Phylogenetic Analysis Reveals New Relationships among Members of the Genera *Microtetraspora* and *Microbispora*

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The 16S rRNA gene sequences of 7 *Microbispora* strains, 14 *Microtetraspora* species, 9 *Streptosporangium* species, and 12 *Actinomadura* species were determined. A phylogenetic analysis showed that *Microtetraspora fusca*, *Microtetraspora glauca*, and *Microtetraspora niveoalba* formed a coherent cluster with the members of the genus *Microbispora*. This cluster is distantly related to two other clusters, one of which consists of the *Microtetraspora* species transferred from the former *Actinomadura pusilla* group and one of which consists of members of the genus *Streptosporangium*. Our results show that it is necessary to review the taxonomic definitions of the genera *Microtetraspora* and *Microbispora*, which have been separated by a single morphological characteristic, the number of spores on spore chains.

The genus *Microtetraspora* was established by Thiemann et al. (26) in 1968 for actinomycetes characterized by formation of chains of four spores on aerial mycelia. Two species, *Microtetraspora glauca* and *Microtetraspora fusca*, were originally proposed. In 1971 Nonomura and Ohara (13, 14) described two more species *Microtetraspora viridis* and *Microtetraspora niveoalba*. On the basis of the results of chemotaxonomic and DNA-DNA homology analyses Miyadoh et al. (12) found that *Microtetraspora viridis* clearly differed from the other three *Microtetraspora* species and resembled the strains belonging to the *Actinomadura madurae* group. *Microtetraspora viridis* was therefore transferred to the genus *Actinomadura*. Recently, Kroppenstedt et al. (7) performed a comprehensive chemical, molecular, and numerical analysis of a large number of *Actinomadura* and *Microtetraspora* type strains. These authors obtained substantial evidence that the *Actinomadura pusilla* group should be reclassified in the genus *Microtetraspora*.

Although the *Microtetraspora pusilla* group and the three original *Microtetraspora* species have very similar chemotaxonomic characteristics, including similar fatty acid and menaquinone profiles (7), there are several lines of evidence which show that these two groups are different. First, a numerical taxonomic analysis revealed that *Microtetraspora glauca* and *Microtetraspora niveoalba* are more closely related to *Microbispora rosea* than to members of the *Microtetraspora pusilla* group (1). Second, the relative electrophoretic mobilities of the ribosomal AT-L30 proteins of the three original *Microtetraspora* species ranged from –6.5 to –5.0, while most of the members of the *Microtetraspora pusilla* group had relative electrophoretic mobilities ranging from –1.5 to 0.0 (15, 16, 18). Third, the three original *Microtetraspora* species are apparently more closely related to each other than to the species belonging to the *Microtetraspora pusilla* group on the basis of chemotaxonomic characteristics and DNA-DNA homology data (7, 11).

Goodfellow et al. (4, 5) proposed the family *Streptosporangiaceae* to accommodate the chemically homogeneous genera *Microtetraspora*, *Microbispora*, *Streptosporangium*, *Planobispora*, *Planomonospora*, and *Spirillospora*. These genera are distin-

guished primarily on the basis of morphological characteristics. For example, the genera *Microtetraspora* and *Microbispora* are distinguished solely by the number of spores on their spore chains. The almost identical chemotaxonomic patterns of *Microtetraspora*, *Microbispora*, and *Streptosporangium* strains has limited the study of the intra- and intergeneric relationships.

Phylogenetic analyses of 16S rRNA gene sequences have been used extensively to resolve relationships among microorganisms at virtually all taxonomic levels (20, 23, 24, 27–29). Stackebrandt et al. analyzed the 16S rRNA genes of several *Streptosporangium* species and described heterogeneity within this genus (23). However, few 16S rRNA gene sequences of other members of the family *Streptosporangiaceae* were available.

In order to add a phylogenetic dimension to the relationships among *Microtetraspora* species and the relationships of these organisms with members of closely related genera, we analyzed the 16S rRNA genes of most *Microbispora*, *Microtetraspora*, *Streptosporangium*, and *Actinomadura* species.

MATERIALS AND METHODS

Organisms and culture conditions. The actinomycete strains used in this study were purchased from the Japan Collection of Microorganisms (Wako, Japan), the Institute for Fermentation (Osaka, Japan), and the American Type Culture Collection (Rockville, Md.). The strains which we used are listed in Table 1. Cells were cultured in the media described by the suppliers.

