alternative phylogenetic position of *M. chalcone*. The numbers on the dendrogram are the percentages of occurrence in 300 bootstrapped trees; only values greater than 20% are shown. Bar = 1 nucleotide substitution per 100 nucleotides.

TABLE 2. Similarity matrix based on 16S rDNA sequence comparisons for the type strains of *Micromonospora* species

Species or subspecies	% Similarity with:																
	<i>M. aurantiaca</i>	<i>M. chalcea</i>	<i>M. brunnea</i>	<i>M. purpureochromogenes</i>	<i>M. carbonacea</i> subsp. <i>aurantiaca</i>	<i>M. carbonacea</i> subsp. <i>carbonacea</i>	<i>M. halophytica</i> subsp. <i>halophytica</i>	<i>M. purpurea</i>	<i>M. coerulea</i>	<i>M. echinospora</i> subsp. <i>pallida</i>	<i>M. rosaria</i>	<i>M. chersina</i>	<i>M. inositola</i>	<i>M. rhodorangea</i>	<i>M. echinospora</i> subsp. <i>echinospora</i>	<i>M. echinospora</i> subsp. <i>ferruginea</i>	<i>M. olivasterospora</i>
<i>M. chalcea</i>	99.1																
<i>M. brunnea</i>	98.4	98.3															
<i>M. purpureochromogenes</i>	99.3	98.5	98.8														
<i>M. carbonacea</i> subsp. <i>aurantiaca</i>	98.4	97.7	97.9	98.6													
<i>M. carbonacea</i> subsp. <i>carbonacea</i>	98.5	97.9	98.0	98.6	99.9												
<i>M. halophytica</i> subsp. <i>halophytica</i>	99.1	98.7	98.3	99.0	98.5	98.6											
<i>M. purpurea</i>	98.5	99.3	97.7	97.9	97.1	97.3	98.0										
<i>M. coerulea</i>	97.8	97.6	97.4	98.2	97.3	97.5	97.8	98.0									
<i>M. echinospora</i> subsp. <i>pallida</i>	98.0	97.8	97.7	98.1	98.1	98.2	98.3	98.1	98.0								
<i>M. rosaria</i>	98.2	98.4	97.9	98.1	97.7	97.8	98.0	98.8	98.0	98.4							
<i>M. chersina</i>	97.8	98.1	98.4	98.2	97.5	97.7	97.8	98.6	98.2	98.4	99.2						
<i>M. inositola</i>	97.8	97.9	98.0	98.1	97.9	97.9	98.1	98.4	98.0	98.9	98.7	99.1					
<i>M. rhodorangea</i>	98.1	98.0	97.7	98.2	98.2	98.2	98.6	98.4	98.4	99.2	98.8	98.9	99.2				
<i>M. echinospora</i> subsp. <i>echinospora</i>	97.7	97.7	97.4	97.9	97.9	97.9	98.2	98.2	98.0	98.9	98.6	98.7	98.8	99.7			
<i>M. echinospora</i> subsp. <i>ferruginea</i>	98.1	98.0	97.7	98.2	98.2	98.2	98.6	98.4	98.4	99.2	98.8	98.9	99.2	100.0	99.7		
<i>M. olivasterospora</i>	97.7	97.2	96.9	97.9	98.0	98.2	97.6	97.6	98.0	98.1	97.8	97.6	97.7	98.0	97.7	98.0	
<i>D. thailandense</i>	95.2	95.4	94.8	95.2	95.4	95.6	95.6	95.7	95.4	95.6	95.8	95.6	95.8	96.0	95.8	96.0	96.3

subspecies is characterized chemotaxonomically by the presence of high concentrations of menaquinones of the MK-12(H_{4,6,8}) type (1), which are not present in other *Micromonospora* type strains [which contain mainly menaquinones of the MK-9(H₄,H₆) and MK-10(H₄, H₆) types (1, 6, 10)]. Apparently, the genus *Micromonospora* is more heterogeneous with respect to menaquinone composition than other actinomycete genera that exhibit similar high levels of intrageneric relatedness.

However, no species exhibits such a high level of 16S rDNA relatedness to another species that the two taxa could be considered synonymous or subspecies. This is also true for most of the strains belonging to taxa considered by Kawamoto (8) to be species incertae sedis. Not only are all of the species genuine members of the genus *Micromonospora*, but considering the isolated position of *Micromonospora purpurea* and *Micromonospora brunnea* on the tree they also do not appear to be subspecies of *M. echinospora* or subjective synonyms of *Micromonospora purpureochromogenes*, as proposed by Szabo and Fernandez (18). *Micromonospora aurantiaca* ATCC 27029^T (= DSM 43813^T) (T = type strain), which was thought to be a member of the genus *Actinoplanes* because of the formation of globose or subglobose sporangia that contain spores having one polar tuft of flagella (8), is an authentic *Micromonospora* species. *Micromonospora rhodorangea* DSM 1039, the type strain of a species whose taxonomic position is uncertain, appears to be a sporeless variant (8) of *M. echinospora* subsp. *ferruginea* (level of 16S rDNA similarity, 100%). To confirm that the sequence of *M. rhodorangea* DSM 1039^T is identical to that of *M. rhodorangea* DSM 43822^T, we analyzed a stretch of 500 nucleotides of the 16S rDNA of the latter strain (there are two Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH numbers for the same type strain because the same strain was obtained independently from two different partial Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH collections prior to the unification of the

West and East German collections). As the sequences of the stretches of the two type strains of *M. rhodorangea* were identical, the authenticity of this species was confirmed. Whether *M. rhodorangea* should be transferred to *M. echinospora* should be decided after a thorough comparative analysis of physiological and chemotaxonomic characteristics and DNA pairing experiments are performed.