Preparation of genomic DNA. Mycelia of actinomycete strains were harvested by centrifugation at $3,600 \times g$ for 5 min in a model 5410 C microcentrifuge (Eppendorf, Hamburg, Germany). Approximately 200- μ l of wet mycelia was transferred to a 1.5-ml Eppendorf microcentrifuge tube. The mycelia were resuspended in 500 μ l of lysozyme buffer containing 25 mM Tris-Cl (pH 8.0), 50 mM glucose, 10 mM EDTA, and 5 mg of lysozyme (Sigma Chemical Co., St. Louis, Mo.) per ml and incubated at 37°C for 30 min. After lysozyme digestion, sodium dodecyl sulfate was added to a final concentration of 1%, and the tubes were incubated at 65°C for 30 min. The cell lysate was extracted twice with an equal volume of phenol and once with chloroform-isoamyl alcohol (24:1). The nucleic acid was precipitated with 1 volume of isopropanol at room temperature until a thread-like precipitate appeared. Then the nucleic acid preparation was centrifuged in an Eppendorf microcentrifuge at $14,000 \times g$ for 5 min. The supernatant was decanted, and the tubes were left inverted on a piece of paper towel for 10 min. The nucleic acid was resuspended in 100 μ l of 10 mM Tris-1 mM EDTA (pH 7.5) containing 100 μ g of DNase-free RNase A (Sigma Chemical Co.) per ml and incubated at 37°C for 30 min. Then the nucleic acid was extracted once with phenol-chloroform (1:1), precipitated, and resuspended as described above.

Oligonucleotides used for PCR and sequencing. The two primers used for PCR amplification of nearly complete 16S rRNA genes were 5'-TTACCTGAT AGCGGCCGACAGAGTTTGATCCTGGCTCAG3' (nucleotides 8 to 27 of the *Escherichia coli* 16S rRNA gene) (2) and 5'-TACAGGATCCGCGGCCGCTAC GG(C/T)TACCTTGTTACGACT3' (nucleotides 1492 to 1513) (the underlined sequences are cleavage sites of restriction enzyme *Not*I). The nonspecific nucleotides on the 5' side of the *Not*I site were included for efficient cleavage by

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TABLE 1. Actinomycete strains used

Taxon	Strain	GenBank nucleotide sequence accession no.
<i>Actinomadura atramentaria</i>	IFO 14695 ^{Ta}	U49000
<i>Actinomadura citrea</i>	IFO 14678 ^T	U49001
<i>Actinomadura coerulea</i>	IFO 14679 ^T	U49002
<i>Actinomadura crenea</i> subsp. <i>rifamycinii</i>	IFO 14183 ^T	U49003
<i>Actinomadura echinospira</i>	IFO 14042 ^T	U49004
<i>Actinomadura fulvescens</i>	IFO 14347 ^T	U49005
<i>Actinomadura kijaniata</i>	IFO 14229 ^T	U49006
<i>Actinomadura libanotica</i>	IFO 14095 ^T	U49007
<i>Actinomadura luteofluorescens</i>	IFO 13057 ^T	U49008
<i>Actinomadura macra</i>	IFO 14102 ^T	U49009
<i>Actinomadura rugatobispora</i>	IFO 14382 ^T	U49010
<i>Actinomadura verrucosospira</i>	IFO 14100 ^T	U49011
<i>Microbispora aerata</i>	ATCC 15448 ^T	U48984
<i>Microbispora amethystogenes</i>	JCM 3021 ^T	U48988
<i>Microbispora chromogenes</i>	IFO 14876 ^T	U48989
<i>Microbispora diastatica</i>	IFO 14041 ^T	U48990
<i>Microbispora parva</i>	ATCC 33326 ^T	U48985
<i>Microbispora thermodiastatica</i>	ATCC 27098 ^T	U14986
<i>Microbispora thermorosea</i>	ATCC 27099 ^T	U48987
<i>Microtetraspora africana</i>	IFO 14745 ^T	U48842
<i>Microtetraspora angiospora</i>	IFO 13155 ^T	U48843
<i>Microtetraspora fastidiosa</i>	IFO 14680 ^T	U48844
<i>Microtetraspora ferruginea</i>	IFO 14094	U48845
<i>Microtetraspora fusca</i>	IFO 13915 ^T	U48973
<i>Microtetraspora glauca</i>	IFO 14671 ^T	U48974
<i>Microtetraspora helvata</i>	IFO 14681 ^T	U48975
<i>Microtetraspora niveoalba</i>	IFO 15239 ^T	U48976
<i>Microtetraspora polychroma</i>	IFO 14345 ^T	U48977
<i>Microtetraspora pusilla</i>	IFO 14684 ^T	U48978
<i>Microtetraspora recticatena</i>	IFO 14525 ^T	U48979
<i>Microtetraspora roseola</i>	IFO 14685 ^T	U48980
<i>Microtetraspora salmona</i>	IFO 14687 ^T	U48982
<i>Microtetraspora spiralis</i>	IFO 14097 ^T	U48983
<i>Streptosporangium corrugatum</i>	IFO 13972 ^T	U48991
<i>Streptosporangium fragile</i>	IFO 14311	U48992
<i>Streptosporangium longisporum</i>	IFO 13141 ^T	U48993
<i>Streptosporangium nondiastaticum</i>	IFO 13990 ^T	U48994
<i>Streptosporangium pseudovulgare</i>	IFO 13991 ^T	U48995
<i>Streptosporangium roseum</i>	JCM 3005 ^T	U48996
<i>Streptosporangium violaceochromogenes</i>	JCM 3281 ^T	U48997
<i>Streptosporangium viridialbum</i>	JCM 3027 ^T	U48998
<i>Streptosporangium vulgare</i>	IFO 13985 ^T	U48999

^a T = type strain.

NotI. The ends of cloned 16S ribosomal DNAs (rDNAs) were sequenced by using M13 forward sequencing primer 5'-GTTTCCAGTCACGAC3' and reverse primer 5'-GCGGATAACAATTCACACAGG3'. The internal regions were sequenced in both orientations by using the following eight oligonucleotide primers whose target sequences were derived either from previous reports (9) or from an alignment of the 16S rRNA gene sequences of many actinomycete species: 5'-GCCTATCAGCTTGTGGT3' (nucleotides 239 to 256), 5'-CGTGCCAGCAGCCGCGGT3' (nucleotides 514 to 531), 5'-TAGATACCCTGGTAGTC3' (nucleotides 789 to 806), 5'-CCGCAACGAGCGCAACCC3' (nucleotides 1097 to 1114), 5'-TACCGCGGC(G/T)GCTGGCAC3' (nucleotides 532 to 515), 5'-GGACTACC(A/C/G)GGGTATCTAAT3' (nucleotides 806 to 787), 5'-GGGTGCGCTCGTTGCGG3' (nucleotides 1114 to 1097), and 5'-ACGGGCGGTGTGTAC3' (nucleotides 1406 to 1392). All of the oligonucleotides were synthesized by Oligos Etc., Inc., Wilsonville, Oreg.

PCR amplification of 16S rRNA genes. Each PCR mixture contained 100 ng of genomic DNA, 20 pmol of each of the two universal primers, 200 μ M dATP, 200 μ M dGTP, 200 μ M dCTP, 200 μ M dTTP, 2.5 U of *Taq* DNA polymerase (Amersham, Aylesbury, Buckinghamshire, England), and 1 $\times$ buffer (the buffer provided with the *Taq* DNA polymerase). The final volume was 100 μ L. The PCR program was 25 cycles consisting of 95°C for 40 s, 52°C for 30 s, and 72°C for 2 min. The PCR products were purified with a QIAquick Spin PCR purification kit

(QIAGEN GmbH, Hilden, Germany) and were quantified by using the Fast-Chek nucleic acid quantification system (GIBCO BRL Life Technologies, Gaithersburg, Md.).

Cloning and sequencing of 16S rDNA. The purified PCR products of 16S rRNA genes were cut with *NotI* (Amersham) and cloned into plasmid Bluescript SK (Stratagene, La Jolla, Calif.). *E. coli* DH5 α F' cells (GIBCO BRL) were prepared and transformed as described previously (10). Recombinant clones were removed and grown in 2 ml of Luria-Bertani (10) medium overnight, and the recombinant plasmid DNA was prepared by using a miniplasmid preparation kit (QIAGEN). Each cloned 16S rDNA was manually sequenced in both orientations by using an Amersham Sequenase kit (version 2.1).