When the 16S rDNA sequence of *D. thailandense* was used to root the phylogenetic dendrograms, *Micromonospora olivasterospora* was the deepest rooting species, followed by *Micromonospora coerulea*. The remaining species formed three subclusters, whose species compositions were the same with all of the treeing programs which we used. Subcluster I contains *M. aurantiaca*, *M. purpureochromogenes*, *M. brunnea*, *Micromonospora halophytica*, and *M. carbonacea*. Subcluster II contains the type species of the genus, *M. chalcea*, and *M. purpurea*, while subcluster III contains *M. echinospora*, *M. rhodorangea*, *Micromonospora inositola*, *Micromonospora rosaria*, and *Micromonospora chersina*. A representative tree based on neighbor-joining method results is shown in Fig. 2. While some of the internal relationships were confirmed by bootstrap values greater than 50%, the branching order of the subclusters remains unresolved. The bootstrap values for the branch points that separate the subclusters are low and thus reveal no confidence in branch topology. However, the species compositions of subclusters I and III are supported by the presence of certain signature nucleotides between 16S rDNA positions 603 and 627 (Table 3). The relationship between the two subcluster II species, *M. chalcea* and *M. purpurea*, is a moot point. As judged from the distribution of the signature nucleotides (Table 3), *M. chalcea* could be considered a member of subcluster I, while *M. purpurea* is a member of subcluster III. Indeed, bootstrap values of 37% support to some extent clustering *M. chalcea* with *M. aurantiaca*, while the bootstrap value for the *M. chalcea*-*M. purpureus* pair is as low as 20%. A possible alternative relationship of *M. chalcea* to members of subcluster

FIG. 2. Neighbor-joining dendrogram (5, 14) showing the intragenetic relationships of *Micromonospora* type strains, based on the results of an analysis of almost complete 16S rDNA sequences. The similarity values are shown in Table 2. The numbers on the dendrogram are the percentages of occurrence in 300 bootstrapped trees; only values greater than 20% are shown. Bar = 2 nucleotide substitutions per 100 nucleotides. The arrow indicates an alternative phylogenetic position of *M. chalicea*.

I is indicated in Fig. 2. The two species that group outside the three subclusters (*M. olivasterospora* and *M. coerulea*) have the subcluster III signature nucleotides, which can therefore be considered the 16S rDNA nucleotides found in the common ancestor of the genus.

Phylogenetic positions of other *Micromonospora* strains and invalid *Micromonospora* species. The inclusion of 19 other *Micromonospora* strains, most of which are members of invalidly described species, did not change the phylogenetic relationships significantly (Fig. 1) (similarity values not shown) when neighbor-joining or maximum-likelihood programs were used to generate dendrograms. The clear distinctions among the three subclusters, however, were blurred by the branch points of the additional strains. On the other hand, the members of subclusters I and III contain the 16S rDNA signature nucleotides of their subclusters. *M. coerulea*, which appears to be isolated in Fig. 2, groups with "*Micromonospora citrea*" DSM 43903 and "*Micromonospora echinobrunnea*" DSM 43913. "*Micromonospora peucetica*" DSM 43363 represents another deep

rooting lineage and contains the subcluster III signature nucleotides.

The only deviation in the branching patterns was observed when the least-squares distance method of De Soete (2) was applied to the full data set. While the neighbor-joining and maximum-likelihood programs clustered *M. chalicea* and *M. purpurea* together with "*Micromonospora melanosporea*" DSM 43126 and strain DSM 43893, the least-squares method separated *M. chalicea* from the other subcluster II strains and placed this organism between *M. aurantiaca* and *Micromonospora halophytica* subsp. *halophytica*. *M. purpurea* and strains DSM 43893 and DSM 43126 remained in an intermediate position as members of an individual branch between subclusters I and III. As indicated above, this grouping of *M. chalicea* within the radiation of members of subcluster I is consistent with the presence of a higher number of common signature nucleotides (Table 3). A bootstrap value of 77% supports the grouping of *M. purpurea* and "*M. melanosporea*," while the values found for these two organisms and *M. chalicea* are not significant. The alternative position of *M. chalicea* is indicated in Fig. 1.

Most of the undescribed *Micromonospora* strains appear to be unrelated to each other, and their relationships resemble the relationships among the valid species. This is also true for the two subspecies of *M. halophytica*, *M. halophytica* subsp. *halophytica* and *Micromonospora halophytica* subsp. *nigra*, which belong to subclusters I and III, respectively. This may be an indication that *M. halophytica* subsp. *nigra*, *M. echinospora* subsp. *pallida*, and each of the invalidly described species actually do represent a new species. As mentioned above, according to the recommendations for descriptions of species (20), any decision concerning taxonomic status should be made only after extensive DNA-DNA hybridization experiments are performed. Considering the similar, sometimes almost identical, 16S rDNA sequences of strains belonging to different species and the few distinguishing phenotypic properties, DNA pairing experiments (15) may show that the genus *Micromonospora* is overclassified and different species may have to be combined.

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TABLE 3. Distribution of 16S rDNA signature nucleotides in members of *Micromonospora* subclusters

Nucleotide positions in 16S rDNA	Base pair in:			
	Subcluster I	Subcluster II		Subcluster III ^a
		<i>M. chalicea</i>	<i>M. purpurea</i>	
603-635	C-G	C-G	U-A	U-A
612-628	U-A	C-G	C-G	C-G
613-627	U-A	U-A	C-G	C-G
615-625	G-C	G-C	C-G	C-G
616-624	G-C	G-C	A-U	A-U(G-U) ^b

^a These base pairs are also found in *M. olivasterospora* and in *M. coerulea* and related strains.

^b The base pair in parentheses occurs in *M. echinospora* subsp. *pallida*.

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