Sequence alignment and phylogenetic analysis. Multiple alignments of sequences and calculations of levels of sequence similarity were carried out by using the CLUSTAL method (6) of the DNASTAR program (DNASTAR, Inc., Madison, Wis.). A phylogenetic tree was constructed by using the maximum-parsimony method of the PAUP program (25) and the neighbor-joining analysis method of Saitou and Nei (21). The confidence level of the phylogenetic tree topology was evaluated by performing 100 bootstrap replications (heuristic search).

RESULTS AND DISCUSSION

Almost complete 16S rRNA gene sequences, corresponding to nucleotides 57 to 1491 of the *E. coli* 16S rRNA gene (2), were determined for the type strains of most species of the genera *Microtetraspora*, *Microbispora*, *Streptosporangium*, and *Actinomadura* (Table 1). The 16S rDNA sequences of several *Streptosporangium* species have been described previously (23), but these sequences contained segments with unknown bases and were 70 bases shorter than the other sequences which we determined. Therefore, we resequenced these 16S rRNA genes. Other than the regions that were absent from the previously described sequences, the two sets of sequences were nearly identical.

Levels of intra- and intergeneric 16S rDNA sequence similarity. Table 2 shows the pairwise levels of sequence similarity for all of the sequences which we studied. The seven *Microbispora* strains used in this study were among the strains of 10 species that were combined by Miyadoh et al. (11) into the single species *Microbispora rosea* on the basis of their high levels of DNA-DNA relatedness. The levels of similarity between the 16S rRNA sequences of these *Microbispora* strains (95.1 to 99.0%) seem to support the hypothesis that this group is homogeneous. The intrageneric levels of sequence similarity for the members of the other genera appeared to vary over wider ranges, as follows: 91.8 to 98.4% for *Microtetraspora* strains, 86.1 to 98.9% for *Streptosporangium* strains, and 90.1 to 98.7% for *Actinomadura* strains. These values imply that each of these groups is heterogeneous. This conclusion is generally consistent with previous observations (11, 17, 18, 19, 23). For example, the sequences of the 16S rRNA gene and the ribosomal AT-L30 protein of *Streptosporangium corrugatum* are known to differ substantially from the sequences of their counterparts in the type strains of *Streptosporangium roseum*, *Streptosporangium nondiastaticum*, and *Streptosporangium pseudovulgare* (17, 23). Significant differences in the relative electrophoretic mobilities of the AT-L30 proteins of *Actinomadura* strains have also been reported (18). The levels of 16S rRNA sequence similarity for the three original *Microtetraspora* species, *Microtetraspora fusca*, *Microtetraspora glauca*, and *Microtetraspora niveoalba*, are 97.9 to 98.4%, but the levels of similarity with the species transferred from the former *Actinomadura pusilla* group are much lower (91.8 to 95.5%), and when the intergeneric levels of sequence similarity were determined, we found high levels of similarity (93.7 to 96%) between these three *Microtetraspora* species and *Microbispora* species. This observation seems to indicate that there is heterogeneity in the genus *Microtetraspora*.

An examination of the intra- and intergeneric sequence similarity values demonstrated that for the genera *Microtetraspora*, *Microbispora*, and *Streptosporangium*, species belonging to dif-

TABLE 2. Levels of 16S rRNA gene sequence similarity for members of the genera *Microbispora*, *Microtetraspora*, *Streptosporangium*, and *Actinomadura*

Organism	% 16S rRNA gene sequence similarity																			
	<i>Actinomadura atramentaria</i>	<i>Actinomadura citrea</i>	<i>Actinomadura coerulea</i>	<i>Actinomadura cremea</i> subsp. <i>rifamycinii</i>	<i>Actinomadura echinospora</i>	<i>Actinomadura fulvescens</i>	<i>Actinomadura kijaniata</i>	<i>Actinomadura libanotica</i>	<i>Actinomadura luteofluorescens</i>	<i>Actinomadura macra</i>	<i>Actinomadura rugatobispora</i>	<i>Actinomadura verrucosospora</i>	<i>Microbispora aerata</i>	<i>Microbispora amethystogenes</i>	<i>Microbispora chromogenes</i>	<i>Microbispora diastatica</i>	<i>Microbispora parva</i>	<i>Microbispora thermodiastatica</i>	<i>Microbispora thermorosea</i>	<i>Microtetraspora africana</i>
<i>Actinomadura atramentaria</i>	100	93.3	93.2	91.8	92.6	93.1	93.9	91.5	93.6	93.9	93.9	93.1	89.6	90.1	89.4	89.1	89.5	89.4	88.9	89.4
<i>Actinomadura citrea</i>		100	97.4	94.1	93.5	95.2	94.8	91.3	97.7	95.2	94.8	96.9	89.6	89.3	89.1	88.8	88.7	89.4	88.2	89.5
<i>Actinomadura coerulea</i>			100	93.5	93.4	94.7	94.7	91.2	98.2	95.6	95.5	97.7	89.6	89.4	89.2	88.8	88.8	89.6	88.2	89.6
<i>Actinomadura cremea</i> subsp. <i>rifamycinii</i>				100	93.2	93.5	94.0	91.6	93.6	92.2	93.3	93.0	88.7	89.5	88.9	88.3	88.2	88.5	87.6	88.8
<i>Actinomadura echinospora</i>					100	94.1	94.4	91.5	94.1	93.4	93.5	93.6	91.3	91.3	91.3	91.4	90.9	90.9	90.1	91.6
<i>Actinomadura fulvescens</i>						100	95.1	92.3	95.5	93.9	94.5	94.8	89.8	90.2	90.3	90.0	90.1	89.6	88.4	91.4
<i>Actinomadura kijaniata</i>							100	92.9	95.3	93.4	94.7	94.8	89.4	89.1	89.6	89.5	88.9	89.1	88.6	90.6
<i>Actinomadura libanotica</i>								100	90.7	90.1	91.2	90.5	89.2	89.0	88.5	88.7	88.5	88.8	88.2	89.7
<i>Actinomadura luteofluorescens</i>									100	95.2	95.4	98.7	89.4	89.1	89.1	88.8	88.5	89.4	88.4	89.6
<i>Actinomadura macra</i>										100	94.7	94.9	90.0	89.8	89.6	89.7	89.4	89.5	88.8	89.5
<i>Actinomadura rugatobispora</i>											100	94.8	89.6	89.4	89.5	89.2	89.6	89.7	88.7	89.4
<i>Actinomadura verrucosospora</i>												100	89.0	88.7	88.7	88.2	88.0	89.0	87.8	89.1
<i>Microbispora aerata</i>													100	96.8	96.3	96.3	97.4	99.2	97.7	92.6
<i>Microbispora amethystogenes</i>														100	97.9	97.8	98.1	96.5	95.1	93.4
<i>Microbispora chromogenes</i>															100	99.0	97.5	96.0	96.3	93.3
<i>Microbispora diastatica</i>																100	97.2	96.0	96.1	93.4
<i>Microbispora parva</i>																	100	97.4	95.8	92.6
<i>Microbispora thermodiastatica</i>																		100	97.5	92.3
<i>Microbispora thermorosea</i>																			100	91.4
<i>Microtetraspora africana</i>																				100
<i>Microtetraspora angiospora</i>																				
<i>Microtetraspora fastidiosa</i>																				
<i>Microtetraspora ferruginea</i>																				
<i>Microtetraspora fusca</i>																				
<i>Microtetraspora glauca</i>																				
<i>Microtetraspora helvata</i>																				
<i>Microtetraspora niveoalba</i>																				
<i>Microtetraspora polychroma</i>																				
<i>Microtetraspora pusilla</i>																				
<i>Microtetraspora recticatena</i>																				
<i>Microtetraspora roseola</i>																				
<i>Microtetraspora salmonea</i>																				
<i>Microtetraspora spiralis</i>																				
<i>Streptosporangium corrugatum</i>																				
<i>Streptosporangium fragile</i>																				
<i>Streptosporangium longisporum</i>																				
<i>Streptosporangium nondiastaticum</i>																				
<i>Streptosporangium pseudovulgare</i>																				
<i>Streptosporangium roseum</i>																				
<i>Streptosporangium violaceochromogenes</i>																				
<i>Streptosporangium viridialbum</i>																				
<i>Streptosporangium vulgare</i>																				

ferent genera often exhibit higher levels of sequence similarity than species belonging to the same genus (Table 2). Clearly, when the sequence similarity values are less than 95%, they cannot be used to reliably assign a species to any of the three genera. The level of sequence similarity between these three genera and the genus *Actinomadura* is less than 92%, which is lower than the levels of intrageneric similarity with only a few exceptions.

Stacketbrandt and Goebel (22) studied the correlation between 16S rRNA sequence analysis results and DNA-DNA reassociation results for defining bacterial species. These authors found that organisms that exhibited less than 97% 16S rRNA sequence similarity did not have DNA-DNA reassociation values of more than 60% and should therefore be placed in different species. Miyadoh et al. (11) combined 10 *Microbispora* species into a single species, *Microbispora rosea*, on the

TABLE 2—Continued

% 16S rRNA gene sequence similarity																					
<i>Microtetrastora angiospora</i>	<i>Microtetrastora fastidiosa</i>	<i>Microtetrastora ferruginea</i>	<i>Microtetrastora fusca</i>	<i>Microtetrastora glauca</i>	<i>Microtetrastora helvata</i>	<i>Microtetrastora nivealba</i>	<i>Microtetrastora polychroma</i>	<i>Microtetrastora pusilla</i>	<i>Microtetrastora recticatena</i>	<i>Microtetrastora roseola</i>	<i>Microtetrastora salmonea</i>	<i>Microtetrastora spiralis</i>	<i>Streptosporangium corrugatum</i>	<i>Streptosporangium fragile</i>	<i>Streptosporangium longisporum</i>	<i>Streptosporangium nondiastaticum</i>	<i>Streptosporangium pseudovulgare</i>	<i>Streptosporangium roseum</i>	<i>Streptosporangium violaceochromogenes</i>	<i>Streptosporangium viridialbum</i>	<i>Streptosporangium vulgare</i>
88.3	88.4	88.6	88.8	88.9	89.9	88.9	89.4	89.6	89.0	89.0	88.3	88.4	88.4	88.9	89.2	88.1	88.6	89.1	88.8	88.8	90.1
88.5	88.7	88.4	88.8	89.3	88.9	89.1	88.2	89.6	88.9	89.5	89.2	86.9	88.9	87.7	88.6	87.8	87.6	88.3	87.4	88.6	88.8
88.7	88.5	89.1	88.5	89.1	89.4	88.9	88.6	89.9	88.9	89.6	89.4	87.3	89.1	86.9	88.9	88.2	88.0	88.4	88.0	88.7	88.9
87.0	87.9	86.2	88.2	88.4	88.6	88.9	87.9	88.6	88.2	88.7	87.7	87.0	88.8	87.5	87.4	87.7	88.1	88.6	85.5	87.9	87.8
89.6	90.4	90.0	90.9	91.0	91.3	90.9	90.9	90.9	91.0	91.4	89.9	89.4	91.0	89.6	89.8	90.5	90.1	90.8	87.4	90.0	90.3
90.5	89.9	89.3	90.4	90.4	91.4	90.3	89.9	90.4	90.3	91.2	90.7	89.7	89.8	88.7	89.4	89.2	89.2	89.5	88.6	89.8	89.6
89.6	88.6	89.0	89.1	89.5	90.2	89.6	89.1	90.5	90.0	90.6	89.8	88.7	89.5	89.5	89.9	89.5	89.8	89.9	89.4	90.8	90.2
87.5	88.2	87.1	88.0	87.9	88.5	87.9	89.0	89.2	89.3	88.8	87.7	87.7	89.0	89.0	89.5	89.4	89.5	89.8	88.7	88.7	89.6
88.7	88.6	89.1	88.4	88.9	89.4	88.9	88.6	89.9	89.0	89.6	89.2	87.3	89.1	86.9	89.0	88.1	87.9	88.6	87.7	88.6	88.9
88.8	89.6	88.5	89.0	89.1	89.5	89.0	89.1	89.0	89.2	89.5	89.2	87.6	89.3	88.1	88.3	88.2	87.9	88.3	86.0	87.6	88.4
87.8	88.3	86.0	88.5	88.8	88.9	88.8	88.7	89.3	88.8	88.7	88.0	87.3	89.7	87.7	87.8	88.0	88.1	88.3	86.1	88.6	88.2
88.1	87.9	88.5	88.0	88.4	88.7	88.1	87.8	89.3	88.5	89.1	88.4	86.7	88.7	87.6	88.4	87.7	87.5	88.0	87.3	88.2	88.4
92.3	93.6	92.3	95.0	95.0	93.7	95.3	93.5	92.6	92.6	91.9	91.9	91.5	94.5	91.2	91.5	91.5	91.9	91.7	91.3	92.3	92.2
92.8	92.6	92.7	96.0	95.8	94.5	95.8	94.3	93.1	92.7	92.4	92.5	92.6	93.7	90.7	90.7	90.7	90.9	91.7	90.9	92.1	91.8
93.5	92.8	92.6	95.3	95.9	94.8	95.5	93.6	94.3	92.6	92.7	92.8	93.6	93.2	92.1	91.2	91.7	91.9	90.8	91.6	93.3	91.6
93.8	92.6	92.1	95.7	95.8	94.5	95.4	93.7	93.7	92.9	92.6	92.8	93.5	93.4	92.1	91.4	91.7	91.7	90.7	91.1	93.3	91.3
92.3	92.1	92.5	95.0	95.1	94.3	94.9	93.4	92.7	92.0	92.1	91.6	92.1	93.7	90.9	90.9	90.4	90.8	91.1	90.9	92.1	91.8
92.1	93.0	92.0	95.0	94.9	93.6	95.3	93.2	92.4	92.3	91.7	91.6	91.2	93.8	91.3	91.5	90.9	91.3	91.0	91.3	91.9	91.9
91.8	93.0	90.9	93.7	94.1	92.8	94.4	92.1	92.6	91.4	90.8	91.7	91.4	93.0	91.4	91.0	91.2	91.8	90.0	89.0	92.3	90.9
96.5	93.6	93.8	94.4	94.0	96.5	93.6	96.0	93.6	98.0	97.6	95.8	95.5	92.2	92.6	93.3	92.6	92.4	92.4	92.4	94.3	93.8
100	94.3	93.5	93.8	93.5	96.6	93.0	95.2	94.6	95.7	95.9	97.4	95.8	91.3	91.6	91.2	91.2	90.9	90.1	91.1	93.3	91.6
	100	93.6	92.0	91.8	94.9	92.2	94.7	93.1	93.3	92.8	94.7	94.3	91.6	90.2	90.3	90.5	90.6	90.0	88.2	90.8	90.0
		100	92.6	92.8	94.9	92.9	95.0	94.7	93.1	93.4	94.1	94.5	92.1	91.5	91.6	90.5	90.8	90.9	90.9	91.7	92.7
			100	98.4	94.6	97.9	94.3	93.6	93.6	93.1	92.7	93.3	94.0	91.9	93.1	91.9	92.1	92.1	92.3	93.6	93.1
				100	95.0	98.2	93.8	94.3	93.3	93.4	92.6	93.1	93.1	91.6	92.3	91.6	91.7	91.2	91.9	93.1	92.5
					100	94.3	97.5	95.0	95.5	96.5	96.0	97.0	92.9	91.6	91.9	91.3	91.6	91.2	91.8	93.5	92.4
						100	93.8	94.0	93.4	93.0	92.6	92.8	93.0	91.2	92.2	91.6	91.7	91.3	91.5	93.1	92.5
							100	94.1	95.3	94.5	95.0	95.9	93.1	91.1	91.6	91.3	91.4	91.8	91.2	92.4	92.4
								100	92.9	92.9	94.6	94.0	92.8	93.3	92.3	92.8	92.8	91.9	91.9	92.8	93.1
									100	97.5	95.5	95.2	91.6	92.4	93.0	92.5	92.2	91.7	91.9	93.6	93.2
											100	95.1	94.4	91.4	92.3	93.1	91.7	91.5	92.0	93.5	93.4
											100	95.6	90.8	91.7	91.9	91.0	90.9	89.7	90.9	93.1	91.6
												100	91.0	91.2	90.8	91.1	91.1	90.3	90.8	92.5	91.1
													100	86.9	92.7	94.2	94.6	94.6	90.6	94.4	93.3
														100	96.7	96.0	96.1	95.5	95.6	95.6	96.7
															100	95.2	95.6	96.0	95.3	95.2	98.2
																100	98.9	96.9	92.6	95.3	95.2
																	100	97.2	93.1	95.6	95.7
																		100	92.3	94.8	96.9
																			100	93.9	95.3
																				100	95.5
																					100

basis of a mean DNA-DNA relatedness value of 56%, and they also defined, on the basis of growth temperatures, two subspecies, *Microbispora rosea* subsp. *rosea* (including *Microbispora chromogenes*, *Microbispora amethystogenes*, *Microbispora parva*, and *Microbispora diastatica* used in this study) and *Microbispora rosea* subsp. *aerata* (including *Microbispora aerata*, *Microbispora thermodiastatica*, and *Microbispora thermorosea*). A total of 9 of the 21 similarity values obtained for the seven

strains which we studied were less than 97%, a finding which does not support the proposal that the 10 species should be combined in a single species. Ochi et al. (16) also found that the 10 *Microbispora* strains which they studied displayed substantially different AT-L30 ribosomal protein relative electrophoretic mobilities and thus raised doubts concerning the conclusions of Miyadoh et al. (11). It should be noted that the evidence that Miyadoh et al. used to combine the 10 *Micro-*

Phylogenetic relationships. All of the nucleotides of the 16S rDNA sequences were included in our phylogenetic analysis without creating significant ambiguity in the multiple-sequence alignment. Our phylogenetic tree (Fig. 1) contains two major clusters, one consisting of *Actinomadura* species (the former *Actinomadura madurae* group species; the type species, *A. madurae*, was not included in this study because of difficulty in amplifying the complete 16S rRNA gene by PCR) (3, 7) and the other consisting of the members of the genera *Microtetraspora*, *Microbispora*, and *Streptosporangium*. The *Actinomadura* cluster contains *Actinomadura echinospora* and *Actinomadura*

rugatobispora, and thus our findings support the transfer of these two species from the genus *Microbispora* proposed by Miyadoh et al. (11). The bootstrap values determined for the two major clusters were 100 and 93%, respectively. This result, in conjunction with the results of the sequence similarity analysis described above, supports the proposal that the genera *Streptosporangium*, *Microtetraspora*, and *Microbispora* should be included in the family *Streptosporangiaceae* (4, 8). The *Streptosporangium* species except *Streptosporangium corrugatum* and the *Microtetraspora pusilla* group species form two distinct sub-clusters. Although *S. corrugatum* exhibits relatively more affinity to the *Microtetraspora pusilla* cluster, its distance from each of the three subclusters is significant, which support the view that this species may merit status as a member of a separate

genus (23). *Microtetrastora glauca*, which is the type species of the genus *Microtetrastora*, together with *Microtetrastora fusca* and *Microtetrastora niveoalba*, belong to a third distant subcluster which contains the members of the genus *Microbispora*. The bootstrap value for the branch point which separates the *Microbispora* subcluster from the *Microtetrastora pusilla* subcluster is significant (88%).

The bootstrap values for phylogenetic separation of the three subclusters are more than 80%. The phylogenetic distance between any two of the three subclusters is comparable to this value. If each of the subclusters represents a genus, obviously *Microtetrastora glauca*, *Microtetrastora fusca*, and *Microtetrastora niveoalba* cannot be accommodated with the members of the *Microtetrastora pusilla* group within the confines of one genus. Chemotaxonomic properties cannot be used to distinguish the genera *Microtetrastora* and *Microbispora*, and differentiation of these taxa has been based on a single morphological property, the number of spores in the spore chains (4). It is generally accepted that morphological characteristics alone are seldom reliable for determining taxonomic positions of bacterial species. However, in this case, a single morphological characteristic apparently plays a dominant role in the separation of two genera. None of the previous chemotaxonomic, numerical, and AT-L30 protein electrophoretic mobility studies included enough species belonging to both genera so that a comprehensive comparison could be performed (1, 7, 16, 18). However, by carefully scrutinizing the results of the previous reports, we obtained some evidence (as described above) which implied that the genus *Microtetrastora* is heterogeneous and that some *Microtetrastora* species and *Microbispora* species are closely related. This seemingly weak evidence became substantial when it was combined with the results of our phylogenetic analysis.

Conclusion. Our phylogenetic analysis based on nearly complete 16S rDNA sequences clearly demonstrated that there are two distantly related clusters in the genus *Microtetrastora* and that three *Microtetrastora* species, represented by the type species of the genus, *Microtetrastora glauca*, are closely related to the genus *Microbispora*. Although the available evidence may not be sufficient to reclassify the genera *Microbispora* and *Microtetrastora* it shows that it will be necessary to carry out a comprehensive analysis in which chemotaxonomic and other approaches are used to redefine the taxonomic positions of these genera.

